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The role of the 3′ terminus of the Sindbis virus genome in minus-strand initiation site selection

Richard W. Hardy *

Department of Biology, Indiana University, 1001 E. Third Street, Bloomington, IN 47405, USA

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Abstract

Genome replication of plus-strand RNA viruses begins with the generation of a minus-strand copy of the genome. Minus-strand synthesis must initiate at or close to the 3′ end of the genome and progress to processive elongation to yield the appropriate template for genomic RNA synthesis. The Sindbis virus genome possesses a 3′ polyadenylate tail preceded by a 19 nucleotide conserved sequence element (3′ CSE). Analyses of in vitro and in vivo synthesized minus-strand RNA presented in this manuscript identify the cytidylate residue immediately preceding the poly (A) tail as the predominant wild-type initiation site. Mutations in the poly (A) tail and the 3′ CSE caused the initiation site to shift to the poly (A) tail. Analysis of the products of non-wild-type initiation events demonstrated that they are not productively elongated. This study indicates that full-length minus-strand RNA synthesis is dependent upon initiation occurring at the appropriate site and suggests a mechanism for selection and maintenance of the wt 3′ CSE and poly (A) tail.

Keywords: Sindbis virus; Minus-strand RNA; Initiation site

Introduction

The process of genome replication for plus-sense RNA viruses begins with the generation of a full-length complementary copy of the genomic RNA. In order to maintain the appropriate passage of genetic information from one generation to the next, synthesis of this anti-genomic minus-strand RNA must begin at or near the 3′ end of the genome, and be processive to result in the production of a full-length copy of the genome. As RNA-dependent RNA polymerase (RdRp) must be recruited to the 3′ end of the plus-strand genome to initiate synthesis of the minus-strand RNA, *cis*-acting elements involved in RdRp recognition and promotion of RNA synthesis are usually found in the 3′ untranslated region (3′ UTR) (Deiman et al., 1998; Jacobson et al., 1993; Kao and Sun, 1996; Miller et al., 1986; Oh et al., 1999; Singh and Dreher, 1997; You and Padmanabhan, 1999). Additionally, mechanisms to repair or restore information at the 3′ end of defective genomes may be manifest at this point in the replication cycle providing an early checkpoint for correction of genetic material (Guan and Simon, 2000). The 3′ UTR of the genome therefore plays a crucial role in virus replication. As the genome of plus-sense RNA viruses acts as mRNA as well as a replicon, the 3′ UTR also functions in a manner typical for cellular mRNA, playing a role in the regulation of translation, and RNA stability (Decker and Parker, 1995). While these functions of the 3′ UTR are common to many plus-strand RNA viruses, this commonality of function is not reflected in a common form, rather this region is diverse in form varying from poly (A) tails, to high order secondary and tertiary structures, to tRNA like structures, to structureless sequences (Dreher, 1999).

Sindbis virus (SIN) is the type species of the alphavirus genus of the Togaviridae, as such it possesses a single-stranded positive sense RNA genome that is capped at the 5′ end and polyadenylated at the 3′ end (Griffin, 2001; Strauss and Strauss, 1994). Alphaviruses possess a highly conserved 3′ sequence element (3′ CSE) immediately preceding the poly (A) tail (Pfeffer et al., 1998; Strauss et al., 1984). Both the poly (A) tail and the 3′ CSE have been demonstrated to be required for virus replication and more specifically for efficient minus-strand RNA synthesis (Hardy and Rice, 2005; Kuhn et al., 1990, 1992; Raju et al., 1999). Using an in vitro assay for SIN

* Fax: +1 812 855 6705.
E-mail address: rwhardy@indiana.edu.

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minus-strand RNA synthesis, it has been shown that the poly (A) tail must be at least 11–12 residues in length and immediately follow the 3′ CSE (Hardy and Rice, 2005). Further, the 3′ 13 nt of the 3′ CSE is critical for efficient minus-strand RNA synthesis. Substitution or deletion of any of these residues led to a dramatic decrease in minus-strand synthesis; however, the role these residues play during the initiation of minus-strand RNA synthesis has not been elucidated (Hardy and Rice, 2005).

The initial interaction between the SIN genome and the replicase complex appears to be mediated by the 5′ end of the genome, as has been shown for a number of other plus-strand RNA viruses (Barton et al., 2001; Chen et al., 2001; Frolov et al., 2001; Gorchakov et al., 2004; Herold and Andino, 2001; You and Padmanabhan, 1999). Also, previous results suggest that the initiation site for minus-strand RNA synthesis is located in the genomic poly (A) tail and that the 5′ end of the minus-strand RNA was a polyuridylate tract averaging 60 residues in length prior to the first non-U residue (Frey and Strauss, 1978; Sawicki and Gomatos, 1976). If this was a true reflection of alphavirus minus-strand RNA initiation, then the 3′ CSE would appear to be required only for promoter clearance and progression to elongation.

Data are presented in this manuscript showing that in a cell free system initiation of minus-strand RNA synthesis on a wild-type template occurs preferentially on the cytidylate residue (−1) immediately preceding the poly (A) tail. In vitro analyses of minus-strand RNA synthesis show that certain mutations in the 3′ CSE and the poly (A) tail lead to a decrease in initiation at the residue preceding the poly (A) tail and an increase in initiations in the poly (A) tail. It is apparent from the data presented that initiation events in the poly (A) tail do not lead to the efficient production of full-length minus-strand RNA in vitro, indicating that the site of minus-strand initiation influences RNA elongation. Additionally, an increase in heterogeneity of the 5′ end of the minus-strand RNA is observed after passaging the virus in vertebrate cells with an increase in the frequency of initiation events occurring in the poly (A) tail. These findings suggest a means of conserving the 3′ sequence of alphavirus genomes and imply that the nascent minus-strand RNA affects polymerase processivity.

Results

Mapping the 5′ end of SIN minus-strand RNA generated in vitro

Previously published data have demonstrated the importance of the 3′ CSE during viral infection and specifically for the production of minus-strand RNA (Hardy and Rice, 2005; Kuhn et al., 1990). In order to further characterize the role of residues in this region during minus-strand synthesis, an in vitro assay was employed. This assay system allows the direct analysis of minus-strand RNA synthesis from characterized templates, and prohibits the introduction of changes to the plus-strand template that may occur in vivo thus complicating analyses.

All RNA templates used in vitro were based on the wt(+) RNA sequence (Fig. 1). This RNA template is analogous to the SIN genome in that the *cis*-acting elements required for virus specific RNA synthesis are maintained, but, with the exception of sequence encompassed by the *cis*-acting elements, the regions of the genome coding for non-structural and structural proteins are deleted. SIN specific replicase activity was provided from membrane fractions prepared from BHK-21 cells in which a proteinase deficient form of SIN P123 and nsP4 was expressed (Lemm et al., 1998; Lemm et al., 1994; Shirako and Strauss, 1994). This form of the replicase efficiently synthesizes minus-strand RNA. Membrane fractions from cells expressing the polyprotein P123, but not nsP4 were used to demonstrate that RNA synthesis in the reactions was dependent upon the presence of the SIN RNA-dependent RNA polymerase (no 4). RNA was analyzed by primer extension with an oligonucleotide (p-ext short) corresponding directly to nucleotides 11,664–11,681 of the SIN genome.

Analysis of RNA extracted from in vitro minus-strand RNA synthesis reactions using wt(+) template shows strong stops mapping to the equivalent of the −1 and A4 positions in the original plus-strand template with more product mapping to the −1 than A4 position (wt(+)+4, Fig. 2). This indicates that the 5′ end of the major minus-strand RNA is a G residue complementary to the C at −1 position in the genomic RNA (residues are numbered in relation to the poly (A) tail, see Fig. 1). Two other products of primer extension are also visible mapping to the A residues at positions 1 and 5 in the poly (A) tail. None of these products of primer extension were observed using RNA isolated from reactions with no nsP4 (wt(+) no4, Fig. 2) showing that they are due to SIN specific RNA synthesis. Products of primer extension are also seen mapping beyond the poly (A) tail; however, these products are also present in the negative control. These data support the conclusion that in vitro initiation of SIN minus-strand RNA synthesis preferentially occurs on the −1 residue of the plus-strand template.

In order to determine if the strong stops observed at the −1 and A4 positions were artifacts of primer extension on a 5′ polyuridylated template control, primer extension reactions were performed using 5 ng or 100 ng of in vitro transcribed minus-strand (wt(−)) RNA with a 5′ poly (U) tract ((−) 5 and 100, Fig. 2), or equimolar amounts of in vitro transcribed wt(−) and wt(+) RNA ((−/+), Fig. 2) as template. These reactions gave rise to a number of primer extension products all of which mapped in the poly (A) tail. The majority of the products did not map precisely to the 5′ end of the input template RNA, most varied around the A25 location, but some mapped around A13. This may be indicative of reverse transcriptase terminating synthesis early or adding non-templated bases due to the long run of U residues being copied. Important observations from these controls are that the reverse transcriptase can copy a run of U residues with observable efficiency, the primer extension conditions allow the U residues to be copied even in the presence of a complementary plus-strand, and no stop is observed at the residue immediately 3′ of the poly (U) tract.

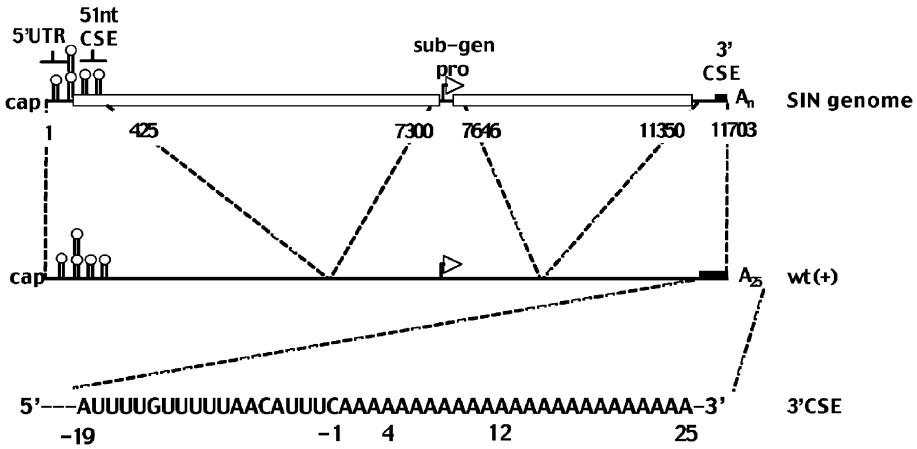

Fig. 1. Diagram of the wt(+) genome analog RNA showing the regions of the SIN genome present in this RNA, and the sequence of the 3′ CSE.

Analysis of the effect of mutations in the 3′ CSE on the site on minus-strand initiation

The results above suggested that the −1 residue in the SIN genome is the preferred site of minus-strand RNA initiation in vitro. In order to verify the importance of the −1 residue in minus-strand RNA initiation, RNA templates with a deletion of the −1 residue (Δ-1), insertion between −1 and the poly (A) tail (insC), and substitutions of the −1 residue (C-1U, C-1G) were generated. In vitro minus-strand synthesis assays to analyze full-length RNA synthesis and for primer extension analyses using these templates were performed a minimum of five times. Quantification of full-length minus-strand RNA and products of primer extension was done by phosphorimagery (see Materials and Methods). For the purpose of these assays, full-length minus-strand RNA was defined as RNA which was extended and migrated as a discrete band of template size during agarose-phosphate gel electrophoresis. These products have been well characterized previously and demonstrated to be SIN specific, de novo synthesized, minus-strand RNA (Hardy and Rice, 2005; Lemm et al., 1998). Results of quantification are shown in Table 1 and representative gels are shown in Fig. 3.

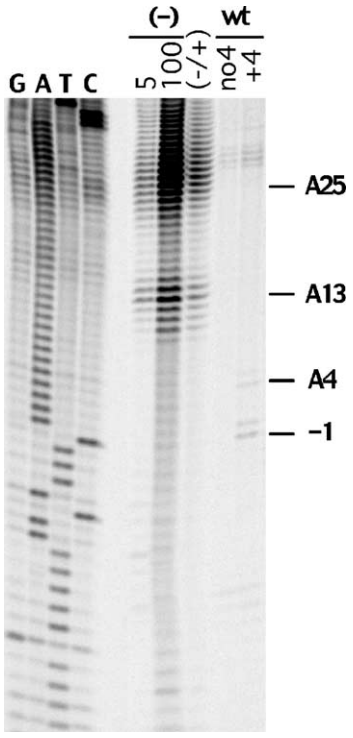

Fig. 2. Primer extension analysis of minus-strand RNA produced from wt(+) template in vitro. Membrane fractions from BHK-21 cells containing SIN P123 and nsP4 (+4) or P123 alone (no4) were used to synthesize minus-strand RNA from in vitro transcribed wt(+) template. RNA isolated from these reactions was analyzed by primer extension using an oligonucleotide corresponding directly to a sequence in the 3′ UTR (p-ext short, nucleotides 11,664–11681). Control primer extension reactions were performed on 5 ng and 100 ng of in vitro transcribed wt(−) RNA, and an equimolar mix of in vitro transcribed wt(−) and wt(+) RNA, (−/+). A sequencing ladder for the 3′ end of the SIN genome using pToto1101 as a template and p-ext short oligonucleotide was generated and run as a marker.

Templates with mutations at the-1 position were compromised in their ability to program full-length minus-strand RNA synthesis (Fig. 3B, Table 1, and Hardy and Rice, 2005). Primer extension analysis of unlabeled RNA isolated from in vitro minus-strand RNA synthesis reactions demonstrated a change in the initiation site for RNA synthesis associated with changes at the −1 position. All changes at this position caused a decrease in 5′ ends detectable by primer extension the extent of which varied depending on the mutation (Table 1). In all cases, a significant decrease in primer extension products mapping to the −1 residue and an increase in primer extension products mapping to the poly (A) tail were observed. The insertion of a C residue between the −1 residue and the poly (A) tail gave rise to low levels of minus-strand RNA one nucleotide longer at the 5′ end than wt minus-strand consistent with limited initiation occurring on the C immediately preceding the poly (A) tail (Fig. 3C).

These data provide strong support for the hypothesis that the C residue at the −1 position is the preferred site of initiation for SIN minus-strand RNA synthesis in vitro. These data also suggest that if this residue is deleted or changed then initiation of minus-strand RNA synthesis defaults to the poly (A) tail.

As mutations in the 3′ 13 nt of the 3′ CSE have been demonstrated to have a deleterious effect on synthesis of full-length minus-strand RNA (Fig. 3B, Table 1, and Hardy and

Table 1
Analysis of the 5′ ends of SIN minus-strand RNA synthesized in vitro from RNA templates possessing mutations in the 3′ CSE and poly (A) tail

Template RNA	Full-length minus-strand	−1 initiations	Total 5′ ends
wt(+)	100	100	100
Δ-1	1.1 (0.5)	1.9 (1.5)	29 (8.5)
C-1U	8.9 (2.7)	4.4 (1.2)	22 (8.4)
C-1G	1.8 (0.7)	0.9 (0.3)	9.3 (3.6)
insC	27 (16.9)	8.5 (2.3)	24 (12.3)
U-2A	3.1 (0.9)	10 (4.9)	32 (9.9)
U-3A	4.8 (2.0)	6.9 (3.3)	28 (9.2)
U-4A	2.0 (0.9)	7.0 (2.6)	10 (3.9)
A-5U	1.6 (0.6)	4.4 (1.4)	7.3 (2.3)
A-7U	1.3 (0.8)	4.3 (1.6)	6.0 (2.2)
Δ-5 > -2	0.6 (0.4)	1.3 (0.7)	6.2 (2.0)
U-12 > -10A	1.5 (1.0)	1.9 (0.9)	2.5 (1.1)
intC3A	7.5 (2.3)	8.8 (2.6)	21 (6.1)
intC6A	3.2 (2.0)	4.2 (1.9)	8.7 (3.1)
intC9A	14 (9.7)	18 (6.7)	29 (7.4)
intC12A	34 (4.2)	27 (5.4)	45 (5.8)
intC15A	74 (8.9)	84 (10.2)	104 (11.8)

Minus-strand RNA was synthesized in vitro from templates described in Figs. 3 and 4. Full-length radiolabeled minus-strand RNA was analyzed by agarose-phosphate gel electrophoresis and products were quantified by phosphorimagery. The efficiency of minus-strand synthesis from a mutant template was expressed as a percentage of that from a wild-type template. The mean (standard deviation) for five independent experiments is shown. The initiations at the −1 residue and total detectable 5′ ends were determined by quantification of primer extension products as described in Materials and Methods. Numbers were calculated as a percentage of wild-type and the mean (standard deviation) for five independent experiments was determined.

Rice, 2005), minus-strand RNA synthesized from templates possessing mutations in residues −12 to −2 (Fig. 3A) was examined by primer extension using oligonucleotide p-ext short. Substitution of uridylate for adenylate residues at the −2 (U-2A) and −3 (U-3A) positions resulted in a decrease in initiation at the −1 residue and an increase in RNA species initiating in the poly (A) tail (Fig. 3C and Table 1). The 5′ sequence preceding the wt initiation site appeared to be longer than seen for the minus-strand produced from the Δ-1 template. Products of primer extension analysis of in vitro synthesized RNA corresponding to the Δ-1 and U-3A minus-strands with a 5′ 25U tract were the same (data not shown). This indicated that the difference in 5′ sequence observed in Fig. 3C is due to incorporation of extra residues in the nascent minus-strand RNA during initiation on the U-2A and U-3A templates, and not a consequence of differences in reverse transcription of the products.

Substitution of the U residue at −4 (U-4A), the A residue at −5 (A-5U), the A residue at −7 (A-7U), and the U residues −10 to −12 (U-12 > -10A) led to a significant decrease in minus-strand RNA detectable by primer extension (Table 1). The pattern of primer extension products for U-4A and A-5U was similar to that for minus-strand produced from wt template with low amounts of product mapping in the poly (A) tail (Fig. 3C). Full-length minus-strand synthesis was almost completely abolished from mutant templates A-7U and U-12 > -10A (Fig. 3B). The 5′ ends of minus-strand RNA produced from these templates mapped to the −1 and −6 residues for A-7U and the −1 and −2 residues for U-12 > -10A (Fig. 3C). However, the major effect of both these mutations was the loss of minus-strand RNA synthesis rather than a change in the initiation site (Table 1).

Deletion of residues −5 to −2 caused initiation to shift to the poly (A) tail. Products of primer extension were predictably smaller than products from other mutant templates (U-2A for example) due to the deletion of 4 residues in the template RNA. The shift in initiation site was probably a consequence of deleting residues −2 and −3 already shown to be important in initiation site selection (see above).

Effects of changes in the poly (A) tail on minus-strand RNA synthesis

It has been previously shown that an uninterrupted run of at least 11–12 A residues following the 3′ CSE is required for efficient synthesis of full-length minus-strand RNA (Hardy and Rice, 2005). If the poly (A) tail is shorter than 11 residues, there is observable, but limited minus-strand synthesis. The wt(+) RNA template has a poly (A) tail 25 residues in length. Minus-strand RNA synthesized from templates with three cytidylate residues inserted into the poly (A) tail following 3, 6, 9, 12, and 15 A residues was analyzed by primer extension to determine the effects of poly (A) sequence on minus-strand synthesis (intC3A, intC6A, intC9A, intC12A, and intC15A, Fig. 4A). For each template, a decrease in minus-strand RNA initiating at the −1 residue and an increase in initiation in the poly (A) tail were observed (Table 1 and Fig. 4B). As the number of uninterrupted A residues increased to 15, initiation at the −1 residue also increased (Fig. 4B and Table 1). Products of initiation at the −1 residue became more readily detectable when 9 A residues (intC9A) followed the 3′ CSE, and when 15 A residues (intC15A) followed the 3′ CSE, the most prominent product of primer extension corresponded to the −1 residue (Table 1).

The variation in the size of the predominant product of primer extension mapping in the poly (A) tail for each mutant indicated that initiation was not occurring in the same position with reference to the −1 residue for all of the mutant templates, nor was there an obvious pattern of primer extension products in reference to the inserted C residues. For intC3A, the predominant product initiated 21 nt 3′ of the −1 residue, for intC6, it was 18 nt 3′ of −1, and for intC9A, intC12A, and intC15A, products mapping 13 nt and 25 nt 3′ of the −1 residue were seen. The products mapping 13 nt 3′ of the −1 residue were more prominent for the intC15A template than others. The different lengths of RNA at the 5′ end of the minus-strands may be explained if the SIN replicase slipped while copying the poly (A) tail, thus incorporating non-templated bases into the nascent minus-strand. In this scenario, the change introduced into the genome could have a significant effect on the ability of the replicase complex to escape from reiterative incorporation of uridylates to template-dependent elongation.

Primer extension analysis of minus-strand RNA produced from a template RNA with no poly (A) tail showed initiation events occurring at the −1, −2, −3, −4, −5, and −6 positions

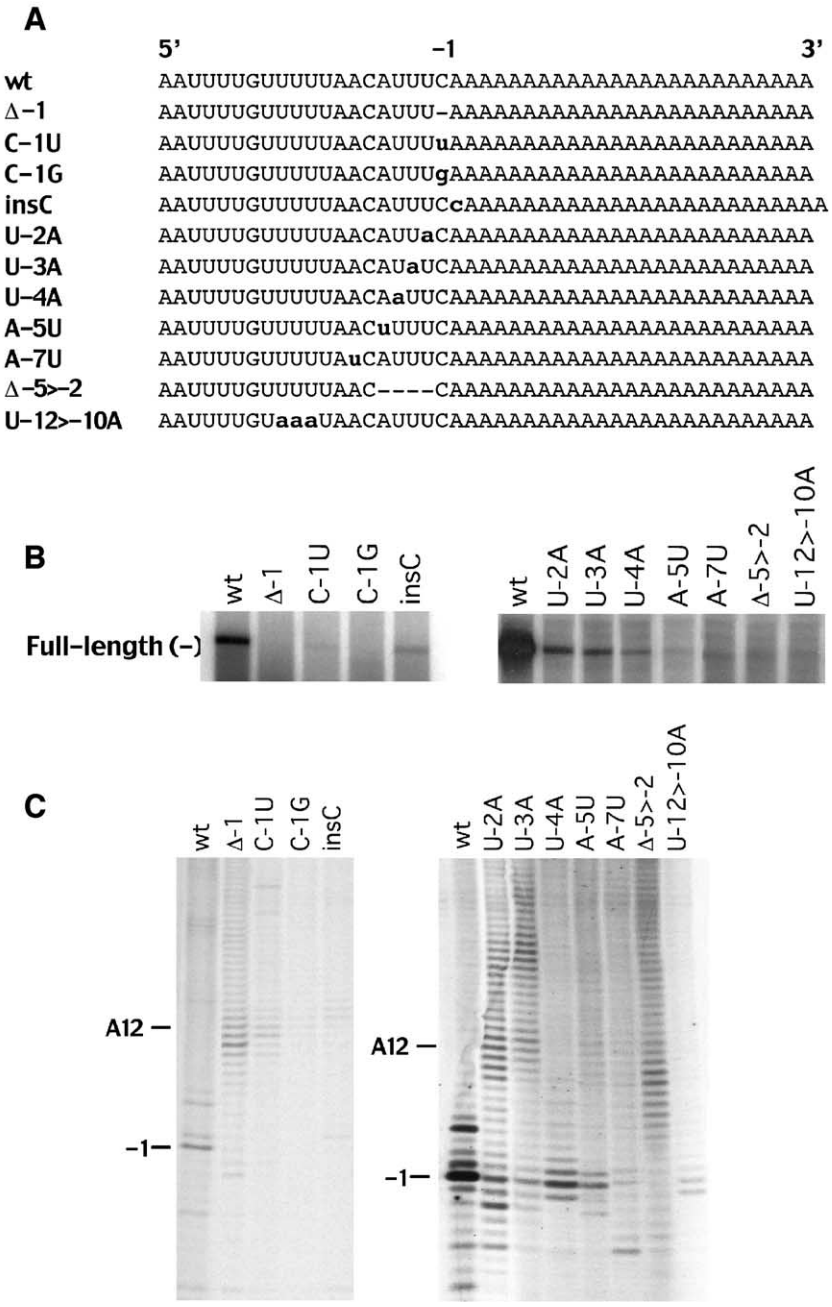

Fig. 3. Analysis of minus-strand RNA synthesized from template RNA possessing mutations in the 3′ CSE. (A) Diagram of the 3′ CSE of RNA templates generated by in vitro transcription from *Bsg*I digested pwt(+) or PCR products encoding a SIN genome analog with specific mutations in the 3′ CSE. Substitutions in the 3′ CSE are shown in bold lower case letters, and deletions are shown by a dash (–). RNA templates were used in an in vitro minus-strand synthesis assay. (B) Analysis of full-length minus-strand RNA synthesis. Minus-strand synthesis reactions were performed in the presence of [α^{32}P]-CTP. Products of the reaction were denatured and separated by agarose-phosphate gel electrophoresis followed by autoradiography. (C) Primer extension analysis of products of minus-strand RNA synthesis reactions. RNA isolated from in vitro minus-strand synthesis reactions was analyzed by primer extension using oligonucleotide p-ext short. Products of primer extension were separated by denaturing polyacrylamide gel electrophoresis and visualized by phosphorimaging.

with the most prominent product corresponding to the −6 position which is the closest C residue 5′ of the C at −1 (Fig. 4B). This indicated a preference for initiation with a G residue in the nascent strand and a requirement not to initiate at the very 3′ terminus of the template RNA. Interestingly, the primer extension analysis showed products of minus-strand synthesis larger than the input template (* in Fig. 4B). This result implied that nucleotides were being added to the non-polyadenylated template possibly facilitating subsequent minus-strand initiation on the newly extended template.

The role of initiation site in synthesis of full-length minus-strand RNA

Examination of the data in Table 1 shows that in most cases the level of full-length minus-strand RNA produced from a

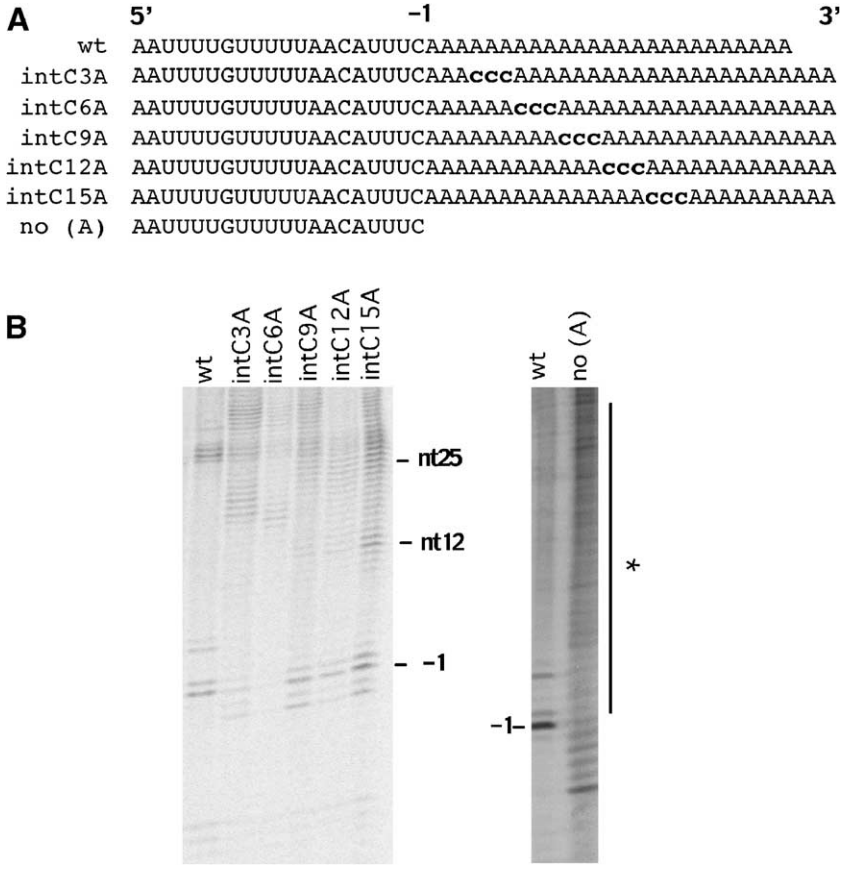

Fig. 4. Analysis of the 5′ ends of minus-strand RNA produced from templates with altered poly (A) tail composition. (A) Diagram of RNA templates generated with no poly (A) tail or 25 3′ terminal A residues interrupted at specific points with three C residues. Residues introduced into the poly (A) tail are shown in bold lower case letters. (B) Primer extension analysis of minus-strand RNA synthesized from templates with altered poly (A) tails. In vitro minus-strand RNA synthesis reactions were performed using the templates described above. RNA was isolated and subjected to primer extension analysis using oligonucleotide p-ext short. * Indicates the location of non-templated residues.

particular template RNA correlates more closely with the level of initiation at the −1 residue rather than the total amount of detectable 5′ ends (the exception to this trend is the insC template which requires further investigation). The question arising from this observation is why do not all initiation events result in the production of full-length minus-strand RNA? One possibility is that the RNA produced from non-wt initiation events is less stable than wt minus-strand RNA. However, analysis of the stability of minus-strand RNA under conditions used for the minus-strand synthesis assay revealed no differences between 5′ oligouridylated RNA and non-oligouridylated RNA (data not shown).

Another possible explanation for the lack of full-length RNA produced from non-wt initiations is that the initiation site affects the ability of the replicase complex to elongate nascent RNA. Minus-strand RNA produced from in vitro reactions using wt and U-3A templates was analyzed by RNase protection. The U-3A mutant was selected because primer extension analysis had demonstrated that it gave rise to minus-strand initiating at the −1 residue and minus-strand initiating in the poly (A) tail, and full-length RNA was seen to be present at approximately 5% the level of wt (Fig. 3 and Table 1). This means that effects on elongation of RNA synthesis as a consequence of the initiation site could be analyzed using this template. A probe corresponding directly to sequence close to the 3′ end of the SIN genome (3′ probe) and a probe corresponding to the subgenomic RNA promoter region (26S probe) were used. Fig. 5 shows the results of the RNase protection assay. To ensure that the probes were protected by minus-strand RNA, control reactions were performed using an in vitro transcript corresponding to the wt minus-strand (con.). The 3′ probe detected minus-strand RNA produced from the U-3A template at approximately 60% the level of that produced from wt(+). However, when the 26S probe was used, the level of minus-strand RNA detected dropped to <10% of wt(+). These quantities were determined by calculating the level of probe protected by minus-strand RNA produced from the wt(+) template compared to the level protected by the control (con) RNA and setting this as 100%. The amount of probe protected by minus-strand from U-3A was compared to that protected by the control RNA and then calculated as a percentage of wt. Quantification was performed by phosphorimagery. Since there appears to be no difference in minus-strand RNA stability, these data indicate that a significant proportion of minus-strand RNA initiating on the U-3A template does not productively elongate.

In order to further determine the role of the initiation site in the production of full-length minus-strand RNA, primer

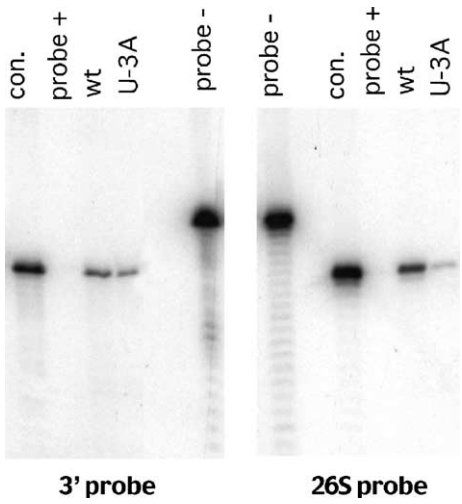

Fig. 5. Analysis of elongated minus-strand RNA produced from wt and non-wt templates. Minus-strand RNA produced from wt and U-3A templates in vitro was analyzed by RNase protection. Isolated RNA was hybridized to [^{32}P]-labeled 3′ probe RNA (corresponding directly to nucleotides 11,662–11,698 of the SIN genome with 6 non-SIN nucleotides) or 26S probe RNA (corresponding directly to nucleotides 7598–7640 of the SIN genome with 6 non-SIN nucleotides). RNA was digested with RNase A and RNase T1 according to manufacturer's protocols (Ambion). Products of digestion were analyzed by denaturing acrylamide gel electrophoresis and autoradiography. In vitro transcribed minus-strand RNA was used as a control (con) for hybridization and digestion. Probe+ is probe incubated with RNase, probe− is probe incubated in the absence of RNase.

extension analysis was performed using oligonucleotides that anneal at different distances from the 5′ end of the minus-strand. Fig. 6 shows the results of primer extensions performed on wt and U-3A minus-strand RNA using oligonucleotides that anneal 40 nt (p-ext short) or 127 nt (p-ext long) from the 5′ end of the wt minus-strand. P-ext short was used and consistent with previous results, the 5′ end of the minus-strand RNA from the wt template maps predominantly to the −1 residue (Fig. 6). The 5′ end of the minus-strand RNA from the U-3A template maps to numerous sites in the poly (A) tail and also to the −1 residue. When the same RNA derived from U-3A was analyzed using the p-ext long oligonucleotide, the major product detected corresponded to initiation at the −1 residue. These data in conjunction with those obtained by RNase protection strongly suggest that initiation events in the poly (A) tail do not progress to productive elongation of full-length minus-strand RNA.

Production of minus-strand RNA with 5′ ends mapping to the poly (A) tail during virus infection

Analyses of SIN minus-strand RNA produced in an in vitro assay indicated that the predominant site of minus-strand initiation was the −1 residue. Additionally, RNA initiating in the poly (A) tail did not efficiently elongate. To determine if initiation of minus-strand RNA synthesis occurred in a similar way in vivo, SIN genomic RNA was transfected into BHK-21 cells and 5 h post-infection cells were harvested and RNA isolated. Primer extension analysis of the isolated RNA using oligonucleotide p-ext short indicated that the majority of the detected 5′ ends mapped to the −1 residue (SIN, Fig. 7A). These products of primer extension were not detected in the uninfected control (cell, Fig. 7A). To address the possibility that base-pairing between minus-strand product and plus-strand template may have resulted in the strong-stop at −1, isolated RNA from SIN transfected cells was heated to 95 °C in the presence of 30pmol of oligo (U) RNA (5′-GGU$_{20}$-3′), rapidly cooled and allowed to incubate at 25 °C for 10 min prior to heating and annealing with the oligonucleotide used for primer extension. The pattern of primer extension products was the same as for SIN RNA alone (SIN + U$_{20}$, Fig. 7A).

The analyses above were performed on RNA isolated from cells transfected with SIN genomic RNA. This method allows little opportunity for the genome sequence to vary from the wild-type consensus. Data presented above have shown that certain mutations in the 3′ CSE and poly (A) tail cause the site of initiation to move to the poly (A) tail in vitro. As RdRps are inherently error prone, the process of replication may lead to the introduction of mutations in the genome that change the site of minus-strand initiation. Therefore, the effect of serially passaging SIN on the initiation site for minus-strand synthesis was analyzed.

Virus was recovered from cells transfected in parallel with those from which RNA was harvested for primer extension analysis in Fig. 7A. This virus was used to infect fresh BHK-21 cells. Infections were performed in duplicate at an m.o.i. of 5

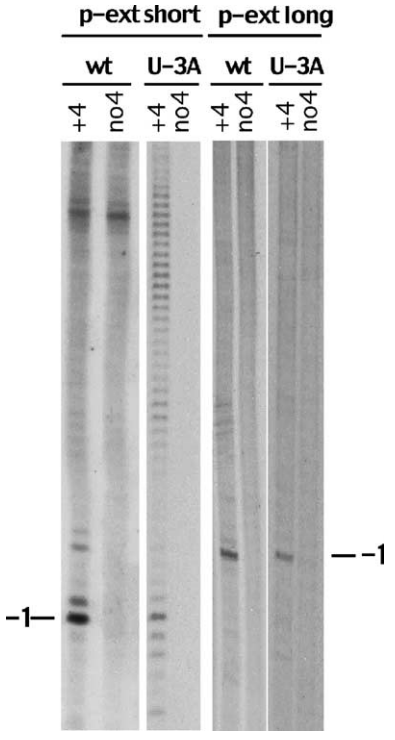

Fig. 6. Analysis of minus-strand RNA elongation by primer extension. Minus-strand RNA produced from wt and U-3A templates was analyzed by primer extension using p-ext short and p-ext long (corresponding directly to nucleotide 11,577–11,595 of the SIN genome). RNA analyzed by primer extension was isolated from in vitro reactions in which SIN nsP4 was present (+4) or absent (no4). Products of primer extension were analyzed as previously described. Exposure time for the gel of p-ext long products from U-3A minus-strand was double that for wt.

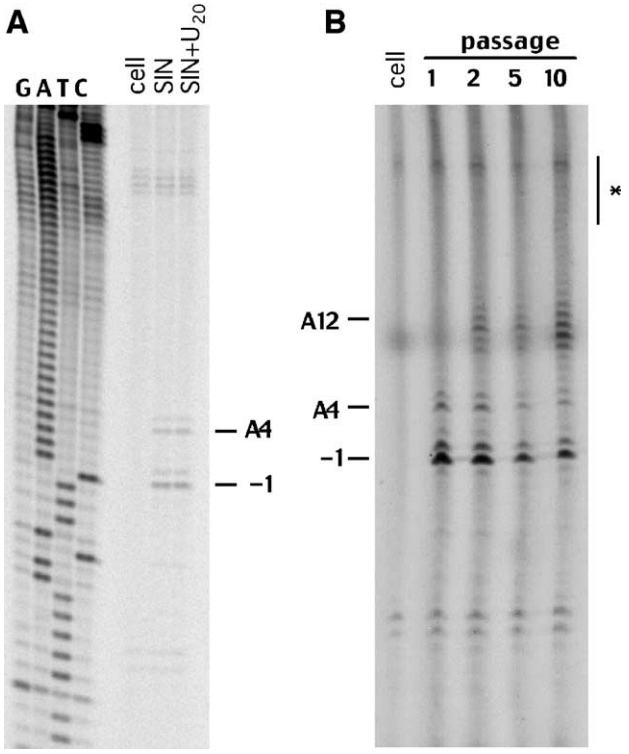

Fig. 7. Analysis of the 5′ end of SIN minus-strand RNA produced in vivo. (A) Primer extension analysis of SIN minus-strand from cells transfected with SIN genomic RNA. In vitro transcribed SIN genomic RNA (Toto1101) was transfected into BHK-21 cells and incubated at 37 °C for 5 h. Cells were harvested into a detergent buffer for lysis, nuclei were removed, and RNA extracted from the lysate. Primer extension was performed using oligonucleotide p-ext short. Products of primer extension on RNA from untransfected cells (cell), cells transfected with SIN genomic RNA (SIN), and RNA from SIN transfected cells heated with oligo (U) prior to primer annealing (SIN + U_{20}) were analyzed on an 8% polyacrylamide gel. A sequencing ladder for the 3′ end of the SIN genome was run as a marker. (B) Analysis of the 5′ end of SIN minus-strand RNA from virus infected cells over multiple passages. RNA extracted from cells infected with SIN passaged 1, 2, 5, or 10 times was analyzed by primer extension using oligonucleotide p-ext short. Products were analyzed as before. * Indicates the presence of low abundance products of primer extension (see text).

p.f.u./cell. Five hours post-infection, cells from one of the duplicate infections were harvested and RNA isolated as described above. The second infection was allowed to proceed for 24 h at which time the medium was removed and virus titer determined by plaque assay. Virus was passaged 10 times and RNA extracted from infected cells during each passage.

Fig. 7B shows the results of primer extension analysis of the RNA from various passages of the virus. The products of primer extension on the RNA isolated from cells during passage 1 again show a strong signal corresponding to the −1 position. In addition, products corresponding to A residues 1, 2, 3, 4, and 5 of the poly (A) tail are visible. Analysis of RNA from passage 2 revealed RNA with 5′ ends mapping further into the poly (A) tail in addition to those products previously observed. The observation of these products indicates the presence of RNA species with oligouridylated 5′ ends with stretches of U varying in length around a predominant size of 11–13 residues. The apparent frequency of these products when compared to the products of initiation at the −1 residue appeared to plateau by passage 5 although larger products were present in low abundance for RNA analyzed from passage 10 (* in Fig. 7B).

While these data show that there is flexibility in where minus-strand synthesis initiates, and that the heterogeneity of the 5′ end increases with passage of virus in cultured BHK-21 cells, the predominant product of primer extension in all cases mapped to the −1 residue. These observations support those made using the in vitro minus-strand RNA synthesis system and indicate that initiation is occurring in a similar manner in vivo as in vitro.

Discussion

The 3′ end of alphavirus genomes has a conserved sequence element, the 3′ CSE, immediately preceding the poly (A) tail. Both the 3′ CSE and the poly (A) tail have been shown to be required for minus-strand RNA synthesis (Hardy and Rice, 2005). However, the precise roles of residues within these elements during minus-strand synthesis have not been elucidated. In order to determine the importance of residues at the 3′ end of the genome during minus-strand initiation, a SIN specific in vitro RNA synthesis system was employed. This system produces adequate amounts of minus-strand RNA for analysis even from templates with mutations in the 3′ CSE. In vivo, such mutations may reduce the low levels of minus-strand beyond the level of detection. Additionally, the in vitro system used allows the examination of minus-strand produced from a characterized template without the possibility of changes being introduced into the template RNA that could further complicate the analysis.

Data presented in this paper show that the predominant minus-strand 5′ end maps to the residue immediately preceding the poly (A) tail indicating that, in vitro, initiation occurs with a G residue on the C at the −1 position relative to the poly (A) tail in the genome. Further examination of minus-strand RNA synthesized from templates with mutations in the 3′ CSE or poly (A) tail showed that changing certain residues in these elements caused a shift in initiation site location from the −1 residue to the poly (A) tail. Although the precise 5′ end of the non-wt minus-strands has not been defined, it was apparent that the site of initiation in the poly (A) tail varied depending on the mutation introduced (i.e. the pattern of 5′ ends observed for Δ-1 is different than that seen for U-3A) suggesting that the 3′ CSE and poly (A) tail are communicating in order to determine the site of initiation.

The requirements for promoting de novo RNA synthesis can be broken into a number of steps: (i) template-polymerase recognition, (ii) promoter/initiation site localization, (iii) nucleotide substrate recognition, (iv) phosphodiester bond formation, (v) promoter clearance, (vi) processive elongation. The data in this paper show that in vitro the poly (A) and the −1, −2, and −3 residues of the 3′ CSE are responsible for promoter/initiation site localization, with the −1 residue representing the predominant initiation site. However, mutations in residues −4 to −12 caused a significant decrease in

both full-length minus-strand RNA synthesis and minus-strands detectable by primer extension. Further work is required to determine the precise role of these residues in the initiation process. Given their location in relationship to the site of initiation, it is possible that they may be required for efficient transition from initiation to elongation, and/or promoter recognition by the replicase.

Examination of the results of primer extension clearly indicated that there were more initiations than full-length minus-strands produced from some of the mutant templates (Table 1 and Fig. 3). RNase protection assays and primer extension analyses of minus-strand RNA produced from mutant templates strongly support the hypothesis that the initiation site and possibly the 5′ end of the nascent strand determine the capacity of the minus-strand to be elongated (Figs. 5 and 6). This means that, by virtue of their role in initiation site selection, the residues at positions −1, −2, −3, and the poly (A) tail significantly influence the process of nascent strand elongation.

The nature of the 5′ end of the nascent strand from mutant templates differs more significantly from wt than do the templates themselves, it therefore seems more likely that the determinant of elongation capacity is in the nascent strand rather than the template strand. Precedent for a eukaryotic viral RNA element that enhances elongation exists in the transactivation response region (TAR) found near the 5′ end of primate lentiviral mRNA. Interaction of the viral transactivating protein, Tat, with the TAR element of the nascent mRNA leads to an increase in cellular RNA polymerase II processivity during transcription of the mRNA (Dingwall et al., 1990; Fisher et al., 1986; Parada and Roeder, 1996).

While the TAR element requires binding of another protein to enhance polymerase processivity, in the lambda-like bacteriophage HK022, an element in the nascent RNA, termed the *put*-site, directly interacts with the transcribing polymerase promoting readthrough of termination signals and reducing the frequency of transcriptional pausing (Banik-Maiti et al., 1997; Sen et al., 2001, 2002). It is possible that the SIN wt nascent minus-strand (initiated at the −1 residue) interacts with the replicase complex, directly or indirectly, to promote its own synthesis or even that the 5′ poly (U) nascent minus-strand RNA produced from mutant templates interacts with the replicase to inhibit elongation. These possibilities require further investigation.

The majority of the work in this manuscript was performed in vitro. Primer extension, mutational analysis, and the elongation capacity of the nascent minus-strand RNA support the conclusion that SIN specific in vitro minus-strand RNA synthesis initiates predominantly at the −1 residue. Analysis of RNA extracted from cells infected with SIN virus mapped the 5′ end of the minus-strand produced in vivo to the −1 residue of the genomic template. Interestingly, the heterogeneity of the 5′ end increases over serial passage with an increase in 5′ ends mapping to the poly (A) tail, possibly indicating that mutations are accumulating in the viral genome causing a change in initiation site location. Another possibility is defective interfering (DI) RNAs have accumulated during passage and minus-strand initiation on these RNAs does not occur at the same place as on the viral genome. The 5′ sequence of DI RNA can be distinctly different from that of the viral genome, and it has been clearly demonstrated that the 5′ end of the plus-sense template RNA is important for efficient minus-strand RNA synthesis (Frolov et al., 2001; Monroe and Schlesinger, 1983, 1984; Monroe et al., 1982). Work is ongoing to determine the role of the 5′ end of the SIN genome in initiation of minus-strand synthesis.

Previously published work used ribonuclease analyses to concluded that the 5′ end of the minus-strand in vivo was a poly (U) tract, and hence initiation of minus-strand RNA occurred in the poly (A) tail (Frey and Strauss, 1978; Sawicki and Gomatos, 1976). It is possible that the 5′ end that was identified was a result of initiation on non-wild-type templates, or that initiation occurs more frequently in the poly (A) tail in vivo due to the presence of a cellular factor that is absent in vitro. In the absence of single molecule studies, it is impossible to definitively identify the functional form of the minus-strand (i.e. that which acts as template for plus-strand synthesis); however, the data presented in this paper strongly suggest that minus-strand initiated in the poly (A) tail does not productively elongate to full-length whereas minus-strand initiating at the −1 residue does.

If the predominant minus-strand initiation site is the −1 residue then this gives rise to the question of how does viral plus-sense RNA become polyadenylated? It had previously been hypothesized that the 5′ poly (U) tract would act as a template for the generation of a poly (A) tail on the plus-sense RNA. In the absence of a 5′ poly (U) tract in the minus-strand, the poly (A) tail must be added in a template-independent fashion. Previous work by Raju et al. (1999) has demonstrated that non-polyadenylated SIN genomic RNA quickly regains the poly (A) tail following introduction into the host cell. The genome may be polyadenylated by cellular cytoplasmic polyadenylation machinery as previously proposed by Raju et al. While the 3′ sequence immediately preceding the poly (A) does not contain a consensus polyadenylation hexanucleotide required for cleavage of the RNA prior to poly (A) addition by poly (A) polymerase, there are a number of U/A rich sequences that closely resemble cytoplasmic polyadenylation elements (Mendez and Richter, 2001; Richter, 1999). During cytoplasmic polyadenylation of cellular mRNA, this element is bound by cytoplasmic polyadenylation element binding protein (CPEB) that recruits the other components of the polyadenylation machinery to the 3′ end of the mRNA (Mendez and Richter, 2001; Richter, 1999). A number of isoforms of CPEB are encoded by many eukaryotes and these variants are expressed in different tissues or at different times during development. Work is continuing to determine whether this mechanism of polyadenylation is required by alphaviruses.

A number of other plus-sense RNA viruses have a polyadenylated genome (Dreher, 1999). Unlike viral genomes that are not polyadenylated, these polyadenylated genomes vary in size as the length of the poly (A) tail for a particular virus can be between 20 and 200 residues. Given this size variation and the probability that the polymerase/replicase

complex interacts with a specific sequence in the 3′ UTR, it may be difficult for the polymerase to find the very 3′ end of the genome that could be hundreds of nucleotides away from the binding site. Studies with bamboo mosaic potexvirus show that the minus-strand has on average 20 U residues at the 5′ end, whereas packaged genomic RNA has 90–170 3′ terminal A residues indicating that the plus-strand generated from the 5′ oligouridylated minus-strand must be polyadenylated in a template-independent fashion (Cheng et al., 2002). Also, hepatitis A virus poly (A) tail is regenerated in a template-independent, but cell cycle-dependent fashion (Kusov et al., 2005). Given the data presented above, it is possible that a template-independent mechanism of polyadenylation is used for alphavirus plus-sense RNA.

Materials and methods

Cells and viruses

BHK-21 cells were obtained from American Type Culture Collection, Rockville, Maryland. These cells were grown in Alpha MEM (Invitrogen) supplemented with 10% fetal bovine serum and vitamins. Recombinant vaccinia viruses encoding T7 DNA-dependent RNA polymerase, the SIN polyprotein $P123_{C>S}$, and a ubiquitin-nsP4 fusion protein were grown individually to high titer in BSC-40 cells cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum, non-essential amino acids, and vitamins (Fuerst et al., 1986; Lemm et al., 1994, 1998; Shirako and Strauss, 1994). Virus titer was determined by plaque assay on BSC-40 cells. SIN was generated by transfection of BHK-21 cells with infectious RNA in vitro transcribed from pToto1101 (Rice et al., 1987).

Plasmids and constructs

Construction of pToto1101 encoding the infectious full-length SIN genome, and pwt(+) encoding a replication competent RNA analogous to the SIN genome, has been described in detail previously (Hardy and Rice, 2005; Rice et al., 1987). RNA produced from these constructs is shown diagrammatically in Fig. 1. Mutations in the 3′ CSE and poly (A) tail of pwt(+) were made by PCR amplification using mutagenic oligonucleotide primers corresponding to the 3′ end of pwt(+) and a primer corresponding to the 5′ end of the SIN genome preceded by a T7 promoter (Hardy and Rice, 2005). PCR amplification was performed using high fidelity Triple Master polymerase (Brinkman–Eppendorf).

RNA transcription

Plasmids pToto1101 and pwt(+) were linearized by digestion with *Xho*I and *Bsg*I, respectively. Products of PCR amplification were gel isolated using a Gel Extraction kit (Qiagen), quantified by spectrophotometry and used directly in transcription reactions. RNA was transcribed in the presence of cap analog (New England Biolabs) using SP6 or T7 RNA polymerase (New England Biolabs). RNA transcribed from pwt(+) and PCR products have no non-SIN sequence at the 3′ end. RNA transcribed from pToto1101 has two cytidylate residues 3′ of the poly (A) tail. DNA templates were removed by DNaseI (Takara) digestion. The integrity and yield of the RNA transcripts were monitored by gel electrophoresis. RNA was purified by phenol–chloroform extraction and ethanol precipitation. Isolated RNA was dissolved in water to a final concentration of 1 μg/μl as determined by spectrophotometry.

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 p.f.u./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×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×g for 5 min at 4 °C). Post-nuclear homogenates were centrifuged at 15,000×g for 20 min at 4 °C. Pellets (P15) were resuspended in 120 μl 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 $MgCl_2$; 10 mM dithiothreitol; 10 μg ActD per ml; 5 mM creatine phosphate; 25 μg creatine phosphokinase per ml; 1 mM ATP, GTP, UTP, CTP; 800 units Ribonuclease Inhibitor (Takara) per ml; 1 μg (3 pmol) template RNA; 18 μl P15; H_2O to total volume of 50 μl. For examination of full-length minus-strand RNA, 1 mM CTP was replaced with 40 μM CTP and 1 mCi [α^{32}P]-CTP per ml. Reactions were incubated at 30 °C for 60 min at which point 5 units 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 μg/ml. RNA was isolated by phenol/chloroform extraction and ethanol precipitated. For examination of full-length products of minus-strand RNA, synthesis RNAs were denatured with glyoxal and separated by electrophoresis and visualized by autoradiography. The quantification of radioactivity was performed using a phosphorimager (Amersham).

Cell lysis and extraction of viral RNA

Approximately 2×10^6 BHK-21 cells were infected with SIN at an m.o.i. of 5 p.f.u./cell, or transfected with 8 μg of in vitro transcribed Toto1101 RNA. Cells were incubated for 5 h at 37 °C then harvested using a mild detergent buffer (10 mM Tris–HCl, pH 7.4, 140 mM NaCl, 1% NP40, 0.4% sodium deoxycholate). Nuclei were removed by low speed centrifuga-

tion and intracellular RNA was purified by phenol extraction in the presence of 0.1% SDS followed by ethanol precipitation.

Primer extension analysis

RNA purified from infections or minus-strand RNA synthesis reactions was resuspended in 20 μl of H_2O. RNA purified from cells transfected with Toto1101 RNA was resuspended in 8 μl of H_2O. Four microliters of RNA solution was mixed with 150 pmol of oligonucleotide, p-ext short, corresponding directly to nucleotides 11,664–11,681 of the SIN genome (5′-TTTCTTTTATTAATCAAC-3′) or oligonucleotide, p-ext long, corresponding directly to 11,577–11,595 of the SIN genome (5′-CATTTATCTAGCGGACGCC-3′). The annealing mix was incubated at 70 °C for 5 min then fast cooled in an ice-water bath for 5 min. Components were added to the annealing mix so the final reaction conditions for cDNA synthesis were 50 mM Tris–HCl (pH 8.3), 10 mM DTT, 75 mM KCl, 2.5 mM $MgCl_2$, 500 μM dGTP, 500 μM dCTP, 500 μM dTTP, 50 μM dATP, 20 μCi [^{35}S]-dATP (Amersham Biosciences), 1 μl ImProm-II reverse transcriptase (Promega) in a total volume of 20 μl. Reactions were incubated at 25 °C for 5 min followed by 45 min at 42 °C. Reactions were stopped by incubation at 70 °C for 15 min. CDNA products were precipitated by the addition of sodium acetate to a final concentration of 300 mM and 3 volumes of 100% ethanol. DNA pellets were resuspended in formamide denaturing buffer and resolved by electrophoresis on an 8% acrylamide–urea–TBE gel.

Quantification of products of primer extension was performed using a phosphorimager. Following background subtraction, individual bands up to 40 nucleotides from the −1 site were quantified. Quantities for each band were adjusted for radiolabel incorporation, and quantities of all products for a particular template were summed and expressed as a percentage of wild-type (set at 100%) to give a quantitative estimate of total 5′ ends. The −1 initiation events were quantified in a similar manner by analysis of a single band mapping to the residue immediately preceding the poly (A) tail for each template RNA. Five independent experiments were performed for each RNA template and a mean value and standard deviation were calculated. The data are presented in Table 1.

Generation of probes and RNase protection analysis

Probe RNAs were generated by T7 transcription from PCR products corresponding to nucleotides 7598–7640 (26S probe), and nucleotides 11,662–11,698 (3′ probe) of the SIN genome. A T7 promoter was added during PCR amplification as were 2 non-SIN 5′ G residues and 4 non-SIN residues at the 3′ end. RNA was labeled by incorporation of [α^{32}P]-ATP and [α^{32}P]-CTP during transcription. Labeled RNA was purified by band excision from a 12% acrylamide–urea–TBE gel. RNA was eluted from the gel slice in 300 mM sodium acetate and precipitated by the addition of 3 volumes of ethanol.

RNA purified from minus-strand RNA synthesis reactions was resuspended in 20 μl of H_2O. RNase protection assays were performed using the RPAIII kit from Ambion according to manufacturer's protocols with minor modification. Four microliters of RNA was mixed with not less than 200 pmol and 30,000 cpm of probe RNA. The RNA/probe mix was incubated at 70 °C for 5 min then fast cooled in an ice-water bath for another 5 min. Hybridization buffer was added and the mix incubated at 42 °C for 16 h. Digestion, inactivation of enzyme, and precipitation of digestion products were performed according to the manufacturer's protocol. Products of digestion were separated on a 12% polyacrylamide–urea–TBE gel and visualized by autoradiography.

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