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Sindbis Virus Expression Vectors: Packaging of RNA Replicons by Using Defective Helper RNAs

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Since the recovery of infectious RNA transcripts from full-length cDNA clones, alphavirus genome RNAs have been engineered to allow expression of heterologous RNAs and proteins. The highest levels of expression of heterologous products are achieved when the viral structural genes are replaced by the heterologous coding sequences. Such recombinant RNAs are self-replicating (replicons) and can be introduced into cells as naked RNA, but they require *trans* complementation to be packaged and released from cells as infectious virion particles. In this report, we describe a series of defective Sindbis virus helper RNAs which can be used for packaging Sindbis virus RNA replicons. The defective helper RNAs contain the *cis*-acting sequences required for replication as well as the subgenomic RNA promoter which drives expression of the structural protein genes. In cells cotransfected with both the replicon and defective helper RNAs, viral nonstructural proteins translated from the replicon RNA allow replication and transcription of the defective helper RNA to produce the virion structural proteins. A series of defective helper RNAs were compared for the ability to package the replicon RNA as well as for the ability to be replicated and packaged. One defective helper RNA not only packaged the replicon but also was itself encapsidated and would be useful under conditions in which extensive amplification is advantageous. Other defective helper RNAs were able to package the replicon efficiently but were packaged very poorly themselves. These helpers should be useful for applications in which expression of the viral structural proteins or virus spread is not desired.

Alphaviruses have been well characterized, not only as disease-causing agents but also as model systems for studies in virology and cell biology. Two members of this genus, Sindbis virus and Semliki Forest virus (SFV), are being developed as vectors for the expression of heterologous genes (2, 9, 19, 32). Their ability to function as expression vectors is based on their genomic organization and replication strategy. The alphavirus genome is a single strand of positive-sense RNA of approximately 12 kb which is capped at the 5′ terminus and polyadenylated at the 3′ terminus (21, 23). The 5′ two-thirds of this RNA encodes the nonstructural proteins; the 3′ one-third encodes the viral structural proteins, i.e., the capsid protein (C), a hydrophobic 6-kDa protein (6K), and two membrane glycoproteins (E1 and E2). In infected cells, only the nonstructural proteins required for RNA replication and transcription are translated from the genomic RNA. In contrast, the virion structural proteins are translated from a subgenomic mRNA (26S RNA) which is identical in sequence to the 3′ terminal one-third of the genomic RNA and produced by transcription of genome-length complementary (minus) strand from an internal promoter. Since the nonstructural protein genes and the structural protein genes are expressed from two different mRNAs, they may be expressed independently of one another.

Two types of RNA expression vectors derived from the infectious alphavirus genome have been described; both take advantage of the high level of subgenomic RNA which accumulates in infected cells. One vector contains two promoters leading to synthesis of two subgenomic mRNAs; one controls expression of the heterologous product, and the other controls synthesis of the Sindbis virus structural proteins (5, 17). This vector is self-replicating, produces infectious virus particles, and can spread from cell to cell in a manner similar to that of the parental virus. Such vectors have proven to be of value for studies in immunology (5) and cell biology (16). In the second type of vector, heterologous sequences replace the structural protein-coding genes (9, 32). Such recombinant RNA replicons can be efficiently introduced into some cell types as naked RNA by electroporation (10) and lead to high levels of heterologous gene expression. However, their use is limited by the efficiency of RNA transfection, which varies greatly among different cell types.

We have been interested in using defective Sindbis virus RNAs to design a helper system for packaging Sindbis virus RNA replicons into infectious virus particles. Defective alphavirus RNAs were originally identified as defective interfering (DI) RNAs. Such DI RNAs are propagated and compete with the parental virus because they contain *cis*-acting sequences essential for replication and packaging (22) and have proven useful for defining these sequence elements (8). Two previous studies are germane to the engineering of DI RNAs to produce defective helper (DH) RNAs useful for replicon packaging. In the first study, DI RNAs were used to define an encapsidation signal for Sindbis virus RNA, which resides between nucleotides (nt) 745 and 1225 in the virion genomic RNA (28). It was also observed that deletion of this region resulted in impaired DI RNA replication. In a second study, it was shown that the subgenomic RNA promoter region was functional when translocated into DI RNAs and led to synthesis of subgenomic mRNAs in cells infected with parental Sindbis virus (7).

Using defective RNAs containing the packaging signal, the subgenomic RNA promoter, and the structural protein genes, Geigenmüller-Gnirke et al. were able to package Sindbis virus replicon RNA expressing the chloramphenicol acetyltransferase (CAT) gene (4). The DI RNA was also packaged, and both RNAs were frequently packaged together, producing

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particles that gave rise to PFU. This defective RNA proved to be both a helper and a DI RNA since it was able to package the vector, but it also interfered with the replication of the replicon. Subsequently, Liljeström and Garoff described a different DH RNA, derived from SFV RNA, that lacks a large portion of the nonstructural region, including the region corresponding to the packaging signal identified for Sindbis virus (9). This defective helper was able to package SFV replicons but was not packaged itself. This provides a useful system for producing suicide vectors, i.e., virus particles which are able to infect cells efficiently but are incapable of forming progeny virions.

We report here investigations with several different DH RNAs derived from Sindbis virus RNAs. One was analogous to the helper described for SFV replicons. When this RNA was used to cotransfect cells with the Sindbis virus replicon RNA, high yields of infectious particles containing the replicon were produced, and the DH RNA was packaged at levels that were essentially undetectable. Another helper RNA was efficiently incorporated into particles along with the replicon to an extent that permitted amplification of the particles. The latter DH RNA may be useful under conditions in which the spread of an infection is of interest.

MATERIALS AND METHODS

Plasmids. (i) Sindbis virus genomic cDNAs. Toto1101 has been described previously (18). Toto1101(5′ $tRNA^{Asp}$) was constructed from Toto1101 and DI25. DI25 is a clone derived from a naturally occurring DI RNA which has a truncated $tRNA^{Asp}$ at its 5′ terminus (8, 14).

(ii) SINrep cDNAs. The Sindbis virus replicon (SINrep) cDNAs contain the first 7,646 nucleotides of the Sindbis virus genomic cDNA (Toto1101) followed by a polylinker, the 3′-terminal 310 nt of the Sindbis virus genome, plus 37 A residues. Directly downstream of the Sindbis virus sequences, there are several unique restriction enzyme sites for linearization of the DNA in preparation for transcription. SINrep5 (Fig. 1) was constructed to provide a variety of sites for the insertion of heterologous genes and for linearization of the plasmid DNA.

(iii) DH cDNAs. Plasmids for transcription of DH RNAs with the same 5′ terminus as the Sindbis virus genome RNA were constructed by using convenient restriction sites for making internal deletions in Toto1101 (Fig. 2). A parallel set of deletion constructs which have the 5′-terminal $tRNA^{Asp}$ sequence was made (Fig. 2). DI(26S) has been described previously (4). DH(26S)5′SIN differs from DI(26S) in two ways: the 5′ terminus of DH(26S)5′SIN is identical to that of the Sindbis virus genome, and the glycoproteins were derived from the Sindbis virus cDNA, TE12. TE12, a derivative of neurovirulent Sindbis virus, has been described elsewhere (11).

Transcriptions and transfections. All of the Sindbis virus cDNAs are positioned downstream from the SP6 DNA-dependent RNA promoter. SP6 RNA transcription reactions were carried out as recommended by the supplier (Bethesda Research Laboratories, Life Technologies Inc., Gaithersburg, Md.), using the 5′ cap analog 7^{m}G5′ppp5′G (New England Biolabs). [^{3}H]UTP was included in the reaction mixture, and the concentration of the RNA was calculated on the basis of the incorporation of tritium. For the experiments reported here, the RNAs were used after transcription without treatment with DNase or further purification. Transcription reaction mixtures containing RNAs were aliquoted and stored frozen at −80°C. Transfections were carried out by electroporation using a Bio-Rad Gene Pulser apparatus as described by

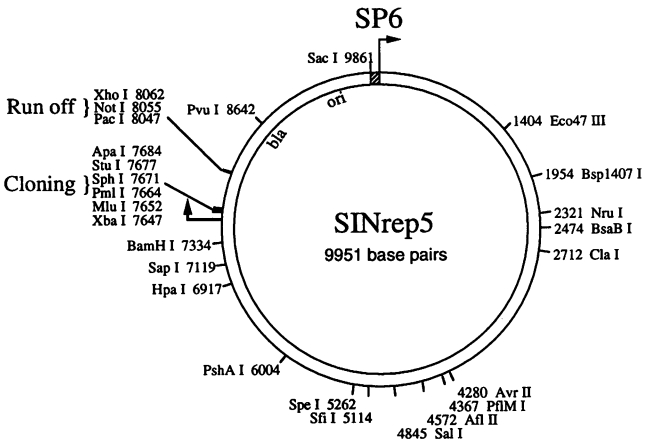


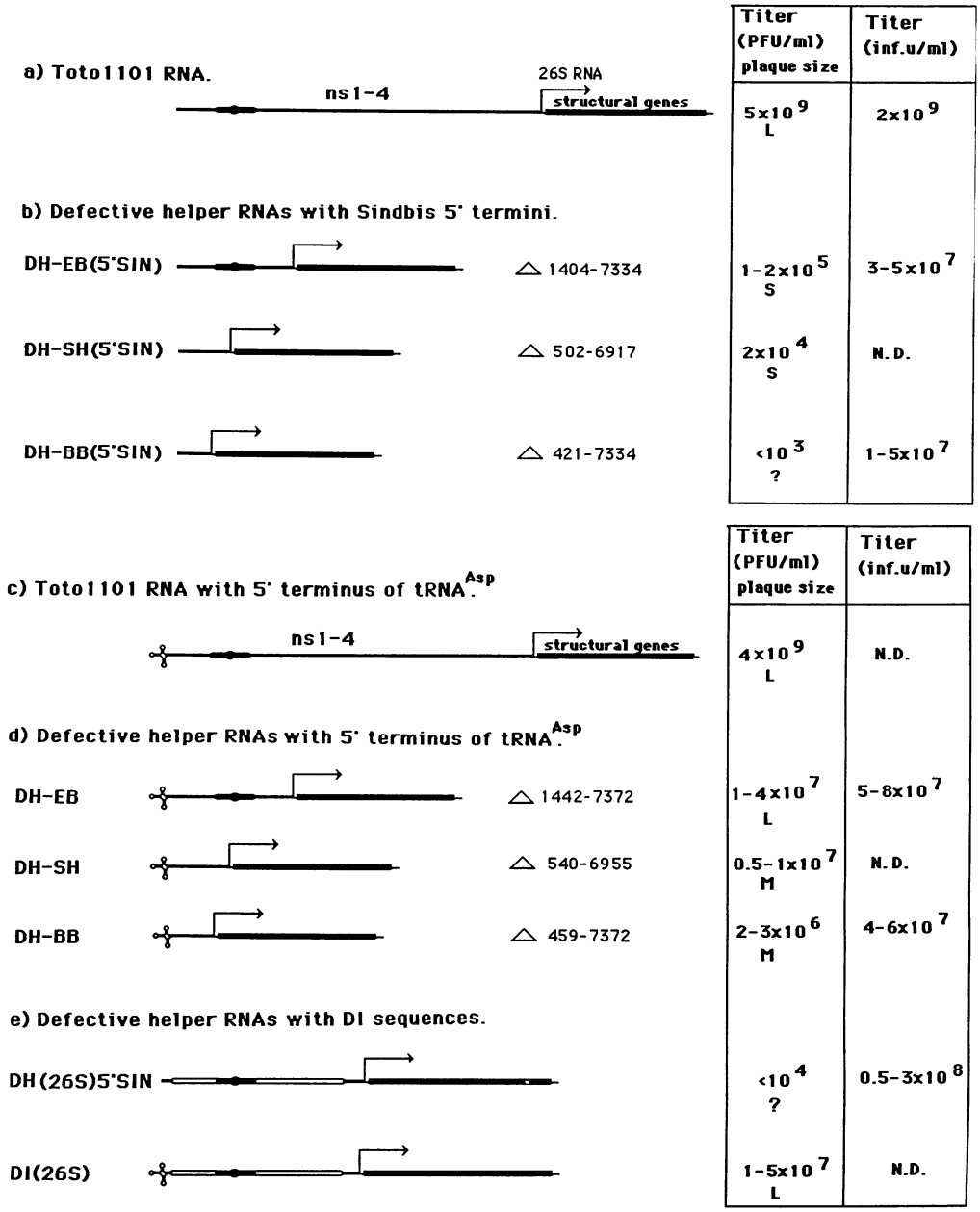
FIG. 1. Map of replicon transcription plasmid pSINrep5. Plasmid numbering begins with the sequence corresponding to the first nucleotide of the Sindbis virus genome RNA sequence. Upstream from the Sindbis virus cDNA is the promoter (hatched box and arrow) for SP6 DNA-dependent RNA polymerase, used for production of RNA transcripts in vitro. Unique restriction sites and their positions in the pSINrep5 sequence, including those which can be used for cloning and expression of heterologous sequences (Cloning) or production of templates for runoff transcription (Run off), are indicated. The position corresponding to the subgenomic mRNA start site (nt 7598) is marked (bold arrow). Also indicated are the regions of the plasmid encoding the ampicillin resistance gene (bla) and the origin of replication (ori). The sequence of the cloning region, located between Sindbis virus nt 7646 and 11394, is 5′-TCTAGACGCGTAGATCT CACGTGAGCATGCAGGCCTTGGG-3′.

Liljeström et al. (10). Virus particles were harvested from the transfected cell monolayers at the time when the cells began to show evidence of cytopathic effects (CPE). When the helper was packaged efficiently [DH-EB and DI(26S)], the particles were harvested between 16 and 20 h posttransfection; when the helper was packaged inefficiently [DH-BB(5′SIN) and DH (26S)5′SIN], the samples were harvested between 30 and 40 h posttransfection.

Analysis of virus-specific RNA and protein. Protocols for the radioactive labeling of cells are described in the appropriate figure legends. RNA was isolated from transfected and infected cells by using RNAzol B as described by the manufacturer (Tel-Test, Inc., Friendswood, Tex.). Cellular RNAs and transcripts were analyzed by agarose gel electrophoresis following denaturation with glyoxal in dimethyl sulfoxide. When cells were to be analyzed for protein, they were rinsed twice with cold phosphate-buffered saline (PBS), removed from the dish by scraping in PBS, pelleted by centrifugation, and dissolved in loading buffer (0.06 M Tris-HCl [pH 6.7], 2% sodium dodecyl sulfate, 5% β-mercaptoethanol, 5% glycerol, 0.05% bromophenol blue). Protein gel analysis was performed as described by Laemmli (6). The stained gels were quantitated by using the IS-1000 Digital Imaging System (Innotech Scientific Corp., San Leandro, Calif.).

β-Galactosidase was purchased from Bethesda Research Laboratories, Life Technologies Inc. We measured an activity of this enzyme of 5×10^{2} U/mg. (One unit hydrolyzes 1.0 μmol of *o*-nitrophenyl-β-D-galactoside per min at 37°C.)

Immunofluorescence assays to determine the titers of vector particles. Vector particles that were packaged in the absence of DH RNAs did not give rise to plaques. In the experiments described here, the titers of these particles (infectious units) were determined in an immunofluorescence assay using sec-



	Titer (PFU/ml) plaque size	Titer (inf.u/ml)
a) Toto1101 RNA.	5×10^9 L	2×10^9
b) Defective helper RNAs with Sindbis 5' termini.		
DH-EB(5'SIN)	$1\text{-}2\times10^5$ S	$3\text{-}5\times10^7$
DH-SH(5'SIN)	2×10^4 S	N.D.
DH-BB(5'SIN)	$<10^3$?	$1\text{-}5\times10^7$

	Titer (PFU/ml) plaque size	Titer (inf.u/ml)
c) Toto1101 RNA with 5' terminus of tRNA Asp	4×10^9 L	N.D.
d) Defective helper RNAs with 5' terminus of tRNA Asp		
DH-EB	$1\text{-}4\times10^7$ L	$5\text{-}8\times10^7$
DH-SH	$0.5\text{-}1\times10^7$ M	N.D.
DH-BB	$2\text{-}3\times10^6$ M	$4\text{-}6\times10^7$
e) Defective helper RNAs with DI sequences.		
DH(26S)5'SIN	$<10^4$?	$0.5\text{-}3\times10^8$
DI(26S)	$1\text{-}5\times10^7$ L	N.D.

FIG. 2. Diagrams of DH RNAs. The nomenclature for these RNAs is based on the restriction enzymes used to make the indicated deletions in the cDNA plasmids. In DH-EB(5'SIN) and DH-EB, the region between the *Eco*47III and *Bam*HI sites was deleted. For DH-SH(5'SIN) and DH-SH, the region between the *Ssp*I and *Hpa*I sites was deleted. In DH-BB(5'SIN) and DH-BB, the region between the *Bsp*MII and *Bam*HI sites was deleted. After digestion of the plasmid DNAs, staggered ends were blunted before ligation and transformation. The presence of the packaging signal in the DH RNAs (nt 745 to 1225 in the genomic RNA) is indicated by the thickened black line near the 5' terminus. The packaging signal is present in both DH(26S)5'SIN and DI(26S) (4). The PFU per milliliter and infectious units (inf.u) per milliliter were determined on secondary CEF. Infectious units per milliliter was determined by immunofluorescence as described in Materials and Methods. The range of values indicates data from several different experiments and with different replicons. The vertical line and horizontal arrow indicate the start of the subgenomic RNA sequences corresponding to 26S RNA, the mRNA for the viral structural proteins. The plaque sizes are divided into L (large; a range of about 3 to 5 mm), M (medium; about 2 to 3 mm), and S (small or pinpoints). N.D., not done.

ondary cultures of chicken embryo fibroblasts (CEF). The cells were seeded on glass coverslips so that they would be subconfluent the following day when they were infected with Sindbis virus, SINrep/LacZ, or SINrep/CAT. Either 4 h after infection with Sindbis virus or 6 h after infection with the SINrep particles, the cells were fixed with cold methanol and subsequently incubated with antibodies. The number of cells infected with the replicon expressing the CAT protein was determined by using rabbit anti-CAT (5 Prime-3 Prime Inc., West Chester, Pa.); cells infected with the replicon expressing β-galactosidase were determined by using mouse monoclonal antibodies directed against this enzyme (a gift from John

Majors, Washington University). Rhodamine-conjugated goat anti-rabbit or rhodamine-conjugated goat anti-mouse antibodies were then used as secondary antibodies. The titer of infectious units was determined from the fraction of the total cells that were immunofluorescent. The total number of cells in the same field was determined by using bisbenzimide for nuclear staining.

RESULTS

Defective Sindbis virus RNAs as packaging helpers. In earlier studies, the defective RNA DI(26S) was shown to provide the structural protein genes for a Sindbis virus RNA replicon in which the CAT gene had replaced the structural protein genes (4). DI(26S) was derived from a naturally occurring Sindbis virus DI RNA and contains the region of the genome required for encapsidation. Hence, this helper is copackaged with Sindbis virus RNA replicons into particles which form plaques (Fig. 2e). This defective RNA not only served as a helper providing the structural proteins for packaging of the replicon but also acted as an interfering RNA decreasing the yield of replicon produced by the cells (4). We have now tested a variety of different defective RNAs as packaging helpers. The ones analyzed in the most detail are listed in Fig. 2. They are divided into three groups with some overlapping features. Those in the first group (Fig. 2b) all have a 5′ terminus identical to that of the Sindbis virus RNA genome (SIN 5′). Those in the second group (Fig. 2d) have nt 10 to 75 derived from the mammalian $tRNA^{Asp}$. This is the 5′ terminus found on some of the naturally occurring DI RNAs of Sindbis virus (14). The first two groups of DH RNAs contain large internal deletions in the nonstructural region of the Sindbis virus genomic RNA. The RNAs in which the deletion starts downstream of nt 1400 retain the encapsidation signal; those RNAs in which the deletion encompasses nt 746 to 1226 are deleted of this element (28).

The third group of defective RNAs (Fig. 2e), which includes DI(26S), was derived from the cDNA of a DI genome that contains both deletions and rearrangements of the Sindbis virus genomic RNA (15). Both contain the packaging signal, but DH(26S)5′SIN differs from DI(26S) in that it contains the normal 5′ terminus of the Sindbis virus genome RNA instead of the truncated $tRNA^{Asp}$.

We compared the different DH RNAs for the ability to be replicated and packaged as well as for the ability to package the replicon RNAs. BHK cells were transfected by electroporation with replicon RNAs, with defective helper RNAs, or with both. The values for infectious units were determined for replicons expressing the *lacZ* gene or the CAT gene (Materials and Methods and Fig. 2). The PFU-per-milliliter values listed in Fig. 2 measure the number of particles in which the replicon and helper RNAs were copackaged (4). The release of PFU in the media varied over a wide range and was dependent on the source of DH RNA, not on the replicon. From the RNA analysis (see below), we assumed that the PFU were due to copackaging or coinfection and not to recombination (29). PFU could be detected with all of the helper RNAs except DH-BB(5′SIN) and DH(26S)5′SIN. These results indicated that removal of the packaging signal was not sufficient to prevent some level of encapsidation of the DH RNAs.

For many of the samples, titers of the particles expressing either β-galactosidase or CAT were determined by immunofluorescence (Fig. 2 and Materials and Methods). These values fell into a much narrower range, although titers were consistently highest when DH(26S)5′SIN was used as the helper. In the previous study using DI(26S), we had determined that the level of particles containing only replicon RNA was twice that of copackaged particles (4). A similar result was obtained with DH-EB RNA. RNA analysis indicated that DH-EB did not interfere with the replication of the replicon to the same extent as had been observed with DI(26S) (data not shown). A comparison of the different DH RNAs showed that the ratio of infectious units to PFU was several orders of magnitude higher when the 5′ terminus of the helper RNA was identical to that of the Sindbis virus genomic RNA.

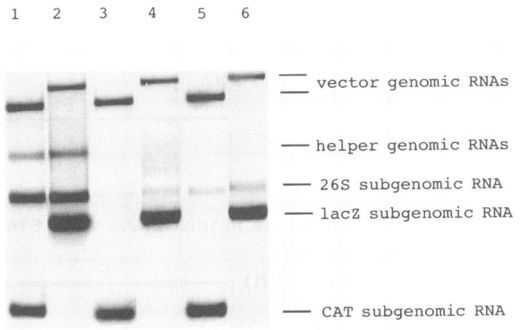


FIG. 3. RNAs synthesized in cells transfected with either the SINrep/LacZ or SINrep/CAT replicon RNA and various DH RNAs. BHK cells were transfected by electroporation with 2 μg of each RNA. Seven hours postelectroporation, dactinomycin (1 μg/ml) and [^{3}H]uridine (20 μCi/ml) were added; 4 h later, cellular RNA was isolated and analyzed by electrophoresis in an agarose gel (see Materials and Methods). The RNA bands were visualized by fluorography. Lanes: 1, 3, and 5, transfection with SINrep/CAT RNA; 2, 4, and 6, transfection with SINrep/LacZ RNA; 1 and 2, transfection with DH-EB RNA; 3 and 4, transfection with DH-BB(5′SIN) RNA; 5 and 6, transfection with DH(26S)5′SIN RNA.

We examined the pattern of Sindbis virus-specific RNAs synthesized in cells cotransfected with replicon RNA and each of the following three DH RNAs: DH-EB, DH-BB(5′SIN), and DH(26S)5′SIN (Fig. 3). Replication of the replicon RNA was observed in the absence of helper RNA, but the DH RNA was not detected in the absence of the replicon (data not shown). Only DH-EB, the defective RNA that has a 5′ $tRNA^{Asp}$ and retained the packaging sequence, was replicated to a level comparable to that of the replicon RNA (Fig. 3, lanes 1 and 2). The other DH genomic RNAs were barely detectable, although their subgenomic 26S RNAs could be seen. Despite the low levels of DH-BB(5′SIN) and DI(26S)5′SIN RNAs, sufficient quantities of the structural proteins were synthesized to allow efficient packaging of the replicon RNAs.

RNA synthesis in cells infected with Sindbis virus vectors. We next analyzed the viral RNAs synthesized in BHK cells that had been infected with particles released from the different cotransfections (Fig. 4). In cases such as DH-EB, for which significant levels of PFU were observed, RNA analysis allows one to discriminate between copackaging of replicon and DH RNAs and RNA recombination to generate plaque-forming virus (4, 29). In cells infected with the SINrep/CAT particles, even those packaged by DH-EB, no RNAs the size of Sindbis virus genomic RNA could be detected (Fig. 4, lanes 1 to 3). The Sindbis virus and SINrep/LacZ genomic RNAs migrated to similar positions on the gel and probably would not be distinguished (Fig. 4, lanes 4 to 6). In previous studies in which recombination has been detected, the recombinant genomic and subgenomic RNAs migrated more slowly than the original parental RNAs (29). These observations indicate that recombination to generate infectious Sindbis virus either had not occurred or was present at a level too low to be detected in our

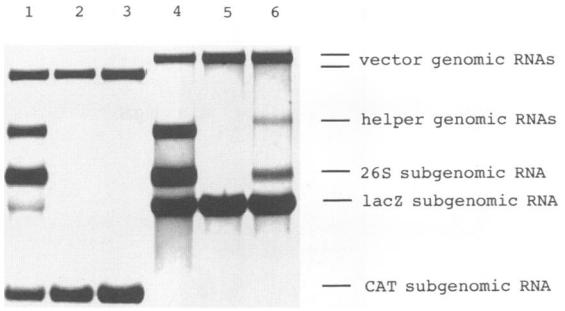


FIG. 4. RNAs synthesized in cells infected with either SINrep/LacZ or SINrep/CAT particles. BHK cells in 12-well dishes were infected at MOIs of 20 to 30 on the basis of infectious units-per-milliliter titers. Two hours postinfection, dactinomycin (1 μg/ml) and [^{3}H]uridine (20 μCi/ml) were added; 4 h later, cellular RNA was isolated and analyzed as described in the legend to Fig. 3. Lanes: 1 and 4, replicons packaged by using DH-EB RNA; 2 and 5, replicons packaged by using DH-BB(5′SIN) RNA; 3 and 6, replicons packaged by using DH(26S)5′SIN RNA.

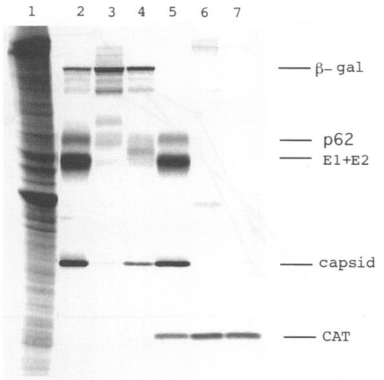


FIG. 5. Radiolabeled proteins in cells infected with either SINrep/LacZ or SINrep/CAT particles. CEF in 12-well dishes were infected at MOIs of approximately 25 infectious units. Twelve hours postinfection, the cells were washed three times with PBS and incubated at 37°C in minimal essential medium minus methionine containing 2% fetal calf serum. Fifteen minutes later, they were pulsed with 10 μCi of [^{35}S]methionine per ml for 15 min and then subjected to a 15-min chase with complete minimal essential medium. Lanes: 1, uninfected cells; 2, cells infected with SINrep/LacZ particles packaged with DH-EB; 3, cells infected with SINrep/LacZ particles packaged with DH-BB(5′SIN); 4, cells infected with SINrep/LacZ particles packaged with DH(26S)5′SIN; 5, SINrep/CAT particles packaged with DH-EB; 6, cells infected with SINrep/CAT particles packaged with DH-BB; 7, cells infected with SINrep/CAT particles packaged with DH (26S)5′SIN. The bands labeled as β-gal (β-galactosidase) and CAT were identical in mobility to the authentic proteins.

experiments. Rather, this analysis showed that the SINrep/LacZ and SINrep/CAT genomic and subgenomic RNAs maintained their correct sizes and showed no evidence of instability. The relatively high levels of the helper RNA DH-EB and its subgenomic RNA (Fig. 4, lanes 1 and 4) were consistent with the levels of PFU obtained with this helper RNA (Fig. 2).

Two of the defective helpers [DH-BB(5′SIN) and DH (26S)5′SIN] produced high titers of particles containing replicon RNA with little or no PFU (Fig. 2). We were only able to put an upper limit on the level of PFU. Plaques could not be detected at the lowest dilutions, since monolayers were destroyed as a consequence of infection by replicon particles. Analysis of radioactive viral RNAs and viral proteins produced in cells infected with replicon particles provided another assay for detecting the presence of packaged DH RNAs. DH (26S)5′SIN genomic and subgenomic RNAs were detected at significant levels in cells that had been infected with SINrep/LacZ particles (Fig. 4, lane 6) and at trace levels in cells infected with SINrep/CAT (Fig. 4, lane 3). The same multiplicity of infection (MOI) of replicon-containing particles (infectious units) had been used. Differences in the amount of the DH RNAs were due to higher titers of DH(26S)5′SIN RNA-containing particles in the SINrep/LacZ stock used for these experiments.

No DH genomic or subgenomic RNA was detected in cells infected with SINrep/LacZ particles or SINrep/CAT particles which had been packaged by using DH-BB(5′SIN), indicating that there was little or no DH RNA present in these particles.

Protein synthesis in cells infected with Sindbis virus replicons. We examined the synthesis of the heterologous proteins in two different host cells, CEF and BHK cells, that had been infected with SINrep particles. The synthesis of radiolabeled proteins in CEF 12 h postinfection is shown in Fig. 5. The autoradiogram demonstrated that the major proteins synthesized were the heterologous protein translated from the replicon subgenomic mRNA and in some cases the viral structural proteins translated from the DH subgenomic RNA. The synthesis of host proteins was inhibited as has been observed in cells infected with Sindbis virus and in cells infected with the SFV replicons (9). There was a direct correlation between the levels of DH RNAs and the levels of the Sindbis virus structural proteins. When DH-BB(5′SIN) was used as the defective helper, no DH RNAs were detected (Fig. 4, lanes 2 and 5) and the level of PFU was less than 10^3/ml. Cells infected with particles produced by this helper appeared to synthesize trace levels of a species which comigrates with the Sindbis virus capsid protein (Fig. 5, lane 3), but we have not verified the identity of this protein by other criteria.

Suppression of host cell RNA and protein synthesis after infection was more rapid in CEF, but CPE also appeared more quickly (data not shown). Dramatic CPE in CEF occurred by 18 to 20 h post infection at 37°C and by 32 h at 30°C. In contrast, CPE in BHK cells could be mitigated by reducing the serum concentration in the medium from 10 to 2%. The synthesis of β-galactosidase continued in these cells for at least 48 h at 37°C when they were infected with particles that did not contain detectable levels of PFU (Fig. 6, curve 1). The integrity of these infected cells was maintained for longer periods at 34°C (Fig. 6, curve 2) or 30°C (data not shown). Maintenance of the infected cells at a lower temperature may be useful for large-scale preparations of some proteins.

Cotransfection of replicon RNA and the defective helper DH-EB produced a significant level of particles that gave rise to PFU (Fig. 2) and contained both DH and replicon RNAs (Fig. 4, lanes 1 and 4). BHK cells infected with relatively low MOIs of plaque-forming particles accumulated levels of β-galactosidase comparable to those in cells infected only with SINrep/LacZ particles (Fig. 6, curve 3; Fig. 7). At higher MOIs, the levels of β-galactosidase decreased (Fig. 7). This decrease may be due to the earlier onset of CPE in cells infected with particles containing significant levels of PFU. When BHK cells were infected at the lower MOIs, it was possible to use particles obtained from this passage to produce β-galactosidase at equivalent levels in a second infection (data not shown).

The expression of heterologous proteins in BHK cells was also determined by staining of protein samples after electro-

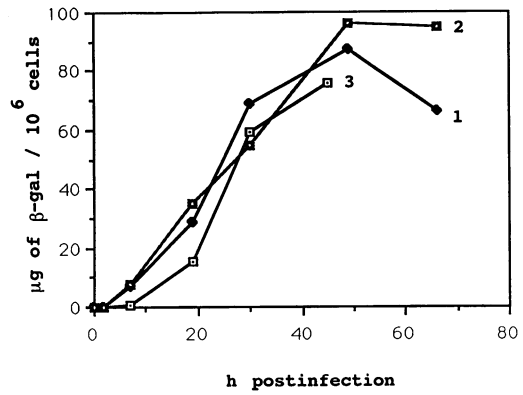


FIG. 6. Synthesis of β-galactosidase in cells infected with SINrep/LacZ particles. SINrep/LacZ replicons were packaged either with DH-BB(5'SIN) RNA to produce replicon-containing particles with no detectable PFU or with DH-EB RNA to produce both particles containing only replicon RNA and particles containing both replicon and DH RNAs. BHK cells in 35-mm-diameter dishes were infected with SINrep/LacZ/DH-BB(5'SIN) particles at 25 infectious units per cell (curves 1 and 2) or with SINrep/LacZ/DH-EB particles at 1 infectious unit and 0.3 PFU per cell (curve 3). Cells were incubated in medium containing 2% fetal calf serum at 37°C (curves 1 and 3) or 34°C (curve 2) and assayed for β-galactosidase (β-gal) activity at the indicated times.

phoresis on acrylamide gels. The amount of β-galactosidase synthesized in infected cells was calculated from a comparison with the purified enzyme (Fig. 8). At 26 h postinfection, the value was 16 μg/10^6 cells (Fig. 8, lane 4); at 42 h, it was 24 μg/10^6 cells (Fig. 8, lane 5). These numbers were considerably lower than those calculated from a comparison of enzymatic activities. One possible explanation for this discrepancy is that the specific activity of the purified enzyme was not as high as that of the newly synthesized protein obtained from the infected cells.

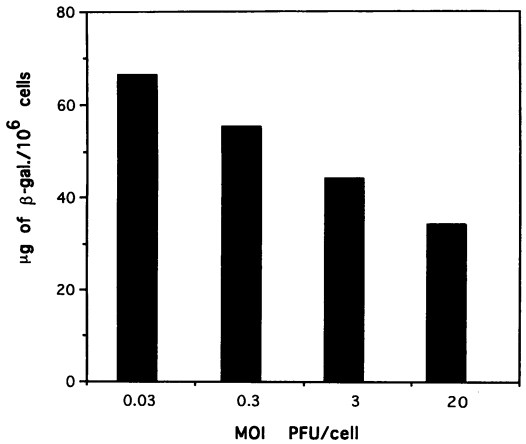


FIG. 7. β-Galactosidase activity in BHK cells infected with SINrep/LacZ/DH-EB particles as a function of MOI. Particles produced after electroporation (passage 0) were used to infect BHK cells at the indicated MOIs. The ratio of infectious units to PFU in these particles was 1:0.3. The cells were harvested at 24 h postinfection for determination of β-galactosidase (β-gal) activity.

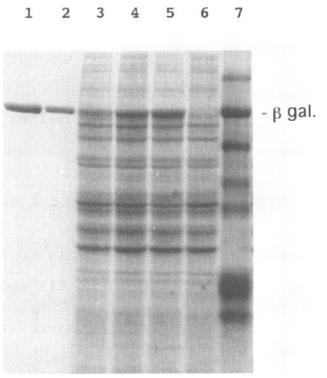


FIG. 8. β-Galactosidase accumulation in BHK cells. Shown are protein patterns in BHK cells as detected by Coomassie blue staining. BHK cells were infected with SINrep/LacZ particles which had been packaged by using DH-BB(5'SIN) as the helper. Lanes: 1 and 2, purified β-galactosidase (β-gal; GIBCO BRL) at 2 and 1 μg, respectively; 3 to 5, samples from cells infected with SINrep/LacZ particles at 18 h (lane 3), 26 h (lane 4), and 42 h (lane 5) postinfection; 6, uninfected cells; 7, molecular weight markers. The BHK cells were infected at an MOI of 25 infectious units per cell. The samples loaded on the gel represented the equivalent of 5×10^4 cells.

DISCUSSION

The concept that alphaviruses can be developed as expression vectors was first established by Xiong et al. (32). Since then, several improvements have made the use of these RNA replicons as expression vectors more practical. A significant advance was the use of electroporation to introduce the RNA into cells (10). With alphavirus RNAs, electroporation can give an efficiency of transfection of close to 100%, a level that was not obtained with DEAE-dextran or Lipofectin. The ability to package the replicon RNA into virion particles under conditions in which it is the predominant RNA in the particles also increases the ease with which they can be used and the variety of cell types which can be used for expression studies. One obvious advantage of the helper RNAs which are themselves inefficiently packaged is to allow expression of the heterologous product in the absence of Sindbis virus structural proteins which, as discussed below, can affect the survival of the host cell. In this report, we compared several different defective Sindbis virus RNAs to determine which would be the most effective as helpers to package Sindbis virus replicons. We also determined the ability of these DH helper RNAs to be replicated and packaged. Many naturally occurring DI RNAs generated by high-multiplicity passaging of Sindbis virus have a truncated $tRNA^{Asp}$ at the 5′ terminus replacing the 5′-terminal 30 nt of the genomic RNA (14). DH RNAs with this $tRNA^{Asp}$ at the 5′ terminus were always replicated to higher levels than were RNAs which differed only by having a 5′ terminus identical to that of Sindbis virus genomic RNA (Fig. 2) (20). They also produced higher levels of PFU, indicating that they were packaged to higher levels than were DH RNAs which had the Sindbis virus genomic 5′ terminus. Although the higher levels of packaging of these defective RNAs were most likely the result of their higher levels in the transfected cells, we cannot rule out the possibility that the 5′ $tRNA^{Asp}$ has some role, perhaps one affecting the structure of the RNA, which allows more efficient encapsidation.

Previous studies had shown that a DI RNA with a deletion that includes the packaging signal was not replicated to the same extent as was a DI RNA containing this region (28).

Some of the DH RNAs described in this report had larger deletions which extended even further upstream (Fig. 2), and these replicated at levels that were barely detectable in our assays. The large deletions may produce some sort of structural abnormality in the RNA that affects its ability to be replicated. Sequences in this region of the genome could also have a more direct function in replication by providing a binding site(s) for proteins involved in viral RNA replication. There is ample precedent for internal sequences in an RNA genome having a role in RNA replication. The best-established example is that of the Qβ RNA bacteriophage: the viral polymerase initiates replication by binding to an internal site on the Qβ RNA (12, 13). Studies with brome mosaic virus (3), flock house virus (1), and turnip crinkle virus (27) RNAs have led to the identification of *cis*-acting internal regions in these genomes which are important in RNA replication.

Several studies have suggested that the capsid protein of alphaviruses may be an important factor in the inhibition of host cell protein synthesis (24–26, 30, 31). Our studies with the Sindbis virus replicons, taken together with those of Liljeström and Garoff (9) for the SFV replicons, establish that host protein synthesis is inhibited under conditions in which essentially little or no capsid protein is being synthesized. These results suggest that some other mechanism is responsible for the shutoff of host protein synthesis. The synthesis of the viral structural proteins, however, did influence survival of BHK cells. When these cells were infected with parental Sindbis virus or with Sindbis virus particles containing a segmented genome and the structural proteins were expressed, CPE was observed by 12 h postinfection. In contrast, BHK cells infected with particles containing replicon RNAs with little or no DH RNA did not show similar CPE until about 48 h.

Our results with packaged Sindbis virus replicon particles indicate that even in those examples in which the viral structural proteins were not detected, low amounts of helper RNAs may be packaged. High levels of particles containing only replicon RNA destroyed the cell monolayers used for plaque titrations, and PFU present at concentrations of less than 10^{-4} those of the replicon could not be assayed. Thus, even replicon particles that are thought to be virus free have the potential for being infectious, and appropriate caution must be used. In addition, recombination between alphavirus replicons and DH RNAs has been observed (29), and the possibility that recombination can occur should be considered. We have detected recombination in transfected BHK cells after electroporation very rarely and only when the transfection efficiency was poor. The replication of recombinants is most probably suppressed under conditions in which most cells are transfected with the replicon RNA.

In some instances, infectious virus expressing a heterologous product may be of value. The possibility of using Sindbis virus expression vectors to generate an immune response has been demonstrated by Hahn et al., who used a virus in which the nonsegmented genomic RNA produced two subgenomic RNAs (5). The segmented genome expression vectors described here can also be used for studies in which virus amplification is desired. The DH RNA (DH-EB) was partially disabled in its ability to be replicated [compared with DI(26S)] and did not interfere significantly with the replication or expression of the replicon RNA. The two RNAs were able to complement each other at a level which permitted both RNAs to undergo many rounds of replication and packaging. Spread of an infection, to augment protein production and cell types infected, could be important for studies in animals. Also, the ability to use low-input MOIs may also be useful for scaling up production of heterologous proteins.

Our results provide further support for the potential of using alphaviruses as vectors for the expression of heterologous proteins (2, 10, 19). BHK cells infected with the SINrep/LacZ replicon or the analogous SFV replicon produce similar levels of β-galactosidase (compare Fig. 6 and results presented in reference 9). The high levels of expression and the relative ease of engineering the expression constructs are two important features. The wide host range of these viruses and the ability to modulate the levels of expression also add to the versatility of this expression system.

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P.J.B. and I.F. contributed equally to this work.

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