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***Cis*-acting RNA elements at the 5' end of Sindbis virus genome RNA regulate minus- and plus-strand RNA synthesis**

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ABSTRACT

Alphavirus genome replication is a multistep asymmetric process. Several lines of evidence suggest that the template preference of the RNA replicase is regulated by proteolytic cleavage of the viral nonstructural polyprotein. *Cis*-acting RNA elements in the viral genome also play crucial roles in regulating genome replication and subgenomic RNA transcription. In this report, a series of RNA templates were analyzed *in vitro* and *in vivo* to define functional elements in the 5' end of the genome. The 5' UTR was shown to contain distinct core promoter elements for both minus- and plus-strand synthesis. In addition, two conserved stem-loop structures within the nsP1 coding sequence enhanced RNA replication but were not required. Studies with chimeric templates and trans-competition experiments suggest that the 5' determinant for minus-strand initiation can differ among alphaviruses and binds to one or more limiting replicase components. The results provide compelling evidence that the 5' and 3' ends of alphavirus genome RNAs must interact to initiate replication and we propose one model for how this interaction might occur. In addition to providing new insight into the initiation of alphavirus genome replication, these results have implications for the development of improved alphavirus vector systems with reduced recombination potential.

Keywords: 5' UTR; alphavirus; core promoter; replicase

INTRODUCTION

The alphavirus genus of the family *Togaviridae* is a group of arthropod-borne viruses, with mosquitoes being the most common vector and a wide variety of birds and mammals serving as primary hosts. The genus is comprised of 28 currently known members with wide geographic distribution. These viruses differ in their ability to cause disease in vertebrate hosts, but have similar replication strategies.

The alphavirus genome is a single RNA molecule of positive polarity approximately 12 kb in length. The viral genome RNA mimics the structure of cellular mRNA in that it possesses a 5' methylguanylate cap structure and a 3' polyadenylate tract (Strauss & Strauss, 1986; Dubin et al., 1977). These features allow translation of

viral proteins by host cell machinery immediately following introduction of the genomic RNA to the cytosol. The 5' two-thirds of the genome is translated into the nonstructural proteins (nsPs) that comprise the viral components of the RNA replicase/transcriptase required for replication of viral genome and transcription of subgenomic RNA (Strauss et al., 1983; Lemm & Rice, 1993a, 1993b; Lemm et al., 1994). The subgenomic RNA corresponds to the 3' third of the genome and encodes the viral structural proteins (Rice & Strauss, 1981). The alphavirus genome is replicated in cells by a two-step process. Initially the viral genomic RNA is used as a template for synthesis of a full-length minus-strand intermediate. Following its production, the minus-strand intermediate serves as a template for production of large quantities of plus-strand genomic and subgenomic RNAs. Synthesis of all three alphavirus RNA species is asymmetric and highly regulated. Minus-strand RNA synthesis occurs only in the first 3–4 h postinfection and is undetectable at late stages, at which time only plus-strand RNA (genomic and subgenomic) synthesis is observed (Sawicki & Sawicki, 1980).

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The prevailing model for regulation of RNA synthesis suggests a dependence on the processing of the non-structural polyproteins. Primary translation of the genomic RNA produces two polyproteins, P123 and P1234, with the latter produced in smaller quantities due to inefficient translation through a termination codon that is present in many alphavirus genomes between the nsP3 and nsP4 genes (Strauss et al., 1983). Processing is mediated by a papain-like protease domain in nsP2 (Ding & Schlesinger, 1989; Hardy & Strauss, 1989) whose substrate specificity is influenced by polyprotein context. Initially P1234 is cleaved in *cis* to yield P123 and nsP4, the viral RNA-dependent RNA polymerase (RdRp). This replicase is active for minus-strand synthesis, but inefficient for the generation of plus-strand RNAs (Lemm et al., 1994; Shirako & Strauss, 1994). Further processing of P123, in particular *trans*-cleavage at the nsP2/nsP3 junction, changes the template specificity of the replicase to increase synthesis of plus-strand RNAs and shut off synthesis of minus strands (de Groot et al., 1990; Lemm et al., 1994; Shirako & Strauss, 1994). In this model, the accumulation of active nsP2 protease prevents accumulation of unprocessed viral replicative enzymes late in infection to favor the synthesis of subgenomic RNAs for structural protein production and genome RNAs for packaging into progeny virions (Strauss & Strauss, 1994).

The synthesis of alphavirus RNAs is also regulated by *cis*-acting RNA elements. Comparison of alphavirus genomes and mutagenesis of conserved RNA elements have identified a number of important *cis*-acting sequences, including a 19-nt conserved sequence element (CSE), which immediately precedes the 3' terminal poly(A) tract (Ou et al., 1981; Kuhn et al., 1990). Mutations within this CSE alter virus replication in cell culture and, based on its 3' location, the 3' CSE has been assumed to function as the core promoter for initiation of minus-strand RNA synthesis. A second conserved 24-nt sequence was identified that spans the start site of the subgenomic RNA (Ou et al., 1982; Levis et al., 1990). The complement of this sequence in the minus-strand replicative intermediate acts as the core promoter for subgenomic RNA transcription (Levis et al., 1990).

The 5' terminal sequence of the alphavirus genome has been provisionally divided into two *cis*-acting elements; the 5' UTR, with low overall homology between different alphaviruses but a conserved predicted secondary structure, and the 51-nt CSE (nt 155–205; Ou et al., 1983). Besides being involved in translation initiation, the complement of the 5' 200–250 nt are believed to be required for synthesis of viral genome RNAs from minus-strand intermediates (Ou et al., 1983; Strauss et al., 1984; Tsiang et al., 1988; Niesters & Strauss, 1990a, 1990b). In addition, based on the observation that the 3' coterminal subgenomic RNA is not a template for minus synthesis (Simmons & Strauss,

1972), the 51-nt CSE was hypothesized to play a role in minus-strand RNA initiation (Monroe & Schlesinger, 1984; Niesters & Strauss, 1990b). The importance of both the 5' UTR and 51-nt CSE in viral replication was confirmed by site-directed mutagenesis (Niesters & Strauss, 1990a, 1990b). However, these early studies measured only virus growth and failed to define the step(s) in virus replication at which these *cis*-acting elements function.

To further define the role of the 5' end of the alphavirus genome in RNA replication, we employed complementary *in vivo* and *in vitro* approaches. These methods allowed us to distinguish between defects in plus- and minus-strand RNA synthesis while separating RNA synthetic events from translation. Our data begin to define the elements required at the 5' end of the genome for initiation of RNA synthesis by the viral replicase. These findings and previously published data suggest a model for initiation of minus-strand synthesis that may be broadly applicable to plus sense RNA viruses (Barton et al., 2001; Herold & Andino, 2001).

RESULTS

A characteristic feature of the 5' terminal sequences of alphavirus genomes and defective interfering (DI) RNAs is that they are predicted to fold into compact structures that bring the 5' UTR and 51-nt CSE into close proximity (Niesters & Strauss, 1990a, 1990b). Figure 1 shows a computer-generated (m-fold) prediction of secondary structures for the 5' ends of Sindbis (SIN) and Semliki Forest virus (SFV) genome RNAs and their minus-strand counterparts. Although the sequences of the 5' ends of these viruses are quite divergent, the models of predicted secondary structure exhibit many similarities. In both cases, there is a short stem-loop (SL) structure in the 5' UTR (SL1) followed by a large stem-loop (SL2) and the 51-nt CSE that is predicted to form two smaller stem loop structures (SL3 and SL4). The model for secondary structure of the 51 nt CSE has been previously refined by comparative analysis of nucleotide sequences from different alphaviruses (Niesters & Strauss, 1990b). Some variability in the predicted loops and interloop regions as compared to conservation of the stems indicated that base-paired regions might play a more important role in the virus life cycle. The 51-nt CSE is preserved not only in complete viral genomes but also in all currently sequenced, naturally occurring DI RNAs of SIN and SFV (Pettersson, 1981; Lehtovaara et al., 1981, 1982; Monroe et al., 1982; Monroe & Schlesinger, 1983, 1984). In contrast to the 51-nt CSE, the 5' UTR is not present in all DI RNAs. Rather, in some SIN DI RNAs, the original UTR is replaced by heterologous sequences, including a portion of tRNA^{Asp} or the 5' UTR from SIN subgenomic RNA (Monroe et al., 1982; Monroe & Schlesinger, 1983,

A **B**

FIGURE 1. (Legend on facing page.)

FIGURE 2. Experimental protocols used to measure plus- and minus-strand RNA synthesis. **A:** In vivo assay. RNA substrates were cotransfected with SIN (SINrep) or SFV (SFVrep) replicons by electroporation. Cells were divided into three 35-mm dishes and harvested for measurement of luciferase, virus-specific RNAs, or total protein as described in the Materials and Methods. **B:** In vitro assay. Membrane fractions containing active SIN P123-nsP4 replicase were prepared from BHK-21 cells as described in Materials and Methods. Minus-strand RNA synthesis was initiated by the addition of NTPs, [^{32}P]-CTP, and template RNA. Reactions were incubated at 30 °C for 1 h, treated with alkaline phosphatase and proteinase K, extracted with phenol/chloroform, and concentrated by ethanol precipitation. Products were separated by agarose-phosphate gel electrophoresis and a phosphorimager (Bio-Rad) used to quantify incorporated radioactivity.

1984). SIN nonstructural proteins efficiently replicate these RNAs with chimeric 5' ends (Tsiang et al., 1985, 1988).

The 51-nt CSE acts as a replication enhancer

The involvement of the 5' terminal 200–250 nt of the alphavirus genome in both replication and translation has complicated its study. In the context of the full-length SIN genome, silent mutations in the 51-nt CSE,

which lies within the nsP1 coding sequence, significantly decreased virus replication (Niesters & Strauss, 1990b). In contrast, studies using SIN DI RNAs found this region to be dispensable for replication, at least in vertebrate cells (Levis et al., 1986). To further investigate the role of this region, we used complementary in vivo and in vitro approaches. For the in vivo system (Fig. 2A), various RNA templates were cotransfected with replicons that express a functional *trans*-acting replicase/transcriptase and replication was analyzed at 3.5 h posttransfection. Due to technical limitations, a

FIGURE 1. Schematic representation of the 5' ends of Sindbis (SIN) and Semliki Forest virus (SFV) genome RNAs and the corresponding minus strand 3' sequences. **A:** Computer-predicted secondary structures (m-fold). Arrows indicate the positions of the initiating AUG codons; large dots indicate the 5' or 3' terminal bases. Shown are the four stem-loop structures, SL1–4, including SL3 and SL4, which are located in the 51-nt CSE (nt 155–205 in SIN). **B:** Linear diagrams of the 5' ends. Boxes indicate positions of the four predicted stem-loop structures. (Figure on facing page.)

single round of minus-strand RNA synthesis is not detectable in this assay. Hence, an increase or decrease in plus-strand RNA accumulation can be due to effects in plus- or minus-strand RNA synthesis or both. To complement the *in vivo* assay, the ability of the same RNA templates to function in minus-strand synthesis was examined using a recently developed *in vitro* system (Fig. 2B). This assay utilizes a vaccinia-expressed SIN minus-strand replicase composed of a protease-defective (and therefore uncleavable) P123 and the nsP4 RdRp. This replicase synthesizes exclusively minus-strand RNAs when programmed with SIN-specific plus-strand RNA templates (Lemm et al., 1998, 1994). Thus far, every RNA template defective for minus-strand synthesis *in vitro* has also been blocked for replication *in vivo*. This suggests that the requirements for minus-strand synthesis *in vitro* and *in vivo* are the same, but this remains to be rigorously established. With this caveat, by combining the results from the two assays, one can deduce whether quantitative changes in plus-strand RNA synthesis detected *in vivo* were due to changes in minus-strand RNA synthesis feeding back into plus-strand synthesis or were preferentially affecting plus-strand synthesis.

Initially, we conducted a deletion analysis of the sequence elements located downstream from the 5' UTR, including SL2 and the 51-nt CSE (SL3 and SL4). The parental RNA template (wild-type or 5'SIN3'SIN) contained the 5' 424 nt of the SIN virus genome, a luciferase gene under the control of the subgenomic RNA promoter, and the SIN 3' UTR (Fig. 3A). Replication of this RNA was measured indirectly by luciferase activity (which requires replication and transcription of subgenomic RNA) and confirmed directly by metabolic labeling of virus specific RNAs. Each sequence element predicted to fold into a stem-loop structure was deleted individually or in combination; Δ SL2, Δ SL3, Δ SL4, Δ SL34, Δ SL234, and Δ SL24. These deletions precisely excised the respective stem-loops based on the predicted secondary structure shown in Figure 1, and were predicted (by m-fold) to cause no alteration in the remaining hairpins (data not shown). These engineered template RNAs were examined for defects in plus- and minus-strand synthesis (Fig. 3).

Deletions of each stem loop did not cause a detectable decrease in RNA replication *in vivo* (Fig. 3A,B, lanes 2, 3, and 4). In fact, the Δ SL2 RNA reproducibly replicated to higher levels than the parental 5'SIN3'SIN

FIGURE 3. Replication of RNAs with modified 5' sequences located downstream of the 5' UTR. **A:** Schematic representation of the 5' ends of substrate RNAs (see Materials and Methods for details) and their replication activity *in vivo* based on luciferase activity. **B:** Autoradiograph of metabolically labeled RNAs separated by agarose gel electrophoresis. **C:** Autoradiograph showing the [32 P]-labeled RNA products of the *in vitro* minus-strand RNA analyses. The lower panel shows the levels of radioactivity as measured by phosphorimaging. **B** and **C:** 5'SIN3'SIN: lanes 1; Δ SL2: lanes 2; Δ SL3: lanes 3; Δ SL4: lanes 4; Δ SL34: lanes 5; Δ SL234: lanes 6; Δ SL24: lanes 7; mut44: lanes 8. The gels shown in **B** and **C** are representative of at least three independent experiments.

RNA (Fig. 3B, lane 2). Deletion of two stem-loops (Δ SL34, Δ SL24) or the entire region downstream of the 5' UTR (Δ SL234), resulted in a 3- to 10-fold decrease in plus-strand synthesis, as measured by luciferase activity (Fig. 3A). This result was confirmed by parallel analysis of metabolically labeled genomic and subgenomic RNAs (Fig. 3B, lanes 5, 6, and 7). The results of the *in vitro* minus-strand assay were consistent with the *in vivo* data in that none of these deletions abrogated minus-strand RNA synthesis. Interestingly all the deletions caused a reproducible decrease in the efficiency of minus-strand RNA synthesis *in vitro* (Fig. 3C, lanes 2–7). This suggests that internal sequence elements near the 5' end of the genome can affect the efficiency of minus-strand RNA synthesis.

These results show that the 51-nt CSE is not absolutely required for RNA replication in mammalian cells. Every structural element of the 5' end, located downstream from SL1 and the 5' UTR, could be deleted without abrogating the template activity of the RNAs. Further experiments were conducted in which the predicted secondary structures downstream from the 5' UTR were disrupted by clustered point mutations. The RNA template mut44 contained 44 silent mutations in the amino-terminal part of the nsP1 coding sequence that were predicted to destabilize SL2, SL3, and SL4. *In vitro* minus-strand RNA synthesis for mut44 was four- to sixfold lower than for parental template (Fig. 3C, lane 8). The products of *in vivo* replication were undetectable on a gel after metabolic labeling (Fig. 3B, lane 8). However, results of the luciferase assay indi-

cated that replication was occurring, albeit at a level 50-fold lower than the parent (Fig. 3A).

Thus, although the 51-nt CSE does not appear to function as an essential core element of the promoter for minus- or plus-strand RNA synthesis, its presence does enhance the production of both. This element may act by stabilizing the structure of the alphavirus 5' end (or the 3' end of the minus-strand intermediate), or by providing additional binding sites for host and viral proteins involved in RNA replication (Pardigon & Strauss, 1992, 1996; Pardigon et al., 1993).

Alphavirus-specific 5'- and 3'-terminal requirements for RNA replication

To look for alphavirus-specific sequence elements required for RNA replication, we made a number of RNA templates containing combinations of 5' and 3' end sequences derived from the genomes of SIN and SFV. We then examined the ability of these RNAs to be replicated either by the SIN or SFV replicase (Fig. 4). As expected from previously published data, the SIN and SFV replicases could recognize homologous as well as heterologous 3' end sequences (Fig. 4A,B, lanes 1, 2, 5, 6, and 8; Kuhn et al., 1991). In addition, RNAs with the 5' end of SIN replicated efficiently in cells expressing either the SIN or SFV replicases (Fig. 4B, lanes 1, 2, 5, and 6). In contrast, the SFV 5' end could be used only by SFV replicase and only if the RNA template contained the SFV 3' UTR (Fig. 4B, lane 8). Replication of the 5'SFV3'SIN occurred inefficiently in

vivo by SIN or SFV enzymes (Fig. 4B, lanes 3 and 7). However, different combinations of the same 5' and 3' ends (5'SIN3'SIN, 5'SFV3'SFV, 5'SIN3'SFV) could be utilized by SFV replicase (Fig. 4B, lanes 5, 6, and 8).

The in vitro-generated data mirrored the in vivo results (Fig. 4C). Minus-strand RNA synthesis by the SIN replicase was undetectable for templates with the SFV 5' end, suggesting that this defect was responsible for their inability to be amplified in vivo (Fig. 4C, lanes 3 and 4). Of note was the observation that 5'SFV3'SIN, which retained the 3' end of SIN genome RNA (the presumed core promoter for minus-strand synthesis), was not a template for minus-strand synthesis by the SIN replicase (Fig. 4C, lane 3).

To rule out the possible influence of RNA stability on our results, we electroporated BHK-21 cells with 5'SIN3'SIN or 5'SFV3'SIN RNA labeled with [³²P]. The intracellular RNAs were isolated at different times post-transfection and the rate of RNA degradation was analyzed by gel electrophoresis. No significant difference in stability of the RNAs was detected (Fig. 5). These

results indicate that the 5' end of the alphavirus genome has a profound effect on the synthesis of complementary minus-strand RNA and may comprise an essential element of the promoter for this process. Furthermore, the 5' SIN determinant could not be functionally replaced by the corresponding region from SFV.

The 5' UTR is a component of the promoter for minus- and plus-strand RNA synthesis

The incompatibility of the SFV 5' end with SIN minus strand synthesis provided an opportunity to map the SIN-specific sequences required for this process. We designed a set of chimeric RNAs with mosaic 5' ends (Fig. 6A) based on the computer-predicted secondary structures for SIN and SFV (Fig. 1). 5'(SIN-SFV)3'SIN contained the 5' UTR of SIN, including SL1, fused with SFV SL2, -3, and -4. 5'(SFV-SIN)3'SIN had SL1 and the interstem fragment of SFV followed by SL2, -3, and -4 of SIN. 5'(SFVSL1-SIN)3'SIN was similar to 5'(SFV-SIN)3'SIN but contained a deletion of the interstem fragment between SFV SL1 and SIN SL2. 5'(SIN-SFV)3'SIN RNA was replicated in cells expressing SIN or SFV nonstructural proteins (Fig. 6B, lanes 2 and 7). In vitro minus-strand synthesis from this template by the SIN minus-strand replicase was not severely affected, being 70–80% of the 5'SIN3'SIN parent RNA (Fig. 6C, lane 2). However, RNA synthesis in vivo from 5'(SIN-SFV)3'SIN driven by the SIN replicase was similar to that observed for Δ SL234 (Fig. 3B, lane 6), in that it was significantly lower than replication of 5'SIN3'SIN. This result suggests that the 5' UTR of the SIN genome is sufficient for directing a basal level of RNA replication, and thus functions as an element of a core promoter. The same 5'(SIN-SFV)3'SIN RNA was replicated in cells transfected with the SFV replicon more efficiently than 5'SFV3'SFV (Fig. 6A,B, lanes 7 and 8). These results suggest that the 51-nt enhancer element of SFV, despite its high homology to the SIN enhancer, could not function with the SIN replication machinery. However, the fact that SFV proteins can efficiently replicate 5'SIN3'SIN and 5'(SIN-SFV)3'SIN indicates that SFV replicative complexes can utilize homologous and heterologous enhancer elements in conjunction with the SIN 5' UTR.

All RNAs containing SFV SL1 and the SIN 3' UTR were replicated inefficiently in vivo by either SIN or SFV nonstructural proteins (Fig. 6A,B lanes 4, 5, 9, and 10). In vitro analysis of minus-strand RNA synthesis revealed that 5'(SFV-SIN)3'SIN and 5'(SFVSL1-SIN)3'SIN could not serve as templates for production of the replication intermediate (Fig. 6C, lanes 3 and 4). These data demonstrate that the 5' end of the SIN genome, in particular the 5' UTR, is an essential element of the promoter for minus-strand RNA synthesis. This observation suggests that an interaction between

FIGURE 5. Stability of RNAs with SIN or SFV 5' ends in BHK-21 cells. RNA templates were synthesized in vitro in the presence of [³²P]-CTP and equivalent amounts, 5 μ g, were transfected into BHK-21 cell by electroporation. Cells were seeded into four 35-mm dishes and incubated at 37°C. RNAs were isolated 0, 1, 2, and 3 h post-transfection using TRIzol, denatured, and analyzed by gel electrophoresis. **A:** Schematic representation of the RNAs used in the experiment. **B:** Autoradiograph of the dried gel. **C:** Quantitation of radioactivity in these RNA bands as determined using a phosphor-imager.

A

RNA replication % of 5'SIN3'SIN	
SINrep	SFVrep
100	100
<0.1	70
10-15	150
<0.1	0.5
<0.1	3

FIGURE 6. Replication of the RNAs with mosaic 5' ends, derived from SIN and SFV. **A:** Schematic representation and nucleotide sequences of the substrate RNA 5' ends (detailed description of the constructs is provided in Results), their replication activity in vivo after coelectroporation with SIN or SFV replicons measured by the luciferase assay. SFV and SIN sequences are shown in bold and plain type, respectively, and underlined type is used to denote the sequences predicted to form the stem-loop structures. **B:** Autoradiograph of the dried gels with the RNAs metabolically labeled with [3 H]-uridine after coelectroporation of the substrate RNAs with the SIN replicon (lanes 1–5) or the SFV replicon (lanes 6–10) into the cells. The detailed conditions of in vivo experiments are described in Materials and Methods. 5'SIN3'SIN: lanes 1 and 6; 5'(SIN-SFV)3'SIN: lanes 2 and 7; 5'SFV3'SFV: lanes 3 and 8; 5'(SFV-SIN)3'SIN: lanes 4 and 9; 5'(SL1SFV-SIN)3'SIN: lanes 5 and 10. **C:** Autoradiograph of the dried gel with the 32 P-labeled RNAs isolated from in vitro minus-strand RNA synthesis reactions. The lower panel shows the amount of radioactivity contained within the displayed RNA bands as measured using a phosphorimager. 5'SIN3'SIN: lane 1; 5'(SIN-SFV)3'SIN: lane 2; 5'(SFV-SIN)3'SIN: lane 3; 5'(SL1SFV-SIN)3'SIN: lane 4.

FIGURE 7. Replication of substrate RNAs with modified SIN 5' UTRs. Nucleotide sequences of the 5' UTRs, their replication activity in vivo based on luciferase assay, and the in vitro minus-strand synthesis activity of the templates are shown. SIN sequences are shown in plain type, tRNA sequences are shown in bold type. Sequences forming stem-loop structures are underlined.

the 5' and 3' ends of the genome occurs to facilitate the initiation of minus-strand RNA synthesis.

To further understand the requirements at the 5' terminus that determine its promoter activities, we created an additional set of RNA templates with modified 5' UTRs and tested their ability to direct plus- and minus-strand RNA synthesis (Fig. 7). The deletion of nt 5 or nt 2–4 in 5'SIN3'SIN did not significantly affect minus-strand synthesis in vitro. However, the ability of these RNAs to replicate in vivo was dramatically reduced. These data suggest that there may be different requirements in the wild-type SIN 5' UTR (or its complement) for initiation of plus- and minus-strand RNA synthesis. Interestingly, 5' tRNA that has tRNA^{Asp}-like sequence at the 5' end, and thus a markedly different sequence from the natural 5' terminus, consistently replicated more efficiently than the 5'SIN3'SIN parent in vivo. Secondary structure predictions for 5' tRNA suggest that it may have some similar features to the SIN 5' UTR. These include a short predicted single-stranded region (5'-AUAUAG-3') followed by stable tRNA-derived secondary structure that may be able to functionally replace SL1.

The 5' end of SIN RNA binds components essential for minus-strand initiation

The results indicated that the 5' ends of alphavirus genome RNA, and the 5' UTRs in particular, are essential elements of the promoter for minus-strand RNA synthesis. To further evaluate their role in the initiation of replication, we performed an in vitro competition experiment. The minus-strand RNA was synthesized in vitro from a SIN-derived RNA template (5'3'SIN) either in the absence or in the presence of competitor RNAs corresponding to the 5' 424 nt and 305 nt of the SIN and SFV genomes, respectively (Fig. 8). These RNAs lacked the conserved 3' 19 nt and poly(A) tract, and

could not be replicated either in vivo or copied in vitro by SIN replication enzymes. Addition of SIN 5' competitor RNA strongly inhibited minus-strand synthesis in vitro in a dose-dependent manner. In contrast, 5' SFV competitor RNA caused significantly less inhibition (Fig. 8B).

FIGURE 8. Template competition assay. In vitro minus-strand RNA synthesis reactions were performed as described in Materials and Methods with minor modifications. Competitor RNA was added to each reaction 5 min prior to the addition of the template RNA. **A:** Schematic representation of the template and competitor RNAs. **B:** Autoradiograph of agarose-phosphate gel of the RNAs isolated from the in vitro minus-strand assay and phosphorimager quantitation. Competitor RNAs were added at the molar ratios indicated.

These data suggest that the 5' end of SIN binds limiting viral or host factor(s), required for minus-strand synthesis, depleting them from the reaction and preventing initiation of minus-strand synthesis.

DISCUSSION

Sequences at the 5' end of genome RNAs and the complementary sequences at the 3' end of minus strand RNAs play crucial roles in translation and replication of positive-strand RNA viruses. For alphaviruses, potentially important RNA elements span not only the 5' UTR (SL1) but also overlap the beginning of the nsP1 coding region (SL2; SL3 and SL4 within the 51-base CSE). Our analyses demonstrate that stem-loops SL2, SL3, and SL4 are dispensable for RNA replication. The deletion of SL2 actually led to a reproducible increase in RNA replication, at least in vertebrate cell lines. However, although RNA synthesis from templates lacking (by deletion or clustered point mutations) SL3 and SL4 did occur, the levels of both plus- and minus-strand RNA synthesis were reduced. These stem-loop structures appear to comprise a replication enhancer that augments but is not essential for RNA replication. However, even a small decrease in the efficiency of minus-strand RNA synthesis and subsequent downregulation of plus-strand RNA synthesis may be an important determinant of virus propagation and pathogenesis in natural hosts. Our results indicate that this enhancer also functions in a virus-specific fashion, as SIN nonstructural proteins cannot utilize the SFV enhancer element.

Although the mechanism by which this replication enhancer functions is unknown, preliminary experiments suggest that the enhancer functions in a host-dependent fashion. We examined the replication of a full-length SIN RNA containing the 44 silent mutations described for mut44 (Fig. 3) that are predicted to destabilize stem-loops SL2, SL3, and SL4. The level of RNA replication was sufficient for virus propagation in mammalian cells, whereas in mosquito cells, virus production was almost completely abolished (I. Frolov & C.M. Rice, unpubl. data). This result is consistent with previous reports demonstrating that cellular factors interact with the 3' terminal sequence of the minus-strand RNA and suggest that this region may confer cell-type specific characteristics (Niesters & Strauss, 1990b; Pardigon et al., 1993).

Elements possessing the ability to increase the basal activity of RNA promoters have been identified in the genomes of other RNA viruses including human rhinovirus 14 (McKnight & Lemon, 1996), brome mosaic virus (Sullivan & Ahlquist, 1999), coronaviruses (Kim et al., 1993), and HIV (Karn et al., 1994). Replication enhancers in RNA genomes may provide an efficient way to regulate the promoter activities during virus propagation in different hosts or tissues.

In contrast to the enhancer, the 5' UTR of the SIN genome appears to be an essential component of the core promoter for both plus- and minus-strand RNA synthesis. Mutations in this region can have distinct and separable effects on plus- and minus-strand RNA synthesis, indicating that there are different requirements for these two processes. For example, deletion of a single A residue at position 5 (del 5; Fig. 7) had no effect on minus-strand RNA synthesis, yet it almost completely abolished plus-strand RNA synthesis. Given this, it is remarkable that the entire 5' UTR can be replaced by heterologous sequence as found for 5' tRNA. This RNA replicates efficiently in cells and is copied efficiently *in vitro*, yet has a 5' UTR sequence quite different from that of the natural 5' end of SIN. This template RNA does, however, possess structural features similar to SIN, including a short 5' single-stranded region followed by a stable secondary structure as well as the wild-type 51-nt CSE. Thus, we postulate that the 5' tRNA sequence or structure can bind viral or host factors required for RNA replication. Defining the common functional elements of the wild-type SIN 5' UTR and the tRNA-like sequence is a complex issue that will require both a detailed structural analysis of these RNAs and, more importantly, the elucidation of factors that bind them.

The most intriguing finding of this study was the discovery that the 5' end of the alphavirus genome plays an essential role in minus-strand RNA synthesis. Although the 3'UTR, specifically the 19-nt CSE, has been considered the core promoter for minus-strand synthesis, it has been previously speculated that there may also be a 5' requirement. This would explain why the subgenomic RNA is not copied in infected cells even though it possesses the same 3' sequences as the genomic RNA (Simmons & Strauss, 1972; Niesters & Strauss, 1990b). We have shown mutations in the 5' UTR to have a profound effect on minus-strand synthesis (Fig. 6), and presented evidence indicating components of the SIN minus-strand replicase interact with the 5' end of the SIN genome (Fig. 8). Currently we do not know if this interaction occurs directly between the replicase and the RNA or via a host factor(s) that binds to the 5' end. In any case, this observation implies that the 5' and 3' ends of the genome must interact to facilitate the initiation of minus-strand synthesis. This is not unique and joins a growing number of examples among positive-strand animal RNA viruses. It was previously shown that flavivirus minus-strand synthesis required an interaction between the 5' and 3' ends of the flavivirus genome RNA. In this case, the interaction occurs by base pairing of conserved complementary RNA sequences near the 5' and 3' ends (You & Padmanabhan, 1999). Alphavirus genome RNAs do not possess obvious complementary sequences but direct RNA–RNA interactions could occur via “kissing” (Haddrick et al., 1996) or by protein stabilized base-pairing

interactions. For poliovirus, two recent reports have shown that the host poly(C) binding protein, PCBP2, and the viral polymerase precursor, 3CD, bind to a 5' cloverleaf structure and interact with poly(A) binding protein (PABP) bound to 3' poly(A). This 5'-3' interaction, mediated by a protein-protein bridge, is required for efficient initiation of poliovirus minus-strand RNA synthesis (Barton et al., 2001; Herold & Andino, 2001).

With this in mind, we propose a model for the initiation of SIN minus-strand RNA synthesis based on the requirement for host translational machinery. Cap-dependent translation initiation requires the formation of a cap-binding complex that consists of initiation factor eIF4F bound to the cap structure of mRNAs (Merrick & Hershey, 1996). The eIF4F complex contains eIF4E, eIF4A, and eIF4G subunits. The eIF4G subunit interacts with PABP bringing the 5' and 3' end of mRNAs together during the initiation of translation (Gallie, 1998; Imataka et al., 1998). We hypothesize that the SIN replicase interacts directly or indirectly with the 5' end of the genome, and then is brought into position for initiation at the 3' end of the genome by the circularization of the template mediated by components of the translational machinery (Fig. 9). In support of this model, SIN minus-strand RNA synthesis is sensitive to the length of the poly(A) tract and the presence of PABP

(R.W. Hardy, R.C. Deo, S.K. Burley, & C.M. Rice, unpubl. data). This mechanism of minus-strand initiation would serve multiple purposes for the virus; it would ensure that the template RNA had both 3' and 5' ends, it could potentially abrogate translation from the template RNA, thus clearing it of ribosomes and facilitating replication, and it may play a role in the shut-off of vertebrate host cell translation, a process that exhibits a strong positive correlation with the level of viral RNA replication. This is just one of many possible models and further work is needed to define specific RNA elements required for minus-strand initiation and the host and viral factors that recognize them.

A replication model involving the interaction of the 5' and 3' ends of the genome may also help to explain the extraordinarily high level of recombination of alphavirus RNAs. Even RNAs incapable of replication can efficiently recombine in infected cells. Recombination has been detected between an RNA lacking the 5' end of the genome and an RNA lacking the 3' end of the viral genome (Raju et al., 1995). These pairs of defective RNAs may be brought together by initiation complexes and recombine using a copy-choice mechanism to produce viable recombinant virus.

In terms of using alphavirus-based vectors for in vitro and in vivo applications, the information obtained in this study can be used to improve existing alphavirus replicon/*trans* packaging systems (Bredenbeek et al., 1993; Lu & Silver, 2001). Current packaging systems involve cotransfection of replicons expressing a functional replicase and a heterologous gene with a defective helper RNA that replicates and expresses the structural proteins for *trans* packaging. Because the deletion of SL2 in the 5' end of SIN caused an increase in RNA synthesis, incorporation of this change into defective helpers could increase replication, structural protein expression, and the titer of *trans*-packaged replicons. In addition, the use of defective helper and replicon RNAs with different combinations of the SFV and SIN 5' and 3' ends could reduce the possibility of producing viable recombinant virus. For example, a SIN5'3' defective helper with a SFV5'3' replicon would require at least two recombination events (or a number of adaptive mutations) to generate efficiently replicating virus.

In conclusion, we have shown that the 51-nt CSE is not essential for SIN RNA replication, but it does enhance its efficiency. Additionally, we have demonstrated that the 5' UTR of the SIN genome is an essential component of the promoter for both plus- and minus-strand RNA synthesis and that the 5' requirements for each of these processes differ. Our data demonstrate that interaction between the 5' and 3' ends of the alphavirus genome is required for efficient initiation of minus-strand RNA synthesis. These data, together with the recent reports for dengue virus and poliovirus (You & Padmanabhan, 1999; Barton et al., 2001; Herold & Andino, 2001), suggest that communication between

FIGURE 9. Model for initiation of SIN genome replication. The preinitiating complex of enzymes, containing the unprocessed P123 and nsP4, cellular translation initiation factors, and unknown host factors at least partially assembles on the 5' end of SIN genome RNA. The interaction of eIF4G with PABP, bound to poly(A), brings the 3' 19-nt conserved region of viral RNA into close proximity with the replication complex, facilitating the initiation of RNA synthesis.

5' and 3' ends of RNAs is a common feature of both host mRNA translation (Gallie, 1998; Imataka et al., 1998) and RNA virus replication.

MATERIALS AND METHODS

Cell cultures

BHK-21 cells were obtained from the American Type Culture Collection, Rockville, Maryland. These cells were grown in Alpha MEM supplemented with 10% fetal bovine serum (FBS) and vitamins.

Plasmid constructs

Standard recombinant DNA techniques were used for all plasmid constructions. Fragments with the deletions of the stem-loop sequences and fragments for creating the RNA templates with mosaic 5' ends were generated by PCR amplification. They were cloned into pRS2 plasmid (a derivative of pUC19), sequenced, and subsequently cloned into p5'SIN3'SIN using convenient restriction sites. Details are available upon request.

The parental construct, p5'SIN3'SIN, was created by cloning the *Bam*HI-*Nco*I fragment of SINrep3/LacZ that contains the SIN subgenomic promoter, the *Nco*I-*Xba*I fragment from pGL3-Promoter Vector (Promega) encoding the luciferase gene, and *Xba*I-*Xho*I fragment from SINrep5 encoding the 3' UTR of the SIN virus genome into pDH-BB(5'SIN) plasmid digested with *Bam*HI-*Xho*I (Bredenbeek et al., 1993). The final construct contained the promoter for SP6 RNA polymerase followed by 425 nt of SIN 5' end (fragment 1), 7335–7646 nt of SIN genome (fragment 2), a TCTAGACC sequence, 1,654 nt sequence encoding the luciferase gene (fragment 3), and 421 nt of sequence (fragment 4) containing the SIN 3'UTR and poly(A) tail followed by *Eco*RI, *Not*I, and *Xho*I restriction sites, useful for plasmid linearization prior to in vitro transcription (Bredenbeek et al., 1993).

The plasmid encoding the indicator RNA template shown in Figure 8 had essentially the same structure as 5'SIN3'SIN, but the luciferase gene was deleted and fragment 2 was fused directly with fragment 4.

pΔSL2 contained a deletion of nt 47–152 in the SIN 5' end coding sequence; pΔSL3 contained a deletion of nt 155–178; pΔSL4 contained a deletion of nt 183–205; pΔSL34 contained a deletion of nt 155–205; and pΔSL234 contained a deletion of nt 46–204.

DNA fragments corresponding to the 3' and 5' ends of the SFV genome were derived from pSF-Helper, kindly provided by Dr. H. Garoff (Liljeström & Garoff, 1991). p5'SFV3'SIN was created by ligating the *Eco*47III-*Bgl*II fragment of pSFV-Helper1 and p5'SIN3'SIN digested with *Ecl*136II and *Bam*HI. The resulting plasmid, p5'SFV3'SIN, contained the SP6 promoter, followed by nt 1–307 (fragment A) and 6399–6714 (fragment B) of the SFV genome and fragments 2, 3, and 4 from p5'SIN3'SIN, as described above. p5'SIN3'SFV was made by ligating the *Bam*HI-*Spe*I fragment of pSFV/LacZ (the *Spe*I site was filled in by T4 DNA polymerase) and p5'SIN3'SIN digested with *Bgl*II and *Xho*I (the *Xho*I site was filled in by T4 DNA polymerase). The *Spe*I site was regenerated by this cloning strategy. The resulting construct con-

tained the SP6 promoter, fragments 1, 2, and 3 of p5'SIN3'SIN, followed by fragment C, comprising the multiple cloning site, nt 11033–11443 of SFV (including the end of the SFV E1 gene and 3' UTR), and the poly(A) tail. The p5'SFV3'SFV contained the SP6 promoter followed by fragments A, B (SFV) 2, 3 (SIN), and C (SFV). The plasmids encoding RNAs with mosaic 5' ends were constructed by PCR using appropriate primers, followed by cloning into p5'SIN3'SIN and confirmation of the sequence. The structures of these RNAs are shown in the figures and described in Results.

The TSG/Pac SIN replicon (called SINrep here) has been described previously (Frolov et al., 1999). The SFV replicon, SFVrep, corresponded to SFV/LacZ and was provided by Dr. H. Garoff (Liljeström & Garoff, 1991).

RNA transcription

Plasmids were purified by centrifugation in CsCl gradients. Prior to transcription, plasmids were linearized with *Eco*RI if they had SIN 3' UTR, or *Spe*I, if they contained SFV 3' UTR. The pTSG/Pac and pSFV/LacZ plasmids were linearized by *Xho*I and *Spe*I, respectively. RNAs were transcribed in the presence of cap analog using SP6 RNA polymerase (Rice et al., 1987). The yield and integrity of RNA transcripts were monitored by gel electrophoresis. Transcription reaction mixtures were aliquoted, stored at –80°C and used for electroporation of BHK-21 cells. For the in vitro system, RNAs were purified by phenol-chloroform extraction, ethanol precipitated, and dissolved in water to a concentration of 1 μg/μL.

In vivo replication assay

To determine the level of RNA replication, each of the experimental RNA templates were cotransfected into BHK-21 cells with TSG/Pac (SINrep) or SFV/LacZ (SFVrep) replicons by electroporation (Liljeström et al., 1991). Cells were divided into three 35-mm dishes and incubated at 37°C. Three and one-half hours postelectroporation cells in one dish were lysed in a Triton X-100 containing buffer to measure luciferase activity. This time point was based on preliminary experiments demonstrating that the number of replicative complexes increased exponentially up to 4 h posttransfection. Also, within this interval, the suppression of host cell protein synthesis is minimal. To confirm the results of the luciferase assay, virus-specific intracellular RNAs in the second dish were metabolically labeled with [³H]uridine (20 μCi/mL) in the presence of ActD between 1 and 3.5 h posttransfection. RNAs were isolated using the TRIzol procedure (Gibco-BRL), denatured with glyoxal, and analyzed by agarose-phosphate gel electrophoresis. Gels were soaked in methanol, impregnated with 2.5% PPO (2,5-diphenyloxazole), equilibrated in water, dried, and exposed to X-ray film at –80°C. Subsaturating amounts of the experimental RNA substrates were used for in vivo experiments to avoid the suppression of replicon replication. Four micrograms of replicon RNA and 4 μg of substrate RNA were found to be optimal. A third dish was used in initial experiments to measure total protein concentration and for the normalization of the results. In later assays, this step was omitted due to high reproducibility of RNA transfection and cell survival after electroporation.

Luciferase assay

Transfected cells in 35-mm dishes were lysed in 1 mL buffer containing 0.025 M Tris-HCl, pH 7.8, 0.002 M DTT, 0.002 M EGTA, and 1% Triton X-100. After pelleting the nuclei by low-speed centrifugation, 1–5 μ L of lysate was used for measuring the luciferase activity (Luciferase Assay System; Promega) on a Lumat LB9501 luminometer (Berthold).

In vitro minus-strand RNA synthesis assay

Polymerase extracts used for in vitro minus-strand RNA synthesis were prepared as previously described (Lemm et al., 1998). Briefly, BHK-21 monolayers (approximately 3×10^7 cells) were infected with recombinant vaccinia viruses expressing T7 DNA-dependent RNA polymerase, SIN polyprotein P123 containing a mutation abolishing nsP2-associated protease activity, and nsP4 with an amino-terminal ubiquitin fusion (m.o.i. = 10 pfu/cell for each virus; Lemm et al., 1994). Infected cells were incubated at 37°C for 6 h in MEM plus 10% FBS, whereupon they were harvested in ice-cold PBS. Cells were collected by low-speed centrifugation ($900 \times g$). Cell pellets were resuspended in 1 mL of hypotonic buffer (10 mM Tris-HCl, pH 7.8, 10 mM NaCl), allowed to swell 15 min on ice, and disrupted with 50 strokes of a tight fitting Dounce homogenizer. Nuclei were removed by centrifugation ($900 \times g$ for 5 min at 4°C). Post nuclear homogenates were centrifuged at $15,000 \times g$ for 20 min at 4°C. Pellets (P15) were resuspended in 120 μ L of storage buffer (hypotonic buffer plus 15% glycerol) and stored at –80°C.

Standard reaction mixtures contained 50 mM Tris-HCl, pH 7.8; 50 mM KCl; 3.5 mM MgCl₂; 10 mM dithiothreitol; 10 μ g ActD/mL; 5 mM creatine phosphate; 25 μ g creatine phosphokinase/mL; 1 mM ATP, GTP, UTP; 40 μ M CTP; 1 mCi [α -³²P]CTP/mL; 800 U RNasin/mL; 1 μ g template RNA; 18 μ L P15; H₂O to total volume of 50 μ L. Reactions were incubated at 30°C for 60 min at which point 5 U of alkaline phosphatase was added and incubation continued for 20 min. Reactions were terminated by the addition of SDS to 2.5% and proteinase K to 100 μ g/mL. RNA was isolated by phenol/chloroform extraction and ethanol precipitated. RNAs were denatured with glyoxal and separated by electrophoresis and visualized by autoradiography. The quantitation of radioactivity was performed using a phosphorimager (Bio-Rad).

Template competition assays

These reactions were performed as described above (In vitro minus-strand RNA synthesis assay); however, prior to addition of template (indicator) RNA, a predetermined quantity of competitor RNA was added to the reaction mix and incubated at room temperature for 5 min. Indicator RNA was then added and the reaction incubated at 30°C for 1 h. Products were isolated and analyzed as described above.

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