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Sindbis Virus DNA-Based Expression Vectors: Utility for In Vitro and In Vivo Gene Transfer

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Several DNA-based Sindbis virus vectors were constructed to investigate the feasibility and potential applications for initiating the virus life cycle in cells transfected directly with plasmid DNA. These vectors, when transfected into mammalian cells, have been used to produce virus, to express heterologous genes, and to produce infectious vector particles. This approach involved the conversion of a self-replicating vector RNA (replicon) into a layered DNA-based expression system. The first layer includes a eukaryotic RNA polymerase II expression cassette that initiates nuclear transcription of an RNA which corresponds to the Sindbis virus vector replicon. Following transport of this RNA from the nucleus to the cytoplasm, the second layer, autocatalytic amplification of the vector, proceeds according to the Sindbis virus replication cycle and results in expression of the heterologous gene. The Sindbis virus DNA vectors expressed reporter genes in transfected cells at levels that were comparable to those of in vitro-transcribed RNA replicons and were approximately 10-fold higher than the levels produced by conventional RNA polymerase II-dependent plasmids in which the promoter and reporter gene were linked directly. Reporter gene expression was also observed in rodent muscle following injection with Sindbis virus DNA vectors. In a second application, packaged vector particles were produced in cells cotransfected with complementing replicon and defective helper DNAs. The Sindbis virus-derived DNA vectors described here increase the utility of alphavirus-based vector systems in general and also provide a vector with broad potential applications for genetic immunization.

Several members of the *Alphavirus* genus, first Sindbis virus (4, 45, 53) and later Semliki Forest virus (1, 23, 24) and other alphavirus members (8, 9), have received considerable attention for use as virus-based expression vectors. Many properties of alphavirus vectors make them a desirable alternative to other virus-derived vector systems being developed, including potential high-level expression of up to 10^8 molecules of heterologous protein per cell (53), a broad host range, and infection of nondividing cells (50). In addition, replication occurs entirely in the cytoplasm of the infected cell as an RNA molecule, without a DNA intermediate. This is in contrast to retrovirus and adeno-associated virus vectors, which must enter the nucleus and usually integrate into the host genome for initiation of vector activity (18, 27, 43). Thus, retrovirus- and adeno-associated virus-derived vectors have application for long-term expression of foreign proteins, while the alphavirus vectors are likely better suited for short-term high-level expression. Furthermore, although vectors derived from poxviruses, adenoviruses, and herpes simplex viruses all express high levels of foreign proteins, these systems are far more complex than alphaviruses and express many highly antigenic virus-specific gene products, including structural proteins (13, 29, 41). In contrast, current alphavirus vectors express only the four viral replicase proteins (nonstructural proteins nsP1 through nsP4) required for RNA amplification in the transduced cell.

The approximately 12-kb single-stranded positive-sense RNA genome of Sindbis virus and other alphaviruses is infectious upon introduction into the cytoplasm of susceptible cells. During viral replication, the Sindbis virus genomic 49S RNA serves as the template for synthesis of a complementary negative strand by the virus-encoded replicase. The negative strand in turn serves as the template for additional genomic RNA and for an abundant internally initiated 26S subgenomic RNA. The nonstructural proteins (nsPs) are translated from the 5′ two-thirds of the genomic RNA, while the structural proteins (sPs) are translated from the subgenomic 26S RNA that represents the 3′ one-third of the genome. The nsP and sP genes are each expressed as polyproteins and are processed posttranslationally into the individual proteins (50). Expression from current alphavirus vectors is based on the same strategy as expression of the sPs of wild-type virus and is initiated by transfection of in vitro-transcribed, self-replicating vector RNA (replicon) molecules (24, 53). The region encoding the virus sPs is replaced with a heterologous sequence or gene of interest, and the viral nsP-encoding region and all sequences required in *cis* for replication and packaging are maintained. Heterologous sequences are synthesized as highly abundant subgenomic mRNA molecules, which in turn serve as the translational template for the heterologous gene. Infectious vector particles have been generated by cotransfection and *trans* complementation of vector RNA replicons with an in vitro-transcribed defective helper (DH) RNA (5). The DH RNA contains the genes encoding the virus sPs and all of the sequences required in *cis* for replication but is deleted in the viral nsP genes and the virus packaging sequence core (1, 51). Thus, replication of the DH RNA and expression of high levels of the sPs occur in the presence of vector-supplied nsPs and result in the production of particles containing vector genomes.

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Despite the current interest in alphavirus-derived expression vectors, further development of the system is required for potential human vaccine and therapeutic applications. The preparation and transfection into animals of in vitro-transcribed expression vector RNA are rather inefficient, due in part to the length and lability of the vector molecules. This limitation has been circumvented somewhat by the production of recombinant vector particles through cotransfection of vector replicon and DH RNA molecules. However, this approach results in the generation of replication-competent virus through mechanisms of copackaging of vector and DH RNAs and/or RNA recombination (12, 52). The level of replication-competent virus has been reduced significantly in the Semliki Forest virus system by DH modifications which result in the production of noninfectious packaged vector particles (1). An alternative approach, which has facilitated the application of Sindbis virus vectors to animal studies, utilizes a duplicated subgenomic promoter within the context of the viral genome (15, 38). However, double subgenomic vectors are propagated as infectious virus, which may be undesirable for applications involving human therapeutics, and the capacity for heterologous genetic material in this system is restricted.

We have been investigating the possibility of using DNA-based plasmid expression vectors to directly initiate the alphavirus RNA replication cascade in transfected mammalian cells. Such vectors are a necessary step towards a goal of developing Sindbis virus-derived DNA-based therapeutic vectors and vector particle-packaging cell lines. In this report, we describe the development of DNA-based Sindbis virus vector replicons. Transfection of these plasmids into cultured cells or animal muscle resulted in high-level expression of foreign genes. The template for gene expression is a self-replicating vector RNA molecule that resulted in synthesis of reporter proteins at levels which were significantly higher than those of conventional expression plasmids in which the promoter and reporter gene were directly linked. Furthermore, we demonstrate that cotransfection of replicon vector and DH plasmid DNAs or in vitro-transcribed RNAs produced similar levels of packaged Sindbis virus vector particles. This work represents the first report detailing the efficient specific initiation of an alphavirus infection in an RNA polymerase II promoter-dependent manner, following transfection of plasmid DNA, and represents a novel approach for the use of Sindbis virus-derived vectors. Similar approaches should facilitate the investigations of other cloned RNA viruses of positive polarity, the utility of their derived expression vectors, and their application to animal studies.

MATERIALS AND METHODS

Virus propagation and purification. A virus stock derived from the HR strain (5) of Sindbis virus was obtained from Lee Biomolecular (San Diego, Calif.). Following growth by passage in BHK-21 cells, virus was cloned by five consecutive rounds of plaque purification as described before (48). A large plaque was selected and expanded by a single low-multiplicity passage in BHK cells to provide a seed stock for subsequent experiments. Virus to be used for RNA isolation was isolated from BHK cell supernatants collected at 18 h postinfection by polyethylene glycol precipitation (48) or by pelleting through a sucrose cushion (35).

Construction of genome-length Sindbis virus cDNA clones. RNA was purified from pelleted virus with RNAzol B (Tel-Test, Inc., Friendswood, Tex.) according to the manufacturer's directions or by poly(A) selection after sodium dodecyl sulfate (SDS) lysis, using a commercially available kit (FastTrack Kit; Invitrogen, San Diego, Calif.). Two rounds of first-strand cDNA synthesis were performed with the purified virion RNA, using a mixture of random primers and primer SIN11703R (Table 1) and SuperScript reverse transcriptase (Gibco-BRL, Gaithersburg, Md.), according to the manufacturer's conditions. The viral cDNA was then amplified in six distinct segments by PCR with six pairs of overlapping primers (sequences may be obtained from the corresponding author). The regions of overlap corresponded to unique enzyme recognition sites within the PCR amplicons and were *Age*I (nucleotide [nt] 3172), *Eco*RI (nt 5870), *Bam*HI (nt 7335), *Bcl*I (nt 9356), and *Bsi*WI (nt 10379). PCR amplification of Sindbis virus cDNA and all subsequent amplifications were performed in separate reactions with the Thermalase thermostable DNA polymerase (Amresco Inc., Solon, Ohio) buffer containing 1.5 mM $MgCl_2$ (provided by the supplier) and the desired primer pair. Additionally, the reactions contained 5% dimethyl sulfoxide and Hot Start Wax beads (Perkin-Elmer, Branchburg, N.J.). The Sindbis virus 5′-end forward primer (SINSP61F [Table 1]) contained a 19-nt sequence corresponding to the bacteriophage SP6 RNA polymerase promoter and the *Apa*I recognition sequence. The SP6 RNA promoter was positioned so that transcription in vitro resulted in the inclusion of only a single nonviral G ribonucleotide linked to the authentic Sindbis virus 5′ end. Inclusion of an upstream *Apa*I recognition sequence facilitated insertion of the PCR amplicon into the plasmid vector (pKSII$^+$; Stratagene, La Jolla, Calif.) polylinker sequence. In addition to sequences that were complementary to the viral RNA 3′ end, the Sindbis virus 3′-end reverse primer (11703R [Table 1]) contained a tract of 25 consecutive thymidylate residues followed by the *Xba*I recognition sequence. The six PCR amplicon products were first digested with the appropriate enzymes and then inserted stepwise into the pKSII$^+$ vector between the *Apa*I and *Xba*I sites.

The Sindbis virus genomic cDNA clone sense strand sequence was determined by the dideoxy-chain termination method (44) and revealed several nucleotide differences from the published Sindbis virus HRsp sequence (49). Reverse transcription-PCR amplicons derived from the corresponding regions in virion RNA were sequenced directly to determine whether the nucleotide differences were the result of cloning artifacts or strain variation. Artifact analysis of silent mutations in the 3′ wobble position of codons was not performed. Such analysis identified cloning artifacts at viral nt 2245, 6193, and 6730 that resulted in the nonconservative amino acid (aa) changes Gly→Glu, Asp→Gly, and Tyr→Cys, respectively, in the nsP gene coding region. These nucleotide changes were repaired by substitution with reverse transcription-PCR amplicons between bases 1407 and 2289 (nt 2245 change, *Eco* 47III-*Bgl*II fragment) and bases 5870 and 6920 (nt 6193 and nt 6730 changes, *Eco*RI-*Hpa*I fragment) derived from genomic RNA of fivefold-plaque-purified virus seed stock. Cloning artifacts which resulted in relatively conservative changes at nt 3822 (nsP2 aa 715, Thr→Ala) and at nt 5466 (nsP3 aa 456, Gly→Ser) were not repaired. Artifact analysis was not performed on base changes at nt 1095 (nsP1 aa 346, Ile→Leu) and at nt 5614 (nsP3 aa 505, Val→Ala), which resulted in conservative changes. These base changes were not repaired. Additionally, artifact analysis of the single noncoding region change observed in our laboratory strain at nt 45 (T→C) was not performed. Strain-specific nucleotide differences in the nsP and sP genes of the genomic cDNA clone are presented in the Results. The full-length cDNA clone was designated pRSINg and could be linearized by digestion at a unique *Xba*I site downstream from the poly(A) tract for in vitro transcription. Relatively conservative changes, on which artifact analysis was not performed or which resulted from a cloning artifact, were not repaired because the growth characteristics of virus derived from BHK cells transfected with RNA transcribed in vitro from pRSINg were indistinguishable from those of our plaque-purified wild-type Sindbis virus stock.

Construction of genome-length Sindbis virus plasmid DNA vectors. For the construction of plasmid DNA vectors, clones of RNA polymerase II promoters linked to Sindbis virus genomic cDNA were inserted into pCDNA3 (Invitrogen) between the unique *Bgl*II and *Xba*I sites. The Moloney murine leukemia virus (MoMLV) long terminal repeat (LTR) U3 region (36), the simian virus 40 (SV40) early region (39), and the cytomegalovirus (CMV) immediate-early (IE) (2, 30, 47) promoters were positioned at the Sindbis virus genomic 5′ end by overlapping PCR (16) with the primers shown in Table 1. The templates for promoter amplification were the BAG vector (36), pBR328/SV40 plasmid DNA (ATCC 45019), and human CMV DNA (Towne strain; ATCC VR-977). A *Bgl*II site was included at the 5′-end forward primer for each promoter to facilitate insertion into the pCDNA3 plasmid. The bovine growth hormone transcription termination/polyadenylation signal was from the pCDNA3 plasmid. These constructions were designated pDLTRSINg, pDSV40SINg, and pDCMVSINg, respectively.

Construction of Sindbis virus DNA and RNA expression vectors and DHs. Sindbis virus plasmid DNA and RNA replicon expression vectors contained viral nt 1 to 7643, pKSII$^+$ polylinker, viral nt 11664 to 11703, and a 25-mer synthetic poly(A) tail and were constructed from the pRSINg, pDLTRSINg, and pDCMVSINg plasmids. The RNA expression vector contained the SP6 promoter at its 5′ end, and the DNA expression vectors contained either the MoMLV LTR, SV40, or CMV IE promoter at their 5′ ends and the bovine growth hormone transcription termination/polyadenylation signal at their 3′ ends. The PCR amplicon product obtained with primer pair SIN3144F and SIN7643R (Table 1) was used to construct a portion of the expression vectors which includes nt 1 to 7643. A unique *Xho*I site was introduced into the 5′ end of primer SIN7643R to facilitate insertion of the amplicon between the *Sfi*I site at Sindbis virus nt 5122 and the *Xho*I site in the pKSII$^+$ polylinker. The primer pair SIN11644F and SIN11703R (Table 1) PCR amplicon product was used to assemble the vector 3′ end between unique *Not*I and *Sac*I sites at the 3′ end of the pKSII$^+$ polylinker. For insertion into DNA expression plasmids, the 3′-end *Sac*I site of the Sindbis virus vector and the unique *Xba*I site of pCDNA3 were digested and blunted with T4 DNA polymerase, and the fragments were ligated. The bacterial *lacZ* gene, obtained from plasmid pSV-β-galactosidase (Promega,

TABLE 1. Primers used to construct Sindbis virus-based vectors

Primer	Sequence (5′→3′)	Enzyme site(s)[a]
Genomic cDNA clones		
SINSP61F	TATATGGGCCCGATTTAGGTGACACTATAGATTGACGGCGTAGTACAC	*Apa*I
SIN11703R	TATATTCTAGA(T_{25})GAAATG	*Xba*I
Genomic plasmid DNA vectors		
MoMLV promoter		
BAGB2F	TATATAGATCTAATGAAAGACCCCACCTGTAGG	*Bgl*II
BAGwt441R2	TCAATCCCCGAGTGAGGGGTTGTGGGCTCTTTTATTGAGC	O.L.
MLV/SIN1F	CCACAACCCCTCACTCGGGGATTGACGGCGTAGTAC	*O.L.*
SIN3182R	CTGGCAACCGGTAAGTACGATAC	*Age*I
SV40 early-region promoter		
B2SVpr250F	TATATATAGATCTGGTGTGGAAAGTCCCCAGGC	*Bgl*II
SINSV5235R	CTACGCCGTCAATGCCGAGGCGGCCTCGGCC	O.L.
SVSIN1F	GGCCGCCTCGGCATTGACGGCGTAGTACACACTATTG	O.L.
SIN3182R	CTGGCAACCGGTAAGTACGATAC	*Bgl*II
CMV IE promoter		
pCBgl233F	TATATATAGATCTTTGACATTGATTATTGACTAG	*Bgl*II
SINCMV1142R	CCGTCAATACGGTTCACTAAACGAGCTCTGCTTATATAGACC	O.L.
CMVSIN1F	GCTCGTTTAGTGAACCGTATTGACGGCGTAGTACACAC	O.L.
SIN3182R	CTGGCAACCGGTAAGTACGATAC	*Bgl*II
Sindbis virus DNA and RNA expression vectors		
Vector nsP region		
SIN3144F	ATACTAGCCACGGCCGGTATC	*Age*I
SIN7643R	TATATCTCGAGGGTGGTGTTGTAGTATTAGTCAG	*Xho*I
Vector 3′ end		
SIN11664F	TATATGCGGCCGCTTTCTTTTATTAATCAACAAAATTTTGTTTTTAA	*Not*I
SIN11703R	TATATGAGCTGGTTTAAACAGGAGCTC(T_{25})GAAATGTTAAAA	*Pme*I, *Sac*I
HDV antigenomic ribozyme sequence		
HDV1F	TATATGAGCTCGGGTCGGCATGGCATCTCCACCTCCTCGCGGTCCG	*Sac*I
HDV17F	TCCACCTCCTCGCGGTCCGACCTGGGCATCCGAAGGAGGACGCACGTCCACT	O.L.
HDV84R	TATATGAGCTCCTCCCTTAGCCATCCGAGTGGACGTGCGTCCTCCTTC	*Sac*I

[a] Amplicon-unique enzyme recognition site present at the primer 5′ end. O.L., overlap; primer is partially complementary with another primer, for use in overlapping PCR.

Madison, Wis.), and the firefly luciferase gene, obtained from plasmid pT3/T7-luc (Clontech, Palo Alto, Calif.), were inserted into the polylinker of the DNA- and in vitro-transcribed RNA-based expression vectors. These constructions were designated pRSIN-luc and pRSIN-β-gal (in vitro-transcribed RNA expression vectors) and pDLTRSIN-luc, pDLTRSIN-β-gal, pDCMVSIN-luc, and pDCMVSIN-β-gal (DNA expression vectors). Linearization of pRSIN-luc and pRSIN-β-gal for in vitro transcription was done with *Sac*I or *Pme*I, respectively.

The synthetic poly(A) tract in plasmid pDLTRSIN-luc was deleted by overlapping PCR to fuse the vector 3′ end, at Sindbis virus nt 11703, with the bovine growth hormone transcription termination/polyadenylation sequence (nt 1132 to 1180, pCDNA3 numbering). Additionally, the vector 3′ end was fused to the SV40 early-region transcription termination signal (nt 2643 to 2588; numbering from reference 10). These constructions were designated pDLTRSIN-lucdlA3′BGH and pDLTRSIN-lucdlA3′SV40.

In other constructions, the hepatitis delta virus (HDV) antigenomic ribozyme (34) was inserted between the synthetic A_{25} tract and the transcription termination/polyadenylation signal of the pDLTRSIN-luc vector. The HDV ribozyme sequence, with *Sac*I sites at each end, was generated by PCR with the primers shown in Table 1. Correct- and reverse-sense HDV insertions were verified by sequence analysis; these constructions were designated pDLTRSIN-lucHDV and pDLTRSIN-lucHDVr, respectively.

Additional plasmids were constructed to compare expression levels with Sindbis virus DNA expression vectors. In plasmid pDLTRdlnsPSIN-luc, nt 1407 to 6920 of the nsP coding region in vector pDLTRSIN-luc were removed by digestion with *Eco*47III and *Hpa*I, which was followed by blunt-end ligation. Plasmid pLTR-luc was constructed by substitution of the MoMLV LTR for the CMV promoter in pCDNA3 by using the BAGB2F and BAGwt441R2 primer pair with the BAG vector template and inserting the luciferase gene from pT3/T7-luc into the polylinker. The LTR promoter and Sindbis virus nt 1 to 2289 were deleted in plasmid pDLTRSIN-lucdlpro by digestion of pDLTRSIN-luc with *Bgl*II and recircularization by ligation.

DNA and RNA DHs for packaging of expression replicons had deleted the nsP coding sequences between nt 422 and 7054 by digestion with *Bsp*EI and ligation at a low concentration of DNA. These vectors were designated pRdlnsPSINg, pDLTRdlnsPSINg, and pDCMVdlnsPSINg. Linearization of pRdlnsPSINg for in vitro transcription was done with *Xba*I.

RNA transcription and DNA and RNA transfections. For in vitro transcription, Sindbis virus cDNA vector plasmids were linearized as described in their construction detail and transcribed in vitro with a commercially available kit (SP6-mMessage mMachine Kit; Ambion, Austin, Tex.) according to the manufacturer's directions. Following in vitro transcription, all RNA reaction mixes were digested with DNase, phenol-chloroform and ether extracted, and precipitated with LiCl. Nucleoside triphosphates (NTPs) were removed by G-50 spin column chromatography (Boehringer Mannheim, Indianapolis, Ind.), and the transcripts were then quantitated by spectrophotometry, aliquoted, and stored frozen at −80°C.

Transfection of BHK-21 cells was carried out by either electroporation or cationic lipid-mediated transfer, as described in the figure legends. Electroporations were performed with a Bio-Rad Gene Pulser (Richmond, Calif.) as described previously (24). DNA transfections were performed with Lipofectamine (Gibco-BRL), with 2 μg of plasmid per 10 μl of lipid. RNA transfections were performed with Lipofectin (Gibco-BRL), with 2 μg of in vitro-synthesized transcript per 8 μl of lipid. Cells were incubated with the cationic lipid-nucleic acid inoculum for a minimum of 6 h before addition of Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum (FBS). Prior to

cationic lipid-mediated transfection, cells were rinsed twice and preincubated with OPTI-MEM medium (Gibco-BRL).

Transcript 5′-end mapping. Total RNA was isolated from the BHK-21 cells with RNAzol B (Tel-Test) at 6 h postinfection, 24 h post-RNA transfection, or 48 h post-DNA transfection according to the manufacturer's directions. RNA pellets were incubated at 56°C with a ^{32}P-labeled 20-nt reverse primer (5′-GGCTTCTCCATTGTGATGGT-3′) complementary to Sindbis virus genomic RNA bases 70 to 51 and transcribed with MoMLV reverse transcriptase (Promega). The pDCMVSINg plasmid was sequenced by a modified dideoxy chain termination method with the fmol DNA sequencing system (Promega). Reverse transcription and sequencing reaction mixtures were electrophoresed on a 6% denaturing polyacrylamide gel, dried, and exposed to film.

Total RNA and Northern (RNA blot) analysis. Total cellular RNA was isolated from transfected or infected BHK cells with Tri-Reagent (Molecular Research Center, Inc., Cincinnati, Ohio) as described by the manufacturer. BHK cells infected with wild-type Sindbis virus at a multiplicity of infection (MOI) of 5 were harvested at 8 h postinfection. RNA from BHK cells transfected with in vitro-transcribed pRSINg or pRSIN-luc RNA was isolated at 24 h posttransfection. RNA from BHK cells transfected with pDLTRSINg or pDLTRSIN-luc plasmid DNA was isolated at 48 h posttransfection. Northern blot analysis was performed as described before (42). RNA was electrophoresed through 0.7% formaldehyde agarose gels and transferred to a Zeta-probe (Bio-Rad) membrane. The blot was hybridized simultaneously with a mixture of random-primed probes corresponding to the capsid gene and the luciferase gene. RNA load was adjusted to normalize the intensity of the hybridizing species.

Transfection efficiency determination. Transfection efficiency was determined by two different methods. In the first method, expressed luciferase protein in transfected cells was detected in situ. Transfected cells were first incubated with a primary rabbit anti-luciferase antibody (Promega) as recommended by the supplier and then incubated with a secondary goat anti-rabbit antibody conjugated to horseradish peroxidase (Vector Laboratories, Burlingame, Calif.). Detection of bound enzyme was done with the colorimetric substrate aminoethylcarbozole. Transfected cells were counterstained with Mayer's hematoxylin. In the second method, the number of bacterial CFU present in Hirt extracts (17) of transfected cells was quantitated (19). Plasmid DNA was isolated from transfected cells at 48 h postinfection, extracted with phenol-chloroform, and ethanol precipitated. Resuspended DNA was used to transform subcloning efficiency XL1-Blue cells (Stratagene), and Amp^r colonies were scored. Plasmid DNA homologous to the test plasmid DNA and treated in parallel was added to Hirt extracts from mock-transfected cells and served as a control to standardize the number of CFU. To compare relative transfection efficiencies among different plasmids, the CFU in transfected cells was determined as a percentage of that in mock-transfected cells.

Virus and DNA and RNA vector assays. The specific infectivity levels of pRSINg in vitro-transcribed RNA and pDLTRSINg plasmid DNA were determined by electroporation of 10^7 BHK cells with 10 μg of nucleic acid as described before (24). Electroporated cells were first diluted into 10 ml of DMEM plus 10% FBS, then serially diluted into 10^6 fresh BHK cells, and plated into 35-mm wells. The cells were overlaid with agarose 4 h after plating. Plaques were visualized by staining with neutral red at 48 h posttransfection.

Lysates from cells transfected with DNA or RNA or infected with Sindbis virus vector particles were tested for expression of luciferase and β-galactosidase reporter proteins by adding 250 μl of reporter lysis buffer (Promega) per 10^6 cells. Luciferase (Promega) and β-galactosidase (Clontech) expression levels in cell lysates were determined by mixture with commercially available substrate detection systems followed by luminometry (Analytical Luminescence Laboratory, San Diego, Calif.). Alternatively, β-galactosidase expression in cells transfected with DNA or RNA or infected with Sindbis virus vector particles was determined by staining in situ with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and counting blue cells, as described previously (26). Expression of reporter proteins was determined at 18 or 48 h post-RNA transfection or DNA transfection, respectively, or at 18 h postinfection except as noted in the figure legends.

Mouse and rat DNA injections. Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, Ind.) or BALB/c or C3H/HeN (Charles River Laboratories, Wilmington, Mass.) mice were injected in either the gastrocnemius or tibialis anterior muscle with 25 μg of pDCMVSIN-β-gal or pDLTRSIN vector containing hepatitis B virus (HBV) core (HBc) and e (HBe) antigen coding sequences in a total volume of 100 μl of isotonic saline. Mice were injected twice at 2-week intervals with HBc- and HBe-expressing vectors. Fluospheres (fluorescently labeled latex spheres; Molecular Probes, Eugene, Oreg.) were added to the injectate to facilitate identification of the injection site. To test for HBV-specific antibody induction, serum was collected 10 days following the second injection and tested for HBV-specific antibodies by an enzyme-linked immunosorbent assay (ELISA; Abbott Laboratories, Chicago, Ill.). For visualization of β-galactosidase expression, the muscles were removed, fixed in toto in 2% formaldehyde in phosphate-buffered saline, and rinsed and stained overnight in X-Gal stain solution (26). The muscles were embedded in paraffin, sectioned, and counterstained with tartrazine (American Histology, Lodi, Calif.).

RESULTS

Cloning and characterization of full-length genomic infectious Sindbis virus cDNA in RNA polymerase II expression cassettes. As a first step towards constructing DNA-based Sindbis virus vectors, a full-length cDNA clone of viral genomic RNA was generated, and the sequence was determined. Sequence analysis revealed nucleotide changes (see Materials and Methods) resulting in 10 amino acid differences between our laboratory strain and the published HRsp strain, located in the nsP2, nsP3, E2, and E1 coding regions. Each of the changes in the nsPs resulted from single differences at nt 2992 (C→T, nsP2 aa 438, Pro→Leu), nt 3544 (T→C, nsP2 aa 622, Val→Ala), nt 3579 (A→G, nsP2 aa 634, Lys→Glu), and nt 5351 (A→T, nsP3 aa 417, Gln→His). Five of the six changes in the sPs resulted from single differences at nt 8637 (A→G, E2 aa 3, Ile→Val), nt 8698 (T→A, E2 aa 23, Val→Glu), nt 9144 (A→G, E2 aa 172, Arg→Gly), nt 9420 (A→G, E2 aa 264, Ser→Gly), and nt 10773 (T→G, E1 aa 237, Ser→Ala). These observed differences in the E2 (except E2 aa 264) and E1 glycoproteins of our laboratory strain are locations of variability among several Sindbis virus strains (25, 35). A fifth change observed in E2 was a 3-nt deletion which eliminated the Glu codon corresponding to aa 160 of E2 and resulted in a genome length for the Sindbis virus strain used in this work of 11,700 nt. The viral genomic cDNA was positioned downstream of the bacteriophage SP6 RNA polymerase promoter, so that in vitro-synthesized transcripts contained a single nonviral G residue at the 5′ end. Additionally, a 25-mer poly(A) tract followed by a unique *Xba*I recognition site were placed downstream of the viral 3′ end in the Sindbis virus genomic cDNA clone designated pRSINg. Transfection of BHK cells with runoff transcripts from *Xba*I-linearized pRSINg plasmid resulted in cytopathic effect within 18 h, which was due to formation of infectious Sindbis virus.

The Sindbis virus genomic cDNA clone was then inserted into several RNA polymerase II expression cassettes to determine whether the Sindbis virus infection cycle could be initiated directly from transfected plasmid DNA. The SV40 early region, MoMLV LTR U3 region, and CMV IE promoters were positioned so that transcription initiation would occur at the precise (or within 1 nt in the case of the LTR promoter) Sindbis virus 5′ end. In addition, the bovine growth hormone transcription termination/polyadenylation signal was positioned downstream from the viral genomic cDNA 3′ end in each construct to generate plasmids pDSV40SINg, pDLTRSINg, and pDCMVSINg. The fidelity of the RNA 5′ ends following transcription in vitro from linearized cDNA clones is important to their overall activity compared with wild-type virion RNA (40). This property likely extends to RNAs transcribed in vivo from transfected Sindbis virus plasmid cDNA constructs. Thus, the major transcript 5′ ends synthesized in BHK cells transfected with pDCMVSINg or pDSV40SINg plasmid DNAs were determined (Fig. 1). The results of this study demonstrated that the primer extension products of RNAs isolated from cells transfected with pDCMVSINg or pDSV40SINg (lanes 1 and 2) were the same lengths as RNAs isolated from cells infected with wild-type Sindbis virus (lane 5). Although direct evidence for capping was not obtained, the two primer extension products observed in all lanes (except lane 4) most likely correspond to the terminal residue and a partial copying of the cap. The lowest-molecular-weight band, labeled on Fig. 1 as genomic length, corresponds to the authentic Sindbis virus RNA 5′ end. Primer extension of the RNAs isolated from cells transfected with in vitro RNA (lane 3) produced three products. The lowest-molecular-weight ge-

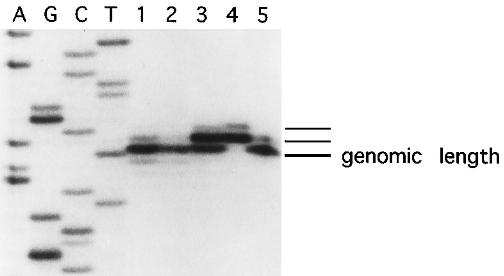

FIG. 1. Transcript 5′-end mapping in cells transfected with Sindbis virus RNA and DNA vectors. Lanes 1 to 5, primer-extended reverse transcription of RNA purified from BHK cells at 48 h posttransfection with pDCMVSINg, BHK cells at 48 h posttransfection with pDSV40SINg, BHK cells at 24 h posttransfection with in vitro-transcribed pRSINg RNA or in vitro-transcribed pRSINg transcription reaction, and BHK cells at 6 h postinfection (MOI of 5) with a Sindbis virus stock derived from in vitro-transcribed pRSINg RNA, respectively. The adjacent sequence ladder was derived from the pDCMVSINg construct with the same primer as used for reverse transcription reactions, and the genome-length band is labeled. The reaction products were electrophoresed on a 6% denaturing polyacrylamide gel. The sequence shown is therefore that of the virus minus strand. Thus, the terminal nucleotide in the reverse transcripts shown in the figure comigrates with the T nucleotide and corresponds to the genomic plus strand A residue at nt 1.

nome-length band and the middle band comigrated with the two extension products from virus-infected cells and most likely correspond to the authentic terminal residue and a partial copying of the cap (which accounts for an undefined fraction of the middle band), respectively. The middle band and the highest-molecular-weight band comigrated with the two extension products from the in vitro transcription reaction (lane 4) and correspond to uncapped (which accounts for an undefined fraction of the middle band) and capped transcripts, respectively, each containing an additional nonviral G ribonucleotide from the SP6 promoter. A significant proportion of the RNA molecules isolated from cells transfected with in vitro-transcribed RNA appeared to have lost the nonviral residue within a single round of viral replication. Also, the additional residue added from the SP6 promoter was not observed in cells infected with a Sindbis virus stock derived from BHK cells transfected with in vitro-transcribed pRSINg RNA. Primer extension products which migrated at a position that was 1 nt shorter than genome length and several lower-molecular-weight species were detected after long exposure and appeared to be indistinguishable between the various samples tested. RNA transcribed in situ from Sindbis virus plasmid DNA vectors containing the MoMLV LTR are expected to have a single G residue at the viral 5′ end, but this was not verified experimentally.

The specific infectivities of pDLTRSINg DNA and pRSINg in vitro-transcribed RNA were determined by an infectious center assay to compare the efficiency of initiation of the virus infection cycle in cells electroporated with in vitro-transcribed RNA and plasmid DNA. In an average of three experiments, the specific infectivity of the in vitro-transcribed Sindbis virus RNA (from pRSINg) was 1.1×10^5 PFU/μg (range, 0.6×10^5 to 1.3×10^5), and the specific infectivity of the Sindbis virus plasmid DNA (pDLTRSINg) was 1.3×10^4 PFU/μg (range, 0.3×10^4 to 3.1×10^4), about 10-fold less. These experiments demonstrated that all of the *cis* and *trans* components of Sindbis virus can be expressed in functional form in vivo from a DNA format.

Production of packaged vector particles in cells cotransfected with Sindbis virus expression vector and DH plasmid DNAs. The full-length genomic cDNA clones were used to construct Sindbis virus-based expression vectors and DH plasmids for in vitro RNA and DNA transfections. As in previous work (22, 53), the expression vectors contained the entire nsP gene coding region and all sequences required in *cis* for viral replication and thus should function as replicons in transfected cells. The DHs contained the entire sP gene coding region and all of the sequences required in *cis* for viral replication but had deleted most of the nsP gene coding region and the Sindbis virus packaging sequence (4). Thus, DH replication and expression of sPs were dependent on nsPs supplied in *trans* by the vector. Expression vectors and DHs were placed into RNA polymerase II expression cassettes used previously for plasmid DNA-based transfection experiments. Expression vectors and DH DNAs were constructed with either the MoMLV LTR or CMV IE promoter to explore a possible relationship between promoter strength and expression levels or packaging efficiencies in cells transfected with Sindbis virus-derived plasmids.

We anticipated that the kinetics of reporter expression from DNA-based expression vectors would be slower than that from the corresponding RNA-based vectors. If so, that would affect the time point chosen in subsequent cotransfection experiments for harvesting packaged vector particles. Therefore, the kinetics of reporter protein expression in cells transfected with in vitro-transcribed RNA or plasmid DNA vectors were compared. As reported previously for in vitro-transcribed RNA vectors containing the chloramphenicol acetyltransferase reporter gene (53), the peak level of luciferase expression occurred at 18 h posttransfection in cells transfected with in vitro-transcribed RNA vectors. In contrast, the kinetics were delayed in cells transfected with pDLTRSIN-luc DNA vectors, and the peak level of expression occurred at approximately 48 h posttransfection (data not shown). On the basis of these results, packaged vector particles were collected at 18 or 48 h posttransfection from cells cotransfected with in vitro-transcribed RNA or DNA expression vector and DH molecules, respectively.

The activities of in vitro-transcribed RNA and plasmid DNA vectors were compared by examining the levels of β-galactosidase expression and the production of packaged vector particles in cells transfected with the expression vectors alone or an expression vector and DH molecules together (Fig. 2). The level of β-galactosidase expressed in transfected cells was affected somewhat by the RNA polymerase II promoter used in the Sindbis virus DNA vector construct. Reporter enzyme activity was 1.5-fold higher in cells transfected with DNA vectors containing the CMV IE promoter than with those containing the LTR promoter (samples 5 and 3, respectively). The levels of β-galactosidase observed in cells transfected with DNA vector containing the CMV IE promoter or with in vitro-transcribed RNA replicon were similar (samples 5 and 1, respectively). The influence of the RNA polymerase II promoter on reporter protein expression was also tested in cells transfected with Sindbis virus DNA vectors containing the luciferase gene. Luciferase activities were about fourfold greater in cells transfected with expression vectors containing the CMV promoter than with the LTR promoter (274,000 ± 52,100 relative light units [RLU] per cell with pDCMVSIN-luc versus 70,600 ± 11,400 RLU per cell with pDLTRSIN-luc).

A separate experiment designed to compare relative activities between the MoMLV LTR and CMV IE promoters in the absence of subsequent RNA amplification was performed. The level of luciferase activity was determined in BHK cells transfected with conventional expression vectors in which the promoter and reporter genes were directly linked. The levels of luciferase activities were approximately 18-fold higher in cells transfected with plasmids containing the CMV IE promoter

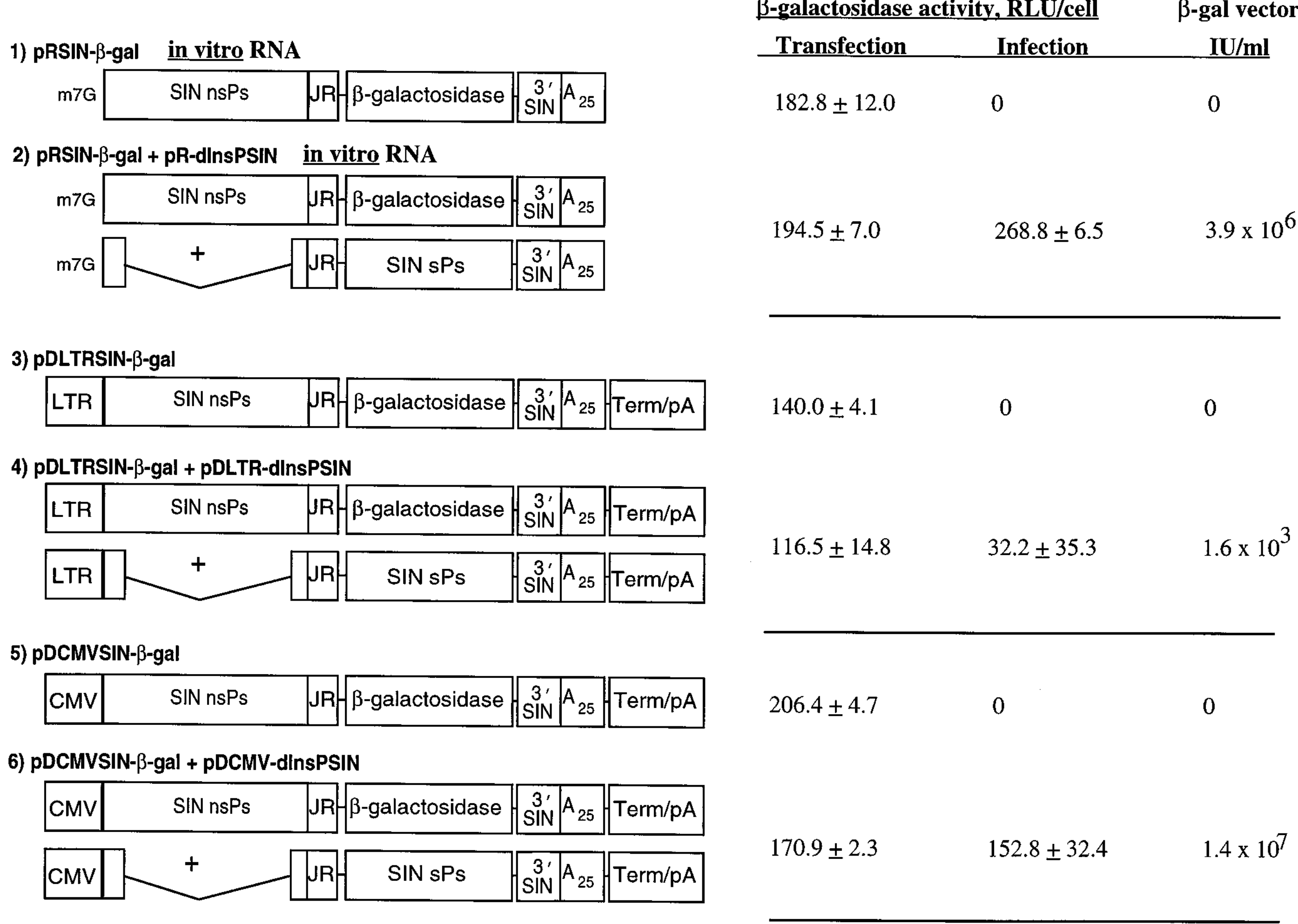

FIG. 2. Expression and packaging of Sindbis virus vectors. BHK cells were transfected with in vitro-transcribed RNA or DNA vectors or cotransfected with in vitro-transcribed RNA or DNA vector and DH molecules. Shown on the left side of the figure are schematic representations of the Sindbis virus RNA and DNA vectors and DH molecules used in the experiment. The vector maps are not drawn to scale. Abbreviations: LTR, MoMLV LTR U3 region promoter; CMV, human CMV IE promoter; JR, Sindbis virus junction region promoter, extending to nt 7643; 3′ SIN, 3′ end of Sindbis virus from nt 11664 to 11703; A_{25}, synthetic 25-mer poly(A) tract; Term/pA, bovine growth hormone transcription termination/polyadenylation signal. Shown on the right side of the figure and adjacent to each vector schematic are the levels of β-galactosidase detected, in RLU per cell, after cotransfection of BHK cells and after infection of fresh BHK cells with 1 ml of clarified culture medium. BHK cells were cotransfected with 2 μg of each vector nucleic acid by lipofection, and β-galactosidase activity was measured in cleared cell lysates at 18 h after in vitro-transcribed RNA transfection and 48 h after DNA transfection. Samples 1 and 2 were cotransfected with in vitro-transcribed RNAs and are marked by the m7G cap on the vector schematics. Packaging was determined by transfer of reporter gene expression for clarified cotransfected cell culture medium to fresh BHK monolayers and was measured at 18 h postinfection. The standard deviation represents six samples. The infectious units (IU) per milliliter were determined by infecting dilutions of clarified medium from cotransfected cells onto fresh BHK cells followed by staining with X-Gal. The IU levels shown are an average for three samples.

versus the LTR promoter (135,000 ± 20,800 RLU per cell versus 7,690 ± 1,660 RLU per cell, respectively). Thus, it appeared that while the promoter strength influenced the level of reporter gene expression in cells transfected with both Sindbis virus-derived DNA vectors and conventional vectors, this effect was less pronounced in the context of autocatalytic RNA amplification arising from the Sindbis replicons.

Paradoxically, the titers of packaged vector did not correlate with the level of β-galactosidase activity in lysates from cells infected with 1 ml of medium from cotransfected cells (Fig. 2). The titer of packaged vector in medium from cells cotransfected with DNAs that contained the CMV promoter (sample 6) was 3.6-fold higher than the titer from cells cotransfected with in vitro RNAs (sample 2). In contrast, the level of β-galactosidase activity in lysates was 1.8-fold lower in cells infected with 1 ml of medium from cells cotransfected with DNAs containing the CMV promoter (sample 6) than with in vitro-transcribed RNAs (sample 2). This result appeared to be related to the level of infectious virus produced, presumably by homologous DNA recombination, in cells cotransfected with plasmid DNAs that contained the CMV IE promoter, which was 2×10^7 to 3×10^7 PFU/ml, compared with 1×10^4 to 5.5×10^4 PFU/ml in cells cotransfected with in vitro-transcribed RNA. RNA analysis of PFU produced in cells cotransfected with in vitro-transcribed RNA or DNA to discriminate between copackaging (12) of replicon and DH RNAs and RNA recombination (52) was not performed. However, the infectious virus in medium from all cotransfected cells had a large plaque morphology, and infection of cultures with undiluted medium from cells cotransfected with DNAs containing the CMV promoter produced rapid cytopathic effect that was indistinguishable from that caused by wild-type Sindbis virus.

The titer of packaged vector particles observed in medium from cells cotransfected with plasmid DNAs that contained the LTR promoter was significantly lower than that in all other nucleic acids that were tested (Fig. 2, sample 4). In these ex-

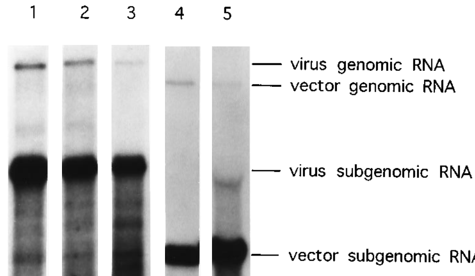

FIG. 3. Sindbis virus-specific RNAs synthesized in BHK cells transfected with various in vitro-transcribed RNA and DNA vectors or infected with wild-type virus. BHK cells were transfected with 5 μg of nucleic acid by lipofection or infected with Sindbis virus at an MOI of 5. Total cellular RNA was isolated at 8 h postinfection or 24 h (in vitro-transcribed RNA vectors) or 48 h (DNA vectors) posttransfection and analyzed by formaldehyde gel electrophoresis, transfer to a nylon membrane, and hybridization with capsid gene- and luciferase gene-specific probes. Lanes: 1, Sindbis virus infection; 2, in vitro-transcribed pRSINg RNA; 3, pDLTRSINg; 4, in vitro-transcribed pRSIN-luc RNA; 5, pDLTRSIN-luc.

periments, the level of β-galactosidase activity in lysates from cells cotransfected with plasmid DNAs that contained the LTR promoter was highly variable and indicated either that the cotransfection frequency of vector and DH was very low or that the frequency of cotransfected cells in which functional replicon and DH RNAs were transported to the cytoplasm was very low.

RNA synthesis in cells transfected with Sindbis virus DNA and RNA vectors. The RNA species synthesized in cells transfected with various Sindbis virus DNA and RNA vector constructs were analyzed by Northern blot and compared with those observed after wild-type virus infection (Fig. 3). The results demonstrated that the genomic and subgenomic RNAs isolated from cells transfected with Sindbis virus genomic in vitro-transcribed RNA or plasmid DNA vectors were comparable to the RNAs synthesized in virus-infected cells. The presence of genomic and subgenomic RNAs in cells transfected with in vitro-transcribed pRSIN-luc RNA or pDLTRSIN-luc plasmid DNA replicons suggested that these vectors function according to the strategy of wild-type Sindbis virus and the genomic clones and that subgenomic RNA is likely the template for luciferase gene expression. Several bands of various molecular sizes which migrated more rapidly than the subgenomic mRNA were observed. However, there is no direct evidence for splicing of Sindbis virus-derived transcripts in plasmid DNA-transfected cells, since these unidentified RNAs that migrated more rapidly than 26S mRNA were also observed in wild-type Sindbis virus-infected cells. Furthermore, analysis of Sindbis virus genomic RNA revealed no cryptic splice sites (14). Thus, it appears that these species may be due to nonspecific RNA degradation.

Enhancement of gene expression by layered Sindbis virus DNA expression vector. The levels of luciferase synthesized in cells transfected with either Sindbis virus plasmid DNA expression vectors or the analogous promoter-reporter plasmid constructions were compared in order to determine the relative enhancement from nsP-catalyzed RNA transcript amplification (Fig. 4). Transfections of individual test plasmids were performed in quintuplicate. The level of synthesis was about 10-fold higher in cells transfected with the pDLTRSIN-luc Sindbis virus vector DNA than in cells transfected with plasmid pLTR-luc. Two additional control constructs also were tested. Plasmid pDLTRdlnsPSIN-luc had most of the nsP genes deleted, between viral nt 1407 and 6920, and serves as a baseline control for the level of vector genomic template-derived luciferase expression. Plasmid pDSIN-luc was an intermediate in the construction of pDLTRSIN-luc, which lacked the LTR promoter and the first 2,289 nt of Sindbis virus and served as a control for background luciferase expression. Measurable reporter gene expression was observed in cells transfected with pDLTRdlnsPSIN-luc, although at levels which were more than 100-fold lower than the level in cells transfected with pDLTRSIN-luc. This low level of activity may be due to alteration

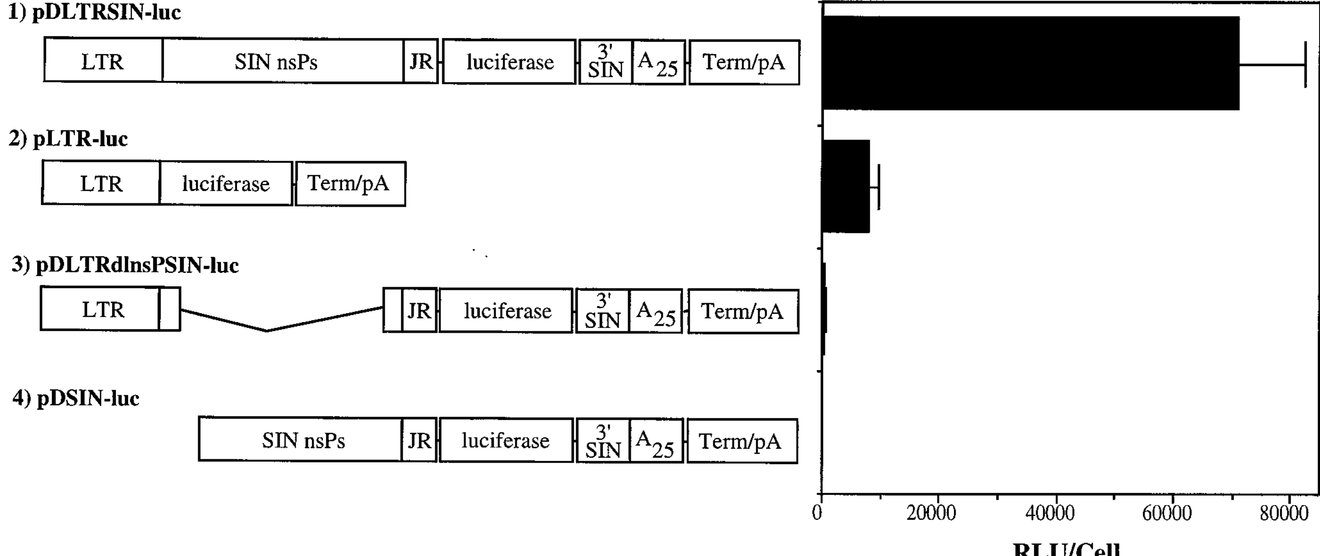

FIG. 4. Comparison of enhancement of luciferase expression by nsP-catalyzed RNA transcript amplification in BHK cells transfected with Sindbis virus DNA expression vectors or with linked promoter-reporter vectors. Shown on the left side of the figure are the schematic representations of the plasmid DNAs used in the experiment. The vector maps are not drawn to scale. Abbreviations are as described in the legend to Fig. 2. Shown on the right side of the figure and adjacent to each vector schematic are the levels of luciferase activity detected in cleared lysates from BHK cells at 48 h posttransfection with 2 μg of plasmid DNA. Error bars represent the standard deviation for five samples.

TABLE 2. Transfection efficiencies of Sindbis virus plasmid DNA and conventional plasmid DNA vectors

Vector	Mean RLU/cell[a] ± SD	Mean % of cells expressing luciferase[b] ± SD	CFU/μg[c] (% of control)
pDLTRSIN-luc	6,310 ± 4,496	3.3 ± 0.8	17
pLTR-luc	200 ± 35	6.2 ± 1.0	21

[a] Luciferase activity present in a lysate from 10^6 cells at 48 h posttransfection. The standard deviation was determined from three individual transfections.

[b] Standard deviation was determined from four fields counted (average of 707 total cells) in three individual transfections.

[c] Bacterial CFU present in Hirt extracts of cells at 48 h posttransfection as a percentage of that in Hirt extracts of cells mock transfected and spiked with test plasmid DNA. Values shown are averages of CFU quantitated from three transfections or plasmid-spiked mock transfections.

of some of the input plasmid DNA or, alternatively, translation directly from degraded vector genomic RNA (6).

Further experiments were performed to investigate the relationship between transfection and expression efficiency in cells into which Sindbis virus DNA vector or the analogous conventional promoter-reporter vector was introduced. The total amount of luciferase expression in transfected cell lysates was compared with the percentage of cells expressing luciferase protein, as determined both by in situ staining with a luciferase-specific antibody and by the total number of transfected biologically active DNA molecules, as determined by quantitating the bacterial CFU of plasmid DNA present in Hirt extracts (19) (Table 2). The data indicate that while the fraction of cells expressing reporter protein was about 2-fold less in pDLTRSIN-luc- than in pLTR-luc-transfected samples, the level of luciferase activity was at least 10-fold greater in pDLTRSIN-luc-transfected cell lysates. It appeared that the relative numbers of biologically active plasmid molecules were similar in cells transfected with either pDLTRSIN-luc or pLTR-luc plasmid DNAs. Taken together, these results indicated that the relative level of nsP-driven reporter gene expression was at least 10-fold higher than the level from the analogous promoter-reporter vector on the basis of transfected cells expressing reporter protein.

Reporter gene expression in rodents inoculated intramuscularly with Sindbis virus DNA expression vectors. Previously (54), RNA derived from the Semliki Forest virus replicon was injected intramuscularly into mice to express the nucleoprotein of influenza virus. To examine in vivo gene expression from DNA-based Sindbis virus vectors, BALB/c mice were injected intramuscularly with 25 μg of pDCMVSIN-β-gal plasmid DNA. Figure 5 demonstrates the in vivo expression of β-galactosidase in mouse muscle stained in toto with X-Gal at 5 days postinjection (Fig. 5A) and a transverse section from a mouse muscle stained in toto with X-Gal at 5 days postinjection (Fig. 5B). The muscle stained in toto shows β-galactosidase protein expression along the length of the mouse muscle. We routinely observed blue fibers which spanned the length of the muscle. After sectioning, the muscle was counterstained with tartrazine, and more than 30 blue fibers can be observed in a transverse section (Fig. 5B). Of these, almost 90% were strongly stained, although a minority of fibers show variable weaker staining. Rats injected with pDCMVSIN-β-gal plasmid DNA also demonstrated positively staining blue muscle fibers (data not shown).

Other experiments were designed to explore further the in vivo expression of clinically relevant genes from Sindbis virus DNA vectors. C3H/HeN mice were injected twice intramuscularly with pDLTRSIN vectors containing either the HBc or

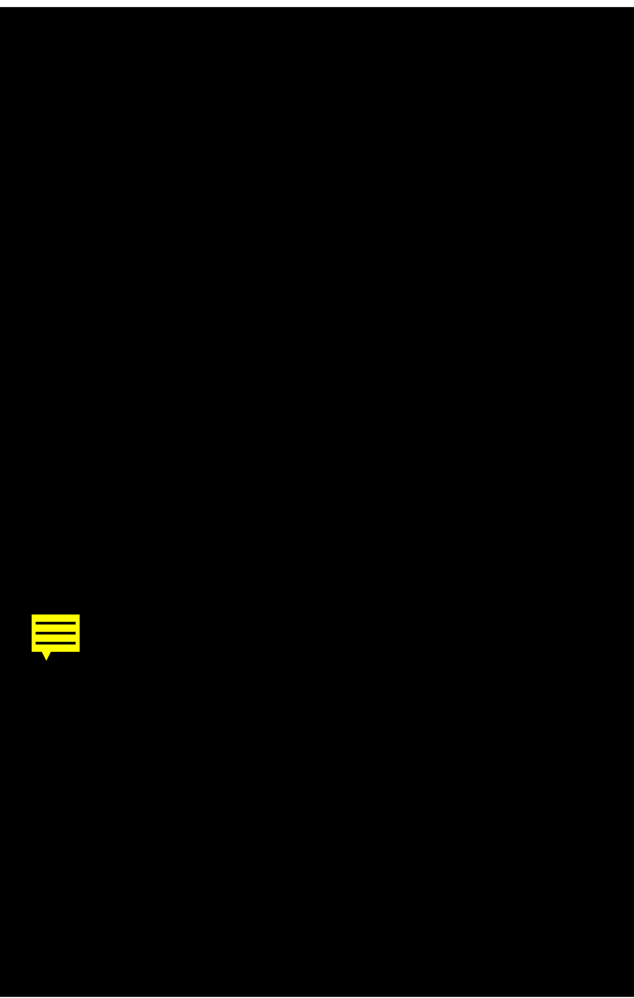

FIG. 5. Expression of β-galactosidase in mouse muscle 5 days after injection with 25 μg of pDCMVSIN-β-gal plasmid DNA contained in phosphate-buffered saline. (A) Photomicrograph of gastrocnemius muscle stained in toto with X-Gal. Five blue fibers are observed in this micrograph. Magnification, ×40. (B) Photomicrograph of transverse cryosection of mouse tibialis anterior muscle. Multiple blue-stained transverse fibers are evident. Magnification, ×400.

HBe gene. With an ELISA detection system, both HBc- and HBe-specific immunoglobulin G antibodies were detected in serum samples collected from the mice 10 days following the second injection with the vectors. Antibody titers of 1:640 were observed in four of five mice inoculated with the HBc vector, and antibody titers of 1:640 or greater were observed in three of five mice inoculated with the HBe vector. Antibodies specific for HBc or HBe were not detected in sera collected from the mice prior to the injection of vector. These experiments demonstrate that Sindbis virus-derived DNA vectors are able to express foreign genes in vivo and that expression levels are sufficient for induction of a humoral immune response. Whether these vectors are also capable of inducing HBV-specific cytotoxic T cells is an area currently under investigation. Indeed, the induction of a vigorous cytotoxic T cell response is believed to play a major role in the clearance of HBV-infected cells (7).

Sindbis virus DNA expression vector modifications. As discussed above, the level of reporter gene expression observed in cells transfected with Sindbis virus plasmid DNA vectors varied with the strength of the RNA polymerase II promoter used

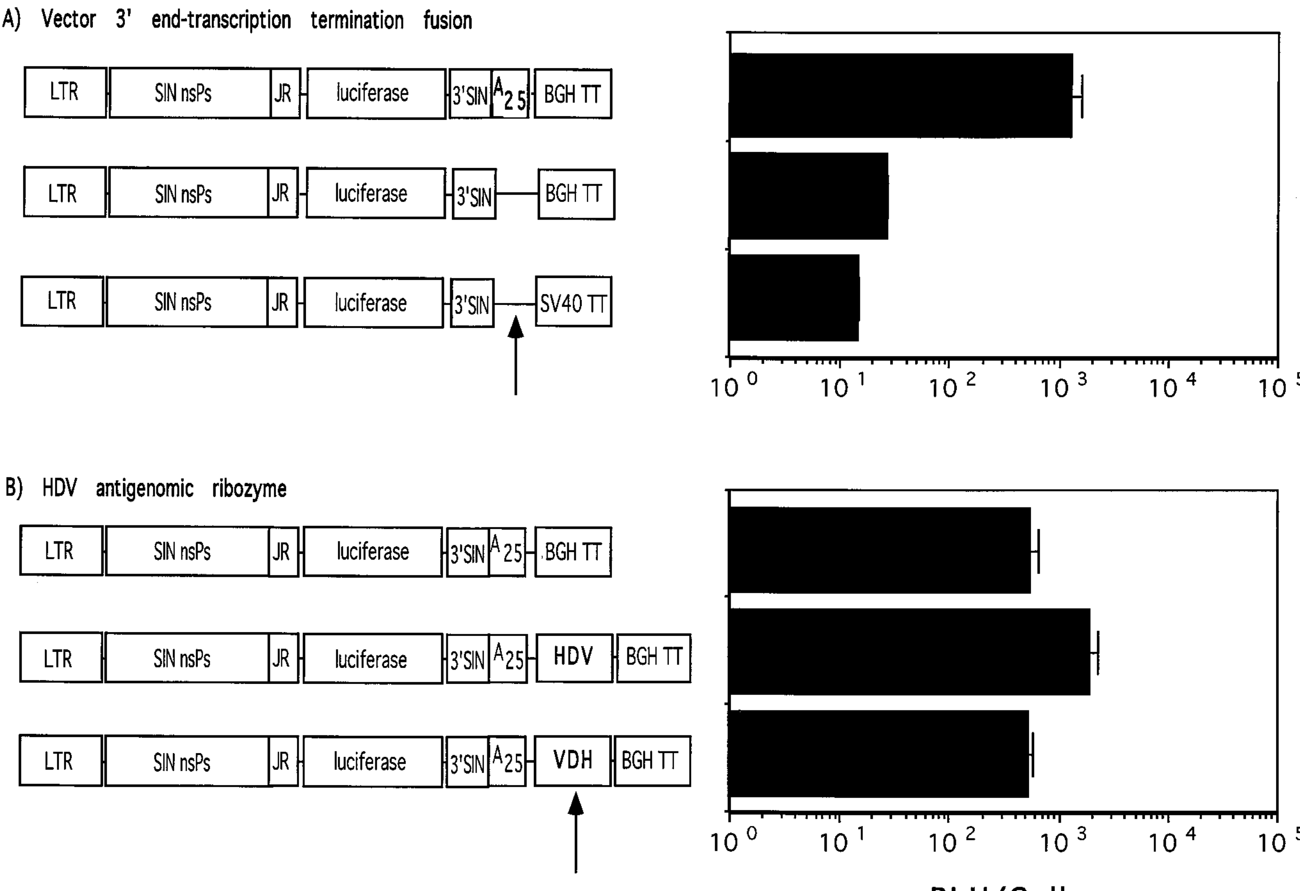

FIG. 6. Luciferase expression in BHK cells transfected with 3′-end-modified Sindbis virus DNA expression vectors. Shown on the left side of the figure are the schematic representations of the plasmid DNAs used in the experiment. The vector maps are not drawn to scale. Abbreviations: BGH TT, bovine growth hormone transcription termination signal; SV40 TT, SV40 early-region transcription termination signal; HDV, HDV antigenomic ribozyme sequence; VDH, HDV antigenomic ribozyme sequence, reverse orientation. Other abbreviations are as described in the legend to Fig. 2. Shown on the right side of the figure and adjacent to each vector schematic are the levels of luciferase activity detected in cleared lysates from BHK cells transfected by lipofection with 2 μg of plasmid DNA in two individual experiments. (A) Deletion of the synthetic poly(A) (A_{25}) tract. The deleted regions of the vectors are denoted by an arrow. Error bars represent the standard deviation for five samples. Luciferase activity was determined at 48 h posttransfection. (B) Insertion of the HDV antigenomic ribozyme sequence immediately downstream of the synthetic poly(A) (A_{25}) tract. Insertions in the correct and reverse orientations are denoted by an arrow. Error bars represent the standard deviation for three samples. Luciferase activity was determined at 36 h posttransfection.

in the vector construct at the time points examined. This level was two- to fourfold greater in Sindbis virus DNA expression vectors that contained the CMV promoter than in those that contained the LTR promoter. In contrast, in cells transfected with conventional vectors in which the promoter and reporter gene were directly linked, the level was about 18-fold greater with plasmids that contained the CMV promoter than with those that contained the LTR promoter. Given the autocatalytic cytoplasmic amplification of transported RNA replicons, it is expected that the expression levels should not be promoter dependent. Our results, however, indicated that expression was partially related to the promoter and therefore suggested that further optimization of the Sindbis virus DNA expression vectors was possible.

Because Sindbis virus plasmid DNA vectors were constructed from the initial SP6-based vectors, the transcription termination/polyadenylation signal follows a 25-mer poly(dA-dT) tract. Thus, the 3′ end of the primary transcript in the nucleus most likely consists of 25 consecutive adenylate residues, followed by the transcription termination sequence cRNA, and terminates with a second poly(A) tract. Figure 6 demonstrates the effect on reporter gene expression for two different vector modifications which were designed to generate a vector RNA 3′ end that was more similar to that of wild-type Sindbis virus. In the first configuration (Fig. 6A), the 25-mer poly(dA-dT) tract was removed, and the viral 3′ end (nt 11703) was linked directly to the core bovine growth hormone or SV40 RNA processing signals. When transfected into cells, the activity of these vectors was decreased by more than 100-fold compared with that of the parental pDLTRSIN-luc prototype vector (Fig. 6A). Although measurable expression of luciferase was detected from the poly(dA-dT) tract-deleted plasmids, it was not determined whether any transported RNA actually replicated in transfected cells. In the second configuration (Fig. 6B), the HDV antigenomic ribozyme sequence (34) was inserted immediately downstream of the 25-mer poly(dA-dT) tract to facilitate autocatalytic processing of the primary transcript and removal of nonviral nucleotides downstream from the vector poly(A) tail. Transfection of cells with DNA vector containing the HDV ribozyme sequence resulted in a three- to fourfold increase in the level of luciferase expression compared with the wild-type vector or an analogous construct with the

HDV ribozyme sequence inserted in the reverse orientation. These data were confirmed by testing the specific infectivity of a genome-length construct derived from pDLTRSINg, which also contains the HDV ribozyme sequence insertion. The specific infectivity of this construct, designated pDLTRSINgHDV, was increased fourfold over that of the parental construct pDLTRSINg (data not shown).

DISCUSSION

In this study, we demonstrated the efficient initiation of an alphavirus infectious cycle in vivo from a genomic cDNA clone contained within an RNA polymerase II expression cassette. The ability to express functional alphavirus genes from a DNA format enabled us to develop two new Sindbis virus-based gene expression systems: (i) a plasmid DNA-based vector with potential application for genetic immunization, and (ii) the production of packaged Sindbis virus particles in cells cotransfected with vector replicon and DH plasmid DNAs. Although previous investigators (21) have used the DNA-based vaccinia virus-T7 polymerase and T7 promoter system to express functional forms of the individual Sindbis virus nsPs, this approach was dependent on infectious vaccinia virus as a helper. Furthermore, these experiments were performed to investigate Sindbis virus replication rather than to develop plasmid DNA-based replicon vectors. Prior to this report, molecular approaches to producing infectious Sindbis virus RNA and its derived complementary vectors were restricted primarily to in vitro transcription of cDNA clones from a bacteriophage RNA polymerase promoter, followed by transfection into permissive cells.

The demonstration of efficient RNA polymerase II promoter-dependent initiation of Sindbis virus infection is in contrast to previous studies (19, 37, 46) with genomic cDNA clones derived from the positive-stranded poliovirus RNA. In these investigations, transfection of cells with the poliovirus cDNA without an RNA polymerase II promoter resulted in the production of low levels of infectious virus (37). Inclusion of SV40 transcription and replication signals in the poliovirus cDNA constructs dramatically improved the production of poliovirus after transfection with plasmid DNA (19, 46). However, it is not clear whether the high level of poliovirus produced in cells transfected with the modified plasmids was related to increased RNA polymerase II-dependent transcription of cDNA amplification, since the increased infectivity of the transfected poliovirus cDNA correlated with the extent of SV40 large-T-antigen-dependent plasmid replication rather than the promoter (19). In these investigations with poliovirus, the requirement for the transfected plasmid to replicate in order to obtain efficient production of virus has several possible explanations, including distant placement of the RNA polymerase II promoter relative to the viral 5′ end, resulting in the relatively poor infectivity of synthesized RNA polymerase II transcripts; cryptic splicing within the viral genome; and poor transport of the viral mRNA. Our current investigation demonstrated that strategic placement of RNA polymerase II promoters in plasmids not containing eukaryotic replication signals resulted in the synthesis of replication-competent Sindbis virus RNA following plasmid DNA transfection of cells. In addition, several examples exist for the RNA polymerase II promoter-dependent initiation of plant virus infection in cells transfected with plasmids in which the cauliflower mosaic virus 35S promoter is linked to the virus genomic cDNA (for a review, see reference 3).

Cotransfection experiments with DNA vectors having the CMV IE promoter or with in vitro-transcribed RNA vectors produced levels of packaged β-galactosidase vector particles that were similar and comparable to those in previous work with cotransfected in vitro replicon and DH RNAs (4). The level of replication-competent PFU produced in cells cotransfected with in vitro-transcribed RNA vectors in this work was similar to that observed in previous work (4) (1×10^4 to 5.5×10^4 PFU/ml versus 2×10^4 PFU/ml, respectively), using a DH deleted of the nsPs gene between nt 502 and 6917. Another DH used in that study, deleted between nt 421 and 7334, resulted in less than 10^3 PFU/ml produced after cotransfection with in vitro-transcribed replicon RNA. The DH used in our current study was deleted between nt 422 and 7054. Additionally, the vector replicons used in the previous work contained 310 nt from the Sindbis virus 3′ nontranscribed region, while the vector replicons used in this investigation contained only the 39 3′-terminal nucleotides.

The level of PFU produced in cells cotransfected with replicon and DH DNAs containing the CMV IE promoter was dramatically higher than that in cells transfected with in vitro-transcribed RNA vectors (2×10^7 to 3×10^7 PFU/ml versus 1×10^4 to 5.5×10^4 PFU/ml, respectively). Although it was not determined whether the PFU produced in cells contained particles which arose from RNA copackaging or recombination between replicon and DH DNAs and/or RNAs, it has been observed that mutations and rearrangements can occur in transfected DNA (20). Thus, it is possible that the culture medium from DNA-cotransfected cells contained significant levels of replication-competent virus generated from homologous recombination. Additionally, the high level of PFU observed here after in vitro-transcribed RNA or DNA cotransfection may be related partially to the transfection efficiencies, which were about 5%. Replication-competent virus produced in cotransfected cells would subsequently be amplified upon infection of naive cells.

The level of reporter gene expression in cells transfected with various Sindbis virus-derived DNA vectors was dependent, in part, on the relative RNA polymerase II promoter strength. This result was unexpected, since the level of translational template, which results from the exponential expansion of RNA, should not depend on the initial number of functional replicon molecules. However, this correlation of reporter gene expression with promoter strength was significantly less with Sindbis virus DNA vectors than with conventional expression vectors. These results suggested that transport of functional replicons from the nucleus to the cytoplasm may be inhibited in some way in cells transfected with Sindbis virus DNA vectors or, alternatively, that transported replicons may be inefficient templates for initiation of replication. Thus, we tested two modifications of the DNA vector to produce RNAs with 3′ ends which were more similar to those of wild-type Sindbis virus. Deletion of the synthetic A_{25} tract by direct linkage of the vector 3′ end (nt 11703) and transcription processing signals resulted in Sindbis virus plasmid DNA expression vectors that were significantly disabled. This observation suggests that the 3′ end and the poly(A) tail of Sindbis virus RNA must be contiguous in order to be recognized by the viral replicase complex and serve as the template for minus-strand RNA synthesis. The 19 3′-terminal viral nucleotides conserved among the alphaviruses (32) appear to be insufficient to catalyze this process. In contrast, juxtaposition of the *cis*-acting HDV antigenomic ribozyme sequence with the Sindbis virus A_{25} tract to generate precise 3′ termini resulted in DNA vectors which produced comparatively higher levels of luciferase expression in transfected cells. Although direct evidence of autocatalytic cleavage by RNA analysis was not obtained, expression levels from vectors containing the HDV ribozyme

sequence in the reverse orientation were unchanged, suggesting that the ribozyme component in the correct orientation was indeed functional. The HDV ribozyme sequence has been used previously to generate precise termini for the replication in situ of the negative-stranded vesicular stomatitis virus (33).

The relative level of nsP-catalyzed enhancement of reporter gene expression was approximately 10-fold, as determined by comparison of luciferase activity between Sindbis virus plasmid DNA vectors and conventional linked promoter-reporter vectors in transfected cells. Current limitations of DNA-based immunization are due, in part, to poor transfection efficiencies and short-lived expression. One approach to mitigate these problems may be to use vectors, such as the Sindbis virus-derived DNA vector described here, which express high levels of the gene of interest through autocatalytic amplification of the vector RNA. We have begun to further modify the Sindbis virus DNA vectors in order to increase the difference in heterologous gene expression compared with conventional vectors (28). We are continuing our efforts by, among other things, exploring the utility of introns to increase the transport efficiency of replicons synthesized from Sindbis virus plasmid DNA vectors. One additional possibility may be to incorporate translation enhancement components, as described previously (11), for Sindbis virus-derived replicons.

The conversion of alphavirus-derived replicon and helper vectors into a plasmid DNA-based expression system is the primary requisite step towards developing alphavirus-based gene transfer systems which parallel the classic retrovirus-based producer cell configurations. The ability to produce packaged vector particles from cotransfected replicon and DH DNAs suggested to us the possibility of developing stable vector-packaging cell lines, in which packaged vector particles are produced following transfection of Sindbis virus-derived plasmid DNA vectors. This notion is supported further by work describing the generation of a vector particle-titering cell line (31) that constitutively synthesizes a defective RNA, which subsequently replicates and expresses luciferase when induced by Sindbis virus nsPs. We have used the plasmid cDNA-based expression system described here to derive a first-generation Sindbis virus vector-packaging cell line (manuscript in preparation). Further goals include developing inducible producer cell lines in which a burst of packaged Sindbis virus vector particles is produced in response to a particular stimulus.

Accomplishment of our first objective has resulted in the development of a new vector which harnesses the expression potential of alphaviruses and appears to lead to increased expression of heterologous genes compared with conventional expression vector plasmids. Finally, the Sindbis virus DNA vector system was shown to express heterologous genes in vivo when injected into mice and rats and is being developed further for general physical gene transfer applications.

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REFERENCES

1. **Berglund, P., M. Sjoberg, G. J. Atkins, B. J. Sheahan, H. Garoff, and P. Liljestrom.** 1993. Semliki Forest virus expression system: production of conditionally infectious recombinant particles. Bio/Technology **11:**916–920.
2. **Boshart, M., F. Weber, G. Jahn, K. Dorsch-Hasler, B. Fleckenstein, and W. Schaffner.** 1985. A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. Cell **41:**521–530.
3. **Boyer, J.-C., and A. L. Haenni.** 1994. Infectious transcripts and cDNA clones of RNA viruses. Virology **198:**415–426.
4. **Bredenbeek, P. J., I. Frolov, C. M. Rice, and S. Schlesinger.** 1993. Sindbis virus expression vectors: packaging of RNA replicons by using defective helper RNAs. J. Virol. **67:**6439–6446.
5. **Burge, B. W., and E. R. Pfefferkorn.** 1966. Isolation and characterization of conditional-lethal mutants of Sindbis virus. Virology **30:**204–213.
6. **Cancedda, R., L. Villa-Komaroff, H. F. Lodish, and M. Schlesinger.** 1975. Initiation sites for translation of Sindbis virus 42S and 26S messenger RNAs. Cell **6:**215–222.
7. **Chisari, F. V., and C. Ferrari.** 1995. Hepatitis B virus immunopathogenesis. Annu. Rev. Immunol. **13:**29–60.
8. **Davis, N. L., K. W. Brown, I. J. Caley, R. I. Swanstrom, and R. E. Johnston.** 1995. Protection against influenza in mice by vaccination with a Venezuelan equine encephalitis virus vector expressing the HA protein. J. Cell. Biochem. Suppl. **19A:**310.
9. **Davis, N. L., L. V. Willis, J. F. Smith, and R. E. Johnston.** 1989. In vitro synthesis of infectious Venezuelan equine encephalitis virus RNA from a cDNA clone: analysis of a viable deletion mutant. Virology **171:**189–204.
10. **Feiers, W., R. Contreras, G. Haegeman, R. Rogiers, A. van der Voorde, H. van Heuverswyn, J. van Herreweghe, G. Volckaert, and M. Ysebaert.** 1978. The complete nucleotide sequence of SV40 DNA. Nature (London) **273:**113–117.
11. **Frolov, I., and S. Schlesinger.** 1994. Translation of Sindbis virus mRNA: effects of sequences downstream of the initiating codon. J. Virol. **68:**8111–8117.
12. **Geigenmuller-Gnirke, U., B. Weiss, R. Wright, and S. Schlesinger.** 1991. Complementation between Sindbis viral RNAs produces infectious particles with a bipartite genome. Proc. Natl. Acad. Sci. USA **88:**3253–3257.
13. **Geller, A. I., and X. O. Breakefield.** 1988. A defective HSV-1 vector expresses *Escherichia coli* β-galactosidase in cultured peripheral neurons. Science **24:**1667–1669.
14. **Green, M. R.** 1991. Biochemical mechanisms of constitutive and regulated pre-mRNA splicing. Annu. Rev. Cell Biol. **7:**559–599.
15. **Hahn, C. S., Y. S. Hahn, T. J. Braciale, and C. M. Rice.** 1992. Infectious Sindbis virus transient expression vectors for studying antigen processing and presentation. Proc. Natl. Acad. Sci. USA **89:**2679–2683.
16. **Heinzinger, N. K., M. I. Bukinsky, S. A. Haggerty, A. M. Ragland, V. Kewalramani, M. A. Lee, H. E. Gendelman, L. Ratner, M. Stevenson, and M. Emerman.** 1994. The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. Proc. Natl. Acad. Sci. USA **91:**7311–7315.
17. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cultures. J. Mol. Biol. **26:**365–369.
18. **Jolly, D.** 1994. Viral vector systems for gene therapy. Cancer Gene Ther. **1:**51–64.
19. **Kean, K. M., C. Wychowski, H. Kopecka, and M. Girard.** 1986. Highly infectious plasmids carrying poliovirus cDNA are capable of replication in transfected simian cells. J. Virol. **59:**490–493.
20. **Lebkowski, J. S., R. B. Dubridge, E. A. Antell, K. S. Greisen, and M. P. Calos.** 1984. Transfected DNA is mutated in monkey, mouse, and human cells. Mol. Cell. Biol. **4:**1951–1960.
21. **Lemm, J. A., T. Rumenaph, E. G. Strauss, J. H. Strauss, and C. M. Rice.** 1994. Polypeptide requirements for assembly of functional Sindbis virus replication complexes: a model for the temporal regulation of minus- and plus-strand RNA synthesis. EMBO J. **13:**2925–2934.
22. **Levis, R., B. G. Weiss, M. Tsiang, H. Huang, and S. Schlesinger.** 1986. Deletion mapping of Sindbis virus DI RNAs derived from cDNAs defines the sequences essential for replication and packaging. Cell **44:**137–145.
23. **Liljestrom, P.** 1994. Alphavirus expression systems. Curr. Opin. Biotechnol. **5:**495–500.
24. **Liljestrom, P., and H. Garoff.** 1991. A new generation of animal cell expression vectors based on the Semliki Forest virus replicon. Bio/Technology **9:**1356–1361.
25. **Lustig, S., A. C. Jackson, C. S. Hahn, D. E. Griffin, E. G. Strauss, and J. H. Strauss.** 1988. Molecular basis of Sindbis virus neurovirulence in mice. J. Virol. **62:**2329–2336.
26. **MacGregor, G. R., A. E. Mogg, J. F. Burke, and C. T. Caskey.** 1987. Histochemical staining of clonal mammalian cell lines expressing E. coli beta-galactosidase indicates heterogenous expression of the bacterial gene. Somat. Cell Mol. Genet. **13:**253–265.
27. **Miller, A. D., D. G. Miller, J. V. Garcia, and C. M. Lynch.** 1993. Use of retroviral vectors for gene transfer and expression. Methods Enzymol. **217:**581–599.
28. **Montgomery, D. L., J. W. Shiver, K. R. Leander, H. C. Perry, A. Friedman, D. Martinez, J. B. Ulmer, J. J. Donnelly, and M. A. Liu.** 1993. Heterologous and homologous protection against influenza A by DNA vaccination: optimization of DNA vectors. DNA Cell Biol. **12:**777–783.
29. **Moss, B., and C. Flexner.** 1987. Vaccinia virus expression vectors. Annu. Rev. Immunol. **5:**305–324.
30. **Nelson, J. A., J. W. Gnann, and P. Ghazal.** 1990. Regulation and tissue-specific expression of human cytomegalovirus. Curr. Top. Microbiol. Immunol. **154:**77–103.
31. **Olivo, P., I. Frolov, and S. Schlesinger.** 1994. A cell line that expresses a reporter gene in response to infection by Sindbis virus: a prototype for

detection of positive strand RNA viruses. Virology **198:**381–384.

32. **Ou, J.-H., D. W. Trent, and J. H. Strauss.** 1982. The 3′ non-coding regions of alphavirus RNAs contain repeating sequences. J. Mol. Biol. **156:**719–730.
33. **Pattnaik, A. K., L. A. Ball, A. W. LeGrone, and G. W. Wertz.** 1992. Infectious defective interfering particles of VSV from transcripts of a cDNA clone. Cell **69:**1011–1020.
34. **Perrotta, A. T., and M. D. Been.** 1991. A pseudoknot-like structure required for efficient self-cleavage of hepatitis delta virus RNA. Nature (London) **350:**434–436.
35. **Polo, J. M., N. L. Davis, C. M. Rice, H. V. Huang, and R. E. Johnston.** 1988. Molecular analysis of Sindbis virus pathogenesis in neonatal mice by using virus recombinants constructed in vitro. J. Virol. **62:**2124–2133.
36. **Price, J., D. Turner, and C. Cepko.** 1987. Lineage analysis in the vertebrate nervous system by retrovirus-mediated gene transfer. Proc. Natl. Acad. Sci. USA **84:**156–160.
37. **Racaniello, V. R., and D. Baltimore.** 1981. Cloned poliovirus cDNA is infectious in mammalian cells. Science **214:**916–919.
38. **Raju, R., and H. V. Huang.** 1991. Analysis of Sindbis virus promoter recognition in vivo, using novel vectors with two subgenomic mRNA promoters. J. Virol. **65:**2501–2510.
39. **Reddy, V. B., P. K. Gosh, P. Lebowitz, M. Piatak, and S. M. Weissman.** 1979. Simian virus 40 early mRNAs. I. Genome localization of 3′ and 5′ termini and two major splices in mRNA from transformed and lytically infected cells. J. Virol. **30:**279–296.
40. **Rice, C. M., R. Levis, J. H. Strauss, and H. V. Huang.** 1987. Production of infectious RNA transcripts from Sindbis virus cDNA clones: mapping of lethal mutations, rescue of a temperature-sensitive marker, and in vitro mutagenesis to generate defined mutants. J. Virol. **61:**3809–3819.
41. **Rosenfeld, M. A., W. Siegfried, D. Yoshimura, K. Yoneyama, M. Fukayama, L. E. Stier, P. K. Paakko, P. Gilardi, L. D. Stratford-Perricaudet, M. Perricaudet, S. Jallat, A. Pavirani, J.-P. Lecocq, and R. G. Crystal.** 1991. Adenovirus-mediated transfer of a recombinant anti-antitrypsin gene to the lung epithelium in vivo. Science **252:**431–434.
42. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
43. **Samulski, R. J., L.-S. Chang, and T. Shenk.** 1989. Helper-free stocks of recombinant adeno-associated viruses: normal integration does not require viral gene expression. J. Virol. **63:**3822–3828.
44. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
45. **Schlesinger, S.** 1993. Alphaviruses—vectors for the expression of heterologous genes. Trends Biotechnol. **11:**18–22.
46. **Semler, B. L., A. J. Dorner, and E. Wimmer.** 1984. Production of infectious poliovirus from cloned cDNA is dramatically increased by SV40 transcription and replication signals. Nucleic Acids Res. **12:**5123–5141.
47. **Stenberg, R. M., P. R. Witte, and M. F. Stinski.** 1985. Multiple spliced and unspliced transcripts from human cytomegalovirus immediate-early region 2 and evidence for a common initiation site within immediate-early region 1. J. Virol. **56:**665–675.
48. **Strauss, E. G., E. M. Lenches, and J. H. Strauss.** 1976. Mutants of Sindbis virus. Virology **74:**154–168.
49. **Strauss, E. G., C. M. Rice, and J. H. Strauss.** 1984. Complete nucleotide sequence of the genomic RNA of Sindbis virus. Virology **133:**92–110.
50. **Strauss, J. H., and E. G. Strauss.** 1994. The alphaviruses: gene expression, replication, and evolution. Microbiol. Rev. **58:**491–562.
51. **Weiss, B., H. Nitschko, I. Ghattas, R. Wright, and S. Schlesinger.** 1989. Evidence for specificity in the encapsidation of Sindbis virus RNAs. J. Virol. **63:**5310–5318.
52. **Weiss, B. G., and S. Schlesinger.** 1991. Recombination between Sindbis virus RNAs. J. Virol. **65:**4017–4025.
53. **Xiong, C., R. Levis, P. Shen, S. Schlesinger, C. M. Rice, and H. V. Huang.** 1989. Sindbis virus: an efficient, broad host range vector for gene expression in animal cells. Science **243:**1188–1191.
54. **Zhou, X., P. Berglund, G. Rhodes, S. E. Parker, M. Jondal, and P. Liljestrom.** 1994. Self-replicating Semliki Forest virus RNA as recombinant vaccine. Vaccine **12:**1510–1514.

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