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Rotavirus VP6 Expressed by PVX Vectors in *Nicotiana benthamiana* Coats PVX Rods and Also Assembles into Viruslike Particles

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The rotavirus major inner capsid protein (VP6) has been expressed in *Nicotiana benthamiana* plants using vectors based on potato virus X (PVX). VP6 was expressed either as a fusion with the PVX coat protein or from an additional subgenomic promoter inserted to enable both VP6 and PVX coat protein to be expressed independently. Both approaches yielded VP6, which retained the ability to form trimers. VP6 expressed from the subgenomic promoter assembled into paracrystalline sheets and tubes. Expression as a fusion protein yielded PVX rods that presented an external "overcoat" of VP6, but unexpectedly, some rotavirus protein also assembled into icosahedral viruslike particles (VLPs). The assembly of viral protein into VLPs suggests that prior display of VP6 on the flexuous PVX rod facilitates the subsequent assembly of VP6 into stable icosahedral particles.

INTRODUCTION

Expression of protein in plants has been proposed as a novel way of generating inexpensive subunit vaccines (Mason and Arntzen, 1995; Johnson *et al.,* 1997). Three general approaches can be employed. First, transgenic plants can be engineered to express foreign proteins through the stable integration of a foreign gene into the plant chromosome, but protein yields achieved by this approach are generally low (Mason and Arntzen, 1995). Second, plant virus vectors can be used to achieve transient expression of foreign protein. This approach results in high yields of either fusion protein or free polypeptide: if a proteolytic cleavage site is included in the vector (Beachy *et al.,* 1996; Santa Cruz *et al.,* 1996), the fusion protein can be cleaved intracellularly to yield free polypeptides. The third approach is to insert appropriate epitopes into polypeptide loops of the coat protein, which are not involved in the subunit interactions required for the self-assembly of virus particles (Johnson *et al.,* 1997). All three approaches have been shown to yield protein able to elicit good immune responses, with protection demonstrated following subsequent challenge with parvovirus (Dalsgaard *et al.,* 1997), FMDV (Carrillo *et al.,* 1998), rabies (Modelska *et al.,* 1998), and cholera (Arakawa *et al.,* 1998).

Although soluble viral proteins presented with an appropriate adjuvant may prime an effective immune response, there is accumulating evidence that assembly of stable viruslike particles (VLPs) enhances the uptake of antigens by the mucosal immune system (reviewed by Sabbaj *et al.,* 1997). Viral proteins expressed in the VLP form are not only more immunogenic, but also more readily purified. Thus when expressing antigens in plants, there are substantial advantages in utilizing viruses such as Hepatitis B (Mason *et al.,* 1992;Thanavala *et al.,* 1995) or Norwalk virus (Mason *et al.,* 1996), for which the coat protein is known to self-assemble into stable VLPs.

Rotavirus is the major cause of viral gastroenteritis in children (Kapikian and Chanock, 1996). As is the case for Norwalk virus and Hepatitis B, rotavirus capsid proteins can also assemble into VLPs, but this requires coexpression of both VP2 and VP6 (Labbé *et al.,* 1991). Rotavirus VP6/VP2 particles are known to retain their physical integrity during passage through the gut (Crawford *et al.,* 1994). Moreover, it is known that secretory IgA antibodies targeted against VP6 can protect mice against infection and resolve chronic rotavirus infection (Burns *et al.,* 1996). These results suggest that the expression of VP6 or VP2/VP6 in plants might represent a feasible method for rotavirus vaccine development, provided that VP6 can be assembled efficiently into the VLP form.

Here we report that rotavirus VP6 can be expressed in plants using appropriate potato virus X (PVX) vectors to produce either free VP6 or a VP6-PVX coat protein fusion. When expressed as free protein from a subgenomic PVX coat protein promoter, VP6 assembles into paracrystal-

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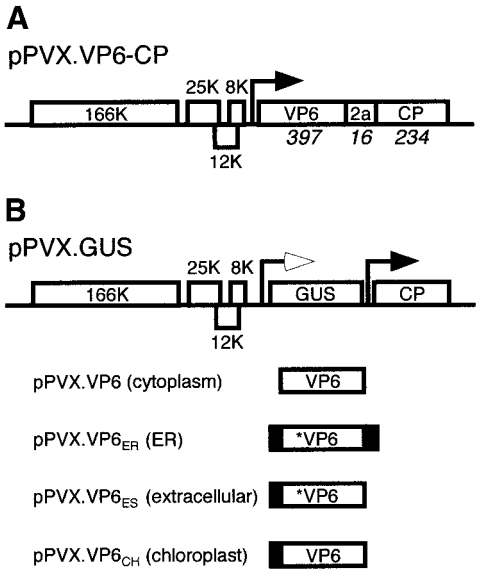

FIG. 1. PVX vectors used for VP6 expression. Boxes represent coding sequences (not to scale). (A) Vector incorporating VP6-PVX coat protein fusions based on pTXS.GFP-CP (Santa Cruz *et al.,* 1996) with VP6 replacing the green fluorescent protein (GFP) present in the original construct. Numbers in italics refer to the amino acid chain length (aa) of murine rotavirus VP6, the FMDV 2A catalytic peptide, and coat protein (CP), respectively. Closed arrow is the CP subgenomic promoter. (B) Vectors for the expression and targeting of free VP6. pPVX.GUS has the backbone of pTXS.GFP-CP with the GUS sequences from pGC3 (Chapman *et al.,* 1992). VP6 expression was achieved by replacing GUS with VP6 and the addition to VP6 of either no leader sequence (pPVX.VP6, targeted to the cytoplasm), the N-terminal signal sequence of the tobacco pathogenesis-related protein S gene (PR-S, 25 aa) and C-terminal KDEL (pPVX.VP6$_{ER}$, targeted to the ER), the N-terminal PR-S signal sequence only (pPVX.VP6$_{ES}$, targeted to the extracellular space), or N-terminal chloroplast transit peptide sequence from the *Malus domestica* small subunit RUBISCO gene (60 aa, pPVX.VP6$_{CH}$, targeted to the chloroplast). Transit/retention sequences are in heavy shading for each construct. *VP6 lacks the N-terminal methionine residue. Closed arrow, CP subgenomic promoter. Open arrow, duplicated CP subgenomic promoter. (See text for further details.)

line sheets. In contrast, expression of VP6 as a fusion with PVX coat protein yields flexuous rods containing a surface overcoat of VP6. Unexpectedly, expression as the fusion protein also yielded icosahedral viruslike particles, implying that presentation of VP6 on the flexuous PVX rod, followed by proteolytic cleavage, promotes its assembly into VLPs.

RESULTS

VP6 can be expressed in *N. benthamiana* using PVX

We first inoculated *N. benthamiana* with RNA transcripts synthesized from pPVX.VP6-CP (Fig. 1A), which resulted in systemic infection of the plants after 15–20 days postinoculation. RT-PCR of leaf samples following systemic infection showed that the VP6 insert is stable in the viral genome for at least 7–10 days following the first evidence of systemic infection (data not shown). Western analysis of leaf sap showed that VP6 is translated as a fusion product with the coat protein, producing a protein of about the expected size (71 kDa), which reacts with both anti-rotavirus and anti-PVX coat protein antibodies. The expected 46-kDa product (VP6.2A), resulting from self-cleavage of the 2A catalytic peptide sequence, was also detected using anti-rotavirus antibodies (Fig. 2A). The ratio of free protein (VP6–2A) to fusion protein (VP6–2A-CP), resulting from the autocatalytic cleavage at the 2A sequence, varied in different infections between 1:1 and 1:5.

The level of VP6 expressed in plant tissue by the PVX.VP6-CP fusion transcripts was determined by comparing the band intensity of plant-expressed VP6 with that of a known concentration of native, baculovirus-expressed VP6. Plants inoculated with transcripts derived from the fusion vector pPVX.VP6-CP produced about 1 μg of VP6 per gram of systemically infected tissue. SDS–PAGE analysis under nondenaturing conditions (37°C, 30 min) demonstrated that the cleaved VP6 product forms trimers (Fig. 2A, lane 2).

To achieve expression of free VP6, we utilized pPVX.VP6 (Fig. 1B), a recombinant PVX vector that was able to express β-glucuronidase (GUS) under the control of a duplicated PVX coat protein subgenomic promoter. Replacing the *uid*A gene (encoding GUS) with the murine VP6 gene yielded pPVX.VP6 (Fig. 1B). When transcripts from this vector were used to inoculate *N. benthamiana*, systemic infection was also achieved after 7–12 days postinoculation. Western analysis confirmed that free VP6 expressed from this vector bound anti-rotavirus antibodies and that trimers of VP6 were visible by SDS–PAGE under nondenaturing conditions (Fig. 2A, lane 3). The yield of VP6 achieved by this approach was much higher at approximately 50 μg/gm of fresh leaf tissue (see below).

VP6 expression is not enhanced by targeting the protein to intracellular compartments

In an attempt to boost VP6 yields, the protein was modified by the addition of N- and C-terminal targeting sequences to target VP6 (Fig. 1B) to the chloroplast (pPVX.VP6$_{CH}$, small subunit of ribulose bisphosphate carboxylase transit peptide sequence), the endoplasmic reticulum (ER) (pPVX.VP6$_{ER}$; PR-S signal sequence and KDEL), or to the extracellular space (pPVX.VP6$_{ES}$; PR-S signal sequence). Nucleotide sequence analysis of pPVX.VP6$_{ER}$, pPVX.VP6$_{E}$, and pPVX.VP6$_{CH}$ showed that one base change had been introduced during PCR amplification. This mutation was located in the PR-S signal sequence at nucleotide position 11 (A $\rightarrow$ T) and resulted in an amino acid change from Gln to Leu; this change was not considered significant because it was predicted to improve signal sequence function.

Infection of *N. benthamiana* plants with RNA tran-

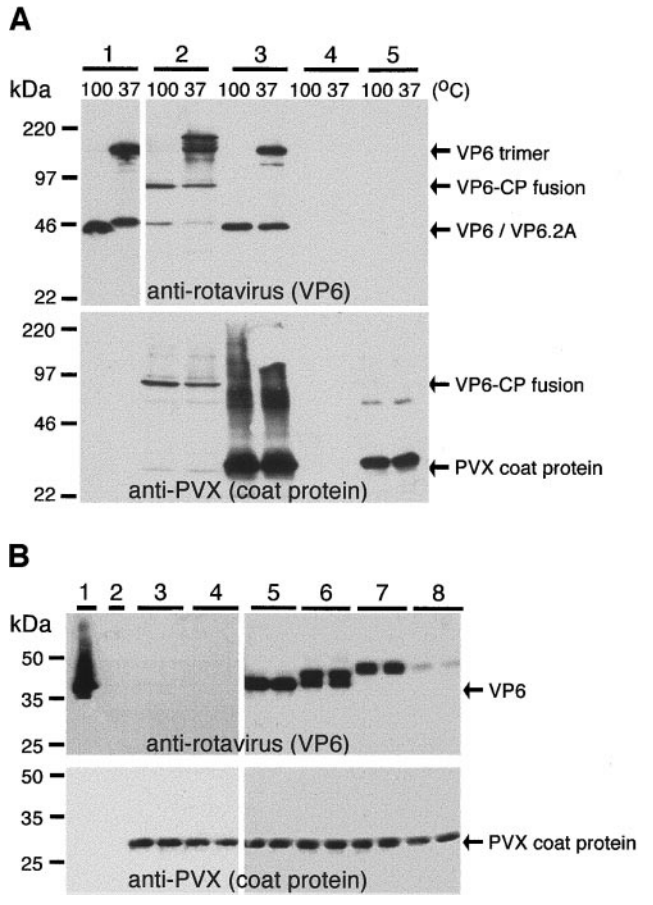

FIG. 2. VP6 is expressed in *N. benthamiana* infected with PVX transcripts. (A) Detection of VP6 and the VP6-CP fusion. Samples were prepared by heating aliquots of sap in Laemmli sample buffer for 5 min at 100°C (denaturing conditions) or 30 min at 37°C (nondenaturing conditions). Gel blots were probed with an anti-rotavirus rabbit polyclonal antibody (upper panel) or an anti-PVX rabbit polyclonal antibody (lower panel). The trimeric form of VP6 (stable at 37°C) binds more antibody than does the monomer. Native VP6 (control) expressed in *Spodoptera* cells using baculovirus (lanes marked 1); pPVX.VP6-CP (VP6-coat protein fusion, lanes marked 2), pPVX.VP6 (free VP6, lanes marked 3), mock-infected (lanes marked 4), PVX UK3 transcripts (wild type, lanes marked 5). Arrows indicate the anticipated locations of VP6, trimeric VP6, PVX coat protein, and VP6-PVX coat protein fusion. (B) Proteins from leaves infected with targeted VP6 constructs. Double lanes represent duplicate plants for each construct. All samples were denatured at 100°C. Blots were first probed with anti-rotavirus polyclonal antiserum (upper panels), then stripped and reprobed with an anti-PVX antiserum (lower panels). VP6 expressed in *Spodoptera* cells using baculovirus (lane 1), mock infected (lane 2), PVX UK3 (wild type, lanes marked 3), pPVX.GUS (lanes marked 4), pPVX.VP6 (free VP6, cytoplasm, lanes marked 5), pPVX.VP6$_{CH}$ (chloroplast, lanes marked 6), pPVX.VP6$_{ER}$ (ER, lanes marked 7), and pPVX.VP6$_{ES}$ (extracellular space, lanes marked 8). The predicted M_r of PR-S-VP6-KDEL (lanes 7), PR-S-VP6 (lanes, 8) and ssuRUBISCO-VP6 (lanes 6) are 49 kDa (425 aa), 48 kDa (421 aa), and 52 kDa (457aa), respectively. The predicted M_r of VP6-KDEL and VP6, the cleavage products derived, respectively, from pPVX.VP6$_{ER}$ and pPVX.VP6$_{ES}$ and pPVX.VP6$_{CH}$ expressed in plants are 45.5, 44.5, and 45 kDa, respectively.

scripts derived from pPVX.VP6$_{ER}$, pPVX.VP6$_{ES}$, and pPVX.VP6$_{CH}$ resulted in visible systemic infection after 12 days, with VP6 expression achieved by all three recombinant viruses (Fig. 2B). Most of the VP6 protein derived from pPVX.VP6$_{CH}$ (with the chloroplast transit peptide sequence), pPVX.VP6$_{ER}$, and pPVX.VP6$_{ES}$ (both with the PR-S sequence) had reduced gel mobility compared with that of native VP6, presumably as a result of incorrect posttranslational processing. The pPVX.VP6$_{CH}$ protein samples (Fig. 2B, lane 6) contained two bands that were consistent with the predicted sizes for VP6 (45 kDa) and the ssu-RUBISCO transit peptide fused to VP6 (52 kDa), indicating that a large portion of the protein was probably not correctly processed. For pPVX.VP6$_{ER}$ (lane 7) and pPVX.VP6$_{ES}$ (lane 8), plant extracts contained a single band that migrated at a higher molecular weight than VP6, indicating that either none of the PR-S signal peptide fused to VP6 is removed or that the protein is glycosylated. All modified forms of VP6 yielded protein able to trimerize under nondenaturing conditions, suggesting that the presence of additional terminal sequences did not interfere with protein folding and assembly (data not shown).

Western blot analysis showed that all the infected plants contained a similar amount of PVX coat protein (Fig. 2B, lower panel), indicating that all the recombinant viruses produced a similar amount of viral protein in infected plants. The amount of VP6 present in *N. benthamiana* plants inoculated with transcripts from pPVX.VP6 was the highest, at approximately 50 μg of VP6 per gram of fresh tissue. Whether the addition of signal sequences achieved the localization desired is unclear, but none of the engineered transcripts improved yields of VP6.

The failure to detect increased protein levels resulting from the targeted expression of VP6 to subcellular compartments could be because of differences in mRNA stability. Levels of PVX genomic RNA in the infected plant were therefore assessed by Northern analysis. All plants, regardless of the transcript used for inoculation, contained full-length viral genomic RNA transcripts in similar amounts, but with minor differences. Genomic PVX RNA, extracted from plants systemically infected with transcripts from pPVX.VP6, appeared to be slightly more abundant than any of the other VP6-containing constructs (Fig. 3, lane 5). Thus, although all constructs yielded satisfactory levels of genomic RNA in the infected cell, none achieved yields of VP6 geater than that achieved by VP6 expression in the cytoplasm.

VP6 expressed in plants forms paracrystalline sheets and tubes

Since VP6 expressed in plant cells assembles into trimers, we examined in the electron microscope sap derived from leaves infected with transcripts from pPVX.VP6, the infectious viral RNA that provided the highest yield of the unmodified form of the protein. A range of tubular and sheet forms of VP6 was observed

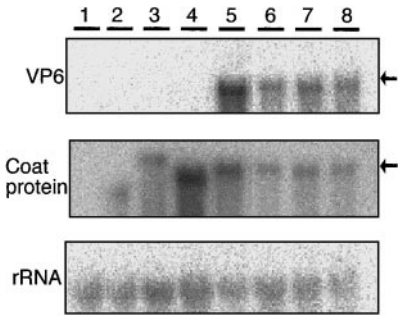

FIG. 3. PVX vectors incorporating modified VP6 genes yield similar levels of genomic RNA in *N. benthamiana* leaves. Total RNA extracted from systemically infected *N. benthamiana* leaves was separated on a 1% agarose gel by electrophoresis and subjected to Northern analysis. ^{32}P-labeled VP6 gene (upper panel), a ^{32}P-labeled PVX coat protein gene (middle panel), or a ^{32}P-labeled wheat 26s rRNA gene (lower panel) were used as probes. The same membrane was used but the probe was stripped after each exposure. Mock inoculation (lane 1), PVX UK3 (lane 2), pPVX.GUS (lane 3), pPVX.GFP-CP (lane 4), pPVX.VP6 (cytoplasm, lane 5), pPVX.VP6$_{CH}$ (chloroplast, lane 6), pPVX.VP6$_{ER}$ (ER, lane 7), pPVX.VP6$_{ES}$ (extracellular space, lane 8). Arrow: 7-kb marker.

which bound to anti-VP6 mouse monoclonal antibody (Fig. 4A). The array periodicity is also characteristic of VP6 expressed in insect cells (Estes *et al.,* 1987) or self-assembled from viral protein (Ready and Sabara, 1987). Extracts of VP6 from infected leaves were then mixed with VP2 particles purified from *Spodoptera* cells expressing the rotavirus VP2 gene under the control of the baculovirus polyhedral promoter (Fig. 4B). The VP6 of plant origin assembled onto the VP2 particles and could be identified as the protein coating the underlying VP2 scaffold when appropriate gold-labeled antibodies were applied to the grid. Thus, we conclude that the VP6 protein expressed in plants is able to assemble into authentic icosahedral structures, provided the appropriate VP2 scaffold is present. It is also clear that any posttranslational modifications introduced into VP6 in the plant have not affected the ability of the protein to assemble into VLPs.

Expression of the fusion protein yields both coated PVX rods and VLPs composed of VP6

We next examined by electron microscopy the flexuous PVX rods from plants infected with transcripts synthesized from pPVX.VP6-CP. This construct incorporates the FMDV 2A catalytic peptide sequence located between the VP6 and the PVX coat protein sequences (Figs 1A). Immunoelectron microscopy revealed that the flexuous PVX rods were coated with VP6 throughout their length (Fig. 5), with the surface display of VP6 clearly detectable by the immunogold-labeled antibody. As anticipated, no VP6 was evident on the wild-type PVX rods and the VP6-coated rods were clearly distinguishable when extracts containing the recombinant and wild-type rods were mixed (Fig. 5, lower two panels). We conclude that the fusion protein assembles to coat the viral RNA in such a manner that rotavirus VP6 is accessible on the surface, as has been described previously for fusions

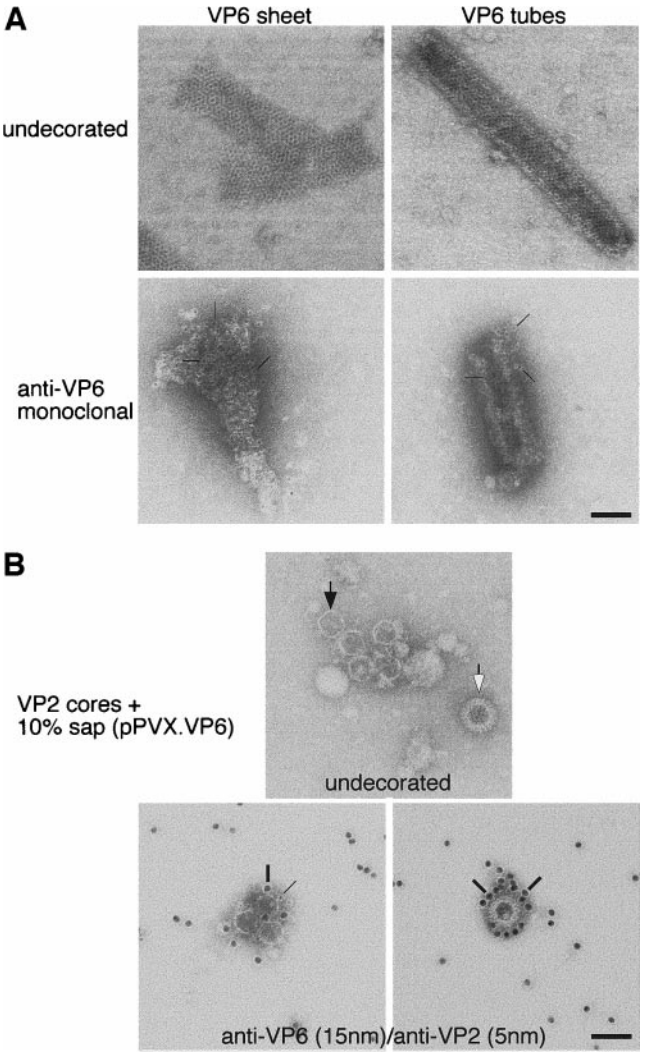

FIG. 4. Murine VP6 expressed in *N. benthamiana* using pPVX.VP6 forms paracrystalline arrays and can assemble on a VP2 scaffold. (A) Paracrystalline arrays and tubes formed from VP6 trimers observed in 10% sap samples. Carbon-coated grids were floated on 10% sap and stained with 2% uranyl acetate. Decoration with anti-mouse gold-IgM (5-nm particles, indicated by thin lines) was performed after applying anti-VP6 monoclonal antibody. (B) Assembly of VP6 as a capsid layer around VP2 cores. VP2 cores were purified from insect cells infected with recombinant baculovirus. Cores were mixed with 10% sap from systemically infected leaves (derived from plants inoculated with pPVX.VP6 transcripts) and incubated overnight at 4°C and then at 37°C for 2 h. Specimens were applied to grids coated with anti-rotavirus polyclonal antibody and stained with 2% uranyl acetate. An assembled VP2/VP6 particle is indicated in the top panel by an open arrow and a VP2 core is indicated by a closed arrow). For gold decoration, samples were applied to antibody-coated grids and then floated on an anti-VP2 (mouse) monoclonal/anti-rotavirus (VP6) polyclonal (rabbit) antibody mix. Samples were then decorated with a mixture of anti-rabbit IgG (15-nm particles, indicated by thick lines) + anti-mouse IgM (5-nm particles, indicated by thin lines) and then stained with 2% uranyl acetate. Scale bar = 100 nm.

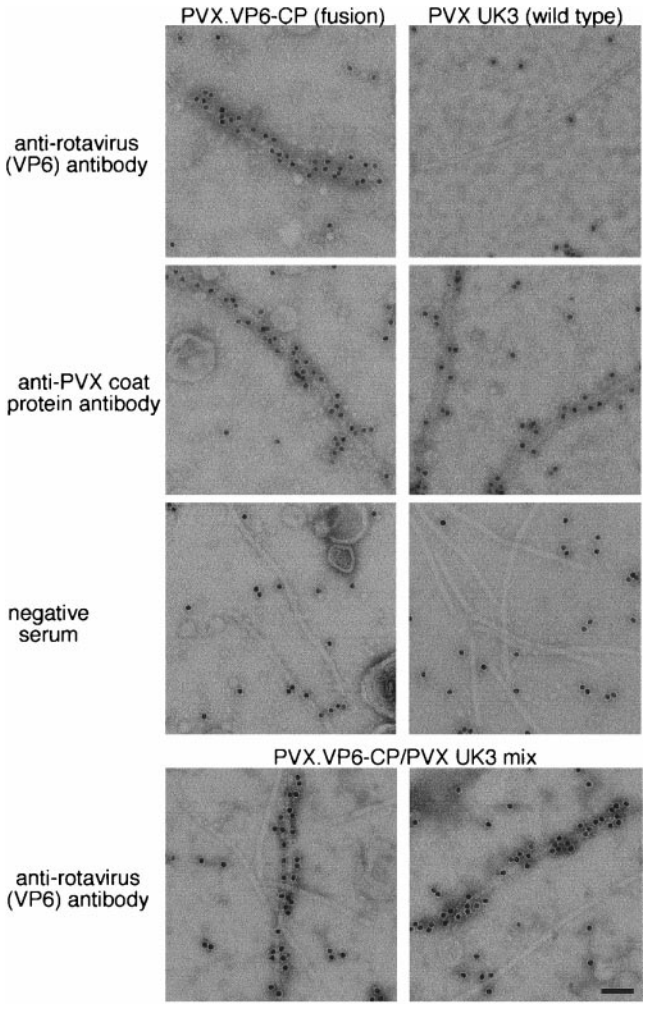

FIG. 5. VP6 is presented on the surface of PVX rods. Purified PVX.VP6-CP virus (see text) was harvested from CsCl gradients, diluted, and applied to carbon-coated grids by flotation. Antibody (as indicated) was applied to the sample by flotation and finally anti-rabbit gold-labeled IgG (ZYMED, 15 nm) was added. The grids were then stained with 2% uranyl acetate. The average number of gold beads per viral rod was determined for each sample (n = 20): PVX.VP6-CP + anti-rotavirus antibody = 25; PVX UK3 + anti-rotavirus antibody = 1; PVX.VP6-CP + anti-PVX coat protein antibody = 23; PVX UK3 + anti-PVX coat protein antibody = 17; PVX.VP6-CP + negative serum = 2; PVX UK3 + negative serum = <1. Scale bar = 100 nm.

incorporating the green fluorescent protein (Santa Cruz *et al.,* 1996).

The 2A catalytic peptide sequence separates the PVX and VP6 protein sequences in the fusion so that free VP6–2A is released by cleavage and is detectable in extracts (Fig. 2A, lane 2). We therefore searched for evidence that this free VP6–2A might assemble into structures such as the tubes and sheets observed in Fig. 4, but no paracrystalline tubes or sheets were observed. However, unexpectedly, VLPs were observed (Fig. 6) and these particles are similar in morphology to those produced when both VP6 and VP2 are expressed in insect cells by dual infection of cells with the appropriate recombinant baculoviruses (compare upper panels of Fig. 6 with lower panels). Immunogold-labeling revealed that the particles produced in plants are indeed icosahedral particles assembled from VP6 protein, thus eliminating the possibility that contamination with some other icosahedral plant virus had occurred in our experiments. The VP6 particles were present at a lower concentration than the recombinant PVX rods with a rod:particle ratio of the order of 50:1. Given that the ratio of fusion protein to VP6 is approximately 5:1 (Fig. 2A) and the VP6-coated rods are approximately 500 nm in length (Fig. 5), the number of icosahedral VP6 shells is about that predicted if most of the free VP6–2A was, in fact, present in VLP (icosahedral) form. VP6–2A assembled as VLPs would subsequently dissociate into trimers when analyzed on SDS–PAGE under nondenaturing conditions (Fig. 2).

DISCUSSION

The use of plants as bioreactors for the synthesis of proteins appears to have the potential to make an important contribution to reducing the cost of vaccine production and delivery. Indeed, recombinant plant virus particles delivered by the oral (Modelska *et al.,* 1998) or intranasal route (Brennan *et al.,* 1999; Durrani *et al.,* 1998) have been shown to be protective in animal models.

The ability of plant-expressed capsid protein to as-

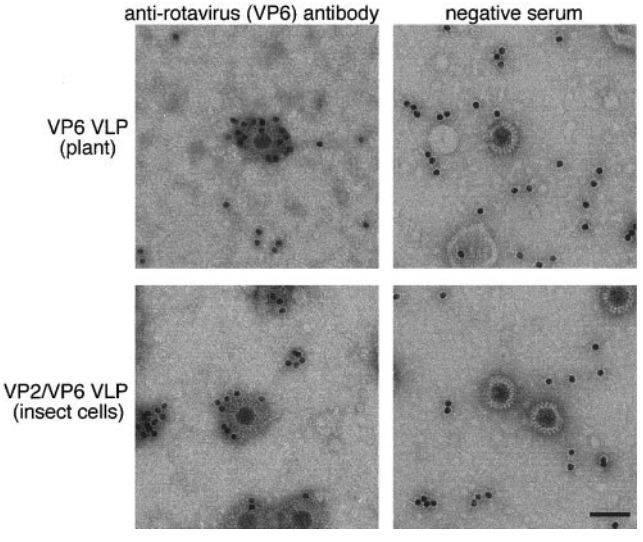

FIG. 6. Viruslike particles composed of VP6 are assembled in plants. Plant VLPs were purified along with recombinant PVX (PVX.VP6-CP) virus preparations. Control samples of VP2/VP6 VLPs were made by baculovirus expression in insect cells. Both samples were diluted and applied to carbon-coated grids by flotation. Samples were coated with anti-rotavirus polyclonal antibody (rabbit) and decorated with anti-rabbit gold-IgG (15 nm), followed by staining with 2% uranyl acetate. The average number of gold beads per viruslike particle was determined for each sample (n = 20): Plant icosahedral particle + anti-rotavirus antibody = 11; Plant icosahedral particle + negative serum = <1; VLP (insect cells) + anti-rotavirus antibody = 6; VLP (insect cells) + negative serum = <1. Scale bar = 100 nm.

semble into VLPs has been demonstrated for the Hepatitis B surface antigen (Mason *et al.,* 1992) and Norwalk virus (Mason *et al.,* 1996). The ability of rotavirus VP6 to self-assemble around a VP2 core is well known from prior work with baculovirus-expressed protein (Crawford *et al.,* 1994). Here we have used PVX-based vectors (Chapman *et al.,* 1992; Santa Cruz *et al.,* 1996) and shown that plant-expressed VP6 not only retains the ability to form trimers and paracrystalline arrays and tubes, but also that it assembles around a VP2 core. All of these forms of VP6 retain the ability to be recognized by anti-rotavirus polyclonal antibodies, confirming that the plant-expressed VP6 is comparable to the VP6 obtained by either insect cell expression (Estes *et al.,* 1987; Crawford *et al.,* 1994) or purification from the virus (Ready and Sabara, 1987).

When expressed as a fusion with the PVX coat protein, VP6 is incorporated into PVX rods. VP6 is displayed on the external surface of the rod and is able to be recognized by anti-rotavirus antibodies. The trimers observed in the PVX.VP6-CP samples (Fig. 2A, lane 2, 37°C) are inferred to form from the VP6–2A cleavage product, because the trimeric molecular weight (~130 kDa) approaches that predicted from the known molecular weight (46 kDa) of the monomer. The ability of VP6 with added N- and C-terminal sequences to trimerize and form paracrystalline arrays has previously been reported (Reddy *et al.,* 1992) and thus the 2A sequence was not expected to inhibit trimerization. However, the assembly of icosahedral rotavirus VLPs was not anticipated, given that previous expression methods employed have yielded paracrystalline tubes and sheets (Estes *et al.,* 1987, Crawford *et al.,* 1994). To our knowledge this is the first report of the intracellular self-assembly of VP6 into icosahedral particles. Ready and Sabara (1987) showed that purified VP6 could self-assemble into particles at pH 4.0, but these structures reverted to tubes when dialyzed to pH 6.0. Since cleavage of the 2A catalytic peptide is cotranslational (Ryan *et al.,* 1994; Halpin *et al.,* 1999), it is reasonable to infer that at least some of the material assembling into VLPs would have been cleaved to yield VP6 accompanied by the C-terminal catalytic peptide. However uncleaved VP6–2A-CP fusion protein would also be available for assembly, leading to the possibility that the PVX sequences rather than those of the 2A catalytic peptide could be acting to nucleate assembly of the VLP.

Expressed proteins can be targeted to specific subcellular organelles using localization sequences (Van den Broeck *et al.,* 1985; Sijmons *et al.,* 1990; Boevink *et al.,* 1996; Bosch *et al.,* 1996) and in some instances high-level expression has been achieved (McBride *et al.,* 1994). A PVX vector has also been developed for the targeting of expressed proteins to the ER of plant cells (Boevink *et al.,* 1996). The protein profile of plant sap infected with transcripts from pPVX.VP6$_{CH}$ showed an additional VP6 band with a higher-than-expected molecular weight (Fig. 2B, lane 6). It is proposed that this band represents VP6, in which the transit peptide has not been cleaved, as has been found in other studies (Wu *et al.,* 1993). The cleavage and targeting of proteins has also been hypothesized to be impaired if the transit peptide is unable to unfold the protein when crossing membranes (Eilers and Schatz, 1986), so the stability of VP6 may have inhibited unfolding and therefore targeting. Preliminary evidence did show that some VP6 was targeted to the chloroplast (data not shown). No evidence of VP6 being targeted to the ER or extracellular space was obtained. The higher molecular weights of the VP6 bands from the sap of plants infected with pPVX.VP6$_{ER}$ and pPVX.VP6$_{ES}$ transcripts (Fig. 2B, lanes 7 and 8) indicate that the VP6 was not processed and so retained the additional N-terminal PR-S sequence. Alternatively there is a possibility that passage of VP6 through the plant cell ER resulted in glycosylation and hence yielded an increased molecular weight. Glycosylation of VP6 resulting in decreased mobility in SDS–PAGE has previously been observed when VP6 passed through the ER of mammalian cells (Reddy *et al.,* 1992).

The yield of VP6 from constructs targeting VP6 expression to subcellular organelles was approximately 15-fold less than that observed for expression using pPVX.VP6 (data not shown). The levels of PVX genomic RNA (Fig. 3) for these constructs did not show more than a fivefold difference when normalized to rRNA loadings, and the levels of PVX coat protein (Fig. 2B) were comparable, indicating that the differences in expression levels probably did not result from a reduction in viral replication. Overall, these results show that, in our hands, maximal expression of VP6 was achieved in the cytoplasm of plant cells.

The rotavirus capsid protein VP6 is a potentially useful antigen because of its inherent stability, ability to self-assemble into VLPs, and the established protective potential of both rotavirus VLPs (O'Neal *et al.,* 1997) and anti-VP6 antibodies (Burns *et al.,* 1996). The ability of plant-expressed VP6 to self-assemble into a VLP structure would potentially enable this protein to be assembled into a VLP without the need for coexpression of a VP2 scaffold. Further refinements of vector construction and cleavage mechanisms would increase the usefulness of PVX as a vector for the expression of VP6 and might improve yields and the efficiency of assembly of VLPs in the plant cell.

MATERIALS AND METHODS

Construction of PVX vectors encoding VP6

The green fluorescent protein (GFP)-coat protein (CP) fusion vector pTXS.GFP-CP, which encodes a *gfp-2a-cp* gene fusion under the transcriptional control of a subgenomic (sg) CP promoter (Santa Cruz *et al.,* 1996), was

TABLE 1

Primers Used to Construct Recombinant PVX Vectors and Monitor Their Stability

Construct/method	Localisation/purpose	Forward primer (5′-3′)	Reverse primer (5′-3′)
pCH231	*in vitro* mutagenesis MORPH primer cv33	GTCAGCACCA*GCTAG***C**GGATCC CCGGGTGG Restriction sites: *Nhe*I (in italic) and *Bam*HI; **C**: nucleotide changed to create a *Nhe*I site	
pPVX.VP6	Cytoplasm	GGGGAGCTCATGGATGTGCTGTA CTCTATC	CAGTATCGATGTCGACTCACTTT ACCAGCATGCTTC
pPVX.VP6$_{ER(int)}$	ER (**KDEL**)	GGGTCGCGATGTGCTGTACTCTATCTC	CAGTATCGATGTCGACTCA**AAG CTCATCTTT**CTTTACCAGCATG CTTCTAATG
pPVX.VP6$_{ER}$	ER (PR-S)	ATGAACTTCCTCAAAAGCTTCCCC TTTTATGCCTTCCTTTGTTTTGG CCAATACTTTGTAGCTGTTACTC ATGC	GCATGAGTAACAGCTACAAAGT ATTGGCCAAAACAAAGGAAGGC ATAAAAGGGGAAGCTTTTGAGG AAGTTCAT
pPVX.VP6$_{CH(int)}$	Chloroplast	GGGCAGTATGCATGGATGTGCTG TACTCTATC	CAGTATCGATGTCGACTCACTTT ACCAGCATGCTTC
pPVX.VP6$_{CH}$	Chloroplast (RUBISCO transit peptide)	GGGATGGCTTCCTCCATGATTTC	GTCAATGCATTGCACTCTTCCTCC
pTXS.VP6-CP	VP6-2A-CP fusion	GCACCAGCTAGCATCGATCCGGC CGATGGATGTGCTGTACTCTATC	CGCAAGCTTAAGAAGGTCAAAAT TCTTTACCAGCATGCTTCTAAT
RT-PCR	Virus stability	ACCTTGTGTCATCAAGATTAC	TCATGTCCTTCCAAATAGCCT

modified by replacing GFP with VP6. Oligonucleotide primers (see Table 1) were generated to amplify the murine EDIM rotavirus VP6 gene from pEWVP6 (Tang *et al.,* 1997), while incorporating compatible restriction sites for cloning into pTXS.GFP-CP. The 1.3-kb PCR fragment was cloned into pGEM-T (Promega) to form pGOBVP6 and sequenced. Insertion of VP6 into the PVX vector was achieved following digestion of pGOBVP6 and pTXS.GFP-CP with *Afl*II and *Nhe*I and ligation. The resulting clone, pTXS-VP6.CP, contained the *vp6–2a-cp* fusion gene under the transcriptional control of the duplicated sg RNA promoter in pTXS.

A PVX vector was constructed to direct the synthesis of free VP6, rather than VP6-PVX coat protein fusions. As a first step, a PVX-GUS vector was derived with a single ATG after the duplicated subgenomic promoter, as follows. The duplicated promoter of pGC3 (pPVX.GUS) (Chapman *et al.,* 1992) was combined with the PVX UK3 backbone of pTXS.GFP-CP (pPVX.GFP-CP) (Santa Cruz *et al.,* 1996). The *Apa*I/*Spe*I fragment of pGC3 encompassing the *uid*A gene was subcloned into pBC KS- to give pCH213. The poly(A) sequence of pCH213 was then eliminated by *Xho*I/*Sst*II digestion, end-filling the fragment using Klenow, and then religation to re-create the *Xho*I site, to generate pCH171. A novel *Nhe*I site was introduced into pCH171 upstream of the *uid*A gene by *in vitro* mutagenesis (primer pCH231, Table 1), resulting in the clone pCH231. Digestion of pCH231 and pCH153 (pTXS.GFP-CP with mGFP5 inserted in the place of GFP) with *Nhe*I/*Xho*I followed by ligation resulted in the clone pPVX.GUS, in which *uid*A with a duplicated subgenomic promoter replaced mGFP5 in pCH153.

Vectors for subcellular targeting of VP6 expression were generated by replacing GUS in pPVX.GUS with PCR-derived VP6 fragments. Oligonucleotide primers were designed to be complementary to the 5′ and 3′ ends of murine VP6 and to include a 5′ *Sma*I recognition sequence and 3′ *Cla*I site for cloning (see Table 1). Three different primer pairs with additional sites or signals were used in separate PCR reactions on pEWVP6, giving rise to pPVX.VP6, pPVX.VP6$_{CH(int)}$ and pPVX.VP6$_{ER(int)}$. After purification, the PCR fragments were blunt-ended (1 unit T4 DNA polymerase, 2 units Klenow fragment, 2 units T4 polynucleotide kinase, 1 mM dNTP T4 DNA Ligase buffer [Life Technologies]) at 37°C for 30 min, cut with *Cla*I and ligated to *Sma*I/*Cla*I digested pPVX.GUS. *E. coli* STBL2 cells were used for transformation. These steps yielded a clone for cytoplasmic expression of VP6 (pPVX.VP6) and intermediate clones for chloroplast targeting (pPVX.VP6$_{CH(int)}$) and for endoplasmic reticulum targeting (pPVX.VP6$_{ER(int)}$). Further modification of pPVX.VP6$_{ER(int)}$ was performed by generating a DNA fragment through the annealing of two complementary oligonucleotides encoding the *N. tabacum* pathogenesis-related protein S (PR-S) sequence (Table 1), and ligating the fragment to *Nru*I-digested pPVX.VP6$_{ER(int)}$, forming pPVX.VP6$_{ER}$. For directing VP6 expression to the chloroplast, the ssu-RUBISCO peptide encoding sequence was amplified

from *Malus domestica* DNA using the primer pair (Table 1). The 180-bp fragment was blunt-ended and mixed with *Sma*I-digested pPVX.VP6$_{CH(int)}$; the DNA mix was digested with *Nsi*I and then ligated. The resulting clone was designated pPVX.VP6$_{CH}$. Both pPVX.VP6 and pPVX.VP6$_{ER}$ were used to construct pPVX.VP6$_{ES}$, the vector for targeting VP6 expression to the extracellular space. A 2-kb fragment from *Apa*I/*Mlu*I-digested pPVX.VP6$_{ER,}$ containing the duplicated promoter, PR-S sequence, and 5′ end of VP6, was gel-purified and ligated to *Apa*I/*Mlu*I-digested pPVX.VP6, to produce pPVX.VP6$_{ES}$.

In vitro transcription and plant inoculation

*Spe*I-linearized DNA of PVX constructs was used as a template for *in vitro* RNA transcription as described by Beck *et al.* (1990), except that T7 polymerase was used instead of SP6 RNA polymerase. The *in vitro* transcripts were mixed with an equal volume of FES buffer (1% celite, 1% bentonite, 1% tetrasodium pyrophosphate, pH 8) immediately prior to inoculation. Three- to 4-week-old *N. benthamiana* plants, which had been subjected to a 24-h dark period, were dusted with Carborundum powder and manually inoculated with transcripts (2 leaves per plant, 10 μL per plant). Plants were grown at 22°C with 16-h light and 8-h dark.

PVX purification

Fresh or stored (−80°C) plant tissue, systemically infected with recombinant PVX, was homogenized in buffer (200 mM Na_2HPO_4, 10 mM EDTA, 0.15% β-mercaptoethanol, pH 7.2; 10–15% plant fresh weight:buffer volume) using a blender. The homogenate was filtered through muslin cloth and then through a Whatman 3-mm paper disc under vacuum. Filtrate aliquots were clarified by one passage through 2- to 3-cm-thick celite pads under vacuum so that the solution was a pale yellow color. Prior to filtration, the celite was suspended in buffer (200 mM Na_2HPO_4, 10 mM EDTA, 1% BSA) overnight and washed three times in buffer without BSA prior to use. PVX rods in the clarified filtrate were pelleted by ultracentrifugation through a 1 mL 10% sucrose cushion using a Beckman SW28 rotor (27,000 rpm, 3 h, 4°C). The pellet was suspended in 10 mM Tris–HCl (pH 7.5) and the virus banded on a preformed CsCl gradient (ρ = 1.1–1.4 g/cm^3 in 10 mM Tris–HCl, pH 7.5) using a Beckman SW40Ti rotor (36,000 rpm, 1.5 h, 4°C). The broad diffuse band of virus that formed in the center of the tube was harvested and CsCl removed by dialysis.

Wild-type PVX UK3 was purified using the method of Dougherty and Hiebert (1980), with the following modification. After the first PEG precipitation (4% PEG, 100 mM NaCl), the virus pellet was resuspended and concentrated by ultracentrifugation over a 35% sucrose cushion using a Beckman SW28 rotor (25,000 rpm, 3 h, 4°C).

SDS–PAGE and Western analysis

The protein profiles of crude 10% sap extracts, purified PVX, or purified rotavirus VLPs were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970) using a 10% resolving and 4% stacking gel, with samples incubated at 100°C for 5 min in Laemmli loading buffer prior to loading. Nondenatured VP6 was visualized using SDS–PAGE by incubating the samples at 37°C for 30 min in the presence of 10% β-mercaptoethanol (Ready and Sabara, 1987). The separated proteins were transferred onto PROTRAN nitrocellulose transfer membrane (Schleicher and Schuell) using the Electro-transfer unit (BioRad). Western analysis was performed using a 1/1500 dilution of rabbit polyclonal anti-rotavirus antibody (this laboratory) or a 1/10,000 dilution of rabbit anti-PVX CP antibody (R. Forster, Hort Research), followed by a 1/2000 dilution of biotinylated donkey anti-rabbit Ig antibody (Amersham Pharmacia Biotech) and a 1/2000 dilution of streptavidin-biotinylated horseradish peroxidase complex (Amersham) using the protocol recommended for the ECL Western blotting analysis system (Amersham). Protein bands were detected using the ECL detection reagents and autoradiography performed using Hyperfilm (Amersham).

The amount of VP6 present in two *N. benthamiana* plants inoculated with viral transcripts was quantified following Western analysis. The density of protein bands from 10% sap (10 μL) and a dilution series of baculovirus-expressed VP6 (25, 50, 100, 150, and 200 ng) was determined using the Luminescent image analyzer LAS-100. Data were plotted as a standard curve and interpolated to determine the amount of VP6 produced in the plants.

RNA extraction and RT-PCR

Total RNA was extracted from plant tissue or purified virus using TRIzol Reagent (Life Technologies) and the RNA precipitated with isopropanol. RT-PCR was performed on 1 μg of total RNA using Ready-To-Go Beads (Pharmacia Biotech) and oligo-(dT) primer (Life Technologies) to synthesize the first strand at 37°C for 1 h. Double-stranded DNA was synthesized by PCR.

To extract RNA for Northern analysis, frozen plant leaf tissue was ground and resuspended in 500 μL RNA extraction buffer (80 mM Tris–HCl, pH 8, 150 mM LiCl, 5 mM EDTA, 5% SDS). After extracting the sample twice with 500 μL phenol/choroform, the aqueous phase was mixed with 50 μL 3 M sodium acetate (pH 5) and 500 μL isopropanol and precipitated at −20°C. After centrifugation the RNA pellet was washed with 70% ethanol, air dried, and resuspended in 50 μL DMDC-treated milliQ water.

Northern blot analysis

RNA samples (5 μg) were separated by electrophoresis using a 1% agarose, glyoxyl/DMSO gel (Sambrook *et al.,* 1989) and the RNA transferred to a nylon membrane (Boehringer Mannheim) using 0.05 M NaOH for 4–6 h. After rinsing in 2× SSC (0.3 M NaCl, 0.03 M tri-sodium citrate, pH 7) the membrane was air-dried overnight and the nucleic acid fixed by UV cross-linking (1200 mJ) using the Stratalinker 2400 (Stratagene).

Probes were prepared from DNA fragments isolated from agarose gels and labeled with [α-^{32}P]dCTP using the Rediprime II Random Prime Labeling System (Amersham) following the manufacturer's instructions. Unincorporated nucleotides were removed using the ProbeQuant G-50 micro column. A wheat 26s ribosomal RNA probe was used to estimate RNA loading for each sample.

Blots were prehybridized for 1 h at 65°C in hybridization buffer (50 mM PIPES, pH 6.5, 100 mM NaCl, 50 mM sodium phosphate, 1 mM EDTA, 5% SDS) with 200 μL of 10 gL^{-1} denatured salmon sperm. Radioactive DNA probe was denatured by boiling, chilled on ice, and added to the membrane in 5 mL of fresh hybridization buffer. After hybridization at 65°C overnight, the membrane was washed twice in 0.5× SSC/0.1% SDS, sealed in a plastic bag, and exposed to Kodak X-Omat film using intensifying screens at −80°C or BAS-MP imaging plates in a BAS cassette 2040 (FUJI) to detect the radioactive signal.

Immunogold-labeling and electron microscopy

Immunogold-labeling of virus was performed using the method of Kistler and Bullivant (1988). Briefly, virus samples were diluted by half in phosphate-buffered saline (PBS) and adsorbed onto carbon-coated grids by flotation for 1 min. The grids were washed by flotation on several drops of PBS. Antibodies (anti-rotavirus rabbit polyclonal, this laboratory), anti-VP2 mouse monoclonal (3A8/M2), and anti-VP6 mouse monoclonal (IEII) were bound to the sample by floating the grid on a 1/500 dilution of antibody for 45 min. Following washing, samples were decorated with gold-labeled goat anti-rabbit IgG (15 nm, ZYMED) or gold-labeled goat anti-mouse IgM (5 nm, AuroProbe EM, Amersham) by floating the grid on a 1/20 dilution for 45 min. The grids were extensively washed and the sample stained using 2% uranyl acetate. Immunoabsorption of samples onto carbon-coated grids was performed by first floating a grid on a 1/500 dilution of appropriate antibody.

Electron microscopy was performed on stained samples (2% uranyl acetate) using a Philips CM12 transmission electron micropscope and micrographs recorded on Copex electron microscope film (Agfa). Images were processed using a Nikon "Coolscan" micrograph scanner.

Production and purification of rotavirus VPLs using baculovirus

Rotavirus VP6/VP2 viruslike particles (VLPs) were produced in SF9 insect cells using the method of Crawford *et al.* (1994) with some modifications. Briefly, 6×10^8 SF9 cells in 50 mL of SF900 media were infected with recombinant baculovirus at a m.o.i. of 10. After a 2-h absorption, fresh media was added up to 100 mL and the cells maintained in spinner flasks (Schott) for 5–6 days. The medium containing VLPs was clarified by low-speed centrifugation in a Sorvall RT 7 centrifuge (4000 rpm, 10 min, 4°C) and the VLPs pelleted by ultracentrifugation through a 35% sucrose cushion using a Beckman SW28 rotor (25,000 rpm, 90 min, 4°C).

Single-shelled particles containing VP2 were prepared as previously described 3 days after infection (Zeng *et al.,* 1996). The cells were collected by centrifugation using a Sorvall RT7 centrifuge (4000 rpm, 10 min, 4°C), washed twice in PBS, and finally suspended in 30 mL of 1% DOC-TED lysis buffer (10 mM Tris–HCl, pH 7.4, 0.1 mM EDTA, 1% sodium deoxycholate) for 2 min. The cells were lysed by vortexing for 1 min and sonicated for 3 min using a Sonifier Converter-L (Branson SonicPower) at setting 6. VLPS were purified by isopycnic centrifugation as described earlier and dialyzed against 10 mM Tris–HCl, pH 7.4, for 2–4 h.

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