Frameshifting in Alphaviruses: A Diversity of 3′ Stimulatory Structures

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Programmed ribosomal frameshifting allows the synthesis of alternative, N-terminally coincident, C-terminally distinct proteins from the same RNA. Many viruses utilize frameshifting to optimize the coding potential of compact genomes, to circumvent the host cell’s canonical rule of one functional protein per mRNA, or to express alternative proteins in a fixed ratio. Programmed frameshifting is also used in the decoding of a small number of cellular genes. Recently, specific ribosomal −1 frameshifting was discovered at a conserved U_UUU_UUA motif within the sequence encoding the alphavirus 6K protein. In this case, frameshifting results in the synthesis of an additional protein, termed TF (TransFrame). This new case of frameshifting is unusual in that the −1 frame ORF is very short and completely embedded within the sequence encoding the overlapping polyprotein.

The present work shows that there is remarkable diversity in the 3′ sequences that are functionally important for efficient frameshifting at the U_UUU_UUA motif. While many alphavirus species utilize a 3′ RNA structure such as a hairpin or pseudoknot, some species (such as Semliki Forest virus) apparently lack any intra-mRNA stimulatory structure, yet just 20 nt 3′-adjacent to the shift site stimulates up to 10% frameshifting. The analysis, both experimental and bioinformatic, significantly expands the known repertoire of −1 frameshifting stimulators in mammalian and insect systems.

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Introduction

Many viruses harbour specific sequences that induce a portion of ribosomes to shift −1 nt and continue translating in the new reading frame. Eukaryotic −1 frameshift sites typically consist of a slippery heptanucleotide fitting the consensus motif X XXY YYYY, where XXX represents any three identical nucleotides, YYY represents AAA or UUU, and Z represents any nucleotide except G. The shift site is followed by a ‘spacer’ region of 5–9 nt, and then a highly structured region, often an RNA pseudoknot or hairpin.¹,² To date, nearly all known −1 frameshift sites in virus genomes have a long downstream −1 frame open reading frame (ORF). One example is the Gag-Pol gene in human immunodeficiency virus (HIV), where Pol is expressed as a −1 frameshift fusion product with Gag.³,⁴ Long coding ORFs are comparatively easy to identify and, in cases where conventional translation initiation appears problematic, the potential for alternative mechanisms, including ribosomal frameshifting, is often investigated. On the other hand, when the −1 frame ORF is very short, such coding ORFs may remain ‘hidden’ despite years of intensive study (e.g., Ref. 5). Recently we demonstrated that ribosomal −1 frameshifting takes place at a conserved U_UUU_UUA motif (spaces represent zero-frame codons) in the sequence encoding the
small 6K protein in alphaviruses. Frameshifting results in the synthesis of an additional low molecular mass protein termed TF (TransFrame protein; ~8 kDa). The alphavirus genomic RNA is single-stranded, positive sense, and about 11–12 kb long. It contains two long ORFs separated by a short noncoding sequence. The 5’-proximal ORF encodes a non-structural polyprotein and often contains an internal stop codon read-through site. The 3’-proximal ORF codes for an ~140-kDa structural polyprotein (C-E3-E2-6K-E1) that is cleaved autocatalytically (to generate the capsid protein C) and by cellular proteases (to yield the envelope glycoproteins E1, E2, and E3) (reviewed in Ref. 7). Both 6K and TF are hydrophobic, cysteine-rich, and acylated. Between them, 6K and TF play roles in envelope protein processing, membrane permeabilization, virus assembly and budding, and virion structure (reviewed in Ref. 6). The N-terminal end of TF retains ~71–83% of 6K, but the C-terminal amino acids (~8–50, depending on species) are encoded by the −1 frame and, in contrast to 6K, tend to be hydrophilic. Frameshifting provides TF with a hydrophilic C-terminal segment, which may play a role in viroporin activity and/or virion structure, while maintaining a hydrophobic C-terminal segment in 6K. The latter acts as the signal peptide sequence for the E1 protein.

Previous bioinformatic analysis of the alphavirus frameshift site suggested a remarkable diversity in the 3’ frameshift stimulatory elements between different alphavirus species. In some species the 3’ sequence potentially forms a simple RNA hairpin structure; other species are predicted to have an RNA pseudoknot; and with still other species a relevant intra-mRNA secondary structure is not simply predicted. Here we present detailed experimental analyses of the 3’-adjacent sequence from several alphavirus species, namely, Sindbis virus (SINV), Semliki Forest virus (SFV), Eastern and Venezuelan equine encephalitis viruses (EEEV, VEEV), Sleeping disease virus (SDV), Middelburg virus (MIDV), and Seal nose virus (SESV).

Results

The slippery heptanucleotide and its flanking sequence

The U UUU UA slippery heptanucleotide at which −1 frameshifting actually occurs is conserved in essentially all sequenced alphaviruses except Bebaru virus. The −1 position (i.e., 5’-adjacent to U UUU UA) is nearly always occupied by a C, although Barmah Forest virus has a U and SESV has a G. However, the −2 position is more variable (cf. Ref. 8), with the following zero-frame codons being present in the E-site at the onset of frameshifting: CCU [VEE and Western equine encephalitis (WEE) complexes], UCU (MIDV, salmonid alphaviruses, SF complex), GCC (Chikungunya virus, EEE complex), ACU (Chikungunya virus, O’nyong-nyong virus, Ndumu virus), AUU (Barmah Forest virus), and UGU (SESV). In a comparison of known viral −1 frameshift sites, it has been noted previously that the three nucleotides immediately 3’ of the slippery site show a strong bias toward a G or U at the +1 position and a G or A at both the +2 and the +3 positions. In the majority of alphavirus species, the nucleotide at the +1 position is a G—a feature also shared by the U UUU UUA frameshift site in HIV-1 and other primate lentiviruses. The main exceptions are the EEE complex viruses, which have a C, and the WEE complex viruses (except SINV), which generally have a U. The nucleotide at the +2 position is a U in the WEE, EEE, and VEE complexes and in MIDV and Ndumu virus, a U or C in the SF complex, a G in the salmonid alphaviruses, and a C in SESV. The nucleotide at the +3 position is a G in the WEE complex, Barmah Forest virus, MIDV, Ndumu virus and the salmonid alphaviruses, a C or G in the VEE and SF complexes, and a U in the EEE complex and SESV.

Analysis of wild-type frameshifting efficiencies

Using dual-luciferase reporter assays, we compared the frameshifting efficiencies of the slippery heptanucleotide U UUU UUA and 3’-adjacent sequences from SINV, VEEV, EEEV, SESV, SDV, MIDV, and SFV in cell culture. The inserts used are defined in Fig. 1. Frameshifting efficiencies were determined relative to in-frame controls in which the shift site U UUU UUA was changed to U UUC UUA, which encodes the same amino acid sequence as the frameshift product, without permitting frameshifting. The frameshifting efficiencies were estimated as follows: SINV ~10%, VEEV ~5%, EEEV ~12%, SESV ~21%, SDV ~42%, MIDV ~48%, and SFV ~15%.

The frameshifting efficiency values above are based on at least three independent tests, each in triplicate (the values varied by a factor of ±30% in some experiments but with the same trends between the different sequences tested). In addition, the dual-luciferase results were verified by Western blotting using antibodies raised against either the 5’-encoded (Renilla) or the 3’-encoded (firefly) luciferase. Appropriately sized specific products for in-frame (IF) and trans-frame decoding were detected by the Renilla antibody and, as expected, only the frameshift-derived products (FS) were detected by the firefly antibody (data not shown). Measurements of frameshifting efficiencies based on the Western blots are not as precise as dual-luciferase measurements but do provide a safeguard against possible internal initiation, cryptic splicing, cryptic promoters, and so on. Densitometry measurements for selected constructs were broadly consistent with the dual-luciferase results (e.g., FS/(IF+FS) ~16% for both SINV and SFV). Finally, Northern blots were...
performed for selected constructs using both Renilla and firefly probes made from the empty dual-luciferase construct, pDluc (Fig. 2). The blots revealed the presence of a single size class of RNA transcript in transfected cells, while no transcripts were detected for mock-transfected controls. (Northern blots also give information about relative mRNA abundance, although because of the dual nature of the reporter assays used this is not a major concern.)

**Analysis of 3′ frameshift stimulatory elements**

The SINV sequence has the potential to form an upper stem (‘stem 1’) and a lower stem (‘potential stem 2’ in Figs. 1 and 3). The potential to form the 8-to-10-bp potential stem 2 (sometimes with a 1-nt symmetric bulge) is preserved throughout the WEE complex (which includes SINV; Fig. 3 of Ref. 6), although there are no compensatory mutations to support its functional conservation at the level of RNA secondary structure rather than just primary sequence. Furthermore, the potential for a similar potential stem 2 (albeit with a larger 3′ bulge) is also present in EEEV and VEEV (Fig. 1). In order to investigate whether stem 2 is important for frameshifting, additional constructs were made for SINV, EEEV, and VEEV with shorter inserts that lack the potential to form stem 2 (Fig. 1; SINV.41, EEEV.32, VEEV.32). However, the frameshifting efficiencies were not reduced for these inserts. In fact, they tended to increase: SINV.WT ∼10%; SINV.41 ∼17%; EEEV.WT ∼12%; EEEV.32 ∼12%; VEEV.WT ∼5%; VEEV.32 ∼11%. Thus, there is no evidence that these potential stem 2’s are functionally important for frameshifting.

The potential to form a highly stable stem 1 is also preserved throughout the WEE complex (Fig. 3 of Ref. 6). In WEEV, stem 1 has 12 consecutive base pairs, with no bulge, and a 5-nt terminal loop. In Fort Morgan virus, stem 1 has 11 consecutive base
Pairs, again with no bulge and a 5-nt terminal loop. The stem is additionally supported by a few compensatory substitutions (i.e., paired substitutions that preserve the base pairings). For example, one position in the stem is occupied by an A:U, G:C or G:U pair depending on the species and strain (Fig. 3 of Ref. 6). Stem 1 is also present in the EEE, MIDV, Ndumu virus, SINV, EEEV, and various other species (Fig. 1; Figs. 3 and 4 of Ref. 6). The precise location, stem length, and terminal loop size vary substantially between different complexes, indicating that it is the conserved presence of a stable stem that is functionally important rather than any necessity for conservation at the level of primary sequence within the stem.

In order to further examine the functional importance of potential structures in SINV, additional constructs were made in which groups of nucleotides were mutated to disrupt predicted structures and/or to maintain predicted structures but with reversed base pairings (Figs. 1 and 3a). Mutants SINV.3S1U ~6% and SINV.3S1L ~3%, which disrupt the ‘upper’ part of stem 1, demonstrate that this stem is important for frameshifting. However, mutant SINV.5S1U ~6%, which was expected to restore the base pairing, did not fully restore wild-type (WT) frameshifting efficiency. This could be due to the precise nature of the structure and not just hydrogen-bond potential being relevant for frameshifting, or specific elements of the primary sequence may be important, or perhaps alternative structures form in the mutated sequence in competition with the frameshifting-stimulating hairpin structure. In fact, such levels of reduced and only partially restored frameshifting activity are typical of this type of mutational analyses (e.g., Ref. 12). Mutants SINV.5SIL ~3% and SINV.3SIL ~6%, which disrupt the ‘lower’ part of stem 1, demonstrate that the lower part of the stem is also important for frameshifting, although perhaps less so than the upper part. In this case also, the mutant that was expected to restore base pairing, SINV.5S1L ~7%, did not fully restore WT frameshifting efficiency. Mutants SINV.5PS3 ~15%, SINV.5PS3 ~13%, and SINV.3PS3 ~14%, besides shortened inserts SINV.5S3 ~15% and SINV.41 ~17%, demonstrate that the potential pseudoknotted stem (‘potential stem 3’ in Fig. 1) is unimportant for SINV frameshifting (Fig. 3a). This is perhaps not surprising, since the potential to form stem 3 is not even conserved throughout the WEE complex (Fig. 3 of Ref. 6). Mutants SINV.5PS2 ~4% and SINV.5PS2 ~4% indicated that the sequence in the 5′ half of the potential stem 2 was important for efficient frameshifting. However, mutant SINV.3PS2 ~13% had near-WT frameshifting efficiency, confirming that it is the primary sequence in this ‘spacer’ region, rather than the ability to form stem 2, that is important. In confirmation of the correct identity of the shift site, a SINV sequence in which the heptanucleotide UUUU UAUA was mutated to U UUC UUA (SINV.NS) had <0.5% frameshifting.

In contrast to the simple hairpin structures in the SINV, EEEV, and VEEV sequences, the potential to form an RNA pseudoknot is apparent for several other species including MIDV, MIDV, and possibly SDV (Fig. 1). The presence and functional importance of the potential pseudoknotted stem 2 in SDV and SESV were investigated using shortened inserts. For SDV, the very high level of frameshifting seen in SDV.WT ~30% was not reduced for the shortened insert, SDV.41 ~39%, which lacks the potential to form stem 2. Formation of the 8-bp stem 2 in SDV involves disruption of the terminal 4 bp of the 13-bp stem 1, so removing the competing potential to form stem 2 (SDV.41) may in fact strengthen stem 1. In contrast, the SESV stem 2 has up to 12 bp and would disrupt at most the terminal 2 bp of the 11-bp stem 1. Here, the frameshifting efficiency in the shortened insert SESV.41, which lacks the potential to form stem 2, was ~6%—a nearly fourfold reduction compared to SESV.WT ~21%. Thus, it appears that the SESV stimulatory structure is indeed a pseudoknot. The SDV and SESV results are in keeping with the minimal free energy (mfe) structures predicted by pknotsRG, namely, a simple hairpin for SDV and a pseudoknot for SESV.

The potential MIDV pseudoknot was investigated in more detail using a number of mutants (Fig. 3b). The importance of stem 1 for efficient frameshifting was supported by the substantial reduction in

![Fig. 2. Total RNA from HEK293 cells transfected with pDluc-based plasmids containing wild-type alphaviruses (wt) and corresponding in-frame controls (if). An additional subset of MIDV plasmids (3S2, 5S1U, 3SS9, and pkdel) was also included. RNA samples were separated by 1.5% agarose-formaldehyde gel electrophoresis and probed by Northern analysis for firefly (F) or Renilla (R). Lanes Wand D show RNA extracted from cells transfected with either water or the empty vector pDluc, respectively. Lane U shows RNA from nontransfected cells.](image-url)
frameshifting seen when stem 1 was disrupted—mutants MIDV.5S1U ~10% and MIDV.3S1U ~17%. Also restoration was to near-WT levels, MIDV.WT ~48%, when stem 1 was restored but with reversed base pairings, mutant MIDV.53S1U ~37%. However, the base pairings in the potential lower extension to stem 1 appear to be not critical for frameshifting. Both complementary mutation of the 5′ sequence, MIDV.5S1L ~10%, and the restorative mutant, MIDV.3S5L ~11%, resulted in an ~4-fold reduction in frameshifting. Revealingly, the mutant tested with altered 3′ sequences, MIDV.3S1L ~43%, is comparable to WT, thus indicating that the spacer region alone is a cis-acting element. This is further supported by mutants where either nucleotides 1–6 or 1–9 3′-adjacent to the shift site were mutated to their complementary nucleotides, MIDV.3S6 ~10% and MIDV.3S9 ~3%, respectively, both of which greatly reduced frameshifting despite leaving stems 1 and 2 of the pseudoknot intact. Thus, the identity of the spacer nucleotides, and not just the stable pseudoknot, is crucial for the highly efficient frameshifting seen in MIDV.

Stem 2 of the predicted pseudoknot was also supported by the compensatory mutation experiments, with frameshifting substantially reduced...
when the stem was disrupted—MIDV.5S2 ~14% and MIDV.3S2 ~9%. However, restoration toward WT levels in the mutant with reversed base pairings, MIDV.53S2 ~19%, was much less pronounced than for the stem 1 restorative mutant. An additional mutant was made to investigate the unpaired adenosine at the junction of the two stems, due to its positional similarity to the critical unpaired adenosine in mouse mammary tumor virus (MMTV).13,14 In contrast to the situation in MMTV, mutation of the adenosine (in this case to a U, which potentially increases the length of stem 1 by 1 bp but, in any case, is expected to alter the geometry of the proposed pseudoknot), had little effect on frameshifting efficiency—MIDV.A29U ~41%. Finally, when the whole pseudoknot was deleted but nucleotides 1–6 of the spacer region were left intact, frameshifting still occurs at ~5% (Fig. 3b; MIDV.PKDEL). The MIDV experiments were repeated in vitro using rabbit reticulocyte lysate and [35S]methionine labelling, which resulted in lower absolute frameshifting values (by ~3 fold), although the overall trends were similar (data not shown).

**Effect of primary sequence on frameshifting in SFV**

SFV frameshifting is intriguing, as no structural analogue of stem 1 is apparent in its RNA sequence. The same is true for Chikungunya virus, Ross River virus, and most other members of the SF complex. Nevertheless, the potential to form a ‘loose’ stem involving more distant nucleotides is present throughout the SF complex (e.g., as shown in Fig. 1 for SFV, ‘potential stem 1’). However, mutants that lack the potential to form this long-range stem have near-WT frameshifting efficiencies (the WT, SFV.WT, frameshifting efficiency is ~16%, compared to shortened inserts SFV.59 ~16%, SFV.38 ~12%, and SFV.20 ~9%, all of which lack the potential to form the potential stem 1). Thus, even if this putative structure forms, it appears irrelevant for frameshifting. This was further borne out by mutants in which the potential pairing of the putative stem 1 was disrupted but the frameshifting efficiency was still similar to that of WT—SFV.3PS1U ~17% and SFV.3PS1L ~19% (Fig. 3c).

These results indicate that frameshifting in SFV is most likely stimulated either by a trans-factor or by the primary sequence downstream of its shift site.

A series of nine substitution mutants was constructed with consecutive blocks of 4 nt 3' of the shift site replaced with complementary nucleotides. The series spans the region nucleotides +3 to +38, where nucleotide +3 is the third base 3' of the UUU UUA shift site. For example, in mutant SFV.3–6, nucleotides +3 to +6 are changed from GCUA to CGAU. The frameshifting efficiencies for these mutants are shown in Fig. 3c. These results, together with the WT frameshifting efficiencies exhibited by SFV.38 ~12% and SFV.20 ~9%, whose 3' ends are at nucleotides +38 and +20, respectively, indicate that while more distal sequences may play some role, just the 3'-adjacent ~20 nt are capable of stimulating reasonably efficient frameshifting in SFV, with nucleotides +3 to +10 being particularly important.

A stimulator 5' of the frameshift site is important for the +1 frameshifting required for antizyme expression and, although generally mediated at the nucleotide level, may act via the encoded nascent peptide in certain fungi (reviewed in Ref. 15). However, constructs that included 90 nt of the WT sequence 5' of the SFV shift site stimulated a similar level of frameshifting as constructs that had no WT sequence 5' of U UUU UUA (constructs SFV.WT90 and SFV.WT; both ~15%). This suggests that frameshifting in WT SFV is unlikely to have a 5' stimulator.

In order to better understand the phylogenetic extent and essential characteristics of the unusual SFV-like frameshifting stimulator, the frameshifting efficiency relative to the respective in-frame controls of just the 20 nt 3'-adjacent to the U UUU UUA shift site of four other viruses from the SF complex was also tested. The following frameshifting efficiencies were determined: Una virus ~8%, Getah virus ~9%, Mayaro virus ~3%, and Chikungunya virus ~1%. Thus, while the 3'-adjacent ~20 nt of Una virus and Getah virus are, like SFV, capable of stimulating efficient frameshifting, in Mayaro virus and Chikungunya virus it appears that additional sequence elements are also required.

**Discussion**

The U UUU UUA motif preserved in the 6K coding sequence of all sequenced alphaviruses except Bebaru virus conforms to the X XXY YYZ heptanucleotide consensus for +1 ribosomal frameshifting. In fact U UUU UUA is one of the most shift-prone of all heptanucleotides in eukaryotes and, even without any 3' stimulatory structure, can induce low levels of frameshifting in vitro.16 The same slippery heptanucleotide is used to produce the Gag-Pol fusion protein in all HIV-1 groups (reviewed in Ref. 4), besides other primate lentiviruses. In HIV-1 group M (subtypes A, B, C, D, F, G, H, J, and K) the 3' stimulatory structure is a simple hairpin with a terminal loop of 4–6 nt, an upper stem of 10–11 bp, a lower stem of ~7 bp that involves base pairing with the ‘spacer’ region, and a 3' bulge of ~3 nt between the upper and lower stems.17 It has been proposed that the lower stem in HIV-1 is unfolded by the time the decoding core of the ribosome reaches the slippery site, but that its presence facilitates interaction of the ribosome with the upper stem.18 The HIV-1 structure is interestingly similar to the predicted structure in several of the alphaviruses, including SINV, WEEV, and VEEV, although the 3' bulge tends to be larger in the alphaviruses than in HIV-1. However, the dual-luciferase results showed that the lower stem was not required for efficient frameshifting in SINV and VEEV. Nonetheless, it is perhaps too early to rule out the predicted lower stem playing some role in frameshifting in the context of the full 26S
subgenomic RNA (sgRNA). In any case, the sequence of the spacer itself does appear to be important—at least in SINV (cf. Refs. 9 and 19). Similar to SINV, frameshifting in SDV, EEEV, and Barmah Forest virus also appears to be stimulated by simple hairpin structures.

In contrast, the stimulatory structure in MIDV is predicted to be a pseudoknot, similar to that in HIV-1 group O and various other viruses.\(^{20,21}\) The level of frameshifting for the WT MIDV insert was extremely high, perhaps partly as a result of the very GC-rich nature of both stems in the WT pseudoknot. However, it appears that the high level of frameshifting in MIDV is not solely stimulated by the presence of a stable pseudoknot, as mutations in the spacer region alone—6 or 9 nt 3' adjacent to the shift site—reduce frameshifting from \(\sim 48\%\) to \(\sim 10\%\) and \(\sim 3\%\), respectively. Moreover, even when one of the pseudoknot stems is disrupted, frameshifting efficiencies of 10–21\% were measured. This suggests that the high level of frameshifting in WT MIDV results from a combination of both the stable pseudoknot and the particular spacer sequence. SESV also appears to use a pseudoknot, although it is unusual in the length of stem 2 (up to 12 bp).

Despite efficient frameshifting, we were unable to locate a compact 3' RNA secondary structure in the SF complex (with the possible exception of a stem-loop or pseudoknot in Mayaro virus). Nonetheless, elements of the 3'-adjacent sequences, in particular the first 10–18 nt after the U UUU UUA motif, are important. As the first \(\sim 15\) nt is within the mRNA entrance tunnel at the onset of frameshifting, direct interaction between certain of these nucleotides and either the ribosomal RNA or the ribosomal proteins may be responsible for stimulating efficient frameshifting (cf. +1 frameshifting in the yeast gene EST3 and retrotransposon Ty3 in Refs. 22 and 23). Alternatively, frameshifting in SFV could be stimulated by an RNA structure involving extensive tertiary interactions (cf. Ref. 24), or potentially a microRNA or other small RNA, or a host or viral protein, binding to the sequence 3'-adjacent to the U UUU UUA motif. On the other hand, since the SFV, WT construct includes no virus sequence 5' of the U UUU UUA motif and only 89 nt of the 3' sequence, but nonetheless exhibits a similar frameshifting efficiency to that observed in virus-infected cells, it is unlikely that SFV frameshifting is affected by sequences 5' of the shift site acting at the mRNA level (cf. +1 frameshifting in antizymes 1 and 2 in Ref. 15), the nascent peptide sequence acting within the ribosomal exit tunnel (cf. Ref. 25), or long-range RNA base pairing to distal sequence elements (cf. +1 frameshifting in Barley yellow dwarf luteovirus in Ref. 26). The improbability of a 5' frameshifting stimulator is further demonstrated by the similar frameshifting levels observed for both SFV.WT and SFV.WT90 (which include, respectively, 0 and 90 nt of virus sequence upstream of the shift site).

Our measurements of frameshifting efficiency involved only a segment of the 26S sgRNA beginning at the U UUU UUA motif and including (depending on species) 56–89 nt 3' of the motif. The facts that our measurements for SINV (viz. 10–17\% for SINV.0, SINV.59, and SINV.41) are similar to the frameshifting efficiency of 10–18\% derived from data presented in Refs. 27 and 28 (see Ref. 6), and our measurements for SFV (viz. 12–16\% for SFV.0, SFV.59, and SFV.38) are similar to the values of \(\sim 15\%\) reported for SFV-infected cells in Ref. 6, lends credence to the supposition that our values may be closely representative of the situation in virus-infected cells. Nonetheless, it is possible that in some species frameshifting efficiency is further modulated in the context of the full 26S sgRNA by distal 3' sequence elements (cf. Refs. 26 and 29) and/or upstream sequence (cf. Ref. 30). Of particular interest for future research will be whether or not the extremely high levels of frameshifting observed for SDV and MIDV (i.e., \(\sim 40–50\%\)) are maintained in virus-infected cells.

The diversity of the stimulatory signals for +1 frameshifting in different alphavirus species is noteworthy, and reminiscent of the diversity of signals that stimulate +1 frameshifting in the eukaryotic cellular gene, antizyme, present from yeast to mammalians.\(^{15}\) Addition of these newly characterised elements substantially increases the repertoire of known +1 frameshifting signals in eukaryotic systems.

**Methods**

**Plasmid constructions**

All dual-luciferase clones were based on pDluc (kind gift from Dr. M. Howard, University of Utah), which is a modified version of p2Luc,\(^ {10}\) and propagated in DH5a cells. All inserts were made from complementary oligonucleotides and cloned between XhoI and BglII sites, except SFV.WT90, which was amplified by PCR from pSP6-SFV.\(^ {4,31}\) Every clone had a corresponding in-frame control, in which the encoded protein sequence is identical to the +1 frameshift product. All plasmid constructions were confirmed by DNA sequencing. Insert sequences were based on GenBank accession numbers: NC_001449 (VEEV), NC_003899 (EEEV), NC_001547 (SINV), NC_001462 (Chikungunya), NC_006558 (Getah), NC_003215 (SFV), NC_003417 (Mayaro), AF339481 (Una), AF339486 (MIDV), AF315122 (SESV), and NC_003433 (SDV). Oligonucleotides used are listed in Table 1, Supplementary material.

**Cell culture and transfections for frameshifting assays**

Human embryonic kidney (HEK) 293T cells were obtained from American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10\% fetal bovine serum (FBS), 1 mM l-glutamine, and antibiotics. Cells were transfected with Lipofectamine 2000 reagent (Invitrogen), using the 1-day protocol in which suspension cells are added directly to the DNA complexes in 96-well plates. In all, 25 ng of DNA and 0.2 \(\mu\)l of Lipofectamine 2000 per well in 25 \(\mu\)l of Opti-Mem (Gibco) were incubated and plated in opaque 96-well half-area plates (Costar). Cells were trypsinized, washed, and added at a concentration of
4×10^4 cells per well in 50 μl of Opti-Mem. Transfected cells were incubated overnight at 37 °C in 5% CO₂, then 75 μl of DMEM and 10% FBS were added to each well, and the plates were incubated for an additional 24 h.

**Dual-luciferase assays for ribosomal frameshifting efficiency**

Luciferase activities were determined using the Dual Luciferase Reporter Assay System (Promega). Relative light units were measured on the Veritas™ Microplate Luminometer (Turner Biosystems). Transfected cells were washed once with 1× phosphate-buffered saline (PBS) and lysed with 20 μl of 1× passive lysis buffer (PLB; Promega), and luciferase activity was determined on 12.5 μl of the cell lysate following injection of 25 μl of luminescence substrate. The total number of independent experiments for each construct varied between 3 and 15. In each experiment, three independent data points were obtained for each construct. Frameshifting efficiencies were calculated by dividing the relative luciferase of the test construct by the relative luciferase of its corresponding in-frame control. Standard deviations were calculated using the quotient formula.

**Northern blots**

For transfection of cells for RNA extraction, 1 μg of a selection of plasmids (SFV.WT, SINV.WT, MIDV.WT, EEEV.WT, VEEV.WT, SESV.WT, SDV.WT, SFV.NF, MIDV.5S1U, MIDV.3S2, MIDV.PKDEL, MIDV.NS), as well as the corresponding in-frame controls, were transfected by using the calcium phosphate method into 8.4×10^4 HEK293 cells in 2 ml of DMEM, 10% FBS. Transfected cells were incubated overnight at 37 °C in 5% CO₂ and the medium was changed for a further 24-h incubation. After 24 h, cells were washed with 1× PBS and trypsinized. Cells were then resuspended in 1 ml of 1× PBS and 10% of cells were spun down and assayed for dual luciferase. RNA in the remaining 90% of cells was extracted by TRIzol (Invitrogen) according to the manufacturer’s instructions and purified using MicroSpin G-50 columns (Amersham). Membranes were washed twice at 42 °C in 2× SSC, 1% SDS for 5 min and then twice at 42 °C in 0.1× SSC, 0.1% SDS for 15 min and exposed to PhosphorImager screens for empirically determined times. ImageQuant (Molecular Dynamics) was used for densitometry analysis.

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**Supplementary Data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2010.01.044.

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