

Genomic Characteristics of eGFP-labeled Senecavirus A Subjected to Forty Serial Passages in Cells

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Abstract

Senecavirus A (SVA) can cause a vesicular disease in swine. This virus belongs to the genus *Senecavirus* in the family *Picornaviridae*. The viral genome is one positive-sense, single-stranded RNA, approximately 7,300 nt in length. It contains 5' and 3' untranslated regions, a single encoding region, and a 3' poly(A) tail. In this study, an enhanced green fluorescent protein (eGFP)-labeled recombinant SVA (rSVA-eGFP) was rescued from its cDNA clone using reverse genetics, and then subjected to forty serial passages *in vitro*. Green fluorescence gradually disappeared with viral passaging. The passage-40 progeny was subjected to next-generation sequencing. The analysis of sequencing data showed that SVA as such did not remove its own elements, but most nucleotides were deleted from the eGFP sequence, implying SVA unable to express stably a foreign gene. Additionally, a total of 26 single-nucleotide mutations were identified in the rSVA-eGFP genome. Out of the 26 mutation sites, a nonsense mutation (G7967A) arose close to the 3' end of 3D sequence. Its emergence made a small proportion of 3D proteins be shortened by six amino acids in the quasispecies pool at P40. The present study lays the foundation for clarifying evolutionary patterns of SVA.

INTRODUCTION

Senecavirus A (SVA) is an emerging pathogen that induces a vesicular disease in swine. SVA has been the only member of the genus *Senecavirus* in the family *Picornaviridae* (1). After SVA infection, pigs may show mild signs characterized by lethargy and lameness, usually followed by the development of vesicles on the snout, dewclaw or (and) coronary band. The clinical signs induced by SVA infection are generally indistinguishable from those of other vesicular diseases in pigs (2). SVA-infected cases have been reported in several countries, including Canada (3), the United States (4), Brazil (5), Colombia (6), Mexico (7), Chile (8), China (9), Thailand (10), Vietnam (11) and India (12). The transmission risk of SVA has attracted a great deal of attention from the pig industry worldwide.

SVA virion is a typical icosahedral particle, encapsulating its own genome that is a positive-sense, single-stranded RNA genome, approximately 7,300 nt in length, composed of 5' untranslated region (UTR), polyprotein open reading frame (ORF) and 3' UTR. Like those of other picornaviruses, the 5' terminus of SVA genome has no cap structure. Alternatively, a genome-linked protein VPg is covalently linked to the 5' terminus, where it plays a key role of protein primer in the synthesis of viral genome. The SVA genome possesses a 3'-end poly(A) tail, whose length is variable (13). In an SVA-infected cell, the viral polyprotein will be translated, and then gradually cleaved into twelve proteins, namely, L, VP4, VP2, VP3, VP1, 2A, 2B, 2C, 3A, 3B, 3C and 3D (14). The VP1 to VP4 are structural proteins, responsible for the viral morphogenesis. The other proteins are nonstructural proteins, which, albeit uninvolved in the virion assembly, play key roles in viral replication (15–17). For example, the 2A oligopeptide generates a ribosome “skipping” effect, separating two proteins without needing a proteinase (18).

The 3D protein is viral RNA-dependent RNA polymerase (RdRp), whereby a picornaviral genome is replicated *via* a negative-sense RNA intermediate (19). Because RdRp of picornaviruses generally lacks

the proofreading ability, the mutation rate of genomes may be relatively high during viral replication (20–22). Mutation events will gradually accumulate to form a viral quasispecies during serial passage (23), as evidenced by our recent report showing a complete mutational landscape of wild-type SVA subjected to eighty serial passages in cells (24). Although the wild-type SVA exhibited a high level of point mutations in its own genome, neither fragment-deleting nor -inserting phenotype was detectable in the passage-80 (P80) progeny (24), implying each genetic element essential in the SVA genome for viral propagation. In other reports, we found that SVA could not tolerate a given foreign sequence stably fixed in a chimeric SVA genome (25, 26). In other words, the foreign sequence will be gradually deleted from the chimeric genome, possibly *via* a mechanism of copy-choice recombination (27, 28). In the present study, an enhanced green fluorescent protein (eGFP)-labeled SVA was rescued from its cDNA clone. The chimeric virus underwent forty serial passages *in vitro*. The P40 progeny was subjected to next-generation sequencing (NGS) analysis for uncovering a complete mutational landscape of the eGFP-labeled genome.

MATERIALS AND METHODS

Cell line and plasmid

Baby Hamster Kidney fibroblasts (BHK cells) were cultured at 37°C with 5% CO₂ in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum. The plasmid M0, containing a recombinant SVA (GenBank: KX751945.1) cDNA clone, was constructed previously (25), into which the eGFP-encoding sequence infused with a *Thosea asigna* virus 2A (T2A) fragment was inserted.

Recovery of recombinant SVA for serial passage

BHK cells were seeded onto a 6-well plate for incubation at 37°C overnight. The M0 cDNA clone was transfected (1000 ng/well) into a cell monolayer at 70% confluency for rescuing viable SVA, *i.e.*, eGFP-labeled recombinant, named rSVA-eGFP. The 6-well plate was incubated at 37°C for 3 days, and then observed using a fluorescence microscope at 72 hr post transfection (hpt), followed by a freeze-and-thaw cycle to collect the supernatant for forty consecutive passages (48 hr/passage) in BHK cells, regardless of the MOI value and the cytopathic effect during passaging. The rSVA-eGFP-infected cell monolayer was observed using the fluorescence microscope at each passage.

NGS analysis

The culture supernatant was harvested at P40 to extract total RNAs for constructing a cDNA sequencing library. The Illumina NovaSeq 6000 System (Illumina, San Diego, CA, USA) was used for NGS, followed by data analysis as described previously (29).

RESULTS

Recovery and passaging of rSVA-eGFP

The rSVA-eGFP cDNA clone M0 was transfected into BHK cells for rescuing the replication-competent virus. Green fluorescence was observable on the plasmid-transfected cell monolayer at 72 hpt (Fig. 1, P0). The P0 stock was collected for serial passaging *in vitro*. Green fluorescence, albeit visible on the virus-inoculated cell monolayer with passaging, gradually disappeared from P8 and completely vanished at P20 (Fig. 1). The P20 stock was continuously passaged until P40. Fluorescence was always unobservable during passaging from P20 to P40.

Sequencing depths of NGS

The P40 progeny was harvested for NGS analysis, and the NGS data were further analyzed. A complete dataset of sequencing depths across the rSVA-eGFP genome was listed in Supplementary 1, and schematically shown in Fig. 2A. The rSVA-eGFP genome contained a foreign sequence, *i.e.*, eGFP-T2A fusion fragment (Fig. 2A, indicated by green-yellow rectangle). The genome layout was described as 5' UTR–L–VP4–VP2–VP3–VP1–2A–eGFP–T2A–2B–2C–3A–3B–3C–3D–3' UTR, as shown proportionally in Fig. 2A. The NGS analysis suggested that the most part of the eGFP sequence was deleted from the chimeric genome (Fig. 2A). Sequencing depth of the deleted fragment was zero (Supplementary 1, nt 3567 to 4123). The average sequencing depth for each genetic element was shown in Fig. 2B. The highest sequencing depth was 2135× at site nt-785, located at the region of L sequence. Because most of its nucleotides were deleted after 40 passages, the eGFP region only revealed its average depth of 29×, the lowest one among all average depths of genetic elements (Fig. 2B).

Mutational landscape of rSVA-eGFP genome

A total of 26 SNMs, including 18 transitions and 8 transversions, were recognized in the rSVA-eGFP genome at P40. Transition sites included U1350C, U2314C, U3168C, G3246A, U3294C, G3505A, G3522A, U4452C, A4668G, U5645C, A6057G, C6158U, G6264A, A6265G, U7399C, G7422A, G7967A and G7971A. Transversion sites included U33G, C558A, G815U, A1521U, A4664U, A4713C, U5078A and A5760C. All 26 SNMs displayed their single nucleotide polymorphisms (SNPs), namely two different nucleotides coexisting at the same site. Figure 2C showed a complete profile of 26 SNP sites with absolute (× reads) sequencing depths in the genome. Wild-type nucleotides were indicated by arrows. Correspondingly, Fig. 2D exhibited relative (%) sequencing depths at the 26 SNP sites.

Approximate locations of SNMs were marked with arrows on the rSVA-eGFP genome (Fig. 3A). Their mutation rates were enclosed within parentheses. Out of the 26 SNMs, five were synonymous mutations, indicated by green arrows (Fig. 3A); seventeen were missense mutations, independently causing single-amino acid mutations (SAAMs), indicated by black and blue arrows in Fig. 3B. Out of the seventeen missense mutations, G6264A and A6265G were adjacent to each other (Fig. 3A), leading to three SAAMs independently arising at a single amino acid site in the 3C protein (Fig. 3B, Blue arrow-indicated).

One nonsense mutation (G7967A) was identified at a site close to the 3' end of 3D sequence, as indicated by a red arrow in Fig. 3A. The SNM G7967A directly resulted in an emerging stop codon, UGA, upstream of the wild-type stop codon. The nucleotide sequence was 5'-AGGGCCUUGUUCGAU-3' between the emerging and the wild-type stop codons. Its emergence made the 3D protein be shortened (Fig. 3B, Red arrow-marked) by six amino acids, WRALFD.

DISCUSSION

As a typical picornavirus, SVA displays a great potential in viral vector for expressing a foreign marker in cells. Unfortunately, chimeric SVAs are prone to loss of their foreign markers during serial passage. The longer a foreign marker is, the more quickly its sequence is lost. The ORF of eGFP is 720 bp in length, longer than that (516 bp) of NanoLuc® luciferase. We found previously that the eGFP was more genetically unstable than the NanoLuc® luciferase during serial SVA passaging (25, 26). It had been unknown what the retention limit of eGFP sequence was in the rSVA-eGFP genome. We initially aimed to clarify this limit in the present study. The result of rSVA-eGFP passaging revealed that the fluorescence signal was becoming weaker and weaker with passaging, and finally unobservable at P20 (Fig. 1), implying that the eGFP ORF was completely or at least partly deleted from the rSVA-eGFP genome. Indeed, the NGS analysis exhibited that most eGFP-derived nucleotides were lost at P40 (Fig. 2A).

There were two non-self sequences in the rSVA-eGFP: in addition to the eGFP, the T2A was also a foreign fragment. Different from that of the eGFP, the role of T2A was essential for the rSVA-eGFP replication, because the T2A functioned to cleave the polyprotein *via* a mechanism of ribosome skipping (30). Therefore, the T2A sequence as a foreign gene was not deleted. The rSVA-eGFP possesses its own 2A polypeptide. During polyprotein translation, the ribosome can skip at the “NPG↓P” motif of SVA 2A polypeptide. Therefore, the motif NPGP is extremely conserved in SVA genomes. Interestingly, the present study displayed an SAAM, G9N (Fig. 3B), arising in the motif NPGP of SVA 2A polypeptide. Due to the introduction of T2A sequence, the function of SVA 2A protein could be replaced with that of the T2A protein. In other words, the G9N-caused inactivity of SVA 2A protein could be functionally compensated by the T2A protein.

The wild-type 3D protein was a 462-aa-long polypeptide (24). In the present study, the G7967A resulted in a stop codon emerging in advance, making the 3D protein be truncated in viral quasispecies. Six amino acid residues (WRALFD) were deficient in the truncated 3D protein. We previously identified a highly conserved fragment located in a region close to the 3' end of 3D sequence (31). This fragment forms a putative hairpin structure, as schematically shown in Fig. 4. The G7967A mutation (Fig. 4, Red arrow-indicated) had no impact on the base pairing within the stem, since the wild-type wobble base-pairing was replaced with the more stable Watson–Crick base pair.

The G7967A was close to the 3' end of polyprotein sequence, causing only six amino acids deficient in the 3D protein. Because the 3D protein was the longest of all structural and nonstructural proteins, we speculated that SVA could tolerate a few amino acids deleted from the 3D protein. In order to unveil the

length limit of the shortest 3D protein, we further modified the M0 through the site-directed mutagenesis (SDM) to reconstruct ten other cDNA clones, M1 to M10 (data not shown). The SDM-caused stop codon was upstream of the wild-type stop codon. If an SDM-caused stop codon advanced the wild-type stop codon by one codon position in an SDM-modified plasmid, this plasmid would be named “M1”, *et cetera*. M1 to M10 cDNA clones were separately transfected into cells in an attempt to rescue viable viruses. Unexpectedly, no virus could be rescued from any of these plasmids (data not shown), suggesting that the 3D protein was highly conserved in length. More precisely, its C-terminal region was highly conserved in length.

In conclusion, rSVA-eGFP was rescued using reverse genetics in this study, and then subjected to forty serial passages *in vitro*. The intensity of fluorescence gradually weakened with serial viral passaging. Green fluorescence completely vanished at P20, implying that the eGFP sequence was at least partly deleted from the rSVA-eGFP genome. The NGS analysis of P40 progeny showed most nucleotides deleted from the eGFP sequence. Additionally, a total of 26 SNMs were recognized in the rSVA-eGFP genome at P40. This study would lay the foundation for clarifying evolutionary patterns of SVA.

DECLARATIONS

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ETHICAL APPROVAL

This article does not contain any studies with human participants or animals performed by any of the authors.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: XXXXXXXXXXXXX

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Author Contribution

ZX, HL and HC: Writing – original draft and Methodology. DE and Fei Liu: Data curation. Fuxiao Liu: Writing – review & editing and Supervision. All authors reviewed the manuscript.

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Data Availability

The datasets presented in the present study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI (accession No.: PRJNA1099315).

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Figures

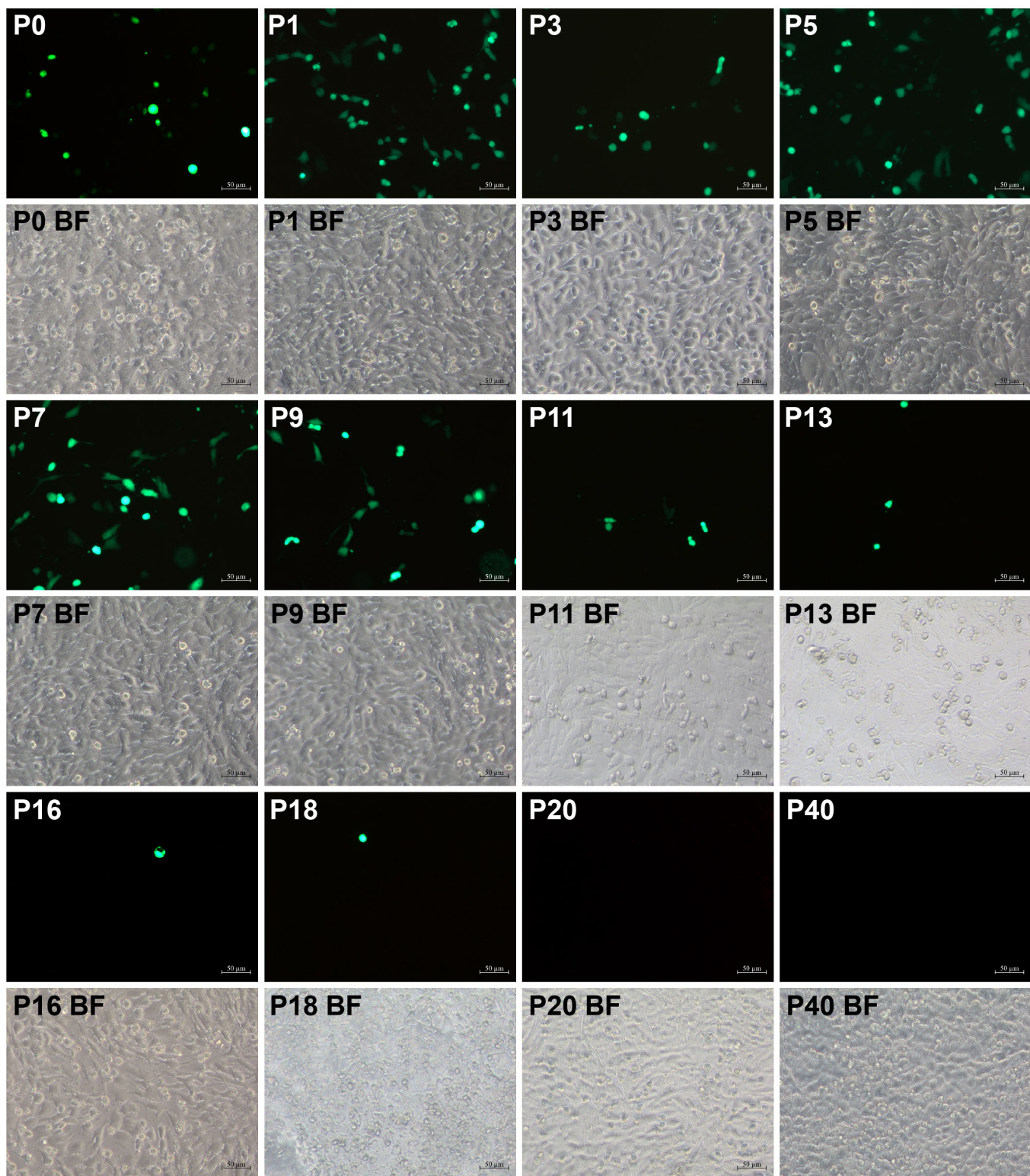


Figure 1

Cell monolayers inoculated with rSVA-eGFP at different passage numbers. The viable rSVA-eGFP is rescued from its cDNA clone, and then subjected to forty serial passages in BHK cells. Green fluorescence is invisible since P20. BF: bright field.

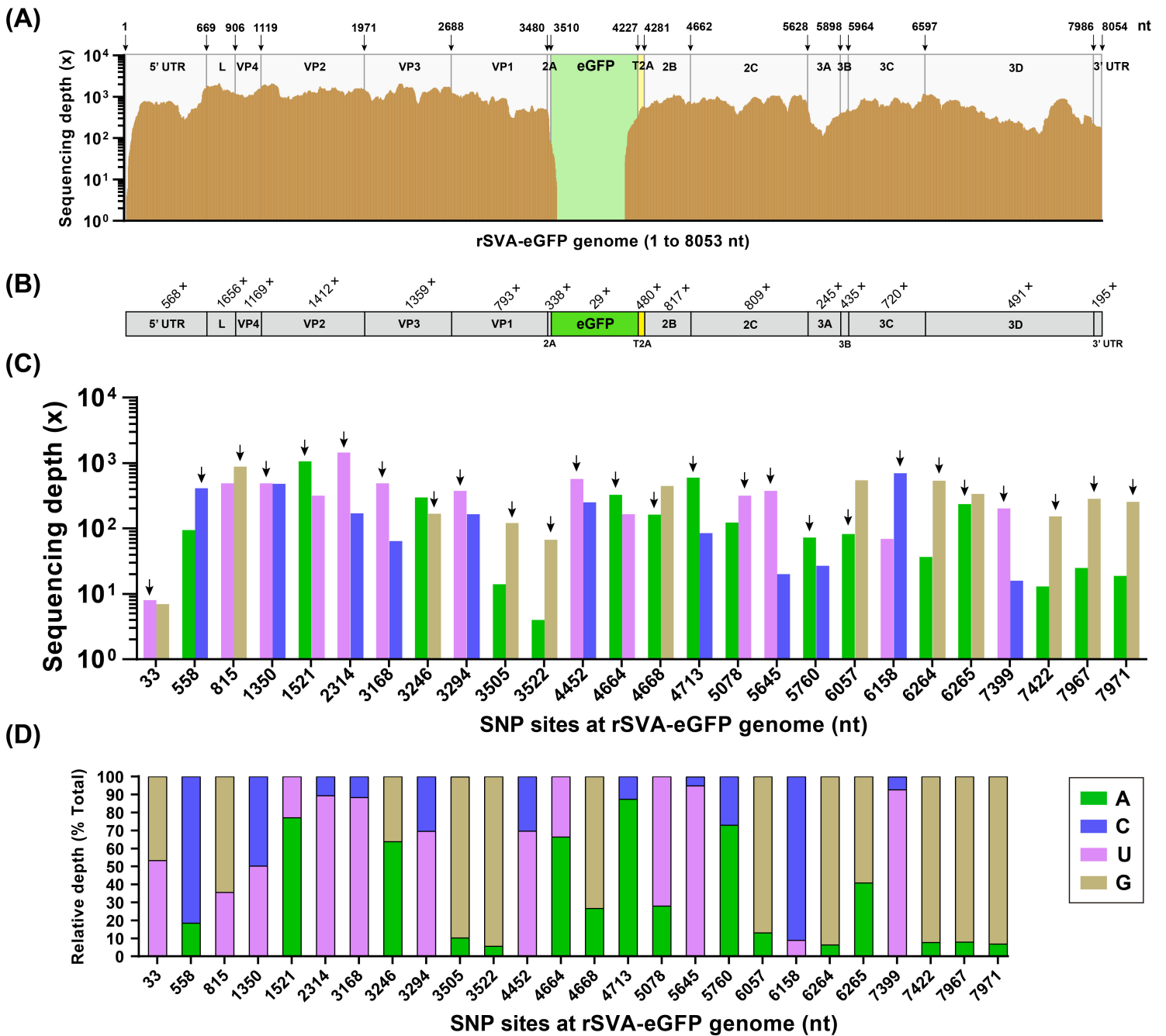


Figure 2

NGS analysis of rSVA-eGFP at P40. Sequencing depth and coverage across the full-length rSVA-eGFP genome (A). All genetic elements proportionally match their actual lengths within the genome. Cross-over sites between two elements are marked by arrows with positions (nt). Average sequencing depth ($\times$ reads) for each element in rSVA-eGFP genome (B). Absolute sequencing depths of 26 SNP sites at rSVA-eGFP genome (C). Wild-type nucleotides are indicated by arrows. Relative (%) sequencing depths of 26 SNP sites at rSVA-eGFP genome (D). Two nucleotides coexist at each SNP site.

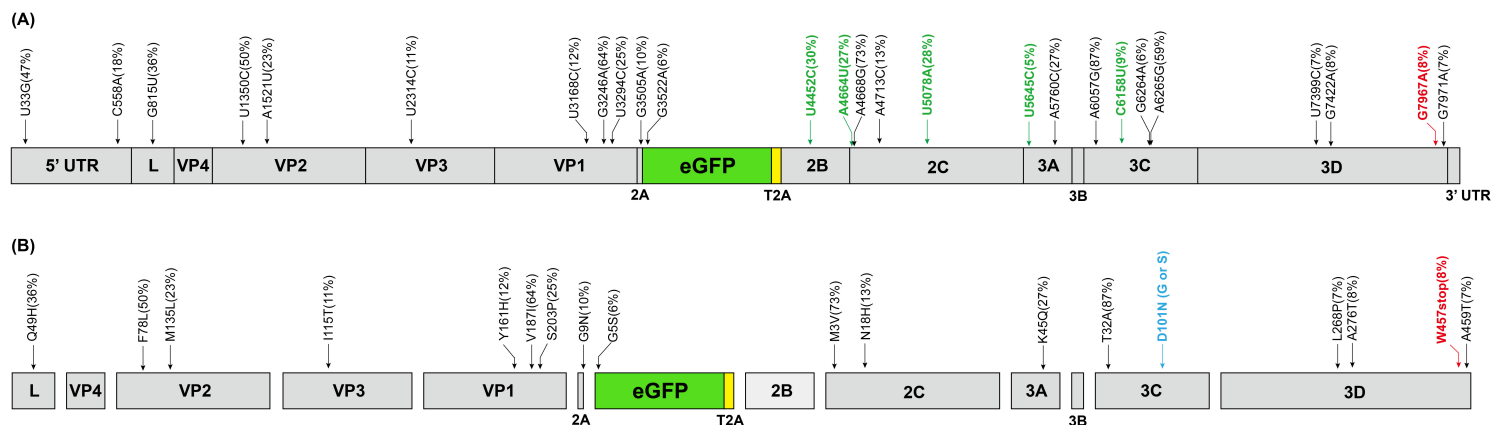


Figure 3

Distributions of SNMs and SAAMs. Distribution of 26 SNMs at the rSVA-eGFP genome (A). The red arrow-indicated site represents a nonsense mutation. Synonymous mutations are indicated by green arrows. Arrow-marked sites never exactly match their definite positions. Mutation rates are enclosed within parentheses. Distribution of SAAMs at viral polypeptides (B). The blue arrow indicates more than one SAAM arising at the same site. The red arrow implies the termination of polyprotein translation in advance. Arrow-marked sites never exactly match their definite positions. Mutation rates are enclosed within parentheses.

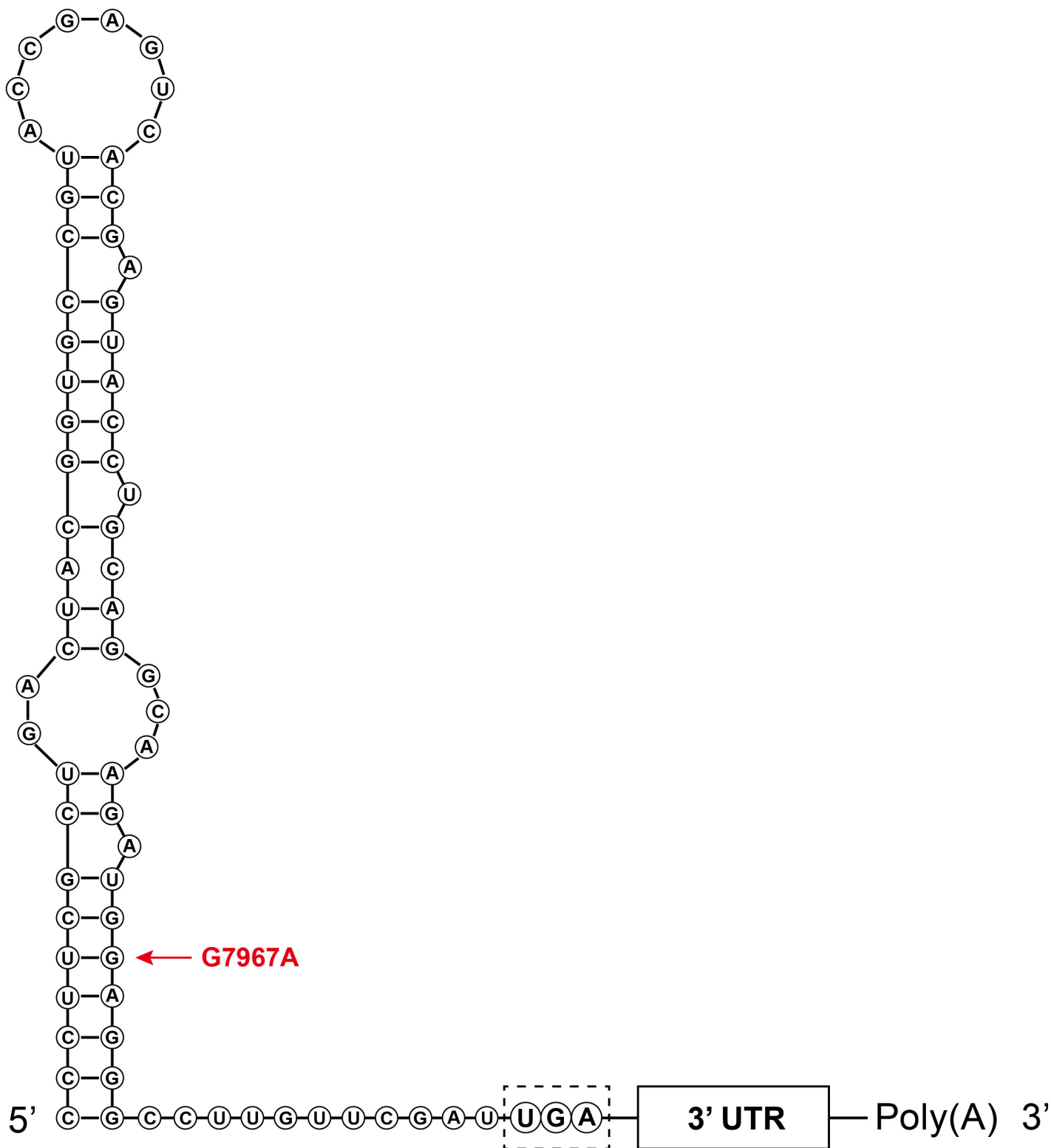


Figure 4

Schematic representation of highly conserved hairpin structure close to the 3' end of 3D sequence. The wild-type stop codon is enclosed by a dotted rectangle. The red arrow indicates the site with nonsense mutation, G7967A.

Supplementary Files

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- [Supplementary1.xls](#)