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Expression and Self-Assembly of Norwalk Virus Capsid Protein from Venezuelan Equine Encephalitis Virus Replicons

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The Norwalk virus (NV) capsid protein was expressed using Venezuelan equine encephalitis virus replicon particles (VRP-NV1). VRP-NV1 infection resulted in large numbers of recombinant NV-like particles that were primarily cell associated and were indistinguishable from NV particles produced from baculoviruses. Mutations located in the N-terminal and P1 domains of the NV capsid protein ablated capsid self-assembly in mammalian cells.

Norwalk-like viruses (NLVs) cause ~85 to 96% of the analyzed outbreaks of acute nonbacterial gastroenteritis in the United States (12, 15, 28, 35, 37, 43, 44). NLVs do not replicate in cell culture, and animal models are not available, hampering the understanding of the replication strategy of these important human pathogens (28). NLVs are classified into two distinct genogroups (genogroups I and II), with genogroup I containing the prototype virus, Norwalk virus (NV) (10, 16, 46). The ~7.6-kb single-stranded, plus-sense RNA genome of NV encodes three large open reading frames (ORFs): a polyprotein of ~1,789 amino acids (aa) (ORF1), a major capsid protein of ~530 aa (ORF2), and a minor capsid protein of 212 aa (ORF3) (14, 26, 27). Baculovirus-expressed NV ORF2 capsid proteins will self-assemble into virus-like particles (VLPs) (BAC-Nor) and have resulted in new diagnostic reagents and vaccine approaches (17, 18, 20, 25, 26, 33, 39, 40, 47). BAC-Nor-based vaccines are being developed for clinical evaluation, but it is not clear whether protective responses will be elicited in humans (2, 3, 34). Because posttranslational modification pathways may process proteins differently in insect and mammalian cells (11), it has been suggested that BAC-Nor capsids may not represent authentic NV capsids (38). Moreover, NLV VLP particle formation in mammalian cells has been unsuccessful thus far. This article describes the successful expression of NLV VLPs in a mammalian system.

Venezuelan equine encephalitis virus (VEE), an alphavirus, has been configured as a replicon vaccine vector. VEE replicon cDNAs are constructed by replacing the VEE structural genes with a foreign gene of interest just downstream from a 26S promoter, which drives high levels of expression of the downstream genes (6, 7, 41). VEE replicon particles (VRPs) are produced by transfecting transcripts of the VEE replicon cDNA along with helper transcripts encoding the VEE structural genes (Fig. 1A). We reasoned that VEE expression of NV capsid proteins might allow for NLV VLP formation in mammalian cells.

Using primer pairs to highly conserved NLV sequences (27, 46), we have cloned and sequenced two NV capsid clones (NV1 and NV2) (Fig. 1B). The amino acid sequence of NV1 was identical with the published sequence, while there were three amino acid changes in the NV2 sequence (26, 27). Both capsid clones were inserted into VEE replicon plasmids (VEE-NV1 and VEE-NV2), and VEE VRPs (VRP-NV1 and VRP-NV2) were generated by coelectroporation of BHK cells with the replicon and helper transcripts (Fig. 1A). Using these conditions, we made the following predictions. (i) VEE VRPs encoding the NV capsid gene will bud from the surfaces of transfected cells. (ii) NV capsid proteins will accumulate and self-assemble into NV virus-like particles (VEE-Nor) (Fig. 1A). In contrast, infection of cells with VRP-NV1 or VRP-NV2 will result in the synthesis of NV recombinant proteins, which should self-assemble into VEE-Nor particles in the absence of VEE structural protein production (Fig. 1A).

VRP-NV1 and VRP-NV2 stocks were prepared, and the titer of the virus stocks was determined at $\sim 1.0 \times 10^9$ infectious units/ml in BHK cells by fluorescent antibody (FA) staining using antiserum from a human volunteer dosed with NV (C. Moe, D. Rhodes, S. Pusek, et al., unpublished data). To determine if recombinant NV (rNV) antigen was expressed following VRP infection in different host cell types, cultures of murine, feline, swine, and human cells were inoculated with VRP-NV1 at a multiplicity of infection (MOI) of 5 and incubated at 37°C for 16 h. By FA staining, VRP-NV1 expressed NV capsid proteins in a variety of different host cell species (Fig. 1C). As animal and human NLVs have been described (45), it seems likely that VRP vectors will allow for NLV capsid expression and vaccine development in the appropriate host species.

Human papillomavirus, calicivirus, rotavirus, and human immunodeficiency virus VLPs have been expressed from recombinant baculoviruses, vesicular stomatitis virus, vaccinia virus, *Escherichia coli*, and other vectors (11, 13, 29, 32). In some cases, the recombinant VLPs are contaminated with the carrier virus, complicating purification, vaccine production, and hu-

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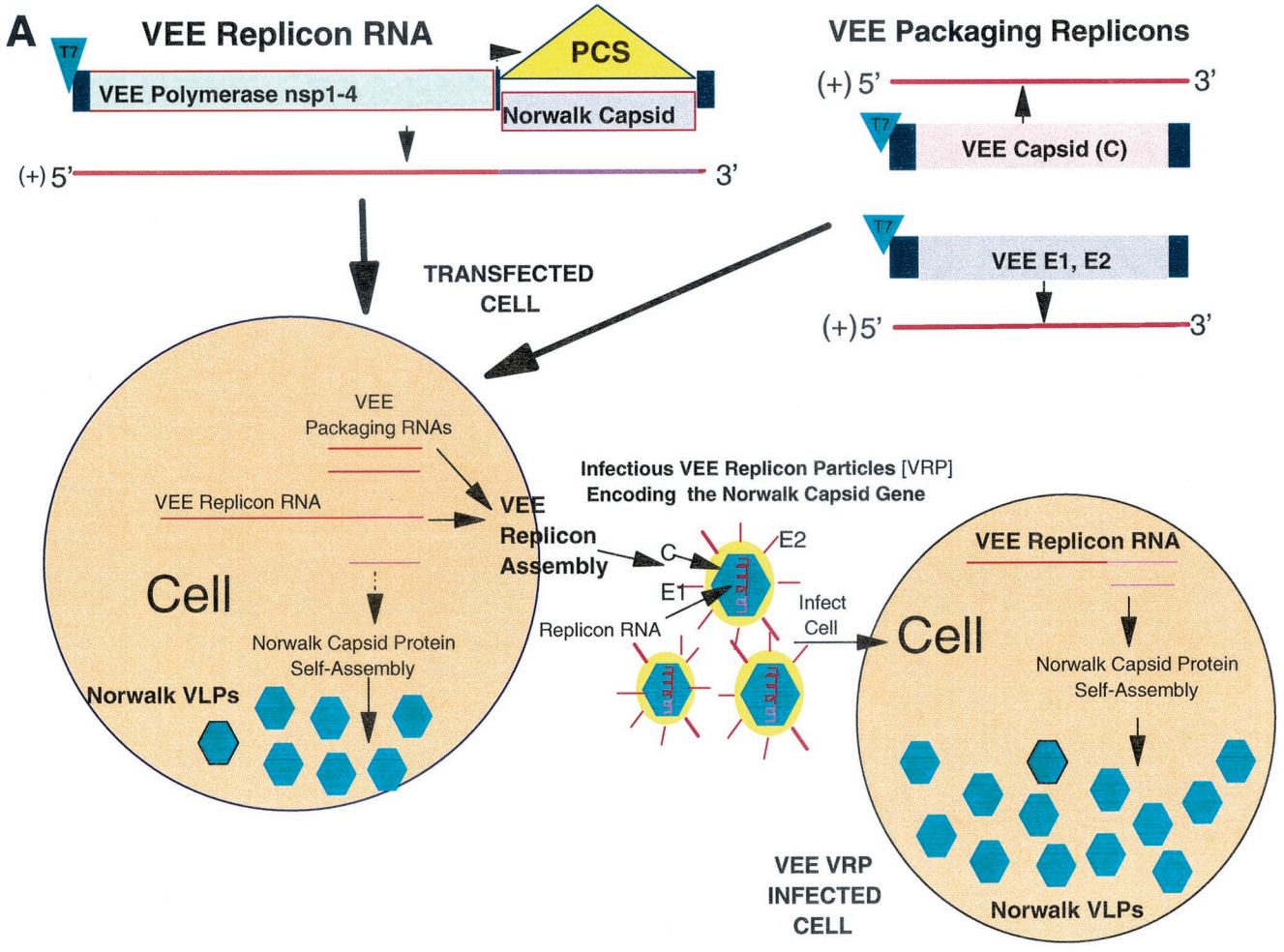

FIG. 1. Expression of NLV capsid genes from VEE replicons. (A) Organization and transcription strategy of VEE replicons. In VEE replicons, the structural genes (capsid, E1, and PE2) are replaced with the NV capsid gene inserted into a polycloning site (PCS) under control of the subgenomic 26S promoter. Replicon transcripts coelectroporated into cells with the VEE defective packaging construct RNAs (VEE capsid and envelope glycoproteins) result in the packaging and release of infectious VEE VRPs that can be used as single-hit vaccine vectors in mammals (30, 41). Transfection will also result in the transient expression of high concentrations of rNV capsid protein which may self-assemble into NV VLPs. Upon infection of cells with VEE VRPs encoding the NV capsid gene, these single-hit replicon vectors will express high levels of the rNV capsid protein that self-assemble into ~38-nm-diameter recombinant NLV particles which should be free of VEE VRPs. (B) Sequence of the NV major capsid gene. The NV capsid gene was cloned from stool samples of two human volunteers challenged with NV. The forward primer (NVCAPF1 [5′-GAT TTCCAGCAAGGTCATAC-3′]) and reverse primer (NVCAPR1 [5′-TTCGCCACCAACCTGTATGC-3′]) were designed to amplify the entire NV capsid region following reverse transcription-PCR (27, 46). The reaction was performed in a 50-μl reaction mixture with 5 μl of purified viral mRNA. Reverse transcription was performed at 50°C for 60 min, and then the temperature was elevated to 94°C for 2 min. Forty amplification cycles were performed, with one cycle consisting of 30 s at 94°C, annealing at 55°C for 1 min, and primer extension at 68°C for 2 min. While the NV1 sequence was identical to the published sequence (26, 27), the sequence of NV2 contained three amino acid changes. Chimeric constructs were assembled in which the N-terminal amino acid alterations in NV2 were joined in frame to the C terminus of NV1 (NV3) and in the opposite orientation (NV4). Using overlapping extension PCR, the NV capsid gene was fused to the VEE subgenomic promoter and inserted into the polycloning site of the VEE PVR21 plasmid vector and sequenced. (C) Expression of NV capsid proteins in cells from different mammals. Cells were infected with VRP-NV1 for 1 h at room temperature at a MOI of 5. At 18 h postinfection, the cultures were fixed and stained by FA staining for the presence of NV capsid proteins using NV antiserum from an infected volunteer using previously described techniques (5). Caco2 human colorectal adenocarcinoma cells, CRFK feline kidney cells, DBT murine astrocytoma cells, and swine testicular (ST) cells are shown.

man use (42). To determine if the rNV capsids self-assemble into VLPs, BHK cells (4.0×10^7 cells) were infected with VRP-NV1 or VRP-NV2 at a MOI of 2 for 1 h. At 36 h postinfection, the cultures were freeze-thawed twice and virus particles were concentrated by ultracentrifugation at 125,000 $\times g$ through 20% sucrose cushions. Using a negative staining technique (21), $\sim 10^{10}$ recombinant NLV virus-like particles (VEE-Nor) with a diameter of about 38 nm were evident in cultures infected with VRP-NV1 but not with VRP-NV2 (Fig. 2). Immunogold labeling was performed using NV-specific monoclonal antibodies (22), and the VEE-Nor particles were clearly labeled with gold beads, indicating that these particles were antigenically intact (data not shown). The smaller 23-nm-diameter rNV particles that were detected in BAC-Nor preparations derived from insect cells were not evident in VEE-Nor preparations (25, 39, 47). With a broad host range, the use of

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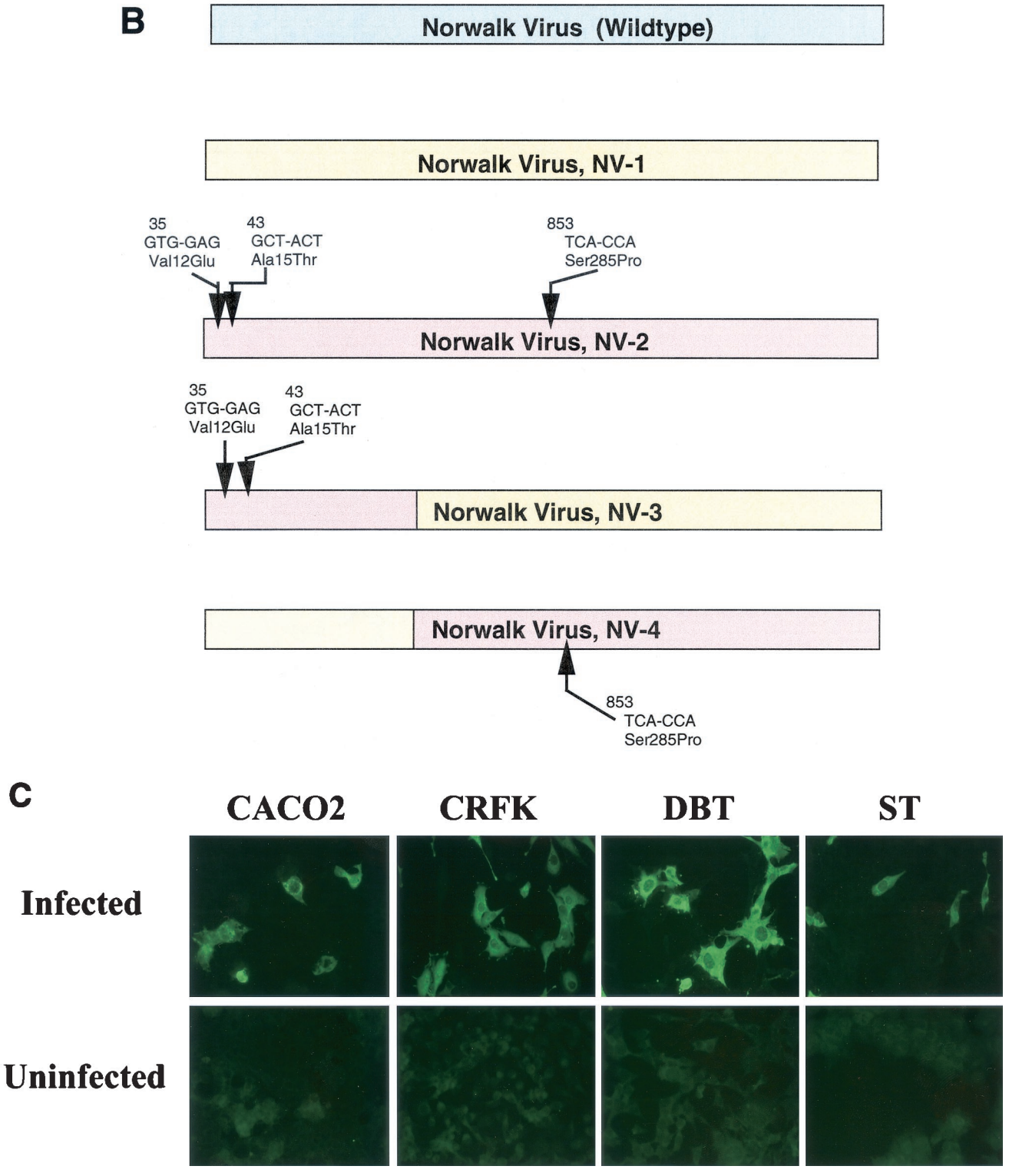

FIG. 1—*Continued.*

single-hit alphavirus replicons provides a safe, alternative vector for the production of high concentrations of VLPs, free from helper viruses and in a mammalian system. We speculate that alphavirus single-hit replicons may serve as vectors for VLP expression and vaccine development for a variety of important human and animal viruses (3, 4, 24, 36).

To determine if rNV VLPs remained cell associated, BHK cultures were infected with VRP-NV1, the culture medium

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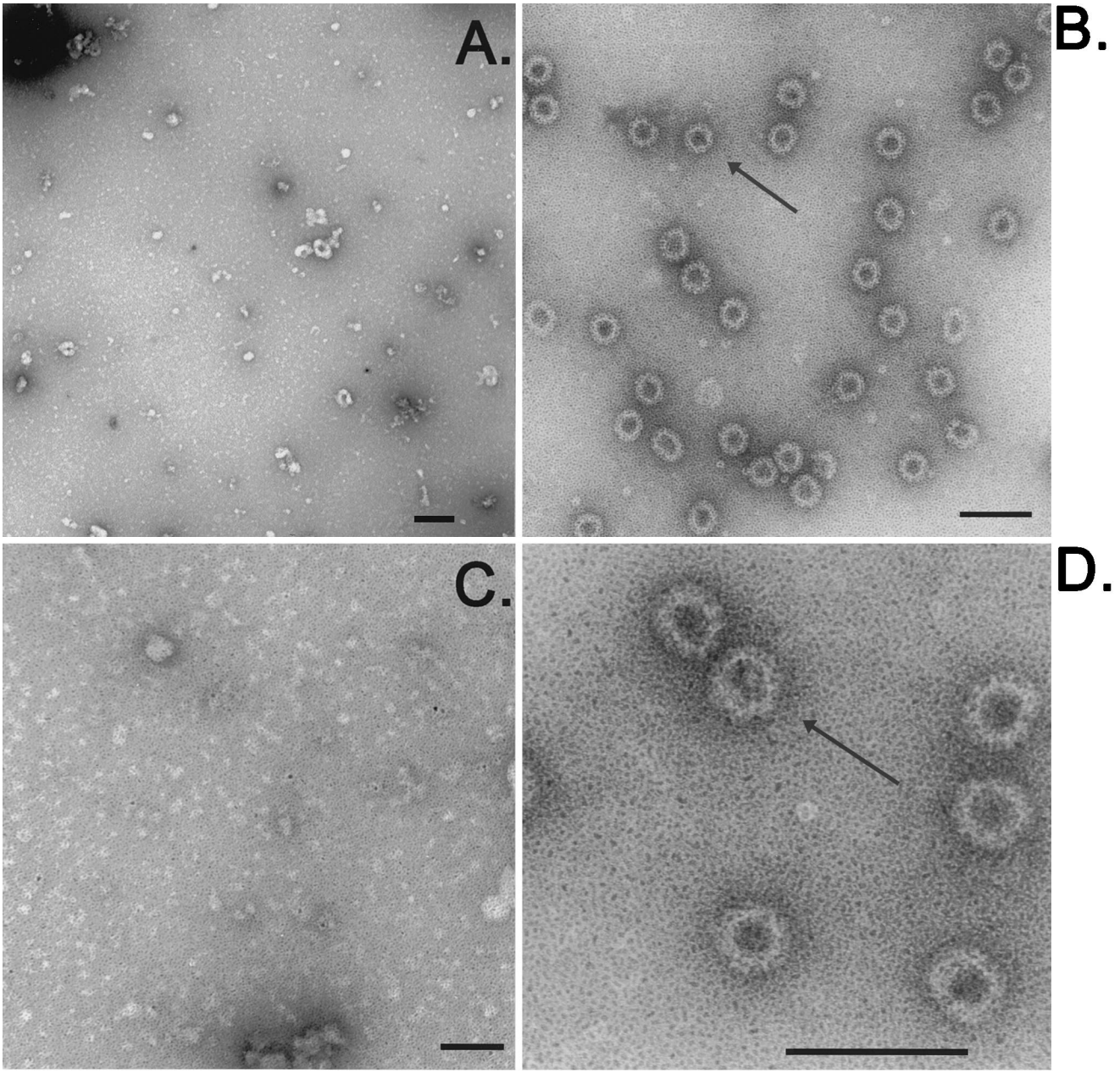

FIG. 2. Assembly of NLV VLPs in mammalian cells. BHK cells (4×10^7) were infected with VRP-NV1 or VRP-NV2. At 36 h postinfection, the cultures were freeze-thawed twice and putative particles were pelleted through a 20% sucrose cushion and resuspended in sterile phosphate-buffered saline. Viral suspensions were examined by negative staining with uranyl acetate (21). Grids were examined at 80 kV in a LEO EM-910 transmission electron microscope (Leo Electron Microscopy, Inc., Thornwood, N.Y.) at magnifications ranging from ×25,000 to ×125,000. Particles were visualized, photographed, and digitized prior to assembly of the images using Adobe Photoshop version 5.5 (Adobe Systems Inc.). VRP-NV2 (A and C) and VRP-NV1 (B and D) lysates at low and high magnifications are shown. Arrows indicate the rNV particles (VEE-Nor). Bars = 100 nm (B, C, and D) and 50 nm (A).

and cell lysates were harvested at 30 h postinfection and clarified by high-speed centrifugation, and VEE-Nor particles were pelleted by centrifugation through a 20% sucrose cushion. While only a few VEE-Nor particles were evident in culture supernatants, the majority were cell associated (Fig. 3A and B). We expect that the few extracellular particles were released as a result of VEE-induced cytopathology.

The 38-nm-diameter BAC-Nor particle exhibited a T3 icosahedral symmetry, with 180 copies of the capsid protein organized into 90 dimers. The NV major capsid protein consists of a protruding (P) domain connected by a flexible hinge to a shell (S) domain that has a classical eight-stranded β-sandwich motif (40). Amino acid changes in VEE-NV2 were noted at positions 12 (Val to Glu) and 15 (Ala to Thr) and at position 285 (Ser to Pro), all of which map at or near loci forming the probable shell domain of the NV capsid (40). Interestingly, the

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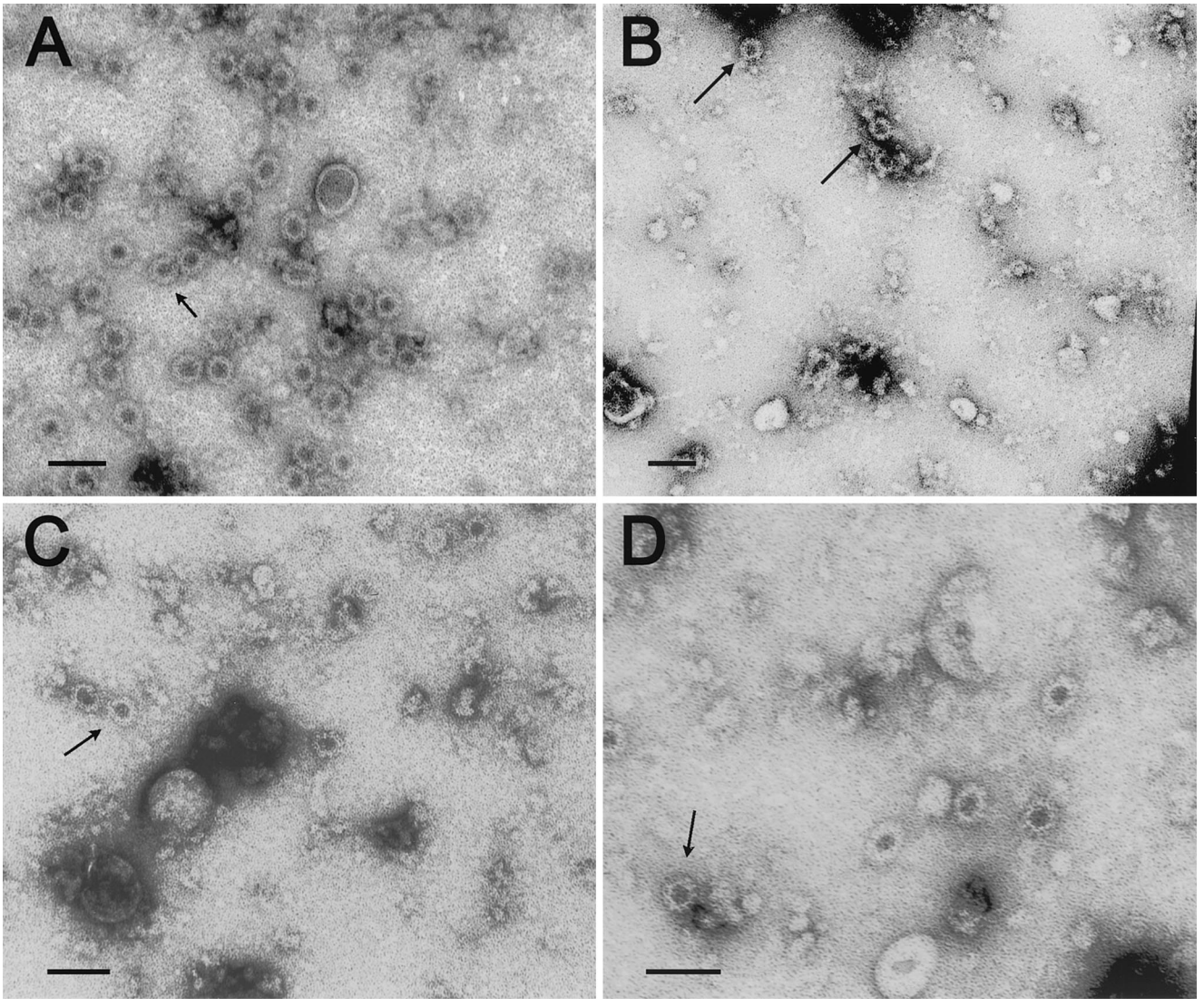

FIG. 3. Cell association of recombinant NLV particles and mapping mutations which ablate particle formation. To determine if VEE-Nor particles are preferentially secreted from infected cells or remain in cellular compartments, BHK cells (4×10^7 cells) were infected with VRP-NV1 at a MOI of 2 for 1 h. At 36 h postinfection, supernatants were harvested, cell monolayers were frozen twice, and cell debris was removed by centrifugation. Virus particles were concentrated from the supernatants or cell lysates by centrifugation through 20% sucrose cushions for 3 h at 28,000 rpm in a Beckman SW28 rotor and analyzed by electron microscopy as described in the legend to Fig. 2. Intracellular (A) and extracellular (B) VEE-Nor and particle formation from VEE-NV3- (C) and VEE-NV4- (D) transfected BHK cells (8.0×10^6). Arrows indicate NLV particles. Bars = 100 nm.

S285P change maps within a predicted dimer-binding site that may be critical for particle assembly (40). To identify the changes that ablated particle formation, we replaced the C-terminal mutation of NV2 (NV3) and the N-terminal mutations of NV2 (NV4) with wild-type NV1 sequences (Fig. 1B). Surprisingly, VEE expression of capsids from either chimera produced rNV VLPs, indicating that the constellation of mutations ablated particle formation (Fig. 3C and D). At this time it is unclear whether the mutations in the NV2 clone affected capsid dimerization, pentamer formation, or a final step in the NV particle assembly cascade.

Posttranslational modification patterns of papillomavirus capsids differ in mammalian and insect cells, and it is possible that these alterations might alter VLP antigenicity and hence, vaccine efficacy (11, 17). To address this question, cultures of BHK cells were infected with VEE VRPs and the biochemical and antigenic properties of the VEE-expressed NV protein were compared to those of BAC-Nor (Fig. 4). Western blotting with human antiserum pre- and post-NV challenge demonstrated that the ~58-kDa capsid protein from VRP-NV1- and VRP-NV2-infected cells was nearly equivalent in size with that from BAC-Nor, although slight differences were noted (Fig. 4 and 5A) (26). To determine if the slight mobility shift was due to protein structure or alternative posttranslational processing, gradient-purified capsid proteins were extensively denatured with 5 M guanidine, reduced with dithiothreitol, and then alkylated with 0.25 M iodoacetic acid (23). Following NV1 sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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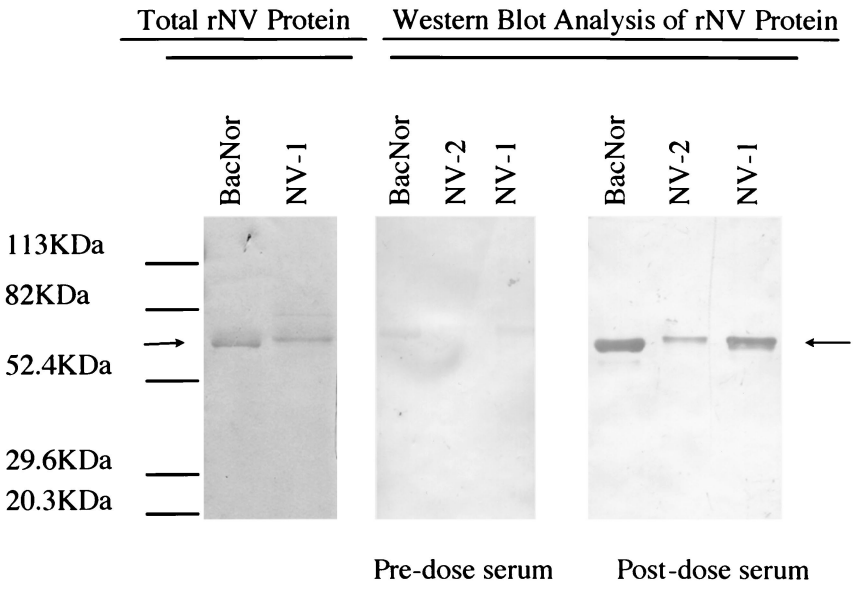

FIG. 4. VEE VRP expression of NV capsid proteins. BHK cells were infected with VRP-NV1 or VRP-NV2, and the cultures were harvested at 36 h postinfection. Cells were freeze-thawed three times, and the particles were purified by ultracentrifugation through 20% sucrose cushions or through 20 to 60% sucrose gradients. The purified particles were then pelleted by ultracentrifugation in an SW28 rotor. (A) Biochemical characterization of rNV capsid proteins synthesized in mammalian cells. Gradient-purified proteins were resuspended in Laemmli loading buffer, and 0.3 μg of purified protein was loaded per lane onto an SDS-10% polyacrylamide gel. After electrophoresis for 1 h at 180 V, the proteins were transferred to nitrocellulose, filters were blocked with 2% bovine serum albumin, and the immobilized proteins were probed following the standard Western blot protocol with a serum sample collected either predose or 21 days postdose from an NV-infected volunteer. Antibody-reactive protein was visualized with an alkaline phosphatase-conjugated anti-human immunoglobulin G secondary antibody (Sigma, St. Louis, Mo.) and 5-bromo-4-chloro-3-indolylphosphate (BCIP)-Nitro Blue Tetrazolium (NBT) substrate (Roche Molecular Biochemicals, Indianapolis, Ind.). The leftmost gel shows Coomassie blue staining of BAC-Nor and VRP-NV1 capsid proteins (Total rNV protein). Western blots using preimmune serum from a human volunteer prior to challenge with wild-type NV and postchallenge serum from the same human volunteer challenged with wild-type NV are shown. Arrows indicate the rNV proteins.

(SDS-PAGE) and Western blot analysis, the sizes of the BAC-Nor and NV1 capsid proteins were found to be identical and not caused by differences in proteolytic digestion (Fig. 5B). As the NV capsid protein contains putative N glycosylation, phosphorylation, and N myristoylation sites (Prosite analysis), we treated concentrated VEE-Nor with various phosphatases and endoglycosidases (Fig. 5C). In agreement with previous studies with the genogroup II Hawaii virus capsid proteins (38), the NV capsid proteins were not phosphorylated or glycosylated in mammalian cells. Under identical conditions, clear differences in the mobilities of the heavily glycosylated and phosphorylated ovalbumin protein were noted (Fig. 5C). To further examine whether the BAC-Nor and VEE-Nor capsid proteins were indistinguishable, gradient-purified proteins were treated as described in the legend to Fig. 5, subjected to exhaustive trypsin digestion, and analyzed by high-pressure liquid chromatography and mass spectrometry at the Protein Core Facility at the University of North Carolina (23). rNV protease cleavage patterns were identical whether expressed from baculoviruses or from VEE (data not shown). Consequently, both the recombinant BAC-Nor and VEE-Nor capsid particles are biochemically indistinguishable and likely represent authentic particles produced during NV infection in human cells.

By SDS-PAGE, BAC-Nor and VEE-Nor separate as major and minor protein doublets of slightly different molecular masses (Fig. 4 and 5). Following complete denaturation and alkylation of VEE-Nor and BAC-Nor particles, these proteins migrated to equivalent positions, suggesting that the observed differences were due to altered conformational structure, rather than from protein modifications or cleavage. In insect cells, BAC-Nor particles of 38 and 23 nm in diameter are often detected, both of which separated as a doublet containing the ~58-kDa capsid protein (25, 47). Similar particle variations have been reported with some but not all NLV capsids expressed in insect cells (13, 17, 20, 25). Different architectures of infectious bursal disease-causing VLPs have also been noted following expression of the identical capsid gene from different baculovirus expression vectors (32). Following VRP-NV1 infection in BHK cells, VEE-Nor particle size was uniform, with the smaller, 23-nm-diameter NV VLP below levels of detectability. Although speculative, these data suggest that folding pathways of the 58-kDa capsid protein may be influenced by the viral vector, capsid protein concentration, temperature of infection, or host cell phenotype. By using a vaccinia virus-based expression system, feline calicivirus capsid proteins were also shown to self-assemble into uniform, similar-sized particles of the appropriate size (19).

A current NLV vaccine approach, based on oral immunization with BAC-Nor or expression of BacNor in plants, has stimulated NV immune responses in experimental rodent models and in humans (2, 3, 33). The VEE system provides an alternative approach for recombinant VLP production and may be useful for a number of viruses, especially when inappropriate biochemical processing is demonstrated following

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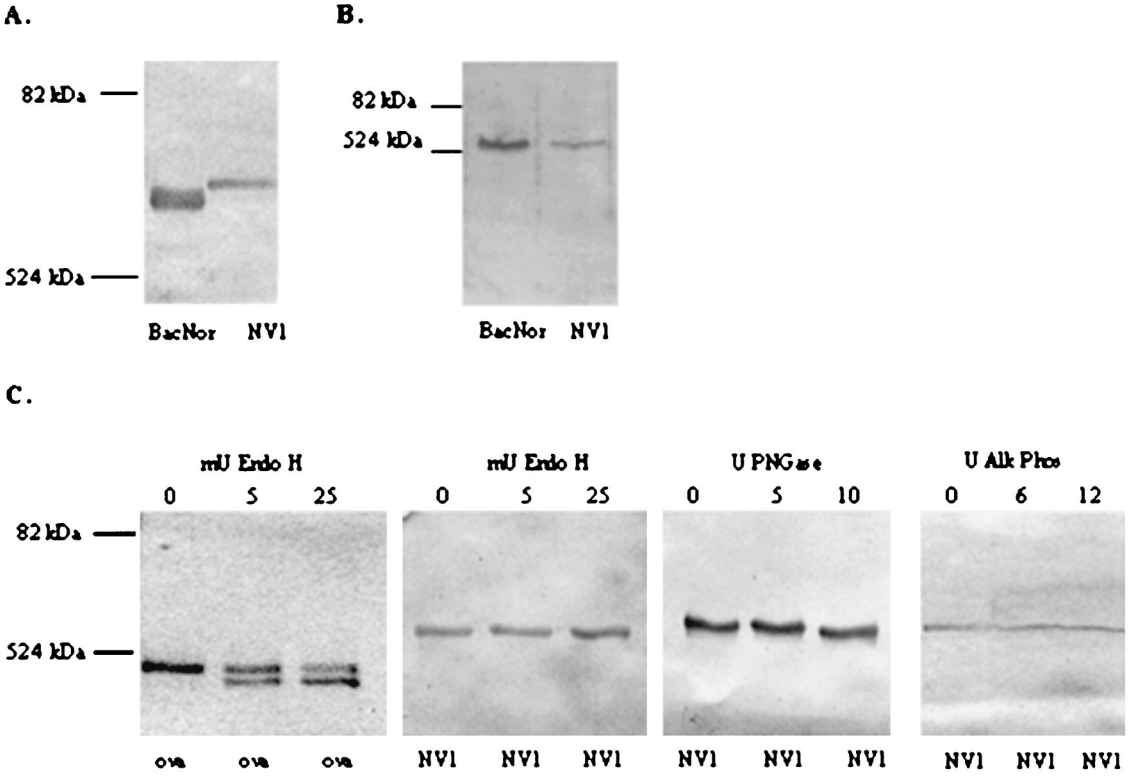

FIG. 5. Posttranslational processing of rNV capsid proteins synthesized in mammalian cells. By Western blot analysis, VEE-Nor displayed a higher apparent molecular mass than that of BAC-Nor (rNV) (A). To remove potential conformational structure, VEE-Nor and BAC-Nor particles were further treated with 5 M guanidine-HCl at room temperature for 5 min to denature the proteins, the pH was adjusted to pH 8.6 with 4.6 M Tris, and the proteins were reduced with 0.1 M dithiothreitol (Sigma) for 45 min in the dark at room temperature before being alkylated by 0.25 M iodoacetic acid (Calbiochem) for 30 min at room temperature (23). The reduced and alkylated capsids were then diluted and analyzed by SDS-PAGE (B). (C) VEE-Nor was incubated with increasing concentrations (shown above the lanes) of endo-β-*N*-acetylglucosaminidase H (Endo H) (Roche Biochemicals) and peptide-*N*-glycosidase (Calbiochem) as previously described in the literature (38). Alkaline phosphatase (Alk Phos) (Roche Biochemicals) digestions were performed for 90 min at 37°C in 0.2 M Tris, pH 9.8. After enzymatic treatment, untreated capsid proteins were added to the reaction mixtures and the reaction mixtures were immediately boiled in loading buffer and separated on an SDS-polyacrylamide gel. A positive reaction is indicated by a doublet in a lane. Enzyme activity was verified using ovalbumin (ova) (Sigma) with each reaction set (data shown for Endo H treatment).

capsid protein expression in insect cells (11). Compared with baculoviruses, the VEE system is somewhat more cumbersome, since cotransfections and BL-3 facilities are needed for VRP production. This could be easily circumvented using other alphavirus vectors, like Sindbis virus or Semliki forest virus (1, 30). VEE VRPs can be used directly as a recombinant virus vaccine vector, which theoretically would drive the production of high concentrations of NV-VLPs in vivo. Alternatively, VRPs could be used to infect cultures of cells to produce high concentrations of NV VLPs to be used separately or in combination with VEE VRP vaccination (Fig. 1B). Parenteral VEE and VEE VRP inoculation is known to target dendritic cells and stimulates potent humoral and cellular immunity that includes high neutralization titers and protection at mucosal surfaces from infection (4, 6–9, 19, 31, 41). The use of VEE vectors may also provide insights into NLV entry, assembly, structure, encapsidation, and release and allow reverse genetic approaches to recover infectious NLV particles from mammalian cells.

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