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Ribosomal Frameshift Signals in Viral Genomes

Ewan P. Plant
Food and Drug Administration
USA

1. Introduction

Viral genomes contain fewer genes and are more compact than eukaryotic or prokaryotic genomes. Overlapping reading frames are often present in viral genomes and this enables multiple proteins to be made from a single transcript. This genomic layout can serve different purposes: more efficient use of the genome; fewer resources needed for making transcripts; and the abundance of each protein can be optimized by regulating translation from one transcript. Programmed ribosomal frameshifting is a mechanism by which viruses can express overlapping reading frames.

Viruses conscript the host translation machinery for the production of viral proteins. Many viruses disrupt transcription and translation of host mRNAs and optimize translation of viral messages. Different methods are used to subvert the fidelity of the translation machinery so that specific viral proteins are produced. These include leaky scanning, reinitiation, suppression of termination, internal ribosome entry sites and programmed ribosomal frameshifting. Normally ribosomes decode the codon triplets with high fidelity. At each step the mRNA passes through the ribosome three nucleotides at a time, from the initiating codon, along the length of the open reading frame, to the termination codon. However, some viruses manipulate the ribosome so that the mRNA does not proceed forward three nucleotides. This disruption is referred to as programmed ribosomal frameshifting.

Programmed ribosomal frameshifting allows for the production of two proteins with the same amino terminus yet differing carboxyl termini. The same initiation codon is used, but at a defined position in the message, the ribosome is stimulated such that it sometimes changes reading frame and continues translating in a new reading frame. The signal that stimulates this change in reading frame is encoded within the message, often comprised of a seven nucleotide slippery sequence followed by a stimulatory element. Here I describe viral frameshift signals with a particular emphasis on -1 frameshift signals. I review the discovery of these signals; provide an update on how the analysis of frameshift signals has broadened our understanding of virus protein translation, virus replication and ribosome fidelity; and describe how frameshift signals can be targeted by antiviral compounds.

2. Ribosome frameshift signals are found in diverse viral genomes

Frameshift signals have been found in the genomes of several double-stranded RNA and plus-strand RNA viruses. Although frameshift signals may be found in one member of a virus family, they are not necessarily ubiquitous among that family. Most of the frameshift

signals are positioned upstream of the open reading frame (ORF) that encodes an RNA-dependent RNA polymerase (RdRP), but signals have been identified that direct ribosomes to translate proteins involved in other functions such as cell-to-cell movement. The sequences that comprise the frameshift signals in viral genomes are diverse and form tertiary structures ranging from simple stem-loops to complex pseudoknots. This section summarizes the discovery, distribution and function of frameshift signals in viral genomes.

Proteins that had the same amino sequence but differed at the carboxyl terminus were first identified by tryptic digests in the 1970's. This led to the development of new ideas about how proteins are translated including programmed ribosomal frameshifting. Frameshifting was first demonstrated in cells infected with Rous sarcoma virus. Analysis of other virus sequences resulted in the rapid identification of more frameshift signals in retroviruses and coronaviruses containing overlapping ORFs. Initially proteins were sequenced to demonstrate that frameshifting occurred. Quickly molecular tools were developed that allowed DNA sequences to be cloned, transcribed and translated so that the ratios of frameshifted and non-frameshifted products could be measured. These methods have continued to evolve and protein expression systems are currently the main method of confirming frameshift signal function. Although recent advances in mass spectrometry have brought protein sequence identification to the fore again.

Over the last two decades considerably more viral sequences have become available for analysis. Many putative frameshift signals have been identified because of homology to an existing virus with a well-described frameshift signal. This approach isn't straightforward because of the diversity of sequences and secondary structures that stimulate frameshifting. For example, there are three very different structures that have been shown to facilitate frameshifting in coronavirues (see Figure 1). Computational efforts have been extended to find new frameshift signals. Two general approaches have been pursued; one looking for potential frameshift signals, that is slippery sites followed by potential stimulatory elements. The second approach is to look for out of frame ORFs and then potential frameshift signals (Belew et al., 2008; Bekaert et al., 2010). Both approaches have resulted in the generation of databases containing numerous potential frameshift signals. Some of these frameshift signals have been validated by functional assays or mass spectrometry analysis. Additional virological studies have helped unravel the function of some of the frameshift proteins although much work remains to be done.

Frameshift signals have been identified in several viral families including *Astroviridae, Flaviviridae, Luteoviridae, Potyviridae, Retroviridae, Togaviridae, Tombusviridae, Totiviridae*, the three families in the Order Nidovirales and a *Paramyxovirus*. Frameshift signals have also been used to support the assignment of viruses to particular families (den Boon et al., 1991; Cowley et al., 2000; Snijder et al., 1990 for example). In addition, frameshift signals have been characterized in several retrotransposons. Recently programmed ribosomal frameshift (PRF) signals were characterized in several invertebrates (Baranov et al., 2011). Currently there is only one characterized example of a frameshift signal in a mammalian genome but it is likely that more will be discovered and described as interest in recoding signals spreads (Manktelow et al., 2005). The proteins produced via frameshifting have varied functions. For example: 1) a frameshift product has a role in the neuro-invasiveness of the Kunjin subtype of West Nile virus (Melian et al., 2010); 2) the ORF3 protein from the Southern Bean Mosaic virus is required for cell-to-cell movement (Sivakumaran et al., 1998) and 3); altering frameshifting

efficiency has been shown to detrimentally affect viral viability for both double-stranded and single-stranded RNA viruses, including the retrovirus HIV (discussed below).

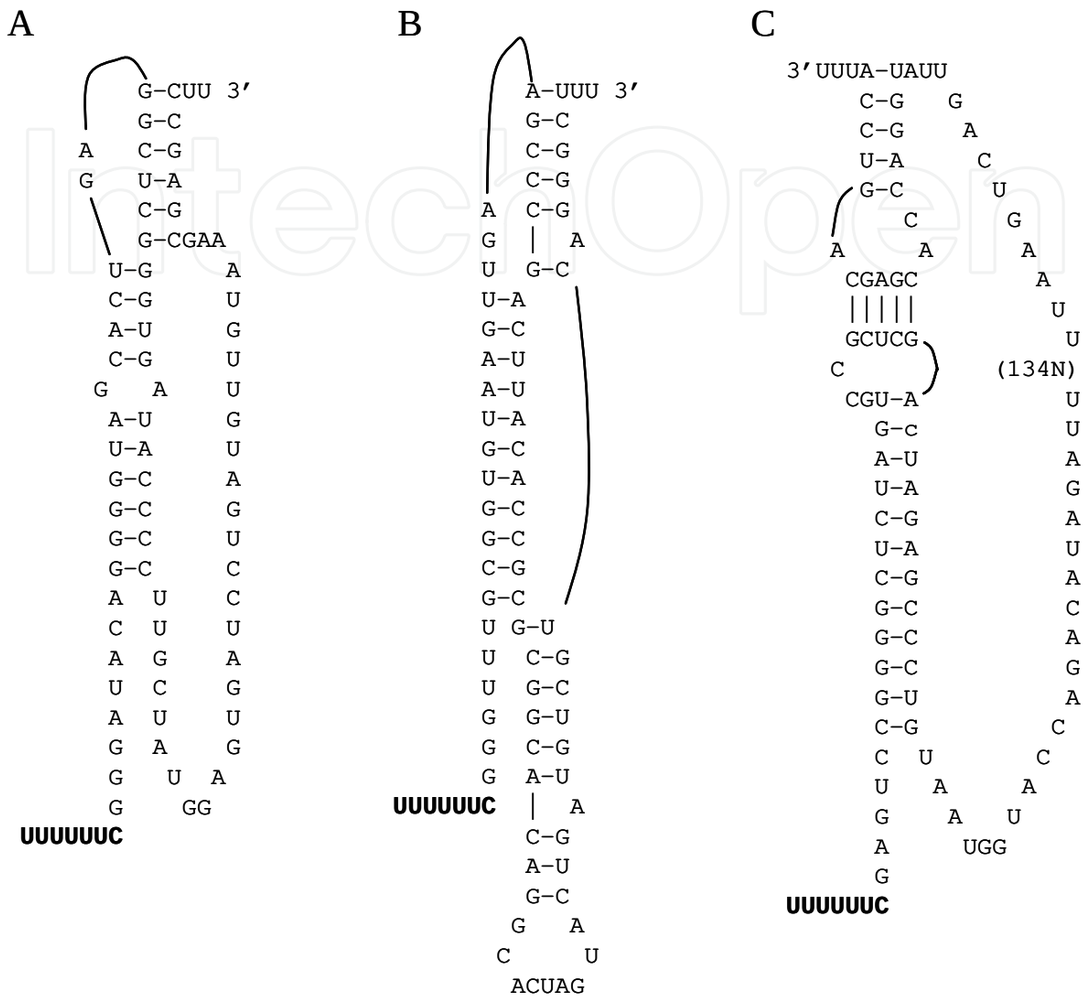

Fig. 1. Diverse stimulatory structures from coronaviruses. Shown are diagrams of the sequence and base-pairing of three different coronavirus stimulatory elements. A) the avian infectious bronchitis virus two-stemmed pseudoknot (Brierley et al., 1989), B) the severe acute respiratory syndrome three-stemmed pseudoknot (Plant et al., 2005), and C) the kissing loops of the human coronavirus 229E stimulatory element (Herold & Siddell 1993). The heptameric slippery sites are shown in bold.

2.1 Frameshift signals in the *Totiviridae* family

The *Totiviridae* are double-stranded RNA viruses infecting arthropod, fungal and protozoan hosts. There are three genera; Totivirus, Giardiavirus and Leishmaniavirus. One of the early frameshift signals detected and well characterized was that of the *Saccharomyces cerevisiae* L-A totivirus (Dinman et al., 1991). It uses frameshifting to control the ratio of structural capsid (gag) protein to enzymatic polymerase (pol) protein. It has been shown that either increasing or decreasing frameshifting efficiency in the L-A genome alters the gag to gag-pol ratio and disrupts propagation of a satellite RNA (Dinman & Wickner, 1992). The yeast cells

harboring the L-A virus are easily manipulated. This allowed many pivotal frameshifting studies to be performed in the Dinman laboratory. The ability to make specific mutants in yeast has permitted the analysis of many different genes that affect frameshifting and virus propagation. Additionally yeast cells are susceptible to many of the drugs that affect higher eukaryotes and this has facilitated the design of assay systems for the investigation of drugs that affect frameshifting (Rakauskaite et al., 2011). The insights from studying the L-A frameshift signal has helped other groups home in on specific regulatory genes, or drugs, that affect other viruses in cell culture systems using mammalian cells.

The Giardiavirus GLV has been shown to have a functional frameshift signal that, like L-A, modulates the gag to gag-pol ratio (Li et al., 2001a). A pseudoknot has been shown to stimulate frameshifting in the L-A virus (Dinman et al., 1991) while a stem-loop structure is used in the GLV virus (Li et al., 2001a). More recent analyses of *Totiviridae* genomes have identified a frameshift signal in the penaeid shrimp infectious myonecrosis virus (Nibert, 2007), Armigeres subalbatus totivirus (Zhai et al., 2010), Omono River virus (Isawa et al., 2011), helminthosporium victoriae virus and trichomonas vaginalis viruses II and 3 (Bekaert & Rousset, 2005). A +1 recoding mechanism for the Leishmania virus LRV1-4 has been reported (Kim et al., 2005). This frameshift also regulates the ratio of the putative polymerase protein to the structural protein. These findings suggest that a common viral problem, that of regulating the abundance of structural and enzymatic viral proteins, is solved in the *Totiviridae* family by manipulating translation fidelity, albeit by slightly different mechanisms.

2.2 Frameshift signals in the *Retroviridae* family

The *Retroviridae* are RNA viruses that produce a DNA copy of their genome that is integrated into the host cell genome. Like the *Totiviridae*, retroviruses use frameshift signals to modulate the ratio of gag protein to gag-pol fusion protein. The ratio of gag to pol proteins has been shown to be important for viral replication and infectivity in several retroviral systems. Altering the ratio has been shown to adversely affect replication, RNA dimerization and particle formation (Biswas et al., 2004; Chen and Montelaro, 2003; Gendron et al., 2005; Hung et al. 1998; Karacostas et al., 1993; Shehu-Xhilaga et al., 2001).

In 1985 it was noted that the 5′ end of the polymerase ORF for the Deltaretrovirus bovine leukemia virus overlapped with the upstream ORF for the gag ORF (Rice et al., 1985). The authors postulated that a recoding event was responsible for the production of the polymerase. The first characterization of a viral frameshift signal was of the Alpharetrovirus Rous sarcoma virus (Jacks & Varmus, 1985). The region of the genome containing the frameshift signal was cloned into an expression vector, transcribed and translated. ^{35}S-labeled products were immunoprecipitated with antiserum against either the gag protein or the polymerase. The result clearly showed that a gag-pol polyprotein was produced from the same transcript as the gag protein (Jacks & Varmus, 1985). Frameshift signals have since been identified in Alpharetroviruses, Betaretroviruses, Deltaretroviruses and Lentiviruses.

Soon after Jacks and Varmus described the first frameshift signal, signals in the Betaretrovirus mouse mammary tumor virus (MMTV) and the Lentivirus human immunodeficiency virus (HIV-1) were characterized by the same group (Jacks et al., 1987, 1988a). The frameshift signal in the MMTV genome is located in the 5′ end of the gag ORF, and when frameshifting occurs a protease encoded in the overlapping pro ORF is produced. The order of the amino acid motifs

that identify the enzyme domains encoded in the frameshift-regulated ORF varies between different retroelements. So, although some commentaries indicate that the frameshift-regulated ORF encodes a protease, the ORF usually codes other enzymatic proteins, including the polymerase, that are produced as a polyprotein which is then cleaved by a protease. The frameshift signal from the Mason-Pfizer monkey Betaretrovirus, (simian retrovirus 1), has also been characterized (ten Dam et al., 1994).

Frameshift signals from the Deltaviruses human T-cell leukemia virus types I and II, have been characterized (Kollmus et al., 1994; Nam et al., 1993) and the putative signals in the primate T-lymphocyte virus 3 and simian T-lymphocyte viruses 1 and 2 have been described (Bekaert & Rousset, 2005; Bekaert et al., 2010). Additional Lentivirus frameshift signals from equine infectious anemia virus, feline immunodeficiency virus and simian immunodeficiency virus have been characterized (Bekaert & Rousset, 2005; Chen & Montelaro, 2003; Morikawa & Bishop, 1992). The HIV-2 genome layout suggests that, like HIV-1, there is a frameshift signal between the gag and pol ORFs. Putative signals in bovine immunodeficiency virus, caprine arthritis virus, ovine lentivirus, Jembrana disease virus and Visna virus have been described (Bekaert & Rousset, 2005; Bekaert et al., 2010). Additional frameshift signals in an Alpharetrovirus genome (avian leukosis virus), and a Deltavirus genome (bovine leukemia virus) have also been identified (Bekaert & Rousset, 2005; Bekaert et al., 2010). A putative signal from a Betaretrovirus, the cancer causing Jaagsiekte sheep retrovirus, has also been been described (Bekaert et al., 2010).

In several retrovirus genomes, the gag and pol genes are separated by a stop codon. It has been shown that readthrough of the stop codon can produce a fusion protein. A pseudoknot facilitates the readthrough of murine leukemia virus and pseudoknots are predicted to be present at the gag-pol junction of several retroviruses (Wills et al., 1994). Interestingly, two competing structures, a stem-loop structure and a pseudoknot, were described for the murine leukemia virus (Alam et al., 1999). Both pseudoknots and stem-loops have been proposed to stimulate frameshifting in HIV. This suggests that secondary structures are important for modulating ribsome fidelity during retroviral protein production in both readthrough and frameshifting, although exactly how they stimulate the ribosome remains a mystery.

2.3 Frameshift signals in the order Nidovirales

The order Nidovirales includes *Coronaviridae*, *Arteriviridae* and *Okavirus*, all of which are positive-stranded RNA viruses that infect animals. They have large single-strand genomes and express structural proteins from subgenomic RNAs transcribed from the 3′ region of the genome. The nonstructural proteins are expressed from the genomic RNA. The coronavirus replicase genes are encoded in the 5′ portion of the genome and a frameshift event is required for the production of several proteins including the RNA-dependent RNA polymerase (RdRP). The replicase proteins are translated as two polyproteins that are processed by self-encoded proteases. The smaller polyprotein has several domains including papainlike cysteine protease, chymotrypsin-like cysteine protease, metal binding and transmembrane motifs. The larger polyprotein, resulting from a frameshift event contains the domains of the smaller polyprotein and the RdRP, helicase, 3′-to-5′ exonuclease and S-adenosylmethionine-dependent ribose 2′-O-methyltransferase domains (Ziebuhr, 2005). The complexities of coronaviral replication are still being unravelled but it is apparent that

frameshifting is essential for the production of the proteins involved. Altering frameshifting efficiency has been shown to be detrimental for coronavirus replication (Ahn et al., 2011; McDonagh et al., 2011; Plant et al., 2010).

The first frameshift signal identified in a coronavirus was by Brierley et al. (1987). The infectious bronchitis virus (IBV) frameshift signal is now perhaps one of the most well characterized frameshift signals along with the HIV frameshift signal. However, although several luteovirus frameshift signals have been crystalized the size of the pseudoknot that promotes frameshifting in IBV has thwarted attempts at crystalization. Additionally there is an example of a kissing stem-loop that promotes coronavirus frameshifting (Herold and Siddell, 1993). A lot of nuclease mapping and NMR data is available for some coronavirus signals but this does not reflect the diversity of nidovirus frameshift signals (Dos Ramos et al., 2004; Napthine et al., 1999; Plant et al., 2005, 2010; Su et al., 2005).

Functional frameshift signals have been identified in some Arteriviridae including equine arteritis virus, lactate dehydrogenase-elevating virus and porcine reproductive and respiratory syndrome virus (Bekaert & Rousset, 2005; den Boon et al., 1991). A frameshift signal has been identified in the simian hemorrhagic fever virus (Bekaert & Rousset, 2005). A frameshift signal in the Gill-associated Okavirus has been shown to be functional (Cowley et al., 2000). Putative frameshift signals have been identified in several coronaviruses including bovine coronavirus, porcine epidemic diarrhia virus, transmissible gastroenteritis virus and human coronavirus OC43 (Bekaert & Rousset, 2005). The functionality of the IBV, human coronavirus 229E, Berne virus, murine hepatitis virus and SARS coronavirus frameshift signals have been demonstrated (Baranov et al., 2005; Brierley et al., 1987, 1989, 1991, 1992; Dos Ramos et al., 2004; Herold & Siddell, 1993; Plant et al., 2005, 2010; Su et al., 2005).

2.4 Frameshift signals in other positive-stranded RNA viruses

The *Astroviridae* are non-enveloped positive sense single-stranded RNA viruses. The genome has three ORFs, with the first two overlapping. A frameshift signal that separates the protease and the RdRP was identified in the Human astrovirus and subsequently shown to be functional (Jiang et al., 1993; Marczinke et al., 1994). More recently frameshift signals have been identified in the Chicken, Mink, Ovine and Turkey astroviruses (Bekaert & Rousset, 2005; Bekaert et al., 2010).

The *Flaviviridae* are positive sense single-stranded RNA viruses with a genome of approximately 10kb. Recently a frameshift signal was identified and characterized in the West Nile virus. Interestingly the frameshift is within an ORF encoding the non-structural protein NS1. The NS1' frameshift protein plays a role in the neuro-invasiveness of the Kunjin subtype of West Nile virus (Melian et al., 2010). Potential frameshift signals have been identified in three other flaviviruses; Japanese encephalitis virus, Murray Valley encephalitis virus and Usutu virus (Firth & Atkins, 2009).

The *Luteoviridae* are positive sense single-stranded RNA viruses that infect plants. The RdRP ORF is downstream from a coat protein (CP) ORF in plant luteoviruses. The expression of the RdRP is regulated by readthrough of a stop codon or by -1 frameshifting. Production of a CP-RdRP fusion protein is required for aphid transmission (Demler & de Zoeten, 1991; Di et al., 1993). Frameshift signals have been found in Enamoviruses, Luteoviruses and Poleroviruses.

A well described example of a Luteoviral signal is upstream from the Barley yellow dwarf virus, PAV serotype polymerase (Di et al., 1993). However, the BYDV frameshift signal differs from other frameshift signals in that a sequence four kilobases downstream from the slippery site is required to stimulate frameshifting (Paul et al., 2001). A similar genome arrangement and frameshift signal has been found for the rose spring dwarf-associated virus (Salem et al., 2008). Interestingly, readthrough signals have been described for the BYDV PAV serotype coat protein which also requires an interaction with a distal sequence (Brown et al., 1996). Other luteoviruses with putative frameshift signals include the Bean leafroll virus and Soybean dwarf virus (Domier et al., 2002; Bekaert et al., 2010).

A frameshift signal has been identified in the Enamovirus pea enation mosaic virus (Demler & de Zoeten, 1991). Like the BYDV virus serotypes there are pea enation mosaic viruses that have been described that have a putative readthrough mechanism (Harrell et al., 2002). The structure of the pseudoknot from the pea enation mosaic virus-1 frameshift signal has been characterized (Giedroc et al., 2003; Nixon et al., 2002).

A number of frameshifting sequences have been identified in Polerovirus genomes including beet mild yellowing virus, cereal yellow dwarf viruses RPS and RPV and turnip yellows virus (Bekaert et al., 2010). The frameshift signals for beet chlorosis virus, beet western yellows virus, cucurbit aphid-borne yellows virus, potato leafroll virus have all been shown to be functional (Bekaert & Rousset, 2005; Kim et al., 2000; Kujawa et al., 1993; Prüfer et al., 1992) and the structure of several stimulatory elements have been elucidated (Cornish et al., 2005; Pallan et al., 2005; Su et al., 1999).

The *Togaviridae* are positive sense single-stranded RNA viruses that include rubella virus and the alphaviruses. The alphaviruses are transmitted by arthropods and many cause encephalitis. The discovery of a functional frameshift signal in the Semliki Forest virus 6K gene is expected to have wide ranging effects on the understanding of alphavirus lifecycle (Firth et al., 2008). The 6K protein is involved in envelope processing, membrane permeabilization, virus budding and virus assembly. Original observations of a 6K protein doublet are now in doubt as Firth et al., (2008) have confirmed the presence of the frameshifted product using amino acid sequencing. The additional protein is refered to as the transframe, or TF, protein. Mutant viruses lacking the ability to frameshift showed reduced growth. Sequence comparisons indicated that frameshift signals are present in other alphaviruses including Seal louse virus, Middleburg virus, Venezuelan equine encephalitis virus, Ndumu virus, Sinbis virus, Barmah Forest virus, Sleeping disease virus and Eastern equine encephalitis virus. The functionality of these frameshift signals has been confirmed (Chung et al., 2010).

The *Tombusviridae* are positive sense single-stranded RNA plant viruses. Some of these viruses are transmitted by fungal species, but either the virion or genetic material are infective. Putative frameshift signals have been identified in Dianthovirus RNA 1-like RNA, Pelargonium line pattern virus, carnation ringspot virus 1, sweet clover necrotic mosaic virus RNA-1, subterranean clover mottle virus, turnip rosette virus, carrot mottle mimic virus, groundnut rosette virus, pea enation mosaic virus-2 and tobacco bushy top virus (Bekaert & Rousset, 2005; Castano & Hernandez, 2005; Ge et al., 1993; Miranda et al., 2001). Frameshifting has been demonstrated from the cocksfoot mottle virus and red clover necrotic mosaic virus RNA-1 (RCNMV) signals (Kim & Lommel, 1994; Tamm et al., 2009; Xiong et al., 1993). It has been shown that the RdRP, which is produced via the frameshifting mechanism, is supplied in a cis-preferential manner for the synthesis of negative-strand

RCNMV RNA (Okamoto et al., 2008). Thus there appears to be some link between control of translation and replication of the virus. Like many other positive-strand RNA viruses the replication process results in a surplus of positive-strand RNA.

3. Programmed ribosomal frameshift signals

Programmed Ribosomal Frameshift (PRF) signals are sequences within a messager RNA (mRNA) that stimulate a portion of translating ribosomes to change reading frames. PRF signals are typically comprised of two features, a heptameric slippery site and a stimulatory element. The slippery site is a series of seven nucleotides in the mRNA from which the tRNAs in the translating ribosome can un-pair from the zero frame and re-pair to in the -1 frame. The stimulatory element has a dual function, it causes the translating ribosome to pause on the message when the slippery site is positioned within the ribosome and it stimulates ribosomal error.

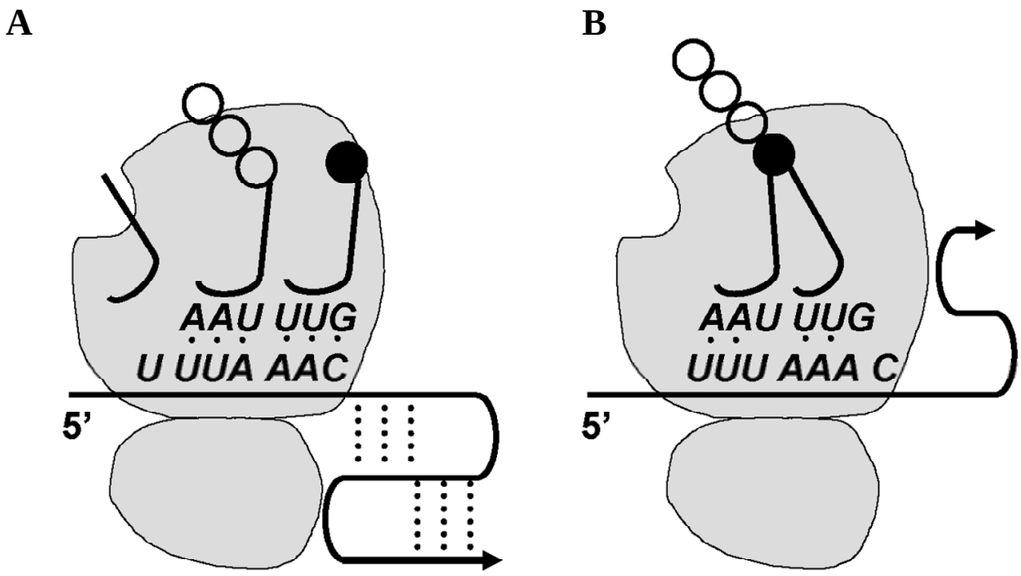

Fig. 2. Simultaneous slippage model of programmed ribosomal frameshifting. A) Cartoon of two tRNAs paired with the mRNA in the zero (AUG-initiated) reading frame before frameshifting occurs. B) Cartoon with the two tRNAs each paired to the mRNA at two of the three codon positions after frameshifting has occured. The 5′ end of the mRNA is indicated and dashed lines indicate secondary structure. Nucleotides involved in the codon:anticodon interaction are shown with dots indicating base pairing. The tRNA in the ribosomal P-site is attached to the elongating peptide chain (open circles) and the aminoacylated tRNA in the ribosomal A-site is shown with a filled circle.

3.1 Heptameric slippery sites

Evidence for the requirement of slippage of both the aminoacyl- and peptidyl-tRNAs has accumulated in the last few decades. The actual point of slippage has been confirmed by protein sequencing for mouse mammary tumor virus (MMTV) (Hizi et al., 1987); HIV (Jacks et al., 1988a); RSV (Jacks et al., 1988b); barley yellow dwarf virus (BYDV) (Di et al., 1993),

human T-cell leukemia virus type 1 (HTLV-1) (Nam et al., 1993) and Semiliki Forest virus (Firth et al., 2008). A number of mutagenesis experiments have been performed that demonstrate that there are specific sequence requirements at both the A- and P-site codons in many viruses and even a eukaryotic gene (for example: HIV virus, Wilson et al., 1988; L-A virus, Dinman et al., 1991; potato leafroll virus (PLRV), Prüfer et al., 1992; red clover necrotic mosaic dianthovirus (RCNMV), Kim & Lommel, 1994; *Edr*, Manktelow et al., 2005). Rules defining the heptameric sequences on which tRNAs could slip were elucidated in part in yeast (Dinman et al., 1991) and more extensively in reticulocyte lysate (Brierley et al., 1992). In general the slippery site can be defined as N NNW WWH, where N is any three identical bases, W is A or U, and H is A, C or U (the frame of the initiator AUG is indicated by the spacing), although there are exceptions (see section 4). This sequence is often described as X XXY YYZ in the literature. The efficiency of frameshifting promoted by each heptameric slippery site varies depending on the system used to assay frameshifting.

3.2 Stimulatory elements 3' of the slippery site

The sequence 3' of the heptameric slippery site has been shown to be required for optimal frameshifting in a number of systems (for example: Brierley et al., 1987; Jacks et al., 1987, 1988a, 1988b). The stimulatory sequences were predicted to fold into stem loops until Brierley et al., (1989) demonstrated that the Avian Infectious Bronchitis virus (IBV) stimulatory element was a pseudoknot. Sequence comparisons indicated that some structures downstream from slippery sites in other viruses could also be pseudoknots (for example: Bredenbeek et al., 1990; Kujawa et al., 1993; Cowley et al., 2000). It has also been shown that the stimulatory structure could involve long range interactions (Herold & Siddell, 1993; Paul et al., 2001). It has also been postulated that stop codons stimulate frameshift events, presumably by pausing translation (Castano & Hernandez, 2005; Horsfield et al., 1995). Mutagenesis or deletion analyses have been used to demonstrate that the 3' stimulatory sequences are stem-loops, pseudoknots or other higher order structures (see Table 1). Nuclease mapping, NMR, crystallography and mass spectrometry has confirmed some of the 3' stimulatory structures but many remain unresolved at the atomic level due to difficulties purifying larger structures. Molecular modeling has been useful in elucidating some of these structures (Ahn et al., 2011). A list of stimulatory elements (with the exception of HIV which is discussed below) is provided in Table 1.

3.3 Stimulatory element for HIV

The structure of one 3' stimulatory element, the HIV structure, remained elusive for many years. Frameshifting efficiencies for many of the viruses listed in Table 1 were dramatically reduced when the 3' sequence was removed. However, removal or alterations in the HIV 3' sequence produced more subtle changes in frameshifting and, as a result, different groups reached different conclusions. Jacks et al., (1988a) proposed that a 3' stem-loop was required for efficient frameshifting. This was refuted later that year by Wilson et al., (1988) who performed experiments in both rabbit reticulocyte lysate and yeast cells in which they obtained efficient frameshifting (5-10% measuring labeled methionine incorporation) from a 26 nucleotide HIV sequence that lacked the proposed stem-loop sequence. Using luciferase reporter plasmids Moosmayer et al., (1991) obtained 2-4% frameshifting in BHK cells using the shorter sequence used by Wilson et al., but did not make comparisons to sequences containing the stem-loop. Parkin et al., (1992) made mutations to disrupt and reform the stem-loop to

demonstrate that, when present, an intact stem-loop stimulated 4-9 fold higher frameshifting in avian and simian cells. This result was also obtained by Reil et al., (1993) in BHK-21 cells (3.2% with the stem loop versus 0.9% without). This difference in frameshifting efficiency was recapitulated in both mouse fibroblasts and human lymphoid cells (Cassan et al., 1994). Additionally it has been shown that HIV frameshifting efficiency is several fold higher in human T-cells than in a bacterial lysate (Plant & Dinman, 2006). Telenti et al. (2002) found that clinical isolates with variations in the stem-loop sequence predicted to disrupt the structure had lower levels of frameshifting. In 1998 Kang used UV absorbance melting and nuclease assays all under the same conditions to show the formation of a stem-loop structure.

Viruses with Stem Loops	Deletion Analysis or Mutagenesis	Nuclease Mapping	NMR, Crystallography or Mass Spectrometry
GLV	Li et al., 2001a	Li et al., 2001a	
HAst-1	Marczinke et al., 1994	Marczinke et al., 1994	
HTLV-II	Falk et al., 1993		
mIAP	Fehrmann et al., 1997		
PLRV-G	Prüfer et al., 1992		
RCNMV	Kim & Lommel, 1998		
Viruses with Pseudoknot			
BWYV	Sung & Kang, 1998		
BYDV	Paul et al., 2001		Su et al., 1999
EIAV	Chen and Montelaro, 2003		
FIV	Morikawa & Bishop, 1992		
IBV	Brierley et al., 1989; 1991	Napthine et al., 1999	Yu et al., 2005
L-A	Dinman et al., 1991 Tzeng et al., 1992		
MHV		Plant et al., 2010	
MMTV	Lee et al., 1995		
PEMV			Shen & Tinoco, 1995 Giedroc et al., 2003
PLRV-P	Kujawa et al., 1993 Kim et al., 2000		Nixon et al., 2002 Pallan et al., 2005
RSV	Marczinke et al., 1998	Marczinke et al., 1998	
SARS	Baranov et al., 2005 Plant et al., 2005	Dos Ramos et al., 2004 Plant et al., 2005 Su et al., 2005	Plant et al., 2005
ScYLV			
SRV	ten Dam et al., 1994 Sung & Kang, 1998	ten Dam et al., 1994	Cornish et al., 2005 Du et al., 1997
TYMV			Michiels et al., 2001
VMV	Pennell et al., 2008	Pennell et al., 2008	Kolk et al., 1998

Table 1. Viral PRF Stimulatory Elements. References are listed that describe different methods used to characterize viral stimulatory elements. GLV, giardiavirus; HAst-1, human astrovirus; HTLV-II, human T-cell leukemia virus type 2; mIAP, mouse interstitial A-type particle; PLRV-G, potato leafroll virus; RCNMV, red clover necrotic mosaic dianthovirus; BWYV, beet western yellows virus; BYDV, barley yellow dwarf virus; EIAV, equine infectious anemia virus; FIV, feline immunodeficiency virus; L-A, *Saccharomyces cerevisiae* virus L-A; MHV, mouse hepatitis virus; MMTV, mouse mammary tumor virus; PEMV, pea enation mosaic virus; RSV, Rous sarcoma virus; SARS, severe acute respiratory syndrome virus; ScYLV, sugarcane yellow leaf virus; SRV, simian retrovirus; TYMV, turnip yellow mosaic virus; VMV, Visna-Maedi retrovirus.

These experiments and observations helped reinforce the idea that a stem-loop was the 3′ stimulatory element in the HIV frameshift signal. However because so many other 3′ stimulatory elements were pseudoknots different groups looked for potential pseudoknot folding downstream from the HIV slippery site. Taylor et al., (1994) analyzed the HIV genome for sequences with the potential to form pseudoknot structures and proposed that a few nucleotides of the sequence 3′ of the stem-loop could fold back, and pair with the loop, creating a pseudoknot. Based on the structure of other known pseudoknots Du et al., (1996) proposed that a small pseudoknot involving the spacer region could form (Figure 3B). Dulude et al., (2002) proposed that the 3′ region was not folding back to form a stem-loop, but was in fact extending stem 1 by pairing with the spacer region (Figure 3G). Dulude et al. supported this hypothesis by mutating each sequence individually and in tandem (to disrupt and reform pairing potential) and assayed frameshifting efficiencies in cultured cells. NMR experiments with the HIV 3′ stimulatory sequence have shown that the extended stem loop proposed by Dulude et al., can form (Gaudin et al., 2005; Staple and Butcher, 2005). Contrasting experiments by Dinman et al. (2002), using comparative genomics, nuclease mapping and frameshifting assays on a variety of mutants, argued that a pseudoknot structure formed. However, instead of the second stem proposed by Taylor et al., (1994) it was suggested that the top portion of stem 1 could form triplex structure with loop 2, in effect creating stem 2. Baril et al., (2003) also used mutagenesis and nuclease mapping to argue that the HIV group O frameshift signal is a pseudoknot. The Brakier-Gingras group has shown that there are a variety of functional structures that stimulate –1 PRF in different HIV strains (Baril et al., 2003; Dulude et al., 2002). Thus it is apparent for HIV at least, that absolute conservation of one particular structure is not essential for frameshifting: there is sequence variation in the stimulatory element between strains, and efficient frameshifting has been demonstrated when proposed stimulatory elements are altered. Examples of these structures are shown in Figure 3.

It is possible that, as suggested by Taylor et al., (1994), the conversion between structures may be important for frameshifting. This notion is important when thinking about the structures proposed by Du et al., (1996) and Dulude et al., (2002) which incorporated the spacer region between the slippery site and stimulatory element. This spacer region needs to be un-paired to fit within the ribosome entry tunnel when the slippery site is correctly positioned inside the ribosome.

In addition to the possibility of different HIV 3′ structures stimulating –1 PRF, there is evidence that the frameshifting efficiencies observed may be the sum of different frameshifting mechanisms. Jacks et al., (1988a) noted that, when they sequenced the frameshifted protein, there was some variation at the position where frameshifting occurs. This variation suggested that different aspects of ribosome fidelity were affected during frameshifting. The effect of complementary DNA sequences on HIV frameshifting efficiency has been analyzed (Vickers and Ecker, 1992). Oligonucleotides predicted to bind to the sequences 3′ of the stem loop, which would disrupt both the proposed extended stem-loop and pseudoknot structures proposed above, actually enhanced frameshifting in a rabbit reticulocyte lysate, as did addition of a second stem-loop downstream from the first. Different groups spent the next two decades analyzing several frameshift signals from a variety of virus families with the goal of understanding frameshift mechanisms.

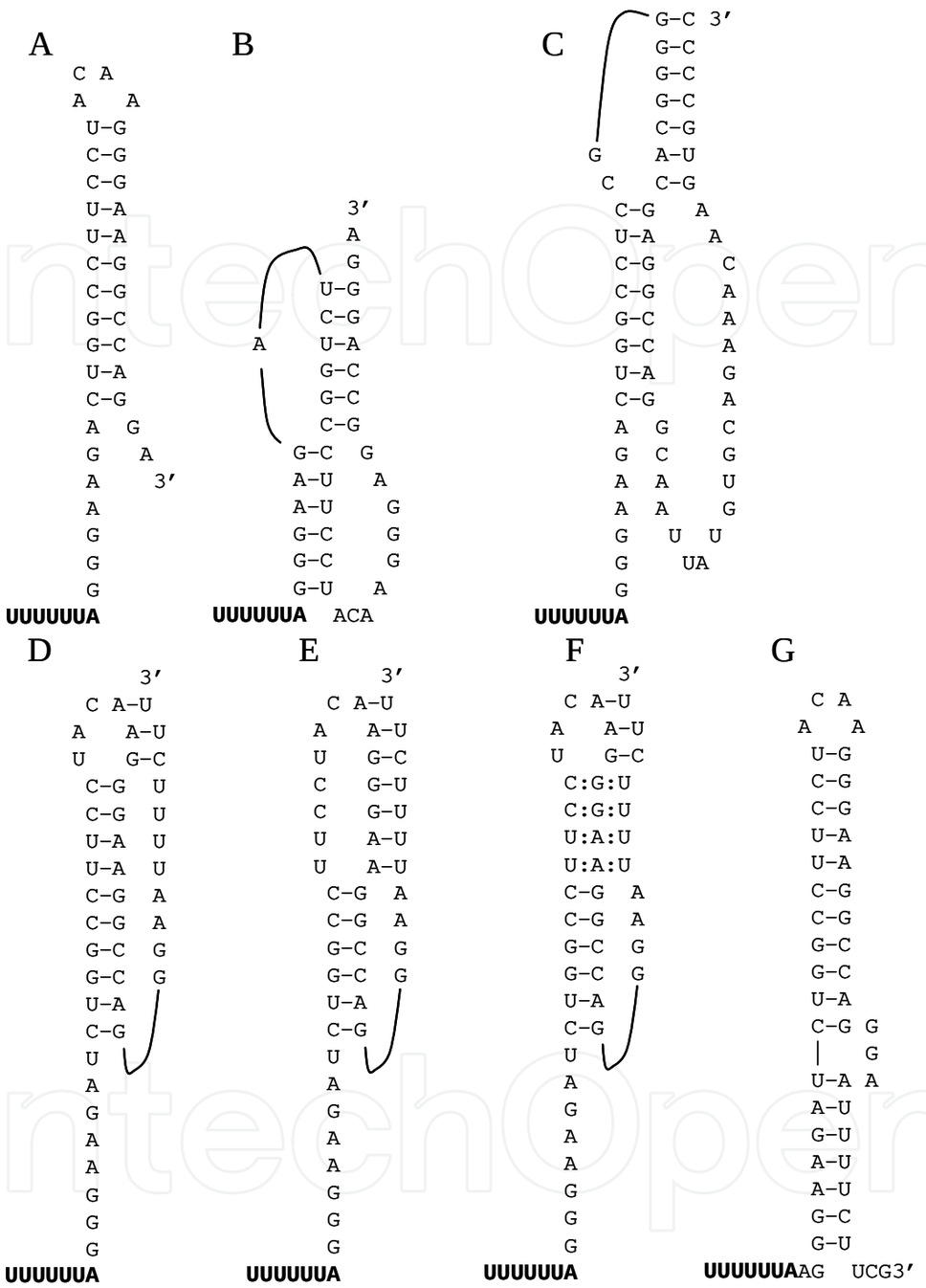

Fig. 3. Different stimulatory elements reported for HIV frameshift signals. There are some minor sequence variations between the subtypes. The stem-loop structure proposed by Jacks et al., (1988a) and pseudoknot proposed by Du et al., (1996) are shown in panels A and B respectivley. Panel C shows the pseudoknots described by Baril et al., (2003) for group O isolates. Panels D and E show two pseudoknots proposed by Taylor et al., (1994) and panel F shows the pseudoknot described by Dinman et al., (2002) using the same sequence. The extended stem-loop structure with a 5′-ACAA-3′ tetraloop described by Dulude et al., (2002) is shown in panel G.

4. Elucidation of frameshifting mechanisms by analysis of viral frameshift signals

Ribosomes translate messager RNA with very low error rates to produce proteins. The availability of error-inducing frameshift-stimulating sequences from viruses has facilitated the study of ribosome function. Next I describe how the studies of virally encoded frameshift signals have had a broad impact on our understanding of protein translation. Further, these analyses have enhanced our understanding of viral replication and opened the door to new antivirals (discussed in section 5).

Translation of mRNA sequence into protein is a universal requirement for living organisms from the smallest single cell organisms through to the most complex mammals. The reaction, directed by the ribosome, is very specific with few errors (reviewed in Ogle & Ramakrishnan, 2005). In some instances, small viral genomes for example, the mRNA is polycistronic, encoding more than one protein. Tryptic analysis of a number of retroviral proteins in the late 1970s indicated that the gag and gag-pol proteins of these RNA viruses had the same amino terminus. It was proposed that the identical amino termini could be achieved by a number of mechanisms including recoding (Pawson et al., 1976). Subsequently gag-pol production for a number of retroviruses and retrotransposons was shown to be due to a recoding event, now known as frameshifting (Jacks & Varmus, 1985; Wilson et al., 1986; Farabaugh et al., 1993). The direction of frameshifting and the mRNA sequences on which frameshifting occurs differ amongst retroelements even though some infect the same host. There are several possible ways that frameshifting sequences overcome ribosome fidelity. Elucidation of these mechanisms has been possible through study of various viral frameshift signals.

The diverse sequence characteristics of different frameshift stimulating signals led many groups to postulate different mechanisms for frameshifting; some are specific to a single frameshift signal, others are more general mechanisms. Frameshifting occurs in the decoding center of the ribosome as part of protein translation. Each translation elongation cycle involves the selection of an aminoacylated-tRNA and it's accommodation into the ribosomal A-site. The amino acid is added to the elongating peptide in the peptidyltransferase center and the tRNA, now referred to as the peptidyl-tRNA, is moved into the P-site of the ribosome. After the addition of the next amino acid, or the termination of protein synthesis, the peptidyl-tRNA is moved into the E-site where it can exit the ribosome. During the elongation cycle the anticodon loop of the tRNA is hydrogen bonded to the corresponding triplex codon on the mRNA in the decoding center of the ribosome. Programmed ribosomal frameshifting is the regulated un-pairing of at least one of the tRNAs from the mRNA and re-pairing of the tRNAs to a different position on the mRNA (see Figure 2).

4.1 Simultaneous slippage

Frameshifting occurs when the anticodon loop of a tRNA binds to an out of frame codon on the mRNA. The first frameshift stimulating sequences described all required a heptameric slippery site suggesting that both the aminoacyl- and peptidyl-tRNAs bind out of frame (Jacks & Varmus, 1985; Jacks et al., 1987, 1988a). Other viruses and retroelements suspected of using programmed –1 ribosomal frameshifting (-1 PRF) also showed a preference for certain heptameric sequences and analysis of these sequences revealed that both the A-site

and P-site tRNAs could potentially un-pair from the zero frame and re-pair in the –1 frame while maintaining "a two-out-of-three base pair, anticodon-codon configuration" (Jacks et al., 1988b). Thus the basis for this model was founded: both the aminoacyl- and peptidyl-tRNAs 'slipped' together on the mRNA one nucleotide from the zero frame register in a 5′ direction to the –1 frame. A 3′ stimulatory element was also required and integrated into the model even though it was not known how it contributed frameshifting at that time.

A 3′ stimulatory structure is required in the simultaneous slippage model and is present in some examples of the P-site slippage model discussed below. Researchers soon began to investigate how these structures were involved in –1 PRF. An early hypothesis was that it could be "the binding site for a ribosomal protein or RNA or soluble elongation factor; this binding could then affect the fidelity of the ribosome-tRNA interaction at the decoding sites" (Jacks et al., 1988b). This is a hypothesis that has been difficult to prove or disprove and only one experiment stands out: tem Dam et al., (1994) added back RNA corresponding to the pseudoknot so that it might quench the supply of potential pseudoknot binding proteins and thus reduce the frequency of –1 PRF in their reporter in an *in vitro* assay. No change in frameshifting efficiency was observed indicating that there is not a pseudoknot binding protein. However, even if there is no pseudoknot binding protein the stimulatory affect of proteins on frameshifting cannot be totally disregarded. An iron response element (stem-loop structure) was positioned downstream from the HIV slippery site and frameshifting promoted when iron regulatory proteins were present (Kollmus et al., 1996). Because this result is from an artificial construct the results may be suggesting a steric interaction with the ribosome is stimulating frameshifting rather than more specific interactions. A single protein (or RNA molecule) capable of binding the diverse array of pseudoknots to direct frameshifting also seems unlikely.

As more frameshift stimulating pseudoknots were discovered, it became apparent that there were different types of pseudoknots that stimulated frameshifting in both eubacterial and eukaryotic systems. The differing size and structures of pseudoknots may reflect specificities for host ribosomes. This is evident from many experiments demonstrating that frameshifting efficiencies from one particular signal are altered when different lysates or cell lines are used to assay frameshifting efficiency (Barry & Miller, 2002; Cassan et al., 1994; Dulude et al., 2002; Garcia et al., 1993; Kim et al., 1999; Lewis & Matsui, 1996; Napthine et al., 2003; Parkin et al., 1992; Plant & Dinman, 2006; Stahl et al., 1995; Tzeng et al., 1992). But even in one host different viruses use different stimulatory elements. For example, the structures described for coronaviruses infecting humans include two-stem pseudoknots, three-stem pseudoknots and kissing loops (see Figure 1).

Another hypothesis was that the function of the 3′ stimulatory structure was simply to cause a translational pause. Adjacent stop codons have also been suggested to promote translational pausing during decoding of the terminator (Rice et al., 1985; Jacks et al., 1988b). The latter suggestion was later supported by the Potato Virus M and Measles virus signals described below (Gramstat et al., 1994; Liston & Briedis, 1995). The ribosome is known to pause at certain positions along an mRNA (Wolin and Walter, 1988) and even pseudoknots (Tu et al., 1992). Time course experiments to demonstrate that ribosomes paused at frameshift stimulating pseudoknots were performed (Somogyi et al., 1993). Translation was stopped by addition of edeine, which hinders mRNA binding and prevents productive peptidyl-tRNA interactions, and methionine incorporation was monitored. Conditions were

altered to change the ability of the pseudoknot structure to form (higher magnesium to promote formation and higher temperatures to discourage formation) and all the results correlated with the formation of a pseudoknot (known to stimulate frameshifting) causing the ribosomes to pause. The final variation in experimental conditions was to test the ability of a stem-loop, predicted to be more stable than the pseudoknot, to stimulate pausing and frameshifting. Although the structure could stimulate efficient pausing like the pseudoknot, it did not promote efficient –1 PRF. In a follow up paper (Kontos et al., 2001) heelprinting was performed to show that the ribosomes paused over the A- and P-site codons with a variety of pseudoknots. The conclusion from these experiments was that a pause was necessary for efficient –1 PRF but insufficient, as stem-loops could also promote pausing.

Experiments that replaced the A-site codon of the HIV slippery sequence with a termination codon also elicited a frameshift event in E. coli (Horsfield et al., 1995). This suggests that a switch from translation elongation to termination can also result in a pause sufficient for frameshifting to occur. However, if the A-site codon has been replaced with a termination codon then frameshifting must occur at the P-site alone, or at the P- and E-sites. Mutagenesis of the HIV frameshift signal by Horsfield et al. (1995) indicated that frameshifting required both the A- and E-site codons in this context. Experiments by a different group using the native HIV stimulatory structure also led to a similar conclusion (Leger et al., 2007). The E-site requirement is also supported by the conservation of certain nucleotides in the E-site position upstream of heptameric slippery sites (Bekaert and Rousset, 2005). Sequencing frameshift protein products showed that the 0-frame or the -1 frame A-site could be decoded (Jacks et al., 1988a, Yeverton et al., 1994, Ivanov et al., 1998). Thus, -1 PRF can occur before the 0-frame A-site is decoded, or if it occurs after the 0-frame A-site decoding, then the aminoacyl-tRNA could be removed and a new tRNA that better matches the -1 frame A-site is inserted before peptidyltransfer occurs. These analyses also show that multiple mechanisms of resolving a frameshift signal with a heptameric slippery site are employed in any one cellular system. The timing of frameshifting within the translation elongation cycle has been further delineated by Liao et al. (2010) using the HIV and HTLV-1 frameshift signals. Their work suggests three different pathways are used when heptameric slippery sites are encountered.

4.2 P-site slippage

When any rule is made, the exceptions are usually revealed shortly thereafter and this was the case with simultaneous slippage. The Potato Virus M (PVM) and Measles virus slippery sites did not conform to the heptameric slippery site rules of the simultaneous slippage model, only one tRNA could re-pair in the new frame while maintaining more than two out of three codon:anticodon base pair interactions. Gramstat et al., (1994) were able to demonstrate that a frameshift protein was made by slippage on a four nucleotide sequence from PVM. This sequence was flanked by two termination codons, the first in the –1 frame and the latter in the zero-frame (U AGA AAA UGA; spacing indicates the zero-frame and stop codons are underlined). Frameshifting was abolished if a single point mutation were made in the poly-A stretch, but maintained at wild type levels if the poly-A stretch was substituted with poly-U. These results demonstrate that programmed frameshifting can occur by slippage of just the peptidyl-tRNA.

Based on these results Gramstat et al., (1994) proposed the P-site slippage model. In this model decoding of the AAA codon by the lysine tRNA occurs in a normal manner and is

followed by pepdidyl transfer and translocation. While the peptidyl-tRNA is in the P-site waiting for decoding of the UGA stop codon in the A-site, a –1 frameshift event occurs at the P-site and translation elongation resumes in the new frame. The peptidyl-tRNA is able to re-pair in the –1 frame with the same amount of hydrogen bonding as the zero-frame. Unlike the frameshift signals that stimulate simultaneous slippage, the PVM frameshift signal does not require a 3′ secondary structure, instead the 3′ termination signal in the zero-frame seems to perform the same function.

A second example of P-site slippage was described that requires a 3′ stimulatory structure. Liston and Briedis (1995) used protein sequencing to pinpoint the frameshift site in the Measles virus P protein coding region to a CCG proline codon in the C UCC CCG sequence. The preceding zero-frame serine codon (UCC) could allow slippage of the decoding proline tRNA while maintaining two of three base-pairing interactions on the –1 frame CCC sequence. However, the nucleotide 5′ of the serine codon is a cytosine so that if the $tRNA^{Ser}$ slipped to the -1 frame CUC codon it would only retain a Watson-Crick interaction in the third codon:anticodon position, the wobble position. Liston and Briedis used deletion mutants to show that –1 PRF in the measles virus requires a downstream stem-loop structure for efficient frameshifting. Thus, the P-site slippage model posits that the A-site is decoded slowly because of a downstream feature or competition from the termination factors and this allows the peptidyl-tRNA time to un-pair from the zero frame and re-pair in an alternative reading frame.

One feature of the P-site slippage model is that if the A-site is not occupied then it is possible that slippage could occur in either direction (Baranov et al., 2004). There is some evidence that this can happen in wheat germ and rabbit reticulocyte lysates and in human hepatoma cells (Choi et al., 2003). The experiments that demonstrate this were performed using a frameshift signal derived from a viral internal ribosome entry site. A protein from the Hepatitis C Virus (HCV) was identified and labeled "F" (Walewski et al., 2001; Xu et al., 2001). It was initially thought that this viral protein was derived from a frameshifting event but this notion was later disproved (Vassilaki and Mavromara, 2003) and it is now thought that protein F is translated from an internal ribosome entry site (IRES) (Baril and Brakier-Gingras, 2005). However, before the IRES function of the HCV RNA was revealed, Choi et al., (2003) set up an *in vitro* expression system that lacked most of the IRES but from which transframe proteins (both –1 frame and a +1 frame products) were detected. They hypothesized that frameshifting occurred at a poly-A stretch (A AAA AAA AAC) and demonstrated that the frequency of either –1 or +1 frameshifting was approximately 2% in Huh7 cells. When the slippery site was mutated such that slippage could occur at only one position (A AGA AAA ACC) +1 and –1 frameshifting still occurred albeit at a reduced efficiency. This lower level of frameshifting could be increased 2-3 fold by adding the elongation inhibitor puromycin, an antibiotic that allows more time for frameshifting to occur by slowing the completion of the elongation cycle. This demonstrated that frameshifting of the P-site tRNA can occur, in either direction, before an aminoacyl-tRNA is accommodated in the A-site.

Some yeast ribosomal L5 mutant alleles affected the frameshifting efficiency of both L-A and Ty-1 and had decreased affinity for the peptidyl-tRNA suggesting that P-site fidelity affects -1 and +1 PRF (Meskauskas and Dinman, 2001). Thus, the nature of the tRNA:mRNA interaction and the ribosome's affinity for the tRNA at the P-site, along with the length of time this interaction is maintained are important determinants of frameshifting efficiency.

4.3 Out of frame binding and +1 slippage

At the same time that evidence for the simultaneous slippage and P-site slippage models were being unraveled a new class of frameshift signals emerged. These were characterized by their lack of homogeneity in the heptameric slippery sites: the peptidyl-tRNAs could not re-pair in the new (+1) reading frame and establish more than one of the three possible codon:anticodon base pair interactions. It was postulated that some frameshift events are due to the incoming aminoacyl-tRNA binding out of frame rather than slipping. The concepts from the initial discoveries and comparisons of frameshifting in yeast retrotransposons can be extended to help explain the frameshifting mechanisms in genomic, mitochondrial and cilate genes as well as those in viruses.

In 1986 Wilson et al. established that the yeast retrotransposon Ty1 used a +1 frameshift to express the protein encoded by the second open reading frame (ORF) TYB. They showed that a 31 nucleotide stretch of sequence from the overlap of ORFs TYA and TYB was required and sequence comparisons suggested that a conserved 11 nucleotide sequence was important. Belcourt and Farabaugh (1990) later showed that frameshifting required only a heptameric sequence (CCU AGG C) and that disruption of either zero frame codon reduced frameshifting. It was established that the leucyl-tRNA in the P-site slipped forward before the A-site is decoded. The proposal that a pause was required was demonstrated when Xu and Boeke (1990) showed that Ty1 retrotransposition, which requires frameshifting, was reduced when a rare $tRNA^{Arg}$ that decodes the A-site codon was expressed at higher levels. In a complementary experiment, frameshifting was reduced when the *HSX1* gene encoding $tRNA^{Arg}$ was deleted (Kawakami et al., 1993). No other *cis*-acting elements are apparently necessary for +1 frameshifting in these constructs.

While sequence homology between the yeast retrotransposons Ty3 and Ty1 initially suggested that the +1 frameshift mechanism might be similar in both retrotransposons (Hansen et al., 1988) it was later shown that the mechanism is quite different. The Ty3 heptameric frameshift sequence (GCG AGU U) is decoded by a peptidyl-$tRNA^{Ala}$ that is unable to slip forward (Farabaugh et al., 1993) implying that the aminoacyl-tRNA binds out of frame. It was also initially suggested that the +1 frameshift signal (UCC UGA U) in the cellular ornithine decarboxylase transcript was due to out of frame binding at the A-site (Matsufuji et al., 1995). This frameshift signal contains a termination codon in the zero frame A-site. It has subsequently been argued that slippage occurs on the ornithine decarboxylase transcript by detachment of peptidyl $tRNA^{Ser}$ from UCC and re-pairing to CCU (Baranov et al., 2004). The scarcity of the aminoacyl-tRNA for the Ty3 is limiting, indicating that a pause is required. Vimaladithan and Farabaugh (1994) showed that increasing the abundance of the tRNA decreased frameshifting and removal of the tRNA enhanced frameshifting. The nature of the codon:anticodon interaction at the P-site is proposed to leave the first base of the A-site codon unpaired at tRNA accommodation (Sundararajan et al., 1999). Displacement of this nucleotide results in the incoming tRNA recognizing the +1 frame codon. This displacement is facilitated by a stimulatory element.

A 3′ stimulatory element enhances Ty3 +1 frameshifting and has been shown to enhance frameshifting from a variety of heptameric frameshift sites (including the Ty1 frameshift sequence) unless there is a termination codon in the A-site (Li et al., 2001b). The positioning of the stimulator was shown to be crucial, moving it as little as one nucleotide downstream

reduced frameshifting. This led to the hypothesis that it may stimulate frameshifting by binding to the 18S rRNA (Li et al., 2001b). A similar stimulatory sequence is present 3′ from the antizyme decarboxylase slippery site (Ivanov and Atkins, 2007). There is no apparent homology between the Ty3 stimulatory element and the diverse 3′ antizyme sequences, and because the antizyme frameshift sites have a conserved termination signal and specific P-site, requirements which the Ty3 frameshift site lacks, this supports the notion that there are subtle differences in the mechanisms that promote +1 out of frame binding.

4.4 A-site contributions

It has been suggested that the equine arteritis virus slippery site is unusual because it does not conform in sequence to other heptameric slippery sites (G UUA AAC) (den Boon et al., 1991). However, this slippery site has been shown to promote efficient –1 PRF in the context of a coronavirus pseudoknot (Brierley et al., 1992). Because of the existence of this and other proposed slippery sites that did not seem to fit with earlier models of –1 PRF Napthine et al., (2003) investigated the importance of the A-site in driving eukaryotic –1 PRF using a series of constructs based on the IBV frameshift signal. In vitro translations reactions were performed in tRNA depleted wheat germ lysates that were supplemented with either *E. coli* or calf liver tRNA populations. Efficient frameshifting was observed for slippery sites X XXA AAC when the lysate was supplemented with eukayotic tRNAs or the *E. coli* tRNAs. Frameshifting was substantially lower when the calf liver tRNAs and the slippery site X XXA AAG were used but not when the *E. coli* tRNAs were used. The reasons for this are thought to be due to the abundance of tRNAs in the different cell types (Napthine et al., 2003 and references within). *E. coli* contains only one $tRNA^{Lys}$ isoacceptor with the anticodon 3′UUU (which is modified at the wobble position) to decode both AAG and AAA, thus explaining the abundance of slippery sites in bacteria ending with A AAG. Mammalian cells contain an additional two $tRNA^{Lys}$ isoacceptors, one with the anticodon 3′UUC could outcompete the 3′UUU anticodon-containing tRNA for the AAG codon explaining the lack of eukaryote slippery sites utilizing A AAG.

Frameshifting (when either eukaryote or eubacterial tRNAs were added) was reduced when mutations were made to the –1 frame P-site positions (underlined) in the U UUA AAC slippery site. Less of an effect was apparent when the U UUA AAG slippery site and P-site mutations were assayed with the *E. coli* tRNAs. However, frameshifting levels were still quite high prompting the suggestion that slippage may be occurring in the A-site alone as the P-site mutations proposed to minimize post-slippage base-pairing interactions did not actually eliminate frameshifting. This result was most apparent with the X XXA AAG slippery sites and *E.* coli tRNAs (Napthine et al., 2003).

From the above experiments, and in conjunction with the slippery site analysis Brierley et al. (1992) performed in rabbit reticulocyte lysates, it appears that the sequence requirements are not absolute. These results suggest that the A-site codon:anticodon interactions are more important for slippage in the system described and that the P-site interactions or peptidyl-tRNAs do not play a pivotal role in –1 PRF. Another possible explanation of these results could be that –1 PRF requires some interaction between the aminoacy-tRNA and the peptidyl-tRNA in addition to the codon:anticodon interactions. That is, the efficiency of frameshifting is dependent, in part, on the type of tRNA used, particularly in the A-site.

5. Frameshift signals as antiviral targets

Most frameshift signals regulate production of an enzyme involved in viral replication. Altering this regulation can disrupt replication. Thus, frameshift signals are ideal targets for antivirals. The diversity of Programmed Ribosomal Frameshift (PRF) signals among, and within, different viral families means that each signal provides specific, unique features that can be used as drug targets. Specific features of both HIV-1 and coronaviruses have been used as antiviral targets and are described below. Modulation of frameshifting affects the viability of both types of viruses even though frameshifting regulates different proteins for each virus.

The generality of the frameshifting mechanism suggests that there could be opportunities to modulate frameshifting by targeting cellular factors that affect PRF (Dinman et al., 1997). A possible caveat with this approach is the demonstrated presence of frameshift signals in non-viral, host genomes (Baranov et al., 2011; Belew et al., 2010; Manktelow et al., 2005). Programmed frameshifting on non-viral transcripts has also been linked to the mRNA decay pathway in yeast cells (Plant et al., 2004). Until we have a better understanding of the extent of host cell usage of PRF caution should be used when designing antivirals that affect general frameshifting mechanisms. That said, cellular factors have been identified that are able to modulate PRF (Koybayashi et al., 2010; Meskauskas and Dinman, 2001).

A screen of cellular factors identified some factors which, when knocked down by RNA interference (RNAi), reduced HIV replication. Further investigation of a subset of these factors, specifically those likely to affect translation, resulted in the identification of eukaryote release factor 1 (eRF1) as a protein of interest (Kobayashi et al., 2010). Characterization of the mode of action demonstrated that it was through the up-regulation of frameshifting. Translation termination factor eRF1 has a tertiary structure that mimics a tRNA structure allowing it to recognise all three termination codons when they enter the ribosomal A-site. Kobayashi et al. observed an increase in reverse transcriptase activity compare to HIV gag protein when eRF1 was knocked down by RNAi. The increase in frameshifting was not due to a change in ribosome pausing indicating that the reduced amount of eRF1 was not affecting translation elongation efficiency. Given the description of the A-site contribution to frameshifting described above, it is plausible that the mode of action is due to an interaction between eRF1 and the P-site tRNA. However, this has not been demonstrated so it is possible that eRF1 is modulating -1 PRF through other mechanisms.

5.1 Targeting retrovirus frameshift signals

Retroviral systems like HIV regulate the ratios of structural (gag) protein and enzymatic (pol) protein via PRF. It has been proposed that maintaining the ratio of structural and enzymatic proteins is important for viral propagation. Increasing frameshifting efficiency activated the HIV-1 protease and inhibited the budding and assembly of virus-like particles (Karacostas et al., 1993). Hung et al. (1998) showed that there was a direct correlation between frameshifting and inhibition of HIV-1 replication; a two- to three-fold increase in gag-pol production inhibited particle formation. Also, it has been shown that maintaining the optimal gag/gag-pol ratio is important for HIV RNA dimerization (Shehu-Xhilaga et al., 2001). Biswas et al., (2004) demonstrated that a decrease in frameshifting levels below 10% of normal activity abolished virus production. It has been shown for another lentivirus, Equine anemia infectious virus, that an 80% reduction in frameshifting abolishes viral replication (Chen and Montelaro, 2003), strengthening the assertion that optimal ratios of gag protein to gag-pol polyprotein are required for successful retroviral replication.

An RNA feature of the HIV frameshift signal has been used as an antiviral target. It has been shown by NMR that the 3′ stimulatory structure for some HIV strains is a stem-loop (Gaudin et al., 2005; Staple & Butcher, 2005). This stem-loop is often refered to as the HIV frameshift stimulatory sequence (FSS) in the literature. The FSS sequence is conserved in most group M subtypes (Baril et al., 2003). It has an uncommon 5′-ACAA-3′ tetraloop capping the stem and a purine bulge that creates a bend in the structure (Figure 3G). A bent stem has also been observed in some other viral frameshift stimulating structures (Chen et al., 1996; Chung et al., 2010). A well defined RNA molecule like the HIV-1 FSS makes a suitable target to screen for compounds able to bind. Indeed, it was found that some compounds are able to bind the upper portion of the HIV stem-loop with high affinity (Palde et al., 2010; Staple et al., 2008). Increasing or decreasing the stability of this stem-loop will likely affect frameshifting. The lower stem was not used in screens as it unfolds more readily than the upper stem-loop (Mazauric et al., 2009) and must be single-stranded to fit into the mRNA entry tunnel of the ribosome so that the slippery site is correctly positioned.

Some molecules have been identified that bind to the FSS and alter the stability of the structure. Staple et al., (2008) investigated the binding of a modified synthetic aminoglycoside to the HIV FSS. Using NMR they were able to show that Guanidinoneomycin B bound to the major groove of the upper stem-loop and this resulted in the repositioning of the 5′-ACAA-3′ tetraloop. It remains to be determined if the binding of the molecule results in a change in frameshifting efficiency. Oligonucleotides designed to match different portions of the HIV FSS also bound to the target (Vickers & Ecker, 1992). Interestingly, only those binding to the 3′ portion of the FSS altered frameshifting. This demonstrates how our lack of understanding of how specific stimulatory elements facilitate frameshifting makes antiviral design difficult.

Marcheschi et al., (2009) replaced the guanosine in the HIV FSS purine bulge with a fluorescent analog, 2-aminopurine. By measuring fluorescence they were able to monitor the stability of the HIV stem-loop as different compounds were applied. This screen led to the identification of Doxorubicin as a frameshift inhibitor. Although it had good affinity with a K_D of 2.8μM Doxorubicin is also unfortunately a general translation inhibitor. However, the identification of a lead compound with some selectivity and specificity demonstrates the utility of this approach.

Another good lead compound was identified by a screen of a resin bound dynamic combinatorial library with the upper stem of the HIV FSS (McNaughton et al., 2007). Subsequent analysis of this compound and derivatives of it demonstrated that it had high affinity and good selectivity (Palde et al., 2010). In solution the K_D of one derivative was 0.18μM. Additionally it was shown that this compound was non-toxic to human cells at concentrations up to 1.0mM. Although it remains to be determined if any of these compounds significantly alter frameshifting and are detrimental to HIV replication, the identification of these compounds, and knowledge of the features that affect selectivity and affinity is encouraging.

5.2 Targeting coronavirus frameshift signals

In contrast to the retroviruses and Totiviruses, which regulate structural and enzymatic protein production by frameshifting, coronaviruses use frameshifting to modulate the ratio of two large polyproteins that both have enzymatic functions. Together two overlapping open reading frames form the replicase gene. The polymerase encoded by coronaviruses is in the

second open reading frame, downstream from a frameshift signal near the end of the first open reading frame. Experiments targeting the frameshift regions of the SARS coronavirus and the feline coronavirus have been performed. For both viruses, disrupting the frameshifting mechanism results in a decrease in infectivity. Given the complexity of the coronavirus lifecycle it is not certain how a reduction in frameshifting leads to the loss of infectivity, but clearly the abundance of proteins translated by the frameshifting mechanism is important.

A minimal level of frameshifting has been shown to be essential for SARS coronavirus replication. Using reverse genetics mutant viruses with varying levels of frameshifting efficacy were made. Reduction of frameshifting to 10% of the normal level completely abrogated the production of infectious virus particles (Plant et al., 2010). Suboptimal frameshifting in the SARS coronavirus reduces the amount of genomic RNA more so than the reduction of subgenomic RNA (Plant et al., 2010). However, this effect was not apparent for the feline coronavirus which has fewer subgenomic RNAs (McDonagh et al., 2011) indicating that more work is needed to understand the mechanics of frameshifting in the context as an antiviral target. Frameshifting occurs during the initial translation of the coronavirus genome early in infection and it is not known if the levels of frameshifting remain the same throughout infection. Therefore timing of an anti-frameshifting treatment may be an important consideration.

The SARS frameshift signal has been used as an antiviral target by several groups using different approaches. Frameshifting efficiency has been altered by four different approaches so far; RNA interfence, peptide-conjugated antisense morpholino oligomers (P-PMOs), antisense peptide nucleic acids (PNAs), and antiviral compounds.

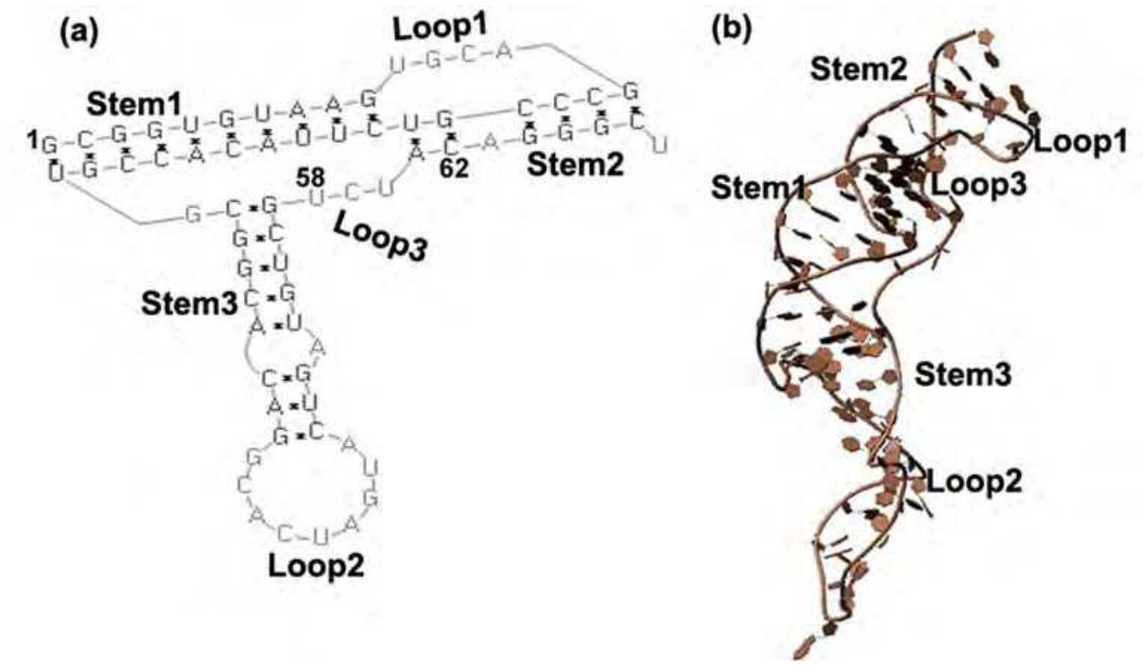

Fig. 4. A) 2-D diagram of the SARS pseudoknot structure. B) 3-D model determined by molecular dynamics simulation. Reprinted with permission from Park et al., 2011, Identification of RNA Pseudoknot-Binding Ligand That Inhibits the -1 Ribosomal Frameshifting of SARS-Coronavirus by Structure-Based Virtual Screening. Copyright 2011 American Chemical Society.

An RNAi approach to inhibit feline coronavirus replication was established by McDonagh et al. (2011). Short interfering RNAs (siRNAs) were designed to target different regions of the genome including the replicase gene. One siRNA targeted the frameshift signal and another targeted the region upstream of the frameshift signal. Both siRNAs reduced the copy number of genomic and subgenomic RNA to a similar extent. However, although both siRNAs also reduced the relative viral titer, the siRNA targeting the frameshift signal caused a significantly greater reduction (McDonagh et al., 2011). P-PMOs directed at the SARS coronavirus frameshift signal and the initiating codon of the replicase gene have been investigated (Neuman et al., 2005). Both P-PMOs resulted in a similar reduction in virus titer. However, in this instance, the reduction in plaque size was more pronounced for P-PMOs targeting the initiating codon of the replicase gene. This suggests that disruption of translation initiation is more effective than an RNAi target within the ORF at limiting viral propagation. Even so, PNAs targeting the pseudoknot caused a significant reduction in the replication of a SARS replicon at concentrations in the low μM range validating the selection of the pseudoknot as an antiviral target (Ahn et al., 2011).

Using molecular dynamics simulation Park et al., (2011) generated a model for the SARS coronavirus frameshift-stimulating pseudoknot. They then used this to screen a database for compounds that might be able to bind to the structure. Several hits were experimentally tested and found to bind to the pseudoknot RNA. The lead compound bound with high affinity and disrupted frameshifting. The effect of this compound on coronavirus replication has not be determined yet.

6. Conclusion

Viruses manipulate host cells in a number of different ways in order to replicate and thrive. The mechanisms used by viruses to manipulate the host cell are varied and evolve as the host defenses evolve. Programmed ribosomal frameshifting is one approach to manipulate host ribosomes that is used by a wide variety of viruses. There are multiple mechanisms that drive frameshifting and a diverse array of viral sequences and structures that stimulate frameshifting. While the position of some frameshift signals upstream of an RNA-dependent RNA polymerase in some viral genomes suggests these signals may have evolved from a common ancestor, the presence of frameshift signals upstream of other genes indicates that frameshifting signals may have developed independently in some instances. The essential nature of frameshifting for many of the viruses described here makes frameshifting an attractive target for antivirals. While some antivirals are being developed for HIV and coronaviruses there are many agriculturally important viruses that have not received attention. Current limitations stem from the diverse nature of frameshift signals amongst virus groups and the different mechanisms driving frameshifting. Although the signals and mechanisms are diverse, an underlying feature of frameshifting is that all these signals alter ribosome fidelity. A clearer understanding of the critical features of frameshift signals and how these alter ribosome fidelity will enhance our ability to develop new antivirals.

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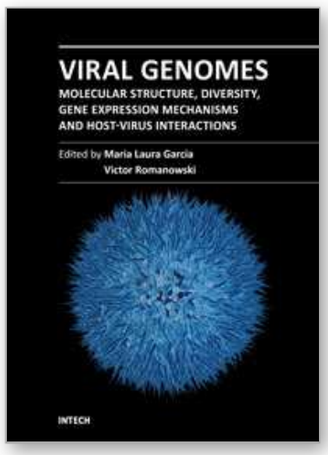

Viral Genomes - Molecular Structure, Diversity, Gene Expression Mechanisms and Host-Virus Interactions

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Viruses are small infectious agents that can replicate only inside the living cells of susceptible organisms. The understanding of the molecular events underlying the infectious process has been of central interest to improve strategies aimed at combating viral diseases of medical, veterinary and agricultural importance. Some of the viruses cause dreadful diseases, while others are also of interest as tools for gene transduction and expression and in non-poluting insect pest management strategies. The contributions in this book provide the reader with a perspective on the wide spectrum of virus-host systems. They are organized in sections based on the major topics covered: viral genomes organization, regulation of replication and gene expression, genome diversity and evolution, virus-host interactions, including clinically relevant features. The chapters also cover a wide range of technical approaches, including high throughput methods to assess genome variation or stability. This book should appeal to all those interested in fundamental and applied aspects of virology.

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Phone: +385 (51) 770 447
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