Identification of Mutations in a Sindbis Virus Variant Able to Establish Persistent Infection in BHK Cells: The Importance of a Mutation in the nsP2 Gene

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Sindbis virus is a positive strand RNA virus that has provided a valuable model for studying virus structure and replication. It is also being developed as a vector for the expression of heterologous proteins. Many studies with this virus are carried out in cultured BHK cells where infection is usually highly cytopathic and within 1 or 2 days after infection all of the cells are dead. Weiss et al. had established a persistently infected culture of BHK cells by infecting the cells with a virus preparation highly enriched in defective interfering (DI) particles and had isolated an attenuated virus, SIN-1 virus, from the culture (Weiss et al. [1980], J. Virol. 33, 463–474). SIN-1 virus, free of DI particles, was able to establish a persistent infection in BHK cells. We initiated studies to determine what changes in the genome of the virus were responsible for this phenotype. We describe here the cDNA cloning and sequencing of the 5' terminus and the four nonstructural protein genes from SIN-1 virus. A single coding mutation in the nsP2 gene (a predicted change of Pro-726 → Ser) produced a virus that was able to establish persistent infection in BHK cells. Additional mutations in the other genes were required to decrease the synthesis of viral RNA to a level similar to that found in cells infected with SIN-1 virus. Incorporation of the nsP2 mutation into a Sindbis virus expression vector led to a higher level of synthesis of the reporter protein, β-galactosidase, than that obtained with the original Sindbis virus replicon. © 1997 Academic Press.

Sindbis virus, the prototype virus of the Alphavirus genus, infects a broad range of cells and in most cases replicates to high titers. Infection is usually highly cytopathic in cultured vertebrate cells, but not in most cultures of mosquito cells. Several years ago we showed that the infection could be modulated in BHK cells by the presence of high concentrations of defective interfering (DI) particles (Weiss et al., 1980). The presence of these particles in the virus population permitted a small number of cells to survive the infection. These cells eventually were able to establish a stable culture that was persistently infected with Sindbis virus. One month after infection the cells had a growth rate indistinguishable from uninfected parental cells. The cells did not produce interferon and the detection of both viral-specific RNA and protein in the vast majority of the cells demonstrated that most of them were infected. We isolated from the culture a virus (SIN-1 virus) that was attenuated and was able to establish a persistent infection in the absence of DI particles. BHK cells infected with SIN-1 virus synthesized about 10-fold less viral-specific RNA than cells infected with the original wild-type virus and there was a delay in inhibition of host cell protein synthesis. In spite of the decrease in viral RNA synthesis, there was not a major effect on virus growth and yield. The properties of SIN-1 virus suggested that it might provide some clues for understanding how Sindbis virus interferes with host cell processes and causes cell death and for this reason we began a study to identify the mutations responsible for the phenotype of this virus.

The genome of Sindbis virus is a single-stranded RNA that contains two open reading frames (ORF). The first, located in the 5' two-thirds of the RNA, codes for the nonstructural proteins; the second, contained in the 3' one-third of the genome, codes for the structural proteins, a capsid protein, a small hydrophobic membrane protein (the 6K protein), and the two glycoproteins which comprise the spikes of the virion particle (reviewed in Schlesinger and Schlesinger, 1996; Strauss and Strauss, 1994). In cells infected with Sindbis virus, the 5' ORF is translated from the genomic RNA into a polyprotein which is proteolytically processed into four polypeptides, referred to as nsP1, nsP2, nsP3, and nsP4. The polyprotein and its cleavage products are required for replication and transcription of the viral RNAs. The 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 is produced by transcription of the genome-length complementary (minus) strand from an internal promoter.

Here we report the cloning of the cDNA of the 5' terminus and the nonstructural protein region of the SIN-1 genome and the mapping of mutations in this part of the genome. The nsP genes individually and collectively...
were cloned into the full-length cDNA clone of Sindbis virus (Toto1101)—a cDNA which can be transcribed in vitro by the SP6 DNA-dependent RNA polymerase to produce infectious transcripts (Rice et al., 1987). The major conclusion of our studies is that a single predicted amino acid change in nsP2 converted Sindbis virus Toto1101 from a virus that kills essentially all of the infected BHK cells to one that was able to establish a persistent infection in some fraction of the cells. Additional mutations were required to decrease the synthesis of viral RNA to a level similar to that found in cells infected with SIN-1 virus.

**MATERIALS AND METHODS**

**Cells and viruses**

BHK-21 cells were maintained in α-MEM containing 10% fetal bovine serum (FBS) supplemented with 100 units/ml of penicillin and 100 μg/ml of streptomycin (BHK medium).

The original SIN-1 virus was described previously (Weiss et al., 1987). SIN-1 virus was plaque purified three times on chicken embryo fibroblasts before preparation of a stock for isolation of the RNA to be used for cloning. After each plaque purification, the eluted virus was used to infect a new dish of BHK cells. Three days after infection, the surviving cells were extensively washed and new BHK medium was added. The next day the virus released into the extracellular medium was harvested and sub-Sequencing of double-stranded DNA was performed into the extracellular medium was harvested and sub-Sequencing of double-stranded DNA was performed.

**cDNA cloning and plasmid constructions**

Oligonucleotides that spanned several different regions of the Sindbis virus genome were used with the Mo-MLV-RT (Gibco-BRL) in the synthesis of first strands of cDNA fragments. These oligonucleotides and those used for second strand synthesis are listed in Table 1. Mo-MLV-RT and the Klenow fragment of DNA polymerase I (Boehringer-Mannheim) were used for second strand synthesis after digestion of the RNA with 0.3 M NaOH for 30 min at 65°. Figure 1a shows the location of these cDNAs in the Sindbis virus genome. The cDNAs were inserted into a Smal-cut pUC18 plasmid by blunt end cloning so that the termini of some of the clones do not always line up precisely with the primers. The cDNA clones were first mapped with restriction enzymes and were then sequenced. The 5′-terminal region of the genome was cloned following amplification of the cDNA by PCR. The cDNA was amplified using oligonucleotides corresponding to regions 1452–1466 and 1–20 (Table 1). The nucleotide sequence of two 5′-terminal clones was determined to establish that the sequence was representative and did not contain PCR-derived mutations. The fragment extending from nt 1404 (Esco7III site) to nt 2162 (BspHI site) was also obtained by PCR and two independent clones were sequenced. A fragment of cDNA containing mutations in only one of the nonstructural protein genes was cloned into the Toto1101 plasmid replacing that segment of the equivalent wild-type gene (see Fig. 1c). The restriction endonuclease sites used for cloning are shown in Fig. 1a. The plasmid pV5′nsP1-3(SIN-1) was cloned using an SstI site located upstream of the sequences encoding the SP6 DNA-dependent RNA polymerase promoter. The virus V5′nsP1-3(SIN-1) contained sequences derived from SIN-1 beginning with nucleotide 21 (nt 1–20 are from the PCR primer).

**Sequence analysis**

Sequencing of double-stranded DNA was performed with Sequenase v.2.0 kit (Amersham), with three-lane data compression as described (Nelson et al., 1993). Sequences were analyzed using the Wisconsin Sequence Analysis Package (Genetic Computer Group, Inc., WI) and AS alignment analysis package (Resenchuk and Blinov, 1995). Virus sequences used for comparison are: Western equine encephalitis virus, Accession No. X74892 (Uryvaev et al., 1994; Yuferov et al., 1992); Eastern equine encephalitis virus, X63135, X67711 (Volchkov et al., 1991); Sindbis virus, J02363, J02364, J02365, J02366, J02367, V00073 (Strauss et al., 1984); Semliki Forest virus, J02361, J02362, L00018, V01399, V01400, V01401, X04129 (Takken, 1986); Ross River virus, M 20162 (Faragher et al., 1987); O’Nyong-nyong virus, M 20303, M 33999 (Levinson et al., 1990; Strauss et al., 1984); Venezuelan equine encephalitis virus, L04653, L00931 (Kinney et al., 1992).

**Transcriptions and transfections**

The Sindbis virus cDNAs were positioned downstream of the SP6 DNA-dependent RNA polymerase promoter. RNA transcription reactions were carried out as recommended by the supplier (Gibco-BRL), using the 5′ cap analog 7′G5′ppp5′G (New England Biolabs). For the experiments reported here the RNAs were used after transcription without further purification. Transfections were carried out by electroporation using a Bio-Rad Gene Pulser apparatus as de-
TABLE 1

Oligonucleotides Used for Cloning of SIN-1 cDNA

<table>
<thead>
<tr>
<th>Name of oligonucleotide</th>
<th>Strand</th>
<th>Start</th>
<th>End</th>
<th>Sequence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Used with:</th>
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<tbody>
<tr>
<td>8</td>
<td>-</td>
<td>8101</td>
<td>8115</td>
<td>TAAATTGAGCTTTG</td>
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<tr>
<td>6602</td>
<td>+</td>
<td>4051</td>
<td>4069</td>
<td>ATTGCCGTATTTGCCTCGT</td>
<td>8</td>
</tr>
<tr>
<td>4302</td>
<td>+</td>
<td>1680</td>
<td>1689</td>
<td>gccatagGCATTAGTTG</td>
<td>8</td>
</tr>
<tr>
<td>4300</td>
<td>-</td>
<td>4204</td>
<td>4221</td>
<td>AACGTTTATAGGCCAC</td>
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</tr>
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<td>4100</td>
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<td>1003</td>
<td>1019</td>
<td>TGACAAAGTTACTGAC</td>
<td>4300, 1675</td>
</tr>
<tr>
<td>1675</td>
<td>-</td>
<td>2806</td>
<td>2823</td>
<td>CTGTCATTCTCATGTC</td>
<td>4100</td>
</tr>
<tr>
<td>8217</td>
<td>+</td>
<td>1</td>
<td>20</td>
<td>ggtggagctctagactcactatagAT</td>
<td>439919</td>
</tr>
<tr>
<td>439919</td>
<td>-</td>
<td>1451</td>
<td>1465</td>
<td>TGACGGCGTAGTACACAC</td>
<td>8217</td>
</tr>
</tbody>
</table>

<sup>a</sup> Bases shown in lowercase are not complementary to Sindbis virus sequences.

scribed (Liljestrom et al., 1991), except that settings for electroporation were 2.0 kV, 25 mF, and 400 Ohm. Virus particles were harvested when the cells showed evidence of cytopathic effects (CPE). Toto1101 and viruses with similar properties were harvested between 20 and 24 hr posttransfection, the other viruses were usually harvested between 40 and 48 hr posttransfection.

Analysis of viral RNA synthesis

BHK-21 cells (2 × 10<sup>5</sup> cells per well of 6-well plate) were infected with SIN-1, Toto1101, or recombinant viruses at an m.o.i. of 20 PFU/cell in phosphate-buffered saline (PBS) containing 1% FBS for 1 hr at 4°C. The inoculum was then removed, BHK-medium was added, and the cells were placed at 37°C. After incubation for 30 min the medium was replaced with the same medium containing dactinomycin (1 μg/ml), and 30 min later [<sup>3</sup>H]uridine was added (10 μCi/ml). At specified times the cells were washed with cold PBS and lysed in TET buffer (10 mM Tris – HCl, pH 8.0, 1 mM EDTA, 0.02% Triton X-100). RNAs were precipitated by addition of an equal volume of 25% (w/v) of trichloroacetic acid and the incorporated radioactivity was quantitated by liquid scintillation counting.

Persistent infections

BHK-21 cells (5 × 10<sup>5</sup> cells) were plated into 60-mm plates and were infected 4 hr later with one of the Sindbis viruses at an m.o.i. of 5 PFU/cell. The number of viable cells at specified intervals was calculated by counting cells in the presence of trypan blue. Cells were immunostained with anti-Sindbis virus antibodies to determine the number of cells expressing Sindbis virus proteins.

Sindbis virus replicons and analysis of β-galactosidase activity

Replicon RNAs were packaged by cotransfection with the defective helper RNA DH-BB (Bredenbeek et al., 1993). BHK-21 cells (2 × 10<sup>5</sup>) in 35-mm dishes were infected at m.o.i.s of 0.1–0.5 infectious units/cell in 0.5 ml of PBS with 1% of FBS for 1 hr at 4°C. The inoculum was then replaced with BHK medium and the cells were placed at 37°C. After incubation times as indicated the cells were washed with PBS and lysed in TET buffer. The activity of β-galactosidase was measured by hydrolysis of o-nitrophenyl-β-D-galactopyranoside. The number of infected cells was determined by immunofluorescence using antibodies directed against β-galactosidase.

RESULTS

Identification of mutations in the SIN-1 virus genome

cDNAs derived from the coding region in the nonstructural protein genes of the SIN-1 RNA were cloned first into the plasmid pUC18 and then into the Toto1101 plasmid (Fig. 1).
FIG. 1. Schematic diagrams of the nsP genes of Sindbis virus. (a) Localization of the cDNA clones in relation to each of the nsP genes. The restriction endonucleases used for substitution of regions of Toto1101 cDNA with fragments from the SIN-1 cDNA are indicated. (b) Position of the mutations in SIN-1. (c) Hybrid genomes. The speckled regions represent the region derived from SIN-1. The open bars and the 3' one-third of the genome were from Toto1101. Division of the nsP2 gene was at nucleotide 2288. The fragments designated by a striped bar contain the mutation in the nsP4 cDNA but its presence in SIN-1 RNA has not been established.

pToto1101 is a plasmid containing the full-length cDNA clone of Sindbis virus positioned downstream of the promoter for the SP6 DNA-dependent RNA polymerase; infectious RNA can be transcribed in vitro from this cDNA (Rice et al., 1987). Restriction fragments from SIN-1 cDNAs that contained the mutations in SIN-1 from the nsP1, nsP2, nsP3, or nsP4 genes were used to replace the equivalent regions in the Toto1101 clone (Fig. 1c). The nsP2 gene was subdivided to further map the position of mutations responsible for a change in phenotype. The complete nonstructural protein region of the virus was sequenced after it had been inserted into pToto1101. In some cases the DNA was also sequenced in pUC18. The specific changes found and comparisons with other alphaviruses are shown in Table 2. The mutation in the cloned nsP4 gene was found in only one of two cDNA clones analyzed. It was not detected in the original SIN-1 RNA and must be derived from a minor species in the original RNA or represent a change that occurred during cloning. All of the other mutations were shown to be present in the recombinant virus RNA and in the original SIN-1 RNA by RT-PCR and cycle sequencing.

Persistent infection of BHK cells with recombinant viruses

One of the hallmarks of the SIN-1 phenotype is its ability to cause persistent infections in BHK cells. Im-
TABLE 2

SIN-1 Specific Mutations in Nonstructural Protein Region: Comparison with Other Alphaviruses

<table>
<thead>
<tr>
<th>Position in genome</th>
<th>Gene</th>
<th>Nucleotide change</th>
<th>Amino acid position in the gene</th>
<th>Amino acid change</th>
<th>Amino acids in other alphaviruses*</th>
</tr>
</thead>
<tbody>
<tr>
<td>45</td>
<td>5′-UTR</td>
<td>T → C</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>120</td>
<td>nsP1</td>
<td>C → A</td>
<td>21</td>
<td>G → A</td>
<td>Q(6/6)</td>
</tr>
<tr>
<td>1775</td>
<td>nsP2</td>
<td>G → A</td>
<td>32</td>
<td>V → V</td>
<td>V(7/7)</td>
</tr>
<tr>
<td>1971</td>
<td>nsP2</td>
<td>T → C</td>
<td>98</td>
<td>F → L</td>
<td>F(7/7)</td>
</tr>
<tr>
<td>3855</td>
<td>nsP2</td>
<td>C → T</td>
<td>726</td>
<td>P → S</td>
<td>P(5/7), T, K</td>
</tr>
<tr>
<td>3866</td>
<td>nsP2</td>
<td>C → T</td>
<td>729</td>
<td>T → T</td>
<td>T(4/6), S(2/6)</td>
</tr>
<tr>
<td>4339</td>
<td>nsP3</td>
<td>A → T</td>
<td>80</td>
<td>E → V</td>
<td>E(7/7)</td>
</tr>
<tr>
<td>4864</td>
<td>nsP3</td>
<td>C → T</td>
<td>255</td>
<td>S → F</td>
<td>S(7/7)</td>
</tr>
<tr>
<td>5854</td>
<td>nsP4</td>
<td>G → A</td>
<td>29</td>
<td>R → H</td>
<td>R(5/7), A, E</td>
</tr>
</tbody>
</table>

* Frequencies of amino acids in alignment are shown in parentheses (number of sequences with particular amino acid/total number of sequences used).

b This mutation was found in one of two cDNA clones.

Important characteristics of this infection are that essentially all of the cells are synthesizing virus-specific products and that low levels of virus continue to be produced (Weiss et al., 1980). The cells are resistant to superinfection by Sindbis virus, but are sensitive to infection by other viruses, demonstrating that interferon is not an important factor in maintaining the infection. Once we obtained infectious virus from the cloned samples described above, we were able to determine if viruses containing mutations derived from SIN-1 in only one of the nsP genes (VnsP1(SIN-1), VnsP2(SIN-1), or VnsP3(SIN-1)) would show the SIN-1 phenotype. Cell survival was only observed with those viruses that contained an nsP2 gene from SIN-1 (Fig. 2) and was essentially the same if only the nsP2 gene was derived from SIN-1 or if additional SIN-1 mutations were also included (VnsP1-3(SIN-1) and V5′nsP1-3(SIN-1)). The surviving cells were resistant to superinfection by Toto1101. (Data not shown.) None of the BHK cells infected with Toto1101, VnsP1(SIN-1), VnsP3(SIN-1) or, VnsP4 survived for more than 24 hr.

In several independent experiments BHK cells that survived infection with VnsP2(SIN-1) were followed for several weeks to establish that the cells began to grow normally and maintained the persistent infection. An immunofluorescence assay in which the cells were treated with anti-Sindbis virus antibodies followed by a fluorescent-labeled second antibody showed that all cells were infected both at 1 and 23 days postinfection. In the earlier studies with SIN-1, we showed that a stable persistent infection was established before any detectable DI RNAs were detected (Weiss et al., 1980). BHK cells persistently infected with VnsP2(SIN-1) virus also showed no evidence of DI RNAs during the period in which a stable persistent infection was established, only the virus genomic and subgenomic RNAs were detected during the first week of cell survival. DI particles were identified in the cultures 3 weeks postinfection (data not shown).

The SIN-1 nsP2 gene has four mutations, but two of them are silent (Table 2). We constructed viruses that contained mutations either in the N-terminal [VnsP2N(SIN-1)] or C-terminal [VnsP2C(SIN-1)] half of nsP2 (Fig. 1c). Only VnsP2C(SIN-1) was able to establish a persistent infection. Cell survival and the establishment of persistent infection with VnsP2C(SIN-1) was identical to that shown for VnsP2(SIN-1) in Fig. 2. A single change in the nsP2 protein, a predicted change from Pro to Ser, was all that was required to convert Sindbis virus from one that killed all of the infected BHK cells into one which permitted many of the infected cells to survive and continue to produce viral RNA and proteins.

Viral RNA synthesis and virus growth

Another property associated with SIN-1 virus was that SIN-1-infected BHK cells synthesized significantly less
viral RNA than did cells that had been infected with the parental virus. This phenotype was not reproduced by VnsP2(SIN-1) or VnsP2C(SIN-1); BHK cells infected with these viruses synthesized about twofold less viral RNA than cells infected with Toto1101 (Table 3). The level of viral RNA synthesis could be reduced further when the SIN-1 mutations in the three nsP genes were combined [VnsP1-3(SIN-1)] and approached that seen with SIN-1 when the mutation in the 5' noncoding region of the genome was also included [V5'nsP1-3(SIN-1)]. The nucleotide change in the nsP4 gene by itself had almost no effect on the synthesis of virus RNA, but in combination with the other mutations in the nonstructural protein genes also produced a virus that had an RNA phenotype similar to that of SIN-1 virus.

For these comparisons the different viruses were all used at the same m.o.i. In addition, we analyzed the cells by immunostaining with anti-Sindbis virus antibodies to determine that they were all infected and that differences in the synthesis of viral RNA were not due to differences in the number of cells infected. We assumed that the relative rates of viral RNA synthesis represented a value that was the average for the population of cells infected by a particular virus; however, the rate of viral RNA synthesis probably reflects a spectrum of rates of viral RNA synthesis in individual cells.

The defects in RNA synthesis did not have major effects on the rate of virus production or virus yield. The growth of VnsP2(SIN-1) was identical to that of Toto1101 and there were minimal effects on the growth of SIN-1 virus (Fig. 3) and in other experiments the difference between Toto1101 and SIN-1 were not as great as that shown here.

Processing of the nsPs in BHK cells

The nsP2 protein is the protease responsible for cleaving the polyprotein containing nsP1, 2, 3, and 4 into the individual polypeptides. A defect in this protein might affect the proteolytic cleavages and this in turn could inhibit the synthesis of new positive strand RNAs. To test this possibility we examined the kinetics of proteolytic processing of the nsPs in BHK cells infected with Toto1101, VnsP2(SIN-1), or SIN-1. Cells were labeled with [35S]methionine at 3 hr postinfection for 15 min and chased for 0, 15, 30, or 60 min (see Materials and Methods). After immunoprecipitation, the nsPs were analyzed by electrophoresis in a polyacrylamide gel. No differences in the rate of processing were detected (data not shown).

Inhibition of host cell protein synthesis and synthesis of viral proteins

A characteristic of infection of BHK cells with Sindbis virus or with Sindbis virus replicons that do not express the virus structural proteins is the rapid inhibition of host cell protein synthesis (Frolov and Schlesinger, 1994; Liljestroem and Garoff, 1991). This inhibition of host cell protein synthesis was clearly seen in cells infected with Toto1101 or with VnsP3(SIN-1) by 6 hr postinfection (Fig. 4). At this time there was almost no reduction in host cell protein synthesis in cells infected with SIN-1 or with VnsP2(SIN-1). By 9 hr postinfection, effects on host cell protein synthesis were beginning to be seen in SIN-1-infected cells and were clearly visible in cells infected

### Table 3

<table>
<thead>
<tr>
<th>Virus</th>
<th>Relative rates of RNA synthesis</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toto 1101</td>
<td>1.0</td>
<td>±0.19</td>
</tr>
<tr>
<td>VnsP1(SIN-1)</td>
<td>1.0</td>
<td>±0.14</td>
</tr>
<tr>
<td>VnsP2(SIN-1)</td>
<td>0.65</td>
<td>±0.19</td>
</tr>
<tr>
<td>VnsP2C(SIN-1)</td>
<td>0.6</td>
<td>±0.19</td>
</tr>
<tr>
<td>VnsP2N(SIN-1)</td>
<td>0.9</td>
<td>±0.1</td>
</tr>
<tr>
<td>VnsP3(SIN-1)</td>
<td>1.0</td>
<td>±0.1</td>
</tr>
<tr>
<td>VnsP4</td>
<td>0.8</td>
<td>±0.1</td>
</tr>
<tr>
<td>VnsP1-3(SIN-1)</td>
<td>0.4</td>
<td>±0.05</td>
</tr>
<tr>
<td>V5'nsP1-3(SIN-1)</td>
<td>0.2</td>
<td>±0.05</td>
</tr>
<tr>
<td>SIN-1</td>
<td>0.1</td>
<td>±0.04</td>
</tr>
<tr>
<td>VnsP1-4(SIN-1)</td>
<td>0.2</td>
<td>±0.1</td>
</tr>
</tbody>
</table>

* These values were obtained at 9 hr postinfection. In most experiments the incorporation of [3H]uridine was determined at several different time points to establish that rates of RNA synthesis were being measured. Toto 1101 RNA synthesis was set at 1.0.

* Calculations of standard deviation were based on a minimum of 3 independent experiments.

![Figure 3](image-url)

**FIG. 3.** Growth curve of SIN-1, VnsP2(SIN-1), and Toto1101 in BHK cells. Infection of BHK cells was identical to that described for the establishment of persistent infection (see Materials and Methods) except that the m.o.i. was 20. At the specified times samples were taken and the titer of the virus was determined on chicken embryo fibroblasts.
decrease in the ability of SIN-1 replicons to complement defective helper RNAs (Weiss and Schlesinger, 1981) and unpublished observations.] BHK cells were infected at m.o.i.s less than 1 so that on the average each cell would be exposed to only one replicon RNA and initially only one template for translation and replication. The number of cells that were expressing β-galactosidase was determined by immunostaining of the infected cells and the data were normalized to 100% of infected cells. The rate of synthesis of β-galactosidase in cells infected with SINrep/nsP2C(SIN-1)/LacZ was significantly higher during the first 9 hr postinfection than the rate in cells infected with SINrep/LacZ (Fig. 5). This is the same time period that the synthesis of host protein synthesis in cells infected with VnsP2(SIN-1) was not as extensively inhibited as in cells infected with Toto1101. It has been demonstrated previously that the capacity for protein synthesis in uninfected cells is greater than that in cells infected with Sindbis virus (Garry et al., 1979; Strauss et al., 1969). Cells infected with a Sindbis virus containing the nsP2 mutation may retain this higher capacity for total protein synthesis.

**DISCUSSION**

SIN-1 virus is an attenuated variant of Sindbis virus that is able to establish persistent infections in BHK cells in the absence of DI particles (Weiss et al., 1980). We undertook the cloning and sequencing of the 5' two-thirds of the genome of SIN-1 RNA to determine if mutation(s) in this region could reproduce this phenotype. We had focused our attention on this part of the genome based on earlier studies with Sindbis virus replicons which showed that inhibition of host cell protein synthesis depended only on the expression of the nonstructural proteins and did not require the synthesis of either the viral structural proteins or the subgenomic RNA (Frolov and Schlesinger, 1994). In addition BHK cells infected with SIN-1 virus are defective in the synthesis of viral-

**FIG. 4.** Autoradiogram of proteins synthesized in BHK cells 6 and 9 hr postinfection. Cells were labeled as described under Materials and Methods. Equal volumes from each extract were loaded on to a 10% polyacrylamide gel. Following electrophoresis the gel was dried and exposed to Kodak BioMax-MR film.

**FIG. 5.** Synthesis of β-galactosidase in BHK cells infected with SINrep/LacZ or with SINrepnsP2(SIN-1)/LacZ. Details are described under Materials and Methods.
specific RNA and we had assumed that this defect would be associated with changes in one or more of the nsPs or the 5′ terminus of the genome. We did not examine the 3′ terminus as the sequence is identical to that of wild-type virus RNA (Monroe et al., 1982). Our results supported these assumptions; a virus in which the genome contained a single coding mutation in the nsP2 gene of SIN-1 virus (a predicted change of Pro-726 → Ser) was able to establish persistent infection in BHK cells. Additional mutations in the nsP genes and in the 5′ noncoding sequence were required to obtain more substantial effects on the synthesis of viral RNA.

The nsP2 protein plays a pivotal role in the life cycle of alphaviruses. The C-terminal half of the protein is a protease (Ding and Schlesinger, 1989; Hardy and Strauss, 1989) and this activity is critical for the processing of the nsP polypeptide— a step that is important in the conversion of the replicase complex from one that synthesizes minus strand RNA to one that synthesizes the positive strand genomic and subgenomic RNA (Lemm et al., 1994; Shirako and Strauss, 1994). This protein contains a helicase motif in the N-terminal half (Gorbalenya et al., 1988) and has both ATPase and GTPase activities (Rikkonen et al., 1994). Mutations in the N-terminus also can affect the conversion of the replicase to the synthesis of plus strands, and this effect is not related to proteolytic processing of the polypeptide (De et al., 1996). Almost half of the nsP2 in cells infected with Semliki Forest virus is localized to the nucleus with most of the protein found within the nucleoli (Peränen et al., 1990; Rikkonen et al., 1992). It is the only one of the nsPs found in the nucleus. The importance of nuclear localization is not understood but appears to be a significant parameter for pathogenicity. A mutant of Semliki Forest virus was constructed in which the Arg649 of the nuclear localization signal (P^Ser^Ser^Arg^Arg^Val) in nsP2 was converted to Asp (Rikkonen, 1996). This virus was distinguished from the parental by two criteria: inhibition of host DNA synthesis was less than that seen in infected cells with the parental virus and, even more striking, its pathogenicity in mice was greatly diminished.

Another strategy for isolating noncytopathic mutants of Sindbis virus was the engineering of replicons to express the dominant selectable marker, puromycin acetyl-transferase (Frolov et al., 1996). Such replicons, which lack the structural protein genes, were transfected into BHK cells and clones resistant to puromycin were selected. Replication of Sindbis virus replicons normally leads to death in BHK cells (Frolov and Schlesinger, 1994) and the hypothesis was that the cell lines that had survived would contain mutations in the replicon which permitted cell survival. Two replicon genomes obtained from these cell lines have been analyzed and they both contain mutations in the nsP2 gene (Frolov et al., 1996). That study, the data presented here and elsewhere (Wang et al., 1994), and the evidence that nuclear localization affects pathogenicity, together suggest that a function or functions of nsP2 are involved in the cytopathic response to infection. In vertebrate cells this response usually leads to cell death which in a number of different cell lines is apoptotic (Levine et al., 1993).

Studies with Sindbis virus and Sindbis virus replicons had shown that expression of the virus glycoproteins on the surface of infected BHK cells cause cytopathic effects within a short time (10 – 12 hr) after infection (Frolov and Schlesinger, 1994). In contrast when cells were infected with replicons or with virus in which the glycoproteins do not reach the cell surface, these effects were delayed but became apparent after about 30 – 40 hr. The rapid cytopathogenicity associated with the expression of the viral glycoproteins may be due to effects of these proteins on cation transport (Carrasco et al., 1988; Despres et al., 1995; Ulug and Bose Jr., 1985; Ulug et al., 1989).

Alphaviruses have also been able to establish persistent infections in cultured vertebrate cells under conditions in which changes in the cell are thought to be responsible for survival. An early example was the establishment of a persistent infection of mouse L929 cells by Semliki Forest virus (Meinkoth and Kennedy, 1980). Only 1–20% of the cells expressed viral-specific antigens and the cells were resistant to infection by both homologous and heterologous virus. Induction of interferon was the major factor in permitting the L cells to survive infection. More recently a persistent infection by Sindbis virus in a rat prostate carcinoma cell line (AT-3 cells) expressing the human bcl-2 gene was reported (Levine et al., 1993). The acute infection of these cells by Sindbis virus is severely restricted with titers ranging from 10- to 100-fold lower in the AT-3 bcl-2 expressing cells than in the controls (Ubol et al., 1994). Only a small fraction of the AT3-cells expressing bcl-2 produced viral-specific antigens, indicating that the low titers were due to a small number of the cells being productively infected (Frolov and Schlesinger, unpublished results). One of the most interesting and important findings in the studies with the AT3 cells expressing bcl-2 is that not only the status of the cells but also the Sindbis virus used for the infection were critical for cell survival. A strain of Sindbis virus in which the E2 glycoprotein has a His at position 55 infects and kills both AT3 cells expressing bcl-2 and the control cells. In contrast when the amino acid at position 55 in the E2 protein is Gin, the AT3 cells expressing bcl-2 replicate the virus poorly and survive infection (Ubol et al., 1994). These results demonstrated that the sequence of the structural proteins, in particular the E2 glycoprotein, can play an important role in cell survival. In this regard, we have not yet investigated the possibility that SIN-1 virus contains mutations in the structural protein genes that have an effect on virus replication. The VnsP2(SIN-1) virus appeared to establish a persistent infection in BHK cells as well as the original SIN-1 virus, but those viruses that contained the SIN-1 mutations in
the 5' 2/3's of the genome synthesized RNA at a level slightly higher than cells infected with the original SIN-1 virus. Other investigators have proposed that the viral glycoproteins can influence the rate of viral replication (Tucker et al., 1993).

Alphavirus replicons are being developed as expression vectors and potentially for use in gene therapy (Bredenbeek and Rice, 1992; Dubensky et al., 1996; Frolov et al., 1996; Herweijer et al., 1995; Schlesinger, 1993; Xiong et al., 1989). Replicons that are able to express a heterologous protein without deleterious effects on the cell would be of particular value for any therapeutic uses. Sindbis virus replicons containing mutations in the nsP2 gene produced heterologous proteins at levels that were even higher than those produced by the original replicons (this work and unpublished observations). Recent studies have indicated that Sindbis virus infection of AT-3 cells (see above) and N18 cells (a neuroblastoma cell line) activate the transcription factor NF-κB (Lin et al., 1995). Treatment of cells with the thiol agents N-acetylcysteine or pyrrolidine dithiocarbamate, known inhibitors of NF-κB, prevented the apoptotic cell death normally associated with infection but did not appear to affect virus replication. Lin et al. (1995) proposed that NF-κB activation may be correlated with the onset of apoptosis and it will be of interest to determine if nsP2 could be involved in this activation.

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REFERENCES


