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Sindbis Virus Replicons and Sindbis Virus: Assembly of Chimeras and of Particles Deficient in Virus RNA

ILYA FROLOV, ELENA FROLOVA, AND SONDRA SCHLESINGER*

Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110-1093

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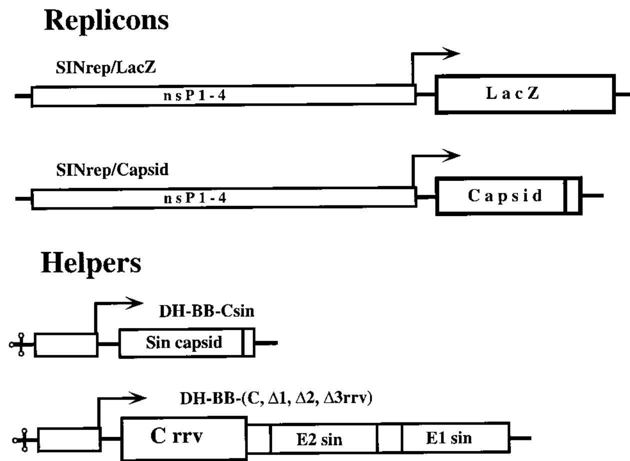
Alphaviruses are a well-characterized group of positive-strand RNA viruses. The identification of *cis*-acting elements in their genomes and their replication strategy have made them useful as vectors for the expression of heterologous genes. In infected cells, the nonstructural proteins, required for replication and transcription of the viral genes, are translated from the genomic RNA; the structural proteins, the capsid protein that interacts with the RNA to form the nucleocapsid and the proteins embedded in the lipid envelope, are translated from a subgenomic mRNA and can be replaced by heterologous genes. Such modified genomes are self-replicating (replicons); they can be introduced into the cells by transfection and can also be packaged into extracellular particles with defective helper (DH) RNAs. The particular DH RNA determines how well it is replicated and to what extent it is packaged. One potential complication of this system has been that recombination between the replicon genome and the DH RNA may occur. The studies described here were designed to prevent recombination by expressing the capsid protein from one DH RNA and the virus membrane proteins from a second helper RNA. Recombination to yield a nonsegmented infectious virus genome would then require several independent crossover events. There is a translational enhancer located downstream of the initiating AUG in the RNA of the capsid gene that had to be conserved in the second helper to achieve high-level expression of the viral glycoproteins. For this reason, we modified the capsid protein gene in two ways: the first was to use the capsid protein gene from a different alphavirus, Ross River virus, and the second was to make deletions in that gene to maintain the translational enhancer in the RNA but to eliminate the positively charged region in the protein that should be essential for the specific and nonspecific interactions with RNA. Transfections with replicon RNA and the deleted chimeric DH RNA as the only helper resulted in the high-level production of particles that were almost completely devoid of RNA. The inclusion of a helper expressing an intact Sindbis virus capsid protein gene led to the production of high levels of packaged replicons. Recombinants were not detected even after several undiluted passages.

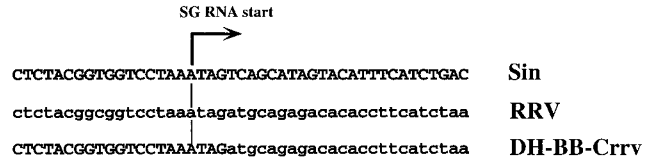
Alphaviruses are enveloped viruses that have a nonsegmented positive-strand RNA genome. The 5′ two-thirds of the genome codes for the nonstructural proteins (nsPs), and the 3′ one-third encodes the structural proteins. In infected cells, only the nsPs are translated from the genomic RNA; these are the proteins required for transcription and replication of the viral RNAs. The structural proteins, the capsid protein, 6,000-molecular-weight protein (6K protein), and the two membrane glycoproteins E1 and E2, are translated from a subgenomic RNA, identical in sequence to the 3′ one-third of the genome. The promoter for the subgenomic RNA, located on the RNA strand complementary to the genome, spans the junction between the nonstructural and structural protein genes (reviewed in reference 34). Several different alphavirus genomes have been cloned as cDNAs, and their RNA transcripts are infectious when transfected into cultured cells (4, 20, 23, 27). The ability to engineer their cDNAs led to the development of alphaviruses, particularly Sindbis virus and Semliki Forest virus, as vectors for the expression of heterologous genes (5, 18, 22, 39). In one type of alphavirus vector, referred to as a replicon, the structural protein genes are replaced by a foreign gene. These replicons can be packaged into extracellular particles by cotransfection of cells with both replicon and defective helper RNAs. The latter are not self-replicating but will be replicated and transcribe the alphavirus subgenomic RNA coding for the viral structural proteins if the required nsPs are expressed from the replicons.

Alphavirus replicons may be valuable for gene therapy, but one potential complication has been that recombination between replicon and helper can occur, giving rise to nonsegmented infectious genomes (1, 15, 26, 38). We thought that it should be possible to reduce the recombination frequency significantly by expressing the virus capsid protein and the membrane proteins from two different helper RNAs. It would then require several independent crossover events to produce viable progeny. Translation of the structural proteins from two different helpers should also decrease the number of particles able to give rise to infectious progeny, due to copackaging of replicon and helper genomes (14). The capsid protein can be translated independently of the other structural proteins. The C-terminal half of the protein contains a protease domain, and it will autocatalytically cleave when only a very few amino acids are located downstream from the cleavage site. The difficulty lies in the expression of the membrane proteins. Sequences in the capsid mRNA downstream of the translation start codon act as a translational enhancer under conditions in which host protein synthesis is suppressed by the infection (10, 11, 29, 30). Conservation of these sequences would be important to obtain a high level of expression of the viral glycoproteins.

The goal of these studies was to design a defective helper that could express the viral glycoproteins at levels that would allow packaging of the virus replicon but would not provide a capsid protein gene that could participate in the formation of viable recombinants. We made two changes in the helper in our attempt to achieve this objective. One was to make dele-

* Corresponding author. Mailing address: Department of Molecular Microbiology, Box 8230, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110-1093. Phone: (314) 362-2746. Fax: (314) 362-1232.





C.

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                     ++ + +   +
Crrv      MNYIPTQTFYGRRWRPRPAFRPWQVSMQPTPTMVTPMLQAPDLQAQQMQQLISAVSALTT 60
CrrvΔ1    MNYIPTQTFYGRRWRPRPAFRPWQVSMQPTPTMVTPMLQAPDLQAQQMQQLISAVSALTT
CrrvΔ2    MNYIPTQTFYGRRWRPRPAFRPWQVSMQPTPTMVTPMLQAPDLQAQQMQQLISAVSALTT
CrrvΔ3    MNYIPTQTFYGRRWRPRPAFRPWQVSMQPTPTMVTPMLQAPDLQAQQMQQLISAVSALTT

          +   +  +    ++  + + ++   +++   +++   + +   ++++  ++ +
Crrv      KQNVKAPKGQRQKKQQKPKEKKENQKKKPTQKKKQQQKPKPQAKKKKPGRRERMCMKIEN 120
CrrvΔ1    KQNVKAPKGQRQKKQQKPKEKKENQKKKPTLK-----------------RRERMCMKIEN
CrrvΔ2    KQNVKAPKGQRQKKQLK--------------------------------RRERMCMKIEN
CrrvΔ3    KQNLK--------------------------------------------RRERMCMKIEN
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FIG. 1. Replicons and defective helpers and sequences in the chimeric defective helpers. (A) The two replicons, SINrep/LacZ and SINrep/capsid, have been described previously (2). nsP1-4 refers to the nonstructural proteins 1 to 4. Both defective helpers contain the 5′ tRNA sequence. The defective helper DH-BB-Csin codes only for the Sindbis virus capsid protein. Four different chimeric defective helpers were used; they differed only in the RRV capsid gene. Crrv is the intact RRV capsid gene; CΔ1, CΔ2, and CΔ3 refer to the three forms with deletions (see panel C). The horizontal arrow and vertical line indicate the start of the subgenomic RNA. (B) Nucleotide sequence of the region spanning the 3′ end of the nsP genes and the start of the subgenomic RNA. The uppercase letters refer to sequences in the Sindbis virus genome; the lowercase letters refer to those from the RRV genome. (C) The sequence of the first 120 amino acids in the RRV capsid protein and in the three different forms with deletions. The basic amino acids are indicated by plus signs.

tions in the capsid protein gene to retain the translational enhancer activity but prevent or decrease those specific and nonspecific interactions between the capsid protein and RNA required for nucleocapsid assembly. The second was to use a capsid protein gene from a different alphavirus to supply the translational enhancer; this change was based on the observation that alphavirus chimeras containing the genes of Ross River virus (RRV) and the capsid protein gene of Sindbis virus were unable to make extracellular particles, although their ability to form intracellular nucleocapsids was unimpaired (24). This result suggested that the nucleocapsid containing the Sindbis virus capsid protein was unable to interact with the cytoplasmic tail of the RRV E2 glycoprotein, preventing the final step in assembly: the release of extracellular particles. These observations were strongly supported by experiments in which the sequence of the RRV E2 cytoplasmic tail was altered by site-directed mutagenesis to be more similar to that of the Sindbis virus E2. Titers of virus produced from transcripts of the chimera genome containing such changes were several orders of magnitude higher than those obtained from the original chimeras. The reciprocal chimera which was derived from the genome of Sindbis virus but had the capsid gene of RRV also was defective in its ability to form particles but was not analyzed further. It was this latter chimera that we planned to convert to a defective helper, reasoning that if recombinants arose they would contain RRV capsid sequences and would not be viable.

Our results, presented here, turned out to be different from what we had expected. First, we found that chimeras in which only the capsid gene was derived from RRV were not defective in particle formation. Chimeric defective helpers containing the intact capsid gene of RRV and the other structural protein genes of Sindbis virus packaged the replicon as efficiently as the original helper. Chimeric viruses, which had the complete nonsegmented alphavirus genome, were almost indistinguishable from Sindbis virus. Second, deletions in the capsid protein of RRV prevented the interaction between viral RNA and protein but did not inhibit the assembly of extracellular particles. These particles, which were formed at almost the same levels as normal virus particles, contained little or no viral RNA. We were able to show that chimeric defective helpers that have a version of the capsid gene of RRV carrying a

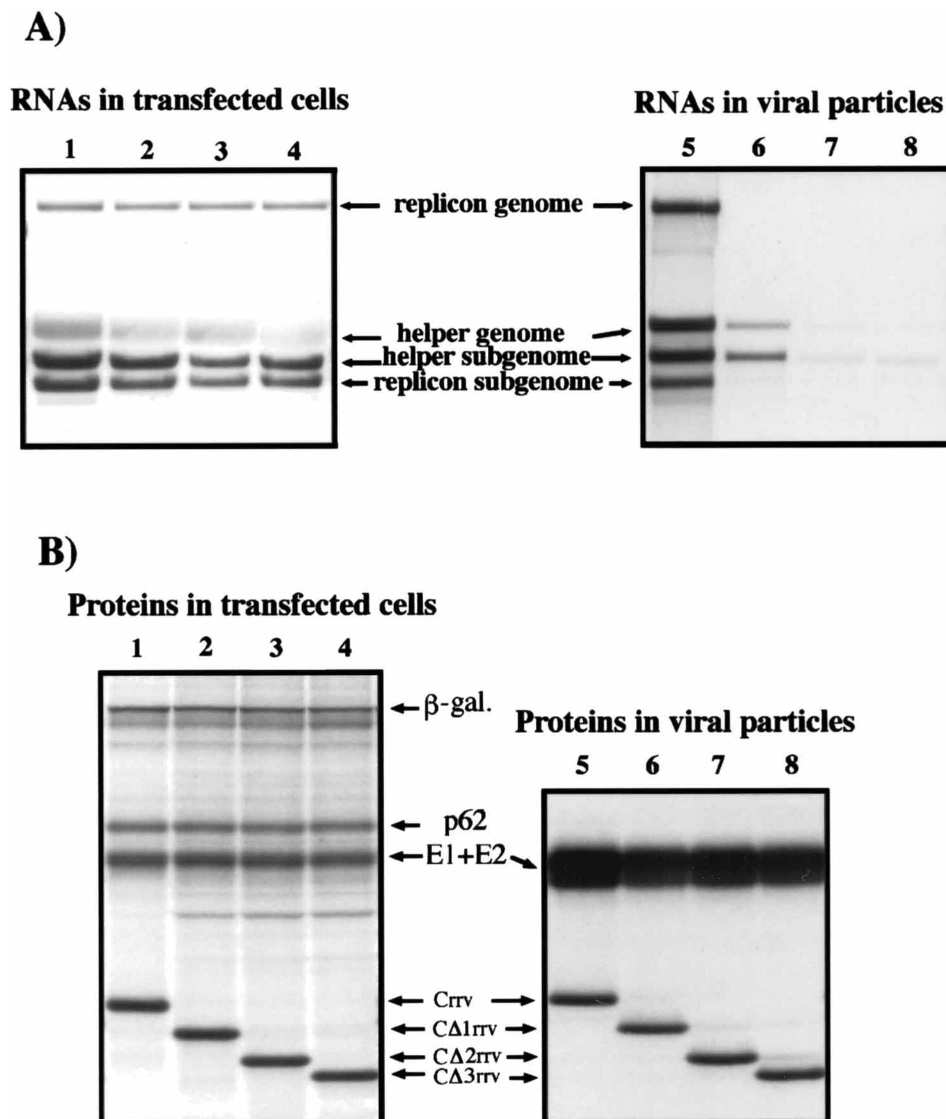

FIG. 2. (A) Viral RNAs synthesized in BHK cells and in the released particles. (B) Proteins synthesized in the transfected cells and in the released particles. The cells were all transfected with the SINrep/LacZ replicon. The helpers used were DH-BB(Crrv) (lanes 1 and 5), DH-BB(CΔ1rrv) (lanes 2 and 6), DH-BB(CΔ2rrv) (lanes 3 and 7), and DH-BB(CΔ3rrv) (lanes 4 and 8).

deletion could be used in conjunction with a second helper expressing the intact Sindbis virus capsid protein to package SINrep/LacZ replicons without producing recombinants.

MATERIALS AND METHODS

Sindbis virus replicons and chimeric defective helpers. The two replicons, SINrep/LacZ and SINrep/capsid, and the defective helper DH-BB are illustrated in Fig. 1A. Details of their construction have been described previously (2, 9). The new helpers constructed for these studies all contained the same 5′ terminus as DH-BB, nucleotides 10 to 75 of $tRNA^{Asp}$ replacing nucleotides 1 to 30 of the Sindbis virus genome, and were identical to DH-BB from the 5′ terminus to the promoter for the subgenomic RNA. They contained the capsid protein gene from RRV. The full-length cDNA copy of RRV(RR6415) was a gift from Richard Kuhn and Jim Strauss (20). The cDNAs for the RRV capsid proteins were obtained from this plasmid by PCR. The primers used for creating the deletions in the RRV capsid gene were designed to create new *Afl*II restriction sites located upstream and downstream of the deletions. All of the fragments were cloned into the pRS2 plasmid (a derivative of pUC18) and sequenced. The cDNAs were inserted into the DH-BB helper plasmid, replacing the Sindbis virus capsid gene. The junction between the Sindbis virus and RRV sequences, verified by sequencing, is shown in Fig. 1B. The amino acids in this region of the RRV capsid and in the forms carrying deletions are shown in Fig. 1C.

Toto1101 cDNAs containing either the full-length RRV capsid gene or this gene with one of the three deletions were obtained by replacing the *Bam*HI-*Xho*I fragment (containing the complete sequence of the subgenomic RNA) in Toto1101 with the corresponding region from the chimeric helper cDNA.

The DH-BB-Csin helper codes for only the Sindbis virus capsid gene in the subgenomic RNA (Fig. 1A). It was constructed by replacing the *Stu*I-*Xho*I fragment (coding for the glycoprotein genes and the 3′ noncoding region) in the DH-BB helper with the *Stu*I-*Xho*I fragment from SINrep5 replicon (coding for only the 3′ noncoding region of Sindbis virus).

Transcriptions and transfections. These procedures were identical to those already described (2, 23).

Analysis of virus-specific RNAs and proteins. After electroporation, the cells were diluted to a total volume of 10 ml (20-fold dilution) in alpha minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and seeded into p35 dishes (approximately 10^6 cells per dish).

(i) RNA. Four hours postelectroporation, the original medium was replaced by 1 ml of the same medium containing dactinomycin (1 μg/ml) and [^{3}H]uridine (35 μCi/ml, final concentration). The cells and medium containing released virus particles were harvested 16 h postelectroporation. The virus particles were pelleted by ultracentrifugation for 1.5 h at 42,000 rpm at 4°C in a TLA 45 rotor

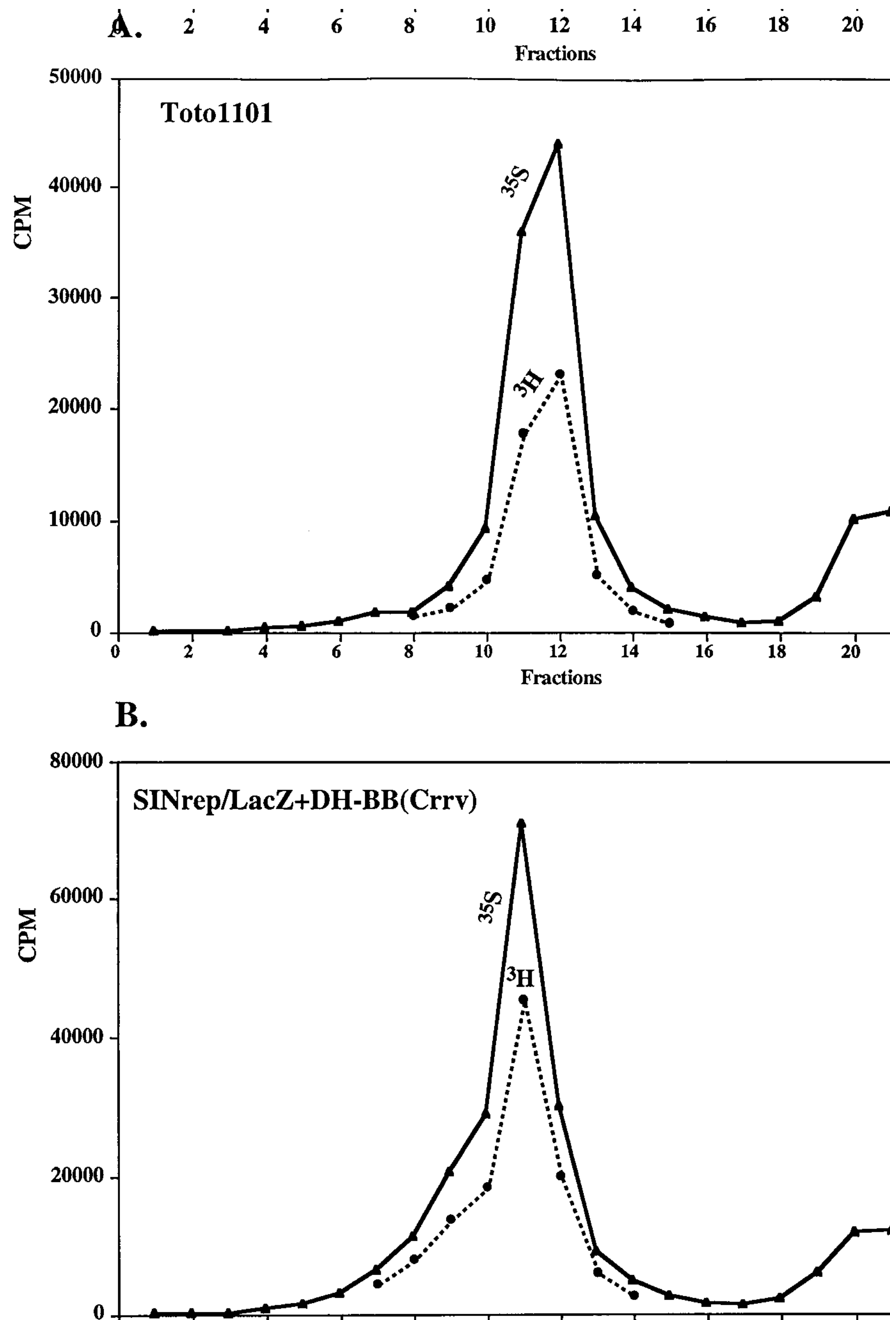

FIG. 3. Rate zonal centrifugation of virus particles released from BHK cells after transfection with Toto1101 RNA (A), cotransfection with SINrep/LacZ and DH-BB(Crrv) RNAs (B), and cotransfection with SINrep/LacZ and DH-BB(CΔ3rrv) RNAs (C). Details are described in Materials and Methods.

(Optima TL ultracentrifuge; Beckman, Palo Alto, Calif.). RNA was isolated from virus pellets and transfected cells with RNAzol B according to the procedures recommended by the manufacturer (Tel-Test, Inc., Friendswood, Tex.). The RNAs were analyzed by agarose gel electrophoresis following denaturation with glyoxal in dimethyl sulfoxide. Usually the entire RNA sample isolated from the virus pellet and 1/10 the cellular RNA sample were loaded onto the gels, so that both samples could be exposed to film for the same time period.

(ii) Protein. The intracellular proteins were labeled between 14 and 16 h postelectroporation by a 30-min pulse. After the cells were washed three times with phosphate-buffered saline (PBS), they were incubated at 37°C in 0.8 ml of MEM minus methionine containing 15 μCi of [^{35}S]methionine for 30 min. They were then washed again with cold PBS, removed from the dish by scraping in PBS, pelleted by low-speed centrifugation, and dissolved in loading buffer. When the proteins in released virus particles were to be labeled, the cells were subjected to the same pulse as that described above at 16 h postelectroporation, but after the 30 min of labeling, the radioactive medium was diluted with one-fourth the volume (0.2 ml) of MEM with methionine, and the cells were incubated for an additional 4 h. Virus particles were isolated by ultracentrifugation as described above or immunoprecipitated with polyclonal antibodies directed against the Sindbis virus structural proteins. For the latter, 5 μl of a rabbit anti-Sindbis virus antiserum was added directly to 1 ml of medium and incubated for 1 h at 4°C. Then 50 μl of a 10% suspension of Pansorbin (CalBiochem-Novabiochem Corp., La Jolla, Calif.) was added to each sample, and incubation was continued for an additional hour. The suspension was pelleted and resuspended in PBS and subjected to repeated washings in this manner. Any radioactively labeled virus protein was then released from the Pansorbin by resuspension in sample buffer (50 mM Tris-HCl [pH 6.7], 2% sodium dodecyl sulfate, 5% β-mercaptoethanol,

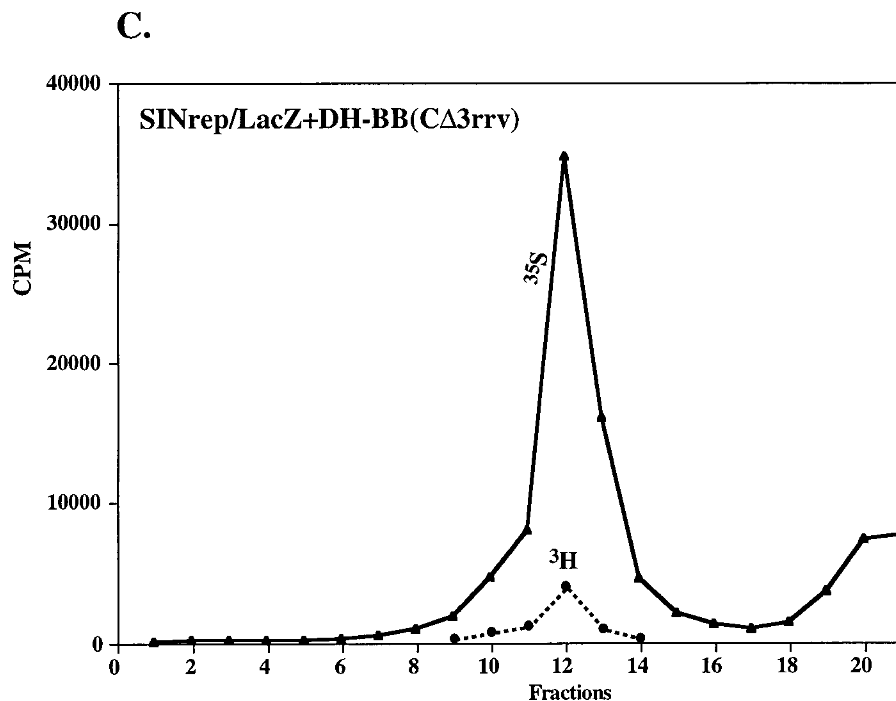

FIG. 3—*Continued.*

10% glycerol, 0.05% bromophenol blue) and boiled for 5 min. Proteins were analyzed by gel electrophoresis.

Preparation of virus particles radioactively labeled with both [^{35}S]methionine and [^{3}H]uridine. After electroporation, 10^7 cells were seeded onto a 150-mm-diameter dish. Four hours later, the medium was changed to MEM plus 2% FBS containing one-fifth the normal concentration of methionine, 15 μCi of [^{35}S]methionine per ml, and 35 mCi of [^{3}H]uridine per ml. The transfected cells were incubated for an additional 14 h until cytopathic effects became apparent, but the cells remained attached to the dish. Virus particles were pelleted by centrifugation for 1.5 h at 25,000 rpm at 4°C in an SW-27.1 rotor and were resuspended in 0.5 ml of MEM containing 1% FBS. The samples were loaded onto a 20 to 40% sucrose gradient in 0.05 M Tris HCl (pH 8.0)–0.1 M NaCl–1 mM EDTA (TNE buffer) and centrifuged for 1.5 h at 35,000 rpm at 5°C in an SW-41 rotor. The gradients were fractionated into 0.5-ml aliquots, and 30 μl from each fraction was taken for determining the amount of ^{35}S-labeled protein by liquid scintillation counting. RNA was isolated from a second 30-μl sample from the peak fractions with RNAzol B, and the incorporation of [^{3}H]uridine into RNA was measured by liquid scintillation counting.

RESULTS

Defective helpers that code for the RRV capsid and for the Sindbis virus membrane proteins. We constructed defective helper cDNAs containing the complete RRV capsid protein gene and three different forms of this gene carrying deletions (Fig. 1C). The first deletion of amino acids 92 to 110 removed 8 lysines (CΔ1), the second (CΔ2) deleted 15 lysines, and the third (CΔ3) deleted 19 lysines. The second and third deletions both caused a significant decrease in the positive charge character of the molecule, as observed in the hydrophobicity profiles of the proteins.

Each of the cDNAs was transcribed in vitro to produce defective helper RNA which was transfected into BHK cells with the SINrep/LacZ replicon RNA. One fraction from each of the transfected cells was labeled with [^{3}H]uridine from 4 to 16 h posttransfection, and the pattern of viral RNAs synthesized in the cells and incorporated into particles released into the extracellular fluid is shown in Fig. 2A. Cells that had been transfected with SINrep/LacZ and the defective helper containing the intact capsid gene from RRV and the glycoprotein genes from Sindbis virus [DH-BB(Crrv)] produced levels of viral particles (Fig. 2A, lane 5) in amounts equivalent to those obtained with the helper containing the capsid gene from Sindbis virus (DH-BB) (data not shown, but see Table 2). This result was different from what we had expected; the previously described chimera containing the capsid of RRV produced low levels of virus (24).

The particles produced in the cells transfected with SINrep/LacZ and DH-BB(Crrv) packaged the helper genome and both the replicon and helper subgenomic RNAs to a much greater extent than is seen when the capsid protein is derived from Sindbis virus. The selectivity that the Sindbis virus capsid protein has for the Sindbis virus replicon genomic RNA is not observed with the RRV capsid protein. Sindbis virus RNAs do not have a packaging signal that is recognized by the RRV capsid protein (12). Although the intact RRV capsid protein packaged the Sindbis virus RNAs successfully, the forms of this protein with deletions did so much less efficiently, and the amount of RNA packaged by the CΔ2rrv and the CΔ3rrv capsid proteins was decreased significantly (Fig. 2A, particularly lanes 7 and 8). The picture was very different when we analyzed the proteins in the extracellular particles. The syntheses of the viral structural proteins in cells transfected with SINrep/LacZ and each of the defective helpers were essentially the same (Fig. 2B), but in contrast to what was seen with the RNAs, the amounts of protein in the released particles were also the same (Fig. 2B). This result suggested that the capsid proteins produced by DH-BB(CΔ2rrv) and DH-BB(CΔ3rrv) were able to interact with the viral membrane proteins to form extracellular particles and that these particles contained very little RNA.

Additional characterization of RNA-deficient particles. To determine if the extracellular particles that lacked RNA were similar to infectious virus particles, we compared Toto1101, particles packaged with DH-BB(Crrv), and particles packaged with DH-BB(CΔ3rrv) in rate zonal centrifugation in a sucrose

gradient. Transfected cells were labeled with both [^{3}H]uridine and [^{35}S]methionine for the same time period (see Materials and Methods). Based on the [^{35}S]methionine label, the samples had similar sedimentation coefficients (Fig. 3). The radioactivity in the [^{3}H]uridine-labeled RNA showed that particles packaged with DH-BB(CΔ3rrv) had at least 10 times less RNA than the others (Fig. 3).

We also compared the particle size and morphology with the electron microscope (Fig. 4). The diameter of the particles packaged with DH-BB(CΔ3rrv) was about 8% smaller than that of particles packaged with either DH-BB or Sindbis virus (the latter not shown). The RNA-deficient particles (Fig. 4a) appeared to take up more uranyl acetate stain than particles containing RNA (Fig. 4b). Although this difference was observed in two independently prepared preparations of the particles, it was almost impossible to distinguish the particles when they were mixed before they were added to the grid and stained.

Chimeric Sindbis viruses containing intact capsid gene or CΔ3 from RRV. We constructed nonsegmented Sindbis virus genomes containing the intact capsid gene of RRV to compare its biological activity with that of the parental Toto1101 virus and the chimeric virus described by Lopez et al. (24). We also inserted the forms of the RRV capsid with deletions into the Toto1101 background to examine the effect of the deletions in the context of the infectious nonsegmented virus genome. BHK cells were transfected with the different transcribed RNAs, and 4 h later [^{35}S]methionine was added. After 12 h, the released virus particles were concentrated by centrifugation, and samples were taken for determining PFU and the number of radioactively labeled particles (Table 1). The ratio of PFU to particles (^{35}S-methionine label) for Toto1101(Crrv) was indistinguishable from that of the parental Toto1101. Toto1101(Crrv) formed large plaques on monolayers of BHK-21 cells, Vero cells, and secondary chicken embryo fibroblasts. The growth rate was almost the same as that of Toto1101 (data not shown). In contrast, the level of PFU

TABLE 1. Titers of Sindbis virus and chimeras containing the capsid protein from RRV or forms of this protein with deletions

Expt no. and virus	Titer (10^9)[a] (plaque diam)	Relative level of particles[b]
1		
Toto1101	3.0 (3 mm)	1.0
Toto1101(Crrv)	2.5 (3 mm)	1.1
Toto1101(CΔ1rrv)	0.1 (~1 mm)	0.2
Toto1101(CΔ2rrv)	0.018 (<0.5 mm)	0.2
Toto1101(CΔ3rrv)	0.001 (<0.5 mm)	0.1
2		
Toto1101	14	ND
Toto1101(Crrv)	4.5	ND
Toto54[c]	0.9	ND
SIN(RRc)[c]	0.005	ND

[a] Titers are reported as PFU per milliliter assayed on chicken embryo fibroblasts. PFU were determined 24 h after plaquing for Toto1101, Toto1101(Crrv), Toto54, and Sin(RRc); PFU were determined after 48 h for Toto1101(CΔ1rrv), Toto1101(CΔ2rrv), and Toto1101(CΔ3rrv). Viruses and titers given were obtained from two different experiments, as indicated.

[b] The relative level of particles was determined by analysis of a sample of each of the [^{35}S]methionine-labeled particles by polyacrylamide gel electrophoresis. The relative amount of radioactivity in the virus structural proteins on the gel was determined with a molecular imager (Bio-Rad Laboratories).

[c] Toto54 and SIN(RRc) were obtained from J. Strauss (24).

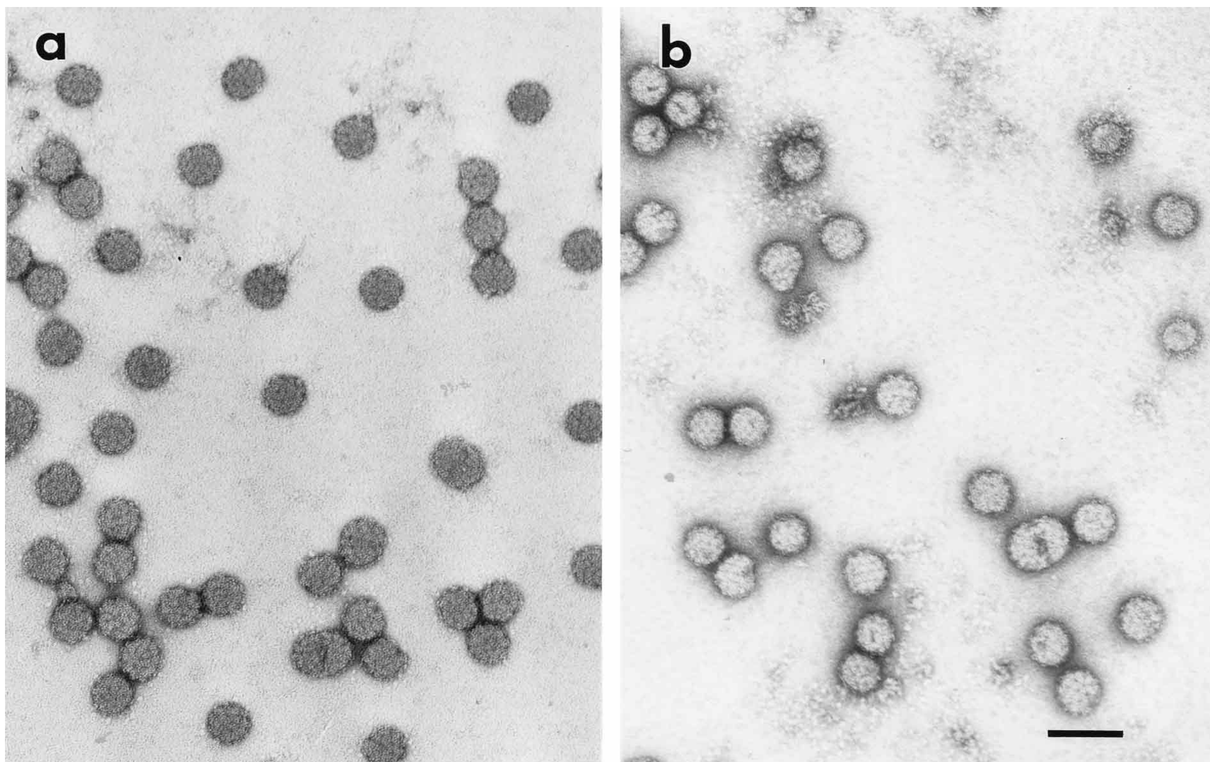

FIG. 4. Electron micrographs of particles packaged with DH-BB(CΔ3rrv) (a) or DH-BB (b). The samples were first concentrated from the medium by sedimentation through a cushion of sucrose. They were then centrifuged to equilibrium in a sucrose gradient. Fractions containing the particles were diluted, the sucrose was removed by centrifugation, and the particles were resuspended in TNE buffer. Bar, 116 nm.

obtained with the previously described chimeric virus [SIN (RRc)] was reduced almost a 1,000-fold (Table 1). This result confirmed the results of Lopez et al. but suggested that, for this chimera, the block was not at the level of assembly. Our preliminary data indicate that there was a block in the transcription of 26S subgenomic RNA and that this may be a consequence of the *Mlu*I site that had been inserted in the genome just downstream of the start of the 26S RNA sequences (8).

BHK cells that were transfected with Toto1101 RNA containing one of the forms of the RRV capsid gene with deletions produced particles at 10 to 20% of the level of cells transfected with either Toto1101 or Toto1101(Crrv) (Table 1). Both Toto1101(CΔ2rrv) and Toto1101(CΔ3rrv) produced a low level of tiny, pinpoint-sized plaques after incubation of chicken embryo fibroblast monolayers under agarose for 2 days. [The large plaques produced by Toto1101 or Toto1101(Crrv) were detected after 24 h.] Virus particles that gave rise to these pinpoint plaques could not be propagated and were lost on further passaging on BHK cells.

Cotransfection of BHK cells with the Sindbis capsid protein and the envelope proteins expressed from two different RNAs. We tested the ability of the defective helpers expressing either the intact RRV capsid or the forms of the RRV capsid with deletions to provide the Sindbis virus envelope proteins when the Sindbis virus capsid protein was being expressed from another subgenomic mRNA. We did this in two ways: one way was to introduce the capsid gene into cells via the replicon; the other was to use a second helper (Fig. 1A). The RNAs and proteins isolated from both the transfected cells and the released particles are shown in Fig. 5. The levels of virus-specific RNAs in the different transfected cells were essentially the same (Fig. 5A and C, lanes 1 to 4). In those examples in which the transfected cells produced an intact Sindbis capsid protein either from SINrep/capsid (Fig. 5B) or from DH-BB-Csin (Fig. 5D), the released particles contained RNA (Fig. 5A and C, lanes 5 to 8).

A comparison of the biological activities (PFU and infectious units) of these particles is summarized in Table 2. For those samples in which the capsid protein was supplied by SINrep/capsid or by helpers carrying the intact RRV capsid gene [DH-BB(Crrv)] or the minimally deleted version [DH-BB(CΔ1rrv)], PFU were detected at significant levels due to copackaging of the replicon and helper (Table 2). When the only source of capsid protein was CΔ2rrv or CΔ3rrv, the level of infectious units (packaged replicons) was low (Table 2), as was expected since these particles contained low levels of RNA (Fig. 2A). The inclusion of the second helper, DH-BB-Csin, increased the level of infectious units by several orders of magnitude (Table 2).

Assays for recombination between replicons and defective helpers. The helper normally used for packaging of the Sindbis virus replicons is DH-BB(5′SIN). Even though the level of copackaged particles obtained with this helper is very low, recombinants can be detected. The most sensitive assay for detecting recombinants is to passage the packaged replicons several times. Recombinants are detected in the harvest from transfected cells only under conditions in which a small percentage of the cells are transfected. A recombinant that arises in a culture in which all of the cells are transfected would not be amplified, because it would not superinfect previously transfected cells (19). When most of the cells are not transfected, a recombinant that infected a cell would be able to replicate in that cell and could then spread to other nontransfected cells.

To assess the possibility of recombination with the two-helper system, we passaged the SINrep/LacZ replicon packaged either with DH-BB(5′SIN) or with the two helpers DH-BB-(CΔ3rrv) and DH-BB-Csin. Approximately 16 h after transfection, the extracellular fluids were harvested, and 1/20 of each of the samples was used to infect a new monolayer of BHK cells. After an overnight incubation, one-fifth of that extracellular fluid was used for a subsequent infection. Recombinants were detected after three passages of the replicon packaged with DH-BB(5′SIN). Under these same conditions, replicons packaged with the two helpers produced no recombinants, and the infectious titers remained low. The continued presence of infectious particles was most likely due to cells being infected with more than one particle. The probability that the three different RNAs—the replicon and the two different helpers—were copackaged seemed unlikely, and when samples were passaged at a low multiplicity of infection, the infectious titer was lost.

DISCUSSION

The original incentive for the studies presented here was to devise a scheme for packaging Sindbis virus replicons that would not give rise to recombinants. Our strategy was to express the viral capsid protein and the membrane proteins from two different helpers so that at least two independent crossovers would be needed to produce a functional nonsegmented virus genome and any recombinational event that connected the capsid and glycoprotein genes would have to be precise to produce functional proteins. (For more detailed discussions of recombination in alphaviruses, see references 15, 26, and 38.) We were constrained by the need to express high levels of the glycoproteins, which in alphavirus-infected cells appears to require the presence of a translational enhancer upstream of the glycoprotein genes and downstream of the capsid AUG initiation codon (10, 11, 29, 30). Our rationale for using the capsid protein gene from RRV was based on the assumption that nucleocapsids containing the RRV capsid protein would not interact with the C-terminal tail of the Sindbis virus E2 glycoprotein—a step that is assumed to be essential for assembly. Detailed studies with the opposite chimera—RRV with the Sindbis virus capsid—clearly demonstrated that the block in assembly was at this step (24). It was rather surprising to find that Sindbis virus with the RRV capsid tolerated the heterologous capsid with essentially no problems, and the same was true for the virus containing the capsid protein from Venezuelan equine encephalitis virus (8). Earlier studies of Lopez et al. (24) had shown that the Sindbis virus-RRV capsid protein chimera was severely defective in its ability to form infectious particles. The authors did not analyze the chimera in more detail, and our initial studies suggested that there was a defect in transcription of the subgenomic RNA (8). More recently, Smyth et al. found that reciprocal chimeras between Sindbis virus and Semliki Forest virus are not equivalent (32). Chimeras with the capsid protein gene derived from Sindbis virus and the glycoproteins from Semliki Forest virus are analogous to the Sindbis virus-RRV capsid chimeras and do not assemble into virus particles. In contrast, the chimeras with the capsid protein of Semliki Forest virus and the glycoproteins of Sindbis virus, comparable to those we describe here, assemble into infectious virus particles at almost the same level as wild-type Semliki Forest virus.

The alphavirus capsid protein plays a multifunctional and crucial role in virus assembly. The 26S subgenomic mRNA that codes for the structural proteins contains a single open reading frame, with the capsid protein gene located at the 5′ terminus of the coding sequences. During translation of the subgenomic RNA, the nascent capsid protein autoproteolytically cleaves

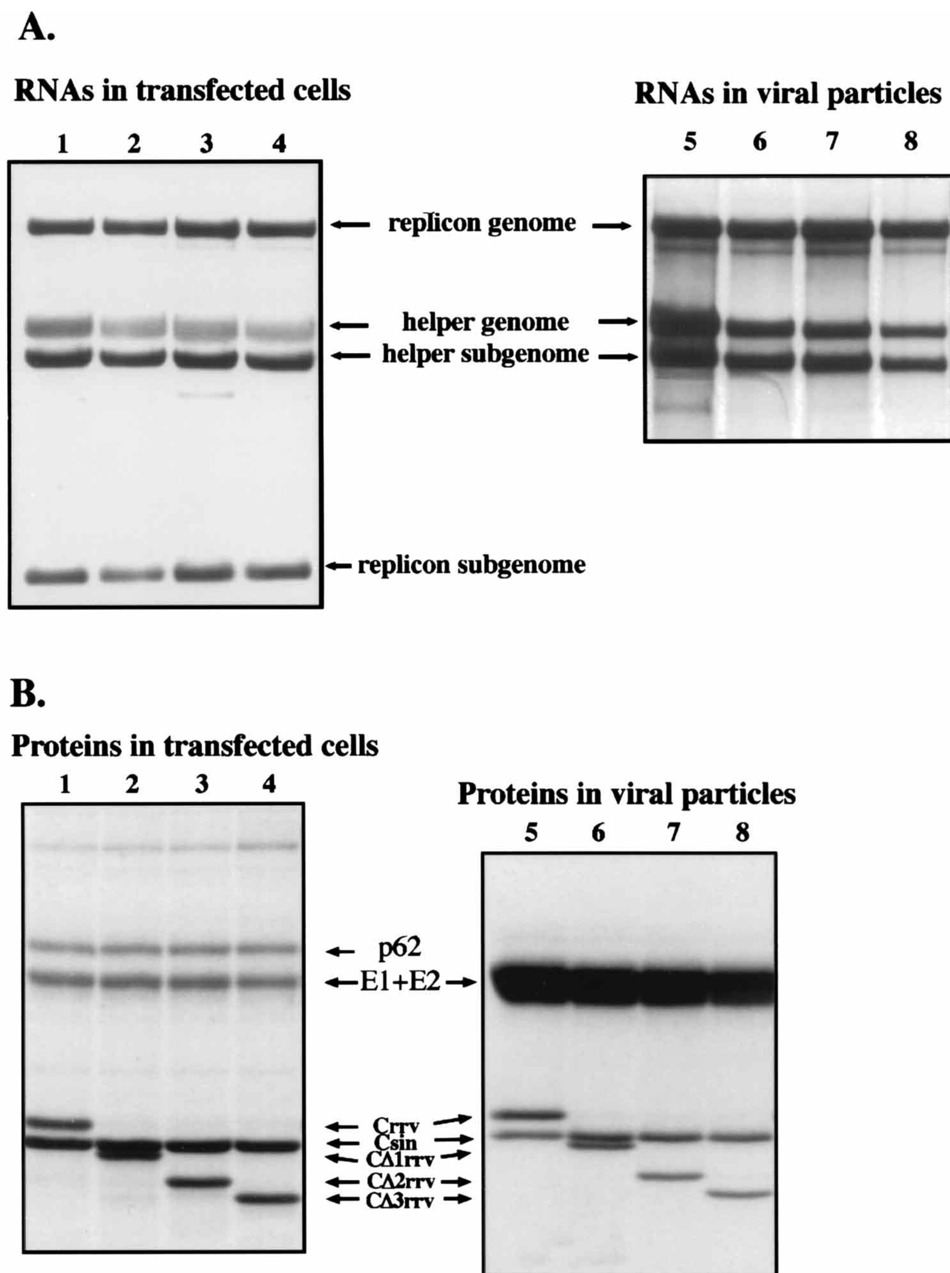

FIG. 5. Analysis of viral RNAs and proteins in cotransfected cells and released particles. (A and B) The replicon was SINrep/capsid. The helpers used were DH-BB(Crrv) (lanes 1 and 5), DH-BB(CΔ1rrv) (lanes 2 and 6), DH-BB(CΔ2rrv) (lanes 3 and 7), and DH-BB(CΔ3rrv) (lanes 4 and 8). (C and D) The replicon was SINrep/LacZ. Two helpers were used. One helper was always DH-BB-Csin; the other was DH-BB(Crrv) (lanes 1 and 5), DH-BB(CΔ1rrv) (lanes 2 and 6), DH-BB(CΔ2rrv) (lanes 3 and 7), or DH-BB(CΔ3rrv) (lanes 4 and 8).

from the growing polypeptide chain. The capsid polypeptide then is assumed to interact with RNA to initiate assembly of the nucleocapsid. Although the subgenomic RNA is present in higher concentrations than the genomic RNA, the latter is selectively packaged due to specific binding between a region in the genomic RNA and the capsid protein (12, 13, 36, 37). This specific interaction is thought to be the nucleation event, which would then be followed by additional nonspecific RNA-protein interactions and by protein-protein interactions that lead to the assembly of the nucleocapsid. These types of interactions have been defined for icosahedral plant viruses such as southern bean mosaic virus and turnip crinkle virus (6, 16, 17, 28, 33) and are now being described in more detail for the alphaviruses (21, 31).

Structural and mutational analyses have identified two domains in the alphavirus capsid protein and are defining specific amino acids involved in the different functions. The C-terminal half of the Sindbis virus capsid protein (residues 114 to 264) contains a serine proteinase activity (3). Constructs of the Semliki Forest virus capsid protein that express this domain in the absence of the N-terminal domain retain proteolytic activity (7). The N-terminal half of the protein is disordered in the electron density map derived from X-ray crystallographic studies, but a structure extending to residue 106 has recently been

FIG. 5—*Continued.*

reported and shows that residues 108 to 111 bind to a specific hydrophobic pocket in neighboring capsid protein molecules (21). This finding, taken together with their mutational studies, led Lee et al. to propose that residues 108 to 111 are important in the interactions between capsid monomers and also in the binding between the nucleocapsid and the C-terminal tail of the E2 glycoprotein (21). Modeling and mutagenesis studies of the Semliki Forest virus capsid protein by Skoging and coworkers identified Tyr-184 and Trp-251 as residues in a hydrophobic pocket that were important in the assembly process (31). Those studies provided a convincing argument that the assembly of alphaviruses may not require prior formation of intact nucleocapsids; instead, virus assembly may involve capsid-capsid and capsid-glycoprotein interactions occurring simultaneously (31).

The N-terminal half of the alphavirus capsid protein is the domain that interacts with RNA. This was an assumption first made based on analogy with icosahedral plant virus coat proteins and by the clustering of basic residues in this part of the molecule. Using forms of the capsid protein with deletions obtained by translation in vitro, Geigenmüller et al. identified a region of 32 amino acids (residues 76 to 107) that was essential for the specific binding of a Sindbis virus RNA to the capsid protein (13). Support for the importance of some of these amino acids in specific RNA binding came from Owen and Kuhn (25), who constructed a Sindbis virus genome that produced a capsid protein lacking residues 97 to 106. Transfection of this genome into BHK cells led to the formation of virus particles, but specificity in encapsidation was lost; both subgenomic RNA and genomic RNA were packaged. Deletion of those amino acids eliminated selectivity in packaging but not the encapsidation of RNA (25). A deletion of residues 66 to 78 in the capsid protein of Semliki Forest virus also permitted the formation of infectious virus, but the specificity of encapsidation was not examined (7).

TABLE 2. Titers of replicons packaged with different helpers

Helper	PFU[a]/ml in cells transfected with:		Infectious units[c]/ml
	SINrep/ LacZ	SINrep/ capsid	SINrep/LacZ
DH-BB(Crrv)	1.5×10^7	8×10^7	
DH-BB(CΔ1rrv)	1.5×10^7	9×10^7	
DH-BB(CΔ2rrv)	ND[b]	7×10^7	
DH-BB(CΔ3rrv)	ND	5×10^7	
DH-BB(CΔ2rrv)			5×10^6
DH-BB(CΔ3rrv)			$<10^6$
DH-BB-Csin + DH-BB(CΔ1rrv)			1×10^9 to 2×10^9
DH-BB-Csin + DH-BB(CΔ2rrv)			1×10^9 to 2×10^9
DH-BB-Csin + DH-BB(CΔ3rrv)			1×10^9 to 2×10^9

[a] PFU were due to copackaging of the replicon and helper (14).
[b] ND, not detected.
[c] Infectious units (packaged replicons) were determined by the assay for cytopathic effects on chicken embryo fibroblasts (9).

The deletions that we made in the N-terminal half of the RRV capsid protein eliminated most of the basic residues and led to an inability of the protein to interact with viral RNAs. These results were reminiscent of those described for icosahedral plant viruses, in particular, southern bean mosaic virus (6) and turnip crinkle virus (33). The proteolytic removal of the N-terminal arm of these coat proteins led to the in vitro assembly of T=1 particles that were free of RNA. Although the large deletions in the alphavirus capsid protein destroyed RNA-protein interactions, they did not prevent the assembly of virus-like particles, demonstrating that the capsid proteins with deletions were able to undergo capsid-capsid interactions and interact with the C-terminal tail of the Sindbis virus E2 glycoprotein. The recent proposal that nucleocapsid formation may be concomitant with budding raises the possibility that the capsid-capsid interactions that occur in the absence of RNA may be stabilized by their interactions with the glycoprotein. The virus particles we have described here contained at least 10-fold less RNA than normal particles (Fig. 4). The possibility that cellular RNAs were replacing the viral RNA seemed unlikely; if the capsid protein could interact with RNA it should interact with the viral RNAs which would be present in high concentrations in the transfected cells. When specificity is lost, either by a deletion in the capsid polypeptide (25) or by using the RRV capsid which doesn't recognize the packaging signal in the Sindbis virus genome (12), the major consequence was an increase in the packaging of subgenomic viral RNAs. In previous studies, virus-like particles were detected when the structural proteins of Semliki Forest virus were expressed from the vaccinia virus-T7 expression system, but the amount was too small to determine if there was RNA present in the particles (35).

The scheme presented here in which the capsid protein was expressed from one defective helper RNA and the viral membrane proteins were expressed from a second helper demonstrated the feasibility of obtaining high titers of packaged replicons in the absence of detectable recombinants. It will be important to test these packaged replicons in other contexts, particularly in infected animals, to determine their potential as expression vectors. In addition, the recent studies identifying amino acids in the capsid protein that are important for capsid-glycoprotein interactions (21, 31) suggest that it will be possible to design defective helpers in which the mRNA contains a translational enhancer but translation of the RNA does not produce a capsid polypeptide able to participate in particle assembly formation.

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