A Mutant of Sindbis Virus that Is Resistant to Pyrazofurin Encodes an Altered RNA Polymerase

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Pyrazofurin (PZF), a cytidine analog and an inhibitor of orotate monophosphate decarboxylase, has been shown to decrease the levels of UTP and CTP in treated cells. When Sindbis virus (SV)-infected Aedes albopictus cells were treated with PZF, the yield of virus was reduced 100- to 1000-fold. By serial passage of our standard SVSTD in Ae. albopictus cells in the presence of increasing concentrations of PZF, a mutant, SV PZF, was derived, which was not inhibited by PZF. SV PZF is also resistant to adenosine, guanosine, and phosphono-acetyl-N-aspartate, all of which have been shown to decrease levels of UTP and CTP. Analysis of chimeric viruses containing sequences from the SV STD and parental genomes showed that the sequence between nt 5262 and 7999 conferred the PZF-resistant phenotype. Sequencing of this region identified four mutations (nt 5750, 6627, 7543, and 7593), which are predicted to lead to amino acid changes, opal550L in nsP3 and M287L, K592I, and P609T in nsP4. Characterization of viruses containing one or more of these mutations demonstrated that all three mutations in the nsP4 coding region are required to produce full resistance to PZF. Using a molecular model of nsP4 based on the structure of HIV reverse transcriptase, we located amino acid change M287L at the tip of the fingers domain and K592I and P609T at the base of the thumb domain of the viral RNA polymerase. We suggest that these three amino acid changes in nsP4 alter the geometry of the NTP binding pocket so as to increase the affinity of the enzyme for CTP and UTP.

INTRODUCTION

Sindbis virus is the prototype virus of the genus alphavirus, family Togaviridae. Its genome is a single-stranded RNA, of messenger polarity, about 11700 nucleotides in length. The 5′ end of the RNA has a type 0 cap, and the 3′ end is polyadenylated. In addition to the genomic RNA, another viral mRNA, the subgenomic or 26S RNA, is found in infected cells. The sequence of the 26S RNA, which encodes the viral structural proteins, is identical to that of the 3′ third of the genomic RNA. Like the genomic RNA, the subgenomic RNA is capped and polyadenylated (see Strauss and Strauss, 1994, for a review).

The genomic RNA encodes the four nonstructural (ns) proteins: nsP1, nsP2, nsP3, and nsP4. These proteins are generated by the processing of two polyproteins: p123, which gives rise to nsP1, nsP2, and nsP3, and p1234, which gives rise to all four of the ns proteins, p123 is made when the ribosome recognizes an opal codon near the 3′ end of the nsP3 coding sequence. At a low frequency (10–20% in vitro), the ribosome reads through the opal codon, and p1234 is made (DeGroot et al., 1990; Li and Rice, 1993). Thus nsP4 is made in lesser amounts than the other three ns proteins. Because there are several stop codons at the end of the nsP4 coding sequence, the structural proteins are not translated from the genomic RNA.

The four ns proteins exist within infected cells as a multiprotein complex (Barton et al., 1991), the function of which is to replicate and transcribe the viral RNAs. Exactly how these proteins interact in this complex is not known. However, there is some information about the functions of the individual proteins. nsP1 has both GTP methyltransferase activity (Mi et al., 1989; Mi and Stollar, 1990) and RNA guanylyltransferase activity (Ahola and Kääriäinen, 1995) and is thus assumed to be responsible for the modification of the 5′ terminus of the two viral mRNAs. nsP2 has an N-terminal domain, with helicase activity (Gomez et al., 1999), which is presumably responsible for the unwinding of the fully or partially dsRNAs during replication and translation, and a C-terminal protease domain, responsible for processing of the p123 and p1234 polyproteins (Hardy and Strauss, 1989). The function of nsP3 is not known. However, it is the only ns protein that is phosphorylated (Peranen et al., 1988; Li et al., 1990). nsP4 contains a GDD sequence, the hallmark of nearly all viral RNA-dependent RNA polymerases (RDRPs) (Kamer and Argos, 1984). There also is good genetic evidence that nsP4 is the RDRP (Barton et al., 1988).

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Synthesis of the different forms of alphavirus RNA is controlled both temporally and quantitatively. Minus-strand RNA is made only early in infection, whereas plus-strand RNA is made both early and late (Sawicki and Sawicki, 1986). Minus-strand RNA is also made in much lesser amounts than plus-strand RNA. Early in infection, when processing of the ns polyproteins is incomplete, the replication complex contains mainly p123 and nsP4, or nsP1, p23, and nsP4. As the infection progresses, the efficiency of processing of the ns polyproteins increases, and the replication complexes then contain only the fully processed ns proteins, nsP1, nsP2, nsP3, and nsP4 (Lemm et al., 1994). These findings indicate that the processing of the ns proteins might govern the type of viral RNA that is made. Based on the results of experiments with ts mutants, Wang et al. (1994) presented a model suggesting that p23 was an essential component of the minus-strand polymerase. Subsequently, by making use of recombinant vaccinia virions to supply both a positive-strand template and the Sindbis virus ns proteins, in either their processed or unprocessed forms, Lemm et al. (1998) showed that minus-strand RNA was made only when the replication complexes contained p123 or p23. When the ns proteins were completely processed, the replication complexes were no longer capable of making minus-strand RNA. The crucial step in this transition appears to be the cleavage of p23 to nsP2 and nsP3.

To synthesize the subgenomic RNA, the replication complex must recognize an internal promoter on the minus-strand RNA. The minimal sequence essential for this promoter extends from nucleotide −19 to +5, relative to the initiation site (nt 7598) for the transcription of the subgenomic RNA (Levis et al., 1990). How the ns proteins are arranged in the replication complex that makes subgenomic RNA and whether this arrangement differs from that in the complex that makes full-length viral RNA are not known.

Additional information concerning the control of viral RNA synthesis and the functions of the ns proteins in this process has emerged from the study of ts mutants of Sindbis virus. For example, ts-11, the causal mutation of which is in the nsP1 coding region, ceases all viral RNA synthesis at the nonpermissive temperature (Barton et al., 1988), supporting the hypothesis that nsP4 is the RDRP.

We began the work described here with the goal of obtaining mutants of Sindbis virus that encoded an altered RDRP, with amino acid changes that would point to residues important for the binding of the ribonucleotide triphosphate (rNTP) substrates. To do this, we used an approach similar to that used previously to select our SVLM21 and SVMPA mutants; we selected for virus able to replicate in cells with decreased levels of a substrate critical for viral replication (Durbin and Stollar, 1985, Scheidel et al., 1987). Thus SVLM21 is able to grow in Aedes albopictus mosquito cells that have been deprived of methionine and should contain low levels of S-adenosylmethionine, and SVMPA is able to replicate in Aedes albopictus cells treated with mycophenolic acid, a compound that interferes with the synthesis of GMP. Such cells therefore are expected to contain low levels of GTP.

To obtain a mutant Sindbis virus that encodes an RDRP with altered substrate binding properties, we decided to select for virus that can grow in cells with low levels of one or more of the rNTP substrates. Pyrazofurin (PZF) is a cytosine analog that, in its monophosphate form, inhibits orotidine monophosphate (OMP) decarboxylase and thus interferes with the synthesis of pyrimidine nucleotides, thereby lowering intracellular levels of UTP and CTP (Cadman et al., 1978; Dix et al., 1979). After demonstrating that PZF inhibited the replication of SVSTD in Ae. albopictus cells, we were able to select a viral mutant, SVPFZ, that is resistant to this drug. In this report, we describe the properties of SVPFZ and identify three mutations found in the nsP4-coding region of the SVPFZ genome.

RESULTS

Isolation and characterization of SVPFZ

Figure 1 shows that in mosquito cells, the replication of SVSTD was readily inhibited by PZF. At 2.5 μM PZF, the yield of virus in the medium was reduced more than 1000-fold, and at 5.0 μM PZF, maximum inhibition was observed. When CEF or BHK cells were similarly infected with SVSTD and treated with PZF, no reduction in virus yield was noted.

To obtain a viral mutant resistant to PZF, Sindbis virus was serially passaged without dilution in Ae. albopictus cells in the presence of increasing concentrations of PZF. We began with SVSTD and carried out a total of 38 passages, each time harvesting the progeny virus 24 h after infection. Infected cells were maintained in the presence of 2.5 μM PZF for 8 passages, 5.0 μM for 10 passages, 10 μM for 12 passages, and 15 μM PZF for 8 passages. At the end of the virus passage, the resulting virus was
plaque-purified on CEF and then grown to a stock in CEF. The titer of this stock was $1.3 \times 10^9$ PFU/ml.

Figure 2 shows that although 2.5 $\mu$M PZF reduced the yield of SV STD from infected mosquito cells by approximately 1000-fold, there was no reduction in the yield of virus from SVPZF-infected cells that were similarly treated. On the contrary, at low concentrations of PZF, the yield of SVPZF was actually increased; in this experiment, the yield increased about 10-fold over that from untreated cells. Similar increases in the yield of infectious SV PZF virus at PZF concentrations of 0.5 to 2.5 $\mu$M were observed in most experiments of this type.

In earlier work, we showed that both adenosine and guanosine reduced the yield of SV from infected Ae. albopictus cells. Because both of these nucleosides, like PZF, lower the intracellular concentrations of UTP and CTP (Stollar and Malinoski, 1981), we wanted to determine whether SVPZF might be cross-resistant to these purine nucleosides. Table 1 shows that although adenosine reduced the yield of SV STD up to 1000-fold, there was no effect on the titer of SV PZF. Similar findings were observed with guanosine. Thus SV PZF is cross-resistant to both adenosine and guanosine. SV PZF was not temperature sensitive, nor was it resistant to mycophenolic acid, an inhibitor of GMP synthesis.

Although we undertook these experiments with the assumption that PZF inhibited viral replication by reducing the intracellular concentrations of UTP and CTP to levels incompatible with viral RNA synthesis, we did consider another possibility to explain the effect of PZF. It has been shown that PZF is phosphorylated to the monophosphate, diphosphate, and triphosphate levels in L1210 leukemia cells (Sant, 1989). Thus it is possible that the triphosphate form of PZF might be recognized by the viral RDRP, interfere with the binding of the other rNTPs at the catalytic site of the RDRP, and inhibit virus replication in this way.

Like PZF, N-phosphonoacetyl-L-aspartate (PALA) is an inhibitor of pyrimidine biosynthesis, but it acts at an earlier stage than does PZF, by preventing the condensation of l-aspartate and carbamoyl phosphate (Collins and Stark, 1971). However, because PALA is not a nucleoside analog, it is not expected to bind to the substrate binding site of the RDRP. Thus if PALA were able to inhibit the replication of viral RNA, it would be expected to do so only by lowering the levels of UTP and CTP. If SVPZF were cross-resistant to PALA, it would be highly

**FIG. 1.** Effect of PZF on the replication of SV STD in *Ae. albopictus* cells. Cells were infected at an m.o.i. of 5 PFU/cell as described in the text and incubated at 34.5°C. Medium was harvested at 24 h p.i. and assayed for infectious virus by plaque assay on CEF.

**FIG. 2.** Replication of SV STD (○) and SV PZF (●) in *Ae. albopictus* cells in the presence of different concentrations of PZF. Cells were infected with either SV STD or SV PZF at an m.o.i. of 5 PFU/cell and maintained as described for Fig. 1. Medium was harvested at 24 h p.i. and assayed for infectious virus by plaque assay on CEF.

**TABLE 1**

Cross-Resistance of SV PZF to Adenosine and Guanosine

<table>
<thead>
<tr>
<th>Virus</th>
<th>Adenosine (µM)</th>
<th>Guanosine (µM)</th>
<th>Yield (PFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SV STD</td>
<td>0</td>
<td>0</td>
<td>$2.2 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>0</td>
<td>$2.0 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>300</td>
<td>$3.9 \times 10^4$</td>
</tr>
<tr>
<td>SVPZF</td>
<td>0</td>
<td>0</td>
<td>$4.3 \times 10^7$</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>0</td>
<td>$5.7 \times 10^7$</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>300</td>
<td>$2.5 \times 10^5$</td>
</tr>
</tbody>
</table>

*Note.* Cells were infected at an m.o.i. of 5 PFU/cell. After adsorption at 28°C for 1 h, cells were re-fed with medium containing 1% dialyzed fetal calf serum and the indicated concentrations of either adenosine or guanosine and were incubated at 28°C. Medium was harvested at 20 h p.i. and assayed for infectious virus by plaque assay on CEF.
likely that both compounds inhibited viral replication via the same mechanism.

Figure 3 shows that PALA inhibits the replication of SVSTD in mosquito cells (as was the case with PZF, PALA had no effect on virus replication in BHK cells; data not shown) and demonstrates that SV_{PZF} is cross-resistant to PALA. We conclude, therefore, that PZF and PALA inhibit the replication of Sindbis virus in mosquito cells in a similar manner (i.e., by reducing the levels of UTP and CTP).

Localization and identification of the mutations responsible for the resistance to PZF

To determine which region of the SV_{PZF} genome is responsible for the drug-resistant phenotype of SV_{PZF}, we constructed chimeric Sindbis viruses containing various SV_{PZF} cDNA sequences in an SV_{Toto} background (Fig. 4) and tested them for resistance to PZF.

The three chimeric viruses that contained the entire SV_{PZF} nsP4-coding region were resistant to PZF (Table 2). These included SV_{Toto}:PZF:41-7999, SV_{Toto}:PZF:2784-7999, and SV_{Toto}:PZF:5262-7999. As was the case with SV_{PZF}, each of these viruses produced a higher yield in the presence of 2.5 μM PZF than in its absence. Chimeric viruses containing the SV_{PZF} nt sequence only from nt 41 to 2784 or from nt 5262 to 7334 (i.e., lacking all or part of the SV_{PZF} nsP4-coding sequence) were sensitive to PZF. The status of SV_{Toto}:PZF:7334-7999, which contains only the SV_{PZF} sequence coding for the C-terminal two thirds of NSP4, remains indeterminate. In contrast to SV_{PZF}, the yield of SV_{Toto}:PZF:7334-7999 did not increase in the presence of 2.5 μM PZF; however, neither did its yield drop very significantly, as is generally seen with virus that is completely sensitive.

Because the SV_{PZF} sequence from nt 5262 to 7999 was the smallest segment that could clearly transfer the PZF-resistant phenotype, we sequenced this entire region.

![FIG. 3. Effect of PALA on the replication of SV_{STD} and SV_{PZF} in Aedes albopictus cells. The experimental procedure was the same as described for Fig. 2 except that PALA was used instead of PZF.](image1)

![FIG. 4. Chimeric Sindbis virus genomes containing various sequences derived from SV_{PZF} RNA. Open boxes represent sequences from pToto. Solid boxes represent sequences from SV_{PZF}.](image2)
Four mutations were found that are predicted to result in amino acid changes (Table 3). The first mutation, at nt 5750, changes the opal codon near the 3' end of the nsP3-coding region to a Cys codon. The other three mutations occur in the nsP4 coding region at nt 6627, 7543, and 7593 and change Met 287 to Leu, Lys 592 to Ile, and Pro 609 to Thr (Sindbis virus nsP4 has 610 amino acids). It is noteworthy that the mutation at nt 7593 occurs at the 25 position of the minimal promoter for the synthesis of the subgenomic 26S RNA (taking the first nt of the transcript as the 11 position; Levis et al., 1990).

There were, in addition, two silent mutations in the nsP3 coding region: C5393U (Ala 431) and U5444A (Ser 448).

Site-directed mutagenesis

To determine which of these mutations is responsible for the resistance to PZF, we used site-directed mutagenesis to introduce the mutations listed in Table 3 into SVSTD, either one at a time or in combinations. For the sake of convenience, in this discussion and in Table 4, the mutations at nt 5750, 6627, 7543, and 7593 are referred to as mutations 1, 2, 3, and 4, respectively.

The introduction of mutations 1, 2, 3, or 4, one at a time, did not in any case produce resistance to PZF. When the mutations were introduced two at a time, the only combinations that led to PZF resistance were 2/4 and 3/4, but in neither case was the resistance as great as that seen with SVpZ.

<table>
<thead>
<tr>
<th>Virus</th>
<th>PZF (μM)</th>
<th>Yield (PFU/ml)</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVSTD</td>
<td>0</td>
<td>2.1 × 10^8</td>
<td>Sensitive</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>1.4 × 10^7</td>
<td></td>
</tr>
<tr>
<td>SVpZ</td>
<td>0</td>
<td>1.9 × 10^8</td>
<td>Resistant</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>1.4 × 10^7</td>
<td></td>
</tr>
<tr>
<td>SVpZ/PZF-41-2784</td>
<td>0</td>
<td>2.8 × 10^8</td>
<td>Sensitive</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>1.1 × 10^7</td>
<td></td>
</tr>
<tr>
<td>SVpZ/PZF-2784-7999</td>
<td>0</td>
<td>4.2 × 10^7</td>
<td>Resistant</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>2.1 × 10^4</td>
<td></td>
</tr>
<tr>
<td>SVpZ/PZF-41-7999</td>
<td>0</td>
<td>1.2 × 10^4</td>
<td>Resistant</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>3.9 × 10^4</td>
<td></td>
</tr>
<tr>
<td>SVpZ/PZF-5262-7999</td>
<td>0</td>
<td>6.0 × 10^7</td>
<td>Resistant</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>3.1 × 10^6</td>
<td></td>
</tr>
<tr>
<td>SVpZ/PZF-5262-7334</td>
<td>0</td>
<td>1.5 × 10^4</td>
<td>Sensitive</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>1.1 × 10^7</td>
<td></td>
</tr>
<tr>
<td>SVpZ/PZF-7334-7999</td>
<td>0</td>
<td>9.8 × 10^7</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>2.2 × 10^7</td>
<td></td>
</tr>
</tbody>
</table>

Note. Cells were infected at an m.o.i. of 5 PFU/cell with SVSTD, SVpZ, or the chimeric viruses shown above. After adsorption at 34.5°C for 1 h, cells were re-fed with serum-free medium containing 0.2% BSA in the presence or absence of 2.5 μM PZF and then incubated at 34.5°C. Medium was harvested at 24 h p.i. and assayed for infectious virus by plaque assay on CEF.

Combinations of the SVpZ mutations three at a time led to resistance only if mutation 4 was present, but a phenotype similar to that of SVpZ was seen only with mutations 2, 3, and 4. The combinations 1/2/4 and 1/3/4 gave only partial resistance. We conclude from these results that mutation 4 is essential for resistance to PZF and that mutation 1 is not required. Mutations 2 and 3 clearly enhanced the effect of mutation 4, and when both mutations 2 and 3 were combined with mutation 4, the full SVpZ phenotype was expressed.

TABLE 2
Phenotypes of Chimeric Viruses Containing Various SVpZ Sequences

| Virus PZF (μM) Yield (PFU/ml) Phenotype |
|------------------|-----------------|-------------|
| SVSTD            | 0               | 2.1 × 10^8  | Sensitive |
| SVpZ             | 0               | 1.9 × 10^8  | Resistant |
| SVpZ/PZF-41-2784 | 0               | 2.8 × 10^8  | Sensitive |
| SVpZ/PZF-2784-7999 | 0       | 4.2 × 10^7  | Resistant |
| SVpZ/PZF-41-7999 | 0               | 1.2 × 10^4  | Resistant |
| SVpZ/PZF-5262-7999 | 0        | 6.0 × 10^7  | Resistant |
| SVpZ/PZF-5262-7334 | 0       | 1.5 × 10^4  | Sensitive |
| SVpZ/PZF-7334-7999 | 0        | 9.8 × 10^7  | ?          |

<table>
<thead>
<tr>
<th>Virus</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVSTD</td>
<td>S</td>
</tr>
<tr>
<td>SVpZ</td>
<td>R</td>
</tr>
<tr>
<td>Virus with mutation(s)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>S</td>
</tr>
<tr>
<td>2</td>
<td>S</td>
</tr>
<tr>
<td>3</td>
<td>S</td>
</tr>
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<td>R</td>
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</tbody>
</table>

Note. Cells were infected at an m.o.i. of 5 PFU/cell with SVSTD, SVpZ, or the chimeric viruses shown above. After adsorption at 34.5°C for 1 h, cells were re-fed with serum-free medium containing 0.2% BSA in the presence or absence of 2.5 μM PZF and then incubated at 34.5°C. Medium was harvested at 24 h p.i. and assayed for infectious virus by plaque assay on CEF.

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Molecular model of nsP4

To understand the effects of the amino acid changes in SVnsP4 on the structure and function of the SV RDRP, it would be necessary to know the location in the protein of the altered amino acid residues and the relationships of these residues to conserved motifs that function in catalysis or binding of substrate or primer. Because no structure has been described for any alphavirus RDRP, we undertook to generate a molecular model of the SV nsP4 using the poliovirus RDRP (Hansen et al., 1997) and the HIV reverse transcriptase (Huang et al., 1998) as the reference molecules. Because the four sequence motifs, A, B, C, and D, common to viral RDRPs (Poch et al., 1989) are all present within the nsP4 sequence from 251 to 610, the first 250 amino acids were not used in constructing our model of nsP4.

In the model of nsP4 obtained using the polio virus 3D RDRP as the reference molecule, residues 592 and 609 were both located near the base of the thumb domain (not shown). However, in determining the structure of the poliovirus RDRP, there were several disordered regions (Hansen et al., 1997), which could not be included in the structure. As a result, the corresponding regions of the SV nsP4 were also missing in our molecular model.

We therefore turned to the HIV reverse transcriptase as the reference molecule. This molecule has been studied extensively, there are no gaps in its structure, and its structure has been determined with bound template-primer as well as bound substrate (Huang et al., 1998). As shown in Fig. 5, in the molecular model of nsP4 that we obtained using the HIV RT as the reference molecule, residues 592 and 609 were again found at the base of the thumb domain; however, with this model, we were now able to locate residue 287 to a loop at the tip of the fingers domain.

DISCUSSION

The first collections of Sindbis virus mutants were generated by means of random chemical mutagenesis (Burge and Pfefferkorn, 1966; Strauss et al., 1976; Strauss and Strauss, 1980). These mutants, which had been selected on the basis of their ts phenotype, were separated into seven complementation groups. Mutants in complementation groups C, D, and E were RNA- (i.e., they were able to make viral RNA at the nonpermissive temperature). The mutations responsible for the phenotypes of these mutants mapped to the genes for the structural proteins. The mutants in complementation
groups A, B, F, and G were RNA" (i.e., they were not able to make viral RNA at the nonpermissive temperature). The mutations responsible for the phenotypes of these mutants were mapped to the regions encoding the ns proteins: nsP1, nsP2, nsP3, and nsP4 (Hahn et al., 1989a,b). Mutants with mutations in the genes coding for the ns proteins have proved to be useful in understanding the regulation of viral RNA synthesis and in associating specific functions with ns proteins. For example, experiments with ts-6 (complementation group F) were important in identifying nsP4 as the viral RDRP (Barton et al., 1988).

We have used a different approach to obtain mutants with alterations in ns proteins. There are only five small molecules that serve as substrates for virus-coded ns proteins: ATP, GTP, CTP, and UTP, which are substrates for the viral RDRP (GTP is also a substrate for the viral guanylyltransferase), and S-adenosylmethionine (AdoMet), which is a substrate for the viral RNA methyltransferase. We reasoned that if the intracellular concentration of any of these fell below a critical value, viral replication would be inhibited, and that if we could isolate viral mutants capable of growth under these conditions, mapping of the sites of such mutations should allow identification of the ns proteins that interacted with these small molecules. Such information should then provide clues as to the function of the ns proteins. We used this approach to determine that mutations in the nsP1 gene allow growth under conditions of low GTP or AdoMet. This was instrumental in identification of nsP1 as the protein responsible for capping viral mRNAs (Durbin and Stollar, 1985; Mi et al., 1989; Scheidel and Stollar, 1991). In the work reported here, our goal was to obtain a mutant that would encode an RDRP (i.e., nsP4) with alterations in its rNTP substrate binding properties. Accordingly, we sought a mutant that could replicate in cells treated with PZF and that therefore should have low levels of UTP and CTP. We obtained the desired mutant (i.e., SV_PZF), and the mutations responsible for the mutant phenotype did, as expected, map to the nsP4 coding region. We anticipate that SV_PZF will be a useful addition to the several ts mutants previously shown to map to the nsP4 coding region and that it will be useful in understanding the structure-function relationships of nsP4.

When we added either adenosine or guanosine to infected Ae. albopictus cells, SV_PZF, in contrast to SV_subplot, was resistant to both of these purine nucleosides. In view of our earlier demonstration that the addition of adenosine or guanosine to Ae. albopictus cells lowers the level of UTP and CTP in these cells (Stollar and Malinoski, 1981) and our demonstration here that SV_PZF can replicate in cells with low levels of UTP and CTP, this result was not surprising. Similarly, our finding that SV_PZF actually produces somewhat higher yields from cells treated with low levels of PZF than from untreated cells is consistent with the idea that SV_PZF is adapted to cells containing lowered levels of UTP and CTP.

The cross-resistance of SV_PZF to PALA reinforces the findings just described with adenosine and guanosine and lends further support to the idea that when mosquito cells are treated with PZF, it is the decrease in the concentration of the pyrimidine nucleotide triphosphates that is responsible for inhibition of viral replication. This being the case, the simplest explanation for the resistance of SV_PZF to PZF is that by virtue of an increased affinity for UTP and/or CTP, the SV_PZF RDRP is able to function in cells with decreased levels of these compounds.

Characterization of chimeric viruses containing various SV_PZF sequences in an SV_toro background indicated that the mutations responsible for the resistance to PZF were contained in the SV_PZF genome sequence extending from nt 5262 to 7999. This sequence includes part of the nsP3 coding region, all of the nsP4 coding region, and the sequence that contains the minimal promoter for the transcription of the subgenomic RNA (nt 7579–7603) (Levis et al., 1990). We identified four mutations in the 5262–7999 sequence that are predicted to result in amino acid changes. Using site-directed mutagenesis to introduce these changes into SV_toro, one at a time or in various combinations, we showed that expression of the full SV_PZF phenotype requires three of the four mutations shown in Table 3, namely those at nt 6627, 7543, and 7593. Thus the mutation at nt 5750, which changes the opal codon to a Cys codon, is not required. The most critical change is that at nt 7593; nevertheless, this mutation alone is not sufficient to produce resistance to PZF. When combined with either the mutation at nt 6627 or that at 7543, partial resistance resulted. When both of these mutations were combined with that at nt 7593, the full SV_PZF phenotype was observed.

The mutation at nt 7593 has two effects: it not only changes the Pro at position 609 to Thr but also changes the −5 position of the promoter for the synthesis of the subgenomic RNA. It is noteworthy that the C at this position is invariant among the alphaviruses (Strauss and Strauss, 1994). However, because it is difficult to understand how a change in the promoter could lead to PZF resistance, we favor the idea that the nt 7593 mutation contributes to PZF resistance because of the resulting amino acid change in nsP4.

Structures have now been described for all classes of nucleic acid polymerases, DNA-dependent DNA and RNA polymerases (Doubli et al., 1998; Sousa et al., 1993), and RNA-dependent DNA and RNA polymerases (Huang et al., 1998; Hansen et al., 1997; Lesburg et al., 1999). It is clear from this body of work that all of these polymerases share common structural features (structures of polymerases are generally represented as a slightly flexed right hand, with fingers, thumb, and palm subdomains; the palm domain contains the catalytic site) and have a common catalytic mechanism (Steitz, 1998). Because no structure has been described for the alpha-
virus nsP4, we generated a molecular model of nsP4, using as reference molecules the poliovirus RDRP (Hansen et al., 1997) and the HIV reverse transcriptase (Huang et al., 1998), an RNA-dependent DNA polymerase. Making use of this model, we located the three positions on nsP4 at which the amino acid changes required for full P2F resistance occurred. In our model, residue 287 is positioned in a loop at the tip of the fingers domain, and residues 592 and 609 are positioned at the base of the thumb domain.

Given that SVp2F, in contrast to SVSTD, can replicate in cells with decreased levels of UTP and CTP, we suggest that the amino acid changes in SVp2F nsP4 alter the binding properties of the SVp2F RDRP for its rNTP substrates. Furthermore, because all DNA and RNA polymerases appear to share a common structure and catalytic mechanism, we anticipate that we will be able to apply what has been learned about other polymerases (e.g., the HIV RT, Huang et al., 1998; the T7 DNA polymerase, Doublie et al., 1998; and the bacteriophage RB69, a close relative of T4 phage, gp43 protein, a DNA polymerase, Brautigam and Steitz, 1998) to our molecular model of the SV RDRP.

When a DNA or an RNA polymerase extends a primer strand, it must choose at each step from a pool of four different substrate molecules, dNTPs or rNTPs, respectively; clearly, the choice as to the correct NTP is determined not only by the amino acid residues surrounding the rNTP binding pocket of the enzyme but also by the positioning of the template-primer that is bound to the polymerase. When the correct NTP is positioned in the binding site and a proper Watson-Crick base-pair is formed, the polymerase undergoes a conformational change to a "closed" form such that the fingers domain is flexed inward toward the palm domain where the catalytic site is located, trapping the template strand and the NTP substrate (Brautigam and Steitz, 1998; Steitz, 1998; Huang et al., 1998). Crystal structures of polymerases to which are bound a template-primer and an incoming NTP molecule clearly show multiple interactions between the NTP and amino acids in the fingers domain.

Furthermore, as has been shown with the HIV-RT, an amino acid change in the fingers domain can alter the specificity of the enzyme with respect to the binding of nucleoside analog inhibitors, thereby making the NTP binding pocket more specific for the natural substrate (Boyer et al., 1994). Thus an amino acid change in the fingers domain of the SVp2F RDRP might reasonably be expected to alter the geometry of the active site of the RDRP, and as a result its binding properties for its rNTP substrate. Less clear is how the amino acid changes at the base of the thumb domain might alter the properties of the RDRP. The thumb domain is known to be important in gripping the template-primer (Jacobo-Molina et al., 1993), and it has been implicated in the processivity of the enzyme. The structure of nevirapine-bound HIV-RT, in which this non-nucleoside RT inhibitor is bound to the base of the thumb domain, suggests that a disturbance of the thumb domain can indirectly influence the conformation of the catalytic site (Kohlstaedt et al., 1992). More specifically, it has been demonstrated that an amino acid change in the thumb domain of the HIV-RT can alter the conformation of the primer grip and affect the precise position of the primer strand in relation to the carboxylate triad (Das et al., 1996). Thus the amino acid changes we have found in the thumb domain of SVp2F nsP4 might well influence, if only indirectly, the geometry of the rNTP-binding pocket.

Because the structure shown in Fig. 5 was obtained by molecular modeling, conclusions concerning the location of the residues that are changed in the SVp2F nsP4 can only be regarded as tentative and must await the crystallization of the protein and the formal determination of its three-dimensional structure. Nevertheless, the molecular model may be useful in providing a preliminary or working model of the Sindbis virus RDRP.

MATERIALS AND METHODS

Cells and viruses

Primary cultures of chicken embryo fibroblasts (CEF), prepared as described previously (Stollar et al., 1976), were grown in Eagle's MEM supplemented with nonessential amino acids, glutamine, 5% tryptose phosphate, and 5% bovine serum. Ae. albopictus mosquito cells (C7-T0) have also been described previously (Durbin and Stollar, 1984); they were grown in Eagle's MEM supplemented with nonessential amino acids, glutamine, and 5% fetal bovine serum.

Our standard Sindbis virus has been described elsewhere (Shenk and Stollar, 1973). Mosquito cell cultures were infected at an m.o.i. of 5 PFU/cell (using the titers as determined on CEF) and maintained after infection, unless otherwise indicated, at 34.5°C in medium containing 0.2% BSA in place of serum. Media from infected cultures were harvested 20–24 h after infection, and virus yields were measured by plaque formation on primary cultures of CEF. Samples to be titrated were diluted in 10-fold steps in PBS containing 0.1% BSA, and 0.5 ml of appropriately diluted samples was adsorbed to the cell monolayers in 60-mm plates for 60 min at 34.5°C. The virus was then removed, and the monolayers were overlaid with 2.5 ml of medium containing 0.9% Noble agar. After 24 h at 34.5°C, 2.0 ml of 0.01% neutral red in PBS was added to the plates, which were then kept for an additional 2–3 h at 34.5°C. After removing the remaining neutral red, the plaques were counted.

Plasmids and bacteria

pToto1101 (13638 bp) contains a cDNA copy of the entire SV genome immediately downstream of an SP6...
promoter (Rice et al., 1987). Infectious RNA transcripts generated from pToto, as described previously, were used to transfect primary cultures of CEF (Mi et al., 1989).

Recombinant DNA techniques and site-directed mutagenesis

DNA manipulations were performed by standard procedures (Sambrook et al., 1989). Site-directed mutagenesis was carried out using the QuikChange kit purchased from Stratagene (La Jolla, CA). SVPZF cDNA was prepared as follows: CEF were first infected with SV PZF at an m.o.i. of 0.01 PFU/ml. After 24 h at 37°C, virus was harvested and the viral RNA was prepared as previously described (Durbin and Stollar, 1986). After clarification of the medium, first at 500 \( g \) for 10 min. and then at 15,000 \( g \) for 10 min, each 30 ml of clarified medium was pelleted through 6.5 ml of 15% (w/w) sucrose in TNE (0.05 M Tris, pH 8.0, 0.1 M NaCl, 0.001 M EDTA) by centrifugation at 26,000 rpm at 4°C in a Beckman SW27 rotor. The pelleted virus was suspended in TNE containing 0.5% SDS and 250 \( \mu \)g proteinase K/ml. After incubation at 37°C for 30 min, the RNA was deproteinized by phenol extraction and then precipitated with ethanol. First-strand cDNA was made by incubating the viral RNA with SuperScript II RT (BRL), and second strand was made using Escherichia coli DNA polymerase in the presence of E. coli DNA ligase and RNase H. The construction of the recombinant pToto plasmids containing cDNA sequences derived from SV \( \text{PFZ} \) is described below.

\[ \text{pToto:SV}_{\text{PFZ}}:5262-7999 \]

A deleted form of pToto, \( \Delta \text{Toto11439} \) (Fig. 6), was made by deleting the sequence between the two \( \text{Stu} \) sites at nt 8571 and 10770. The nucleotide numbering for pToto begins with the first base of the SV RNA, followed by the vector sequence. \( \text{SV}_{\text{PFZ}} \) cDNA and \( \Delta \text{Toto11439} \) were digested with \( \text{SpeI} \) (nt 5262) and \( \text{AatII} \) (nt 7999). The 2737-bp fragment from the \( \text{SV}_{\text{PFZ}} \) cDNA digestion was then ligated to the 8702-bp piece from the \( \Delta \text{Toto11439} \) digestion to give \( \Delta \text{Toto:SV}_{\text{PFZ}}:5262-7999 \). The \( \text{AatII}–\text{SacI} \) fragment (nt 7999–13552) from pToto 1101 was then substituted for the corresponding fragment in \( \Delta \text{Toto:SV}_{\text{PFZ}}:5262-7999 \) to give a complete infectious clone.

\[ \text{pToto:SV}_{\text{PFZ}}:41-2784 \]

The cloning strategy for \( \text{pToto:SV}_{\text{PFZ}}:41-2784 \) was similar to that used to make \( \text{pToto:SV}_{\text{PFZ}}:5262-7999 \) except that the \( \text{SV}_{\text{PFZ}} \) cDNA and \( \Delta \text{Toto11439} \) were digested with \( \text{MfeI} \) (restriction sites at nucleotides 41 and 2784, see Fig 6) instead of with \( \text{SpeI} \) and \( \text{AatII} \). The orientation of the \( \text{SV}_{\text{PFZ}} \) cDNA insert was checked by digestion with \( \text{EcoRV} \) (restriction sites at nt 2750 and 6878).

\[ \text{pToto:SV}_{\text{PFZ}}:2784-7999 \]

\[ \Delta \text{Toto11439} \) and \( \text{SV}_{\text{PFZ}} \) cDNA were digested with \( \text{MfeI} \) (nt 41 and 2784) and \( \text{AatII} \) (nt 7999). The nt 7999–41 fragment from the former was ligated to the nt 2784–7999 fragment from the latter, giving the intermediate plasmid \( \Delta \Delta \text{Toto:SV}_{\text{PFZ}}:2784-7999 \). The deleted sequence between the \( \text{Stu} \) sites (nt 8571–10774) was replaced as described above, and the deletion between the \( \text{MfeI} \) sites (nt 41–2784) was filled by inserting the corresponding sequence from \( \Delta \text{Toto11439} \).

\[ \text{pToto:SV}_{\text{PFZ}}:41-7999 \]

This plasmid was assembled by digesting \( \Delta \text{Toto11439} \) and \( \text{SV}_{\text{PFZ}} \) cDNA with \( \text{MfeI} \) (restriction sites at nt 41 and 2784) and \( \text{AatII} \) (nt 7999). The nt 7999–41 fragment from the former was ligated to the nt 2784–7999 fragment from the latter, giving the intermediate plasmid \( \Delta \Delta \text{Toto:SV}_{\text{PFZ}}:2784-7999 \). The deleted sequence between the \( \text{Stu} \) sites (nt 8571–10774) was replaced as described above, and the deletion between the \( \text{MfeI} \) sites (nt 41–2784) was filled by inserting the corresponding sequence from \( \Delta \text{Toto11439} \).

\[ \text{pToto:SV}_{\text{PFZ}}:5262-7334 \text{ and } \text{pToto:SV}_{\text{PFZ}}:7334-7999 \]

Both pToto1101 and \( \Delta \text{Toto:SV}_{\text{PFZ}}:5262-7999 \) (see above) were digested with \( \text{BamHI} \) (restriction sites at nt 4633 and 7334). The 2701-bp fragment from the latter was then ligated to the 10937-bp fragment from the former to give \( \text{pToto:SV}_{\text{PFZ}}:5262-7334 \). To construct \( \text{pToto:SV}_{\text{PFZ}}:7334-7999 \), the \( \text{BamHI} \) fragment from \( \Delta \text{Toto11439} \) was ligated to the 10937-bp sequence that was produced by \( \text{BamHI} \) digestion of \( \text{pToto:SV}_{\text{PFZ}}:5262-7999 \).

Molecular modeling of SV nsP4

The amino acid sequence of Sindbis virus nsP4 was translated from the nucleotide sequence (gb_vi:sinclg). Amino acid sequences for poliovirus and HIV-1 were taken from accession numbers p03301 and p03366, respectively. Conserved motifs from the three sequences were aligned according to Poch et al. (1989). The molecular model of SV nsP4 was generated with Look 3.0 (Molecular Application Group) either using Polio3D (PDB: 1RDR) or HIV-RT (PDB: 1RTD) as template. The resulting
structures were then energy refined and displayed with Ribbons.

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