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Viral Gastroenteritis
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V, 2. Ribosomal frameshifting in astroviruses

Ian Brierley and Marijana Vidakovic

Division of Virology, Department of Pathology, University of Cambridge, Cambridge CB2 1QP, U.K.

Introduction

For the vast majority of eukaryotic mRNAs, translation initiation is a 5'-end dependent process beginning with recognition of the cap structure by the cap-binding complex eIF4F (Pestova *et al.*, 2001). Subsequently, a scanning ribosome complex is assembled which migrates along the mRNA until an AUG codon is encountered in a suitable context (Kozak, 1983). At this point, the elongation phase of protein synthesis begins and the ribosome translates along the mRNA in triplet steps, the reading frame being set by that of the initiator AUG. Upon encounter of an in-frame termination codon, the polypeptide is released and the ribosome dissociates from the mRNA. Thus for most mRNAs, only the 5'-most open reading frame (ORF) is translated and downstream ORFs, at least in principle, are not reached by the ribosome. This 5'-end dependence is a problem faced by many RNA viruses with polycistronic genomes and elaborate strategies have been developed to overcome the translational limitation. Some viruses produce subgenomic-length mRNAs in which the relevant downstream ORF is effectively moved to the 5'-end of the RNA from where it can be efficiently translated. Where the replication cycle involves a nuclear step, RNAs can be spliced by the cellular machinery, and many cytoplasmically-replicating viruses have evolved mechanisms to produce subgenomic mRNAs during transcription. In other viruses, the 5'-end problem is obviated simply by encoding all of the required information in a single ORF and subsequently processing the encoded polyprotein proteolytically.

However, viruses have also evolved a number of unconventional translation strategies to express distal ORFs. These include leaky scanning, where the AUG of the 5'-most ORF is inefficiently recognised and ribosomes scan on to initiate at a downstream ORF; ribosomal re-initiation, where a post-termination ribosome remains associated with the mRNA and reinitiates translation at a downstream ORF, and translational fusion, where two (or more) ORFs separated by a stop codon or in an overlapping configuration are translated as a single protein following termination codon suppression or programmed ribosomal frameshifting respectively (reviewed in Pe'ery and Mathews, 2000). Of these unconventional mechanisms, the best studied is programmed –1 ribosomal frameshifting, a process where specific signals in the mRNA instruct the ribosome to change reading frame from the zero to the –1 frame (movement 5'-wards) at a certain efficiency, and

to continue translation in the new frame (see Chandler and Fayet, 1993; Brierley, 1995; Dinman, 1995; Futterer and Hohn, 1996; Farabaugh, 2000; Brierley and Pennell, 2001 for reviews). A growing number of viruses are found to employ frameshifting during their replication cycle, including many retroviruses, several eukaryotic positive-strand RNA viruses, double-stranded RNA viruses of yeast, some plant RNA viruses and certain bacteriophages. In most of the systems studied to date, frameshifting is involved in the expression of replicases. In retroviruses, it allows the synthesis of the Gag-Pol and Gag-Pro-Pol polyproteins from which reverse transcriptase is derived, and for most other viruses, frameshifting is required for expression of RNA-dependent RNA polymerases. In this article, we will review ribosomal frameshifting with an emphasis on the frameshifting process in astroviruses.

Programmed –1 ribosomal frameshifting signals

Eukaryotic ribosomal frameshift signals contain two essential mRNA elements: a "slippery" sequence, where the ribosome changes reading frame, and a stimulatory RNA secondary structure, usually an RNA "pseudoknot", located a few nucleotides downstream (Jacks *et al.*, 1988a; Brierley *et al.*, 1989; ten Dam *et al.*, 1990). A spacer region between the slippery sequence and the stimulatory RNA structure is also required, and a precise length of this spacer must be maintained for maximal frameshifting efficiency (Brierley *et al.*, 1989, 1992; Kollmus *et al.*, 1994). The slippery sequence is a heptanucleotide stretch that contains two homopolymeric triplets and conforms in the vast majority of cases to the motif XXXYYYZ (for example, UUUAAAC in the coronavirus infectious bronchitis virus (IBV) *1a/1b* signal). In eukaryotic systems, frameshifting at this sequence is thought to occur by "simultaneous-slippage" of two ribosome-bound tRNAs, presumably peptidyl and aminoacyl tRNAs, which are translocated from the zero (X XXY YYZ) to the -1 phase (XXX YYY) (Jacks *et al.*, 1988a; see Fig. 1). The homopolymeric nature of the slippery sequence seems to be required to allow the tRNAs to remain base-paired to the mRNA in at least two out of three anticodon positions following the slip. Frameshift assays, largely carried out *in vitro*, have revealed that the X triplet can be A, C, G or U, but the Y triplet must be A or U (Jacks *et al.*, 1988a, Dinman *et al.*, 1991; Brierley *et al.*, 1992). In addition to these restrictions, slippery sequences ending in G (XXXAAAG or XXXUUUG) do not function efficiently in *in vitro* translation systems (Brierley *et al.*, 1992), nor in yeast (Dinman *et al.*, 1991) or mammalian cells (Marczinke *et al.*, 2000). At naturally-occurring frameshift sites, of the possible codons which are decoded in the ribosomal aminoacyl (A)-site prior to tRNA slippage (XXXY<u>YYN</u>), only five are represented in eukaryotes, AAC, AAU, UUA, UUC, UUU (Farabaugh, 1996). Together with the *in vitro* data, it seems that the sequence restrictions observed are a manifestation of the need for the pre-slippage codon-anticodon complex in the A-site to be weak enough such that the tRNAs can detach from the codon during the process of frameshifting. G-C pairs are therefore avoided.

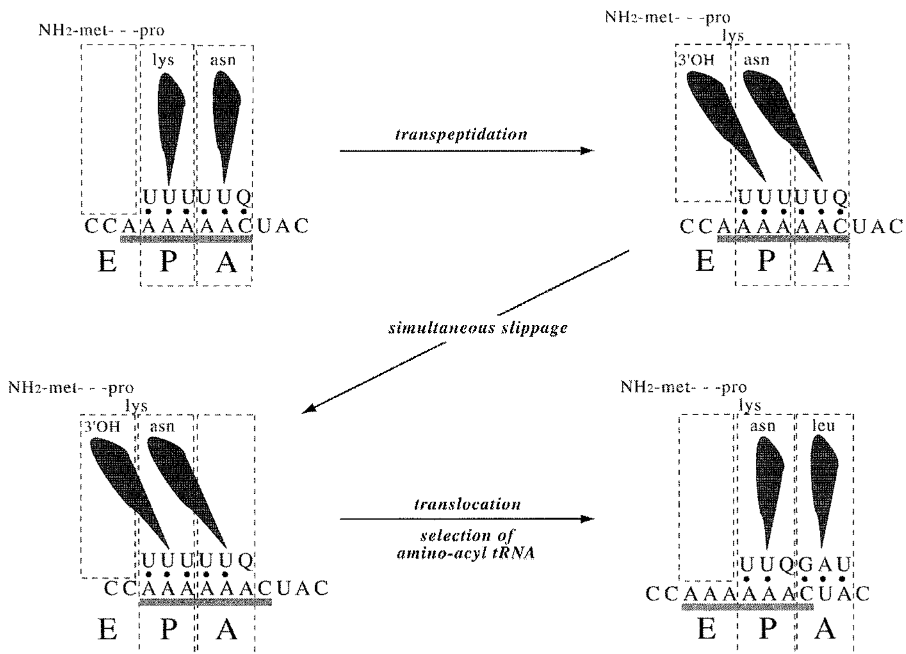

Fig. 1. The simultaneous slippage model of programmed –1 ribosomal frameshifting. The model shown is a variant of the original (Jacks *et al.*, 1988a) as proposed by Weiss *et al.* (1989). The first stage shows the amino-acyl- (A) and peptidyl- (P) tRNAs base-paired to the slippery sequence (AAAAAAC) in the zero frame before transpeptidation. In the second stage, the tRNAs are still in the zero frame but occupy hybrid sites (P-A and E (exit)-P), based on the displacement model for the peptidyl transfer reaction (Moazed and Noller, 1989). The third stage shows the tRNAs slipping back by one nucleotide, retaining (in the case of this slippery sequence) a total of five of six anticodon-codon base-pairs. In the fourth step, the incoming $tRNA^{Leu}$ decodes the –1 frame codon, completing the cycle. Q represents the base queuosine, the hypermodified derivative of guanosine present at the wobble position of $tRNA^{Asn}$ (Björk *et al.*, 1999). The specific modification of the wobble base of mammalian $tRNA^{Lys}$, 5-methylcarboxymethyl-2-thiouridine (mcm^5s^2U), is not shown.

Efficient frameshifting requires the presence of a stimulatory RNA structure located a few nucleotides downstream of the slippery sequence. In some cases this is a stem-loop, but more commonly, an RNA pseudoknot is present (an example from the coronavirus IBV is shown in Fig. 2, alongside a selection of stem-loop-containing signals). These structures are sometimes referred to as frameshifter stem-loops or frameshifter pseudoknots to distinguish them from related structures in cellular RNAs. Pseudoknots are formed when nucleotides in the loop of stem-loop base-pair with a region elsewhere in the mRNA to create a quasi-continuous double-helix joined by single-stranded connecting loops (Pleij *et al.*, 1985; Hilbers *et al.*, 1998; Giedroc *et al.*, 2000).

The interaction of the ribosome with the stimulatory RNA is thought to pause ribosomes in the act of decoding the slippery sequence, allowing more time for the tRNAs to realign in the -1 reading frame (Jacks *et al.*, 1988a). Our knowledge of the folding of these RNAs has been derived from site-specific mutagenesis, chemical and enzymatic structure probing and more recently from NMR and X-ray crystallography. The topic has recently been the subject of several reviews (Hilbers *et al.*, 1998; Giedroc *et al.*, 2000; Brierley and Pennell, 2001).

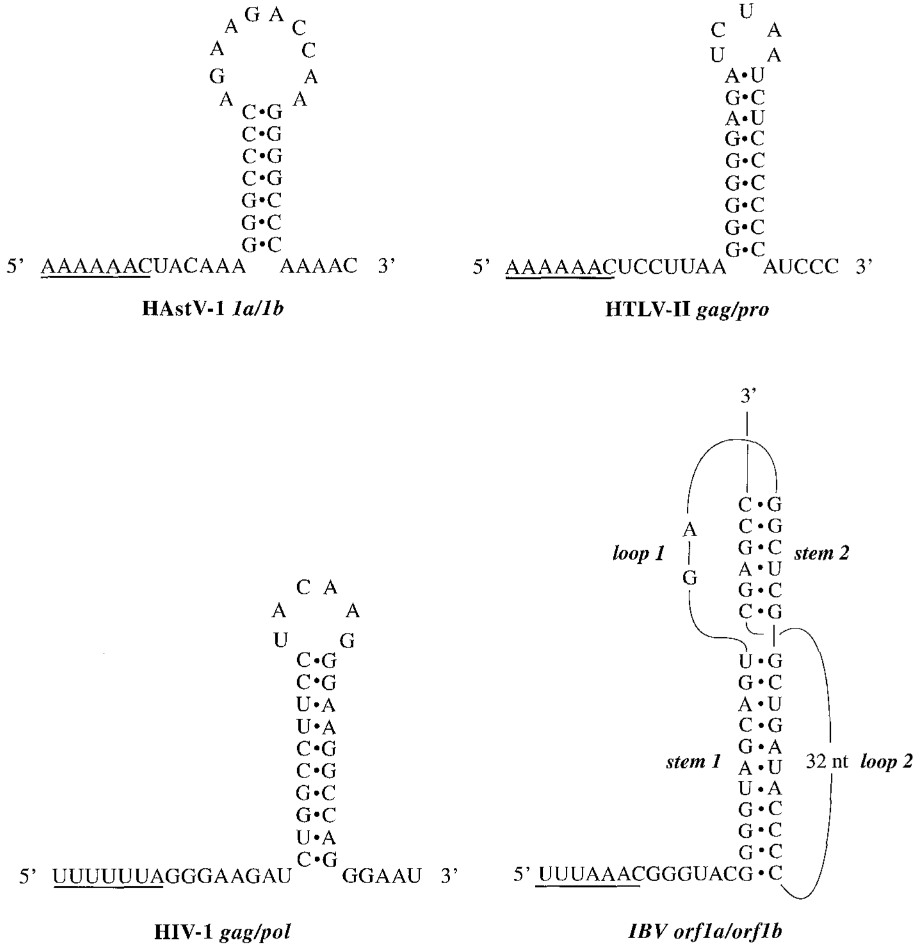

Fig. 2. Examples of stem-loop- and pseudoknot-containing ribosomal frameshift signals. Illustrated are the stem-loop signals of HAstV-1 *1a/1b* (Marczinke *et al.*, 1994), HTLV-II *gag/pro* (ten Dam *et al.*, 1990), HIV-1 *gag/pol* (Jacks *et al.*, 1988b; Kang, 1998) and the pseudoknot of the coronavirus IBV (Brierley *et al.*, 1991). The slippery sequences are underlined.

Astrovirus frameshifting signals

Astroviruses are small, non-enveloped viruses with a positive-sense, single-stranded RNA genome of about 7kb in length. Human astrovirus serotype 1 (HAstV-1; Willcocks *et al.*, 1994), like all astroviruses, contains three sequential open reading frames (ORFs) designated ORFs 1a, 1b and 2. The most 3' coding region, ORF 2, encodes the viral structural proteins (Matsui *et al.*, 1993; Lewis *et al.*, 1994) and is expressed from a sub-genomic mRNA (Monroe *et al.*, 1991; Matsui *et al.*, 1993; Geigenmüller *et al.*, Section V, Chapter 1 of this book). ORFs 1a and 1b contain amino-acid motifs indicative of non-structural proteins (reviewed in Cubitt, 1996), and a characteristic YGDD motif found in the RNA-dependent RNA polymerases of a variety of RNA viruses (Kamer and Argos, 1984) is located in ORF 1b. Initially, the mechanism of expression of this ORF was uncertain, since it overlapped ORF1a by 71 nucleotides and was in the –1 reading frame with respect to ORF1a. An examination of the sequence information present within the overlap region of the two ORFs suggested that ORF1b was expressed as a translational fusion with the upstream 1a ORF following a –1 ribosomal frameshift event within the ORF1a/1b overlap region (Jiang *et al.*, 1993; Lewis *et al.*, 1994; Willcocks *et al.* 1994). This was subsequently confirmed experimentally in *in vitro* translation systems; the HAstV signal was shown to cause some 5-7% of ribosomes to frameshift (Marczinke *et al.*, 1994; Lewis and Matsui, 1995). The frameshift signal of HAst-1 is one of the simplest described to date, comprising the slippery sequence AAAAAAC and a small GC-rich stem-loop located some six nucleotides (nts) downstream (see Fig. 2). Frameshifting at this signal was tested by cloning a region of the astrovirus genome containing the putative frameshift signal into a plasmid-borne reporter gene and performing *in vitro* transcription and translation studies using the rabbit reticulocyte lysate (RRL) system (Marczinke *et al.*, 1994). Site-specific mutagenesis confirmed the site of frameshifting as the AAAAAAC sequence, the presence of the stem region and also that the primary sequence of the loop nucleotides was unimportant, arguing against the presence of a pseudoknot. Further experimental evidence that the HAst-1 stimulatory RNA is a stem-loop and not a pseudoknot was obtained by RNA structure mapping of the *in vitro* transcripts, which indicated that the loop nucleotides were accessible to single-strand specific chemical and enzymatic probes (Marczinke *et al.*, 1994). A study of astrovirus frameshifting in an infection-transfection transient expression system also found that a minimal cassette of slippery sequence and downstream stem-loop was sufficient to induce efficient frameshifting both *in vitro* and *in vivo* arguing against the need for pseudoknot formation (Lewis and Matsui, 1996). The astrovirus signal closely resembles that present at the human T cell lymphotropic virus type II (HTLV-II) *gag/pro* junction and is similar to that used by human immundodeficiency virus type 1 (HIV-1) at the *gag/pol* junction, although the latter virus employs a U-rich rather than an A-rich slippery sequence (Fig. 2).

The complete genomic sequences of several human and animal astroviruses have been determined in the last few years. Fig. 3a shows an alignment of the sequences of the frameshift region of these astroviruses, and in Fig. 3b the predicted folding of

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HAstV-1 [W]          ---GCAGGUUUGGAAGGUUUUCUCCAAAGGGUUAAAUCGAAAAACAAGGCCCCAAAAAAC 57
HAstV-1 [L]          ---GCAGGUUUGGAAGGUUUCCUCCAAAGGGUUAAAUCAAAAAACAAGGCCCCAAAAAAC 57
HAstV-2              ---GCAGGUCUAGAAGGUUUCCUCCAAAGAGUUAAAUCGAAAAACAAGGCCCCAAAAAAC 57
HAstV-5              ---GCAGGUCUAGAAGGUUUCCUCCAAAGAGUUAAAUCGAAAAAUAAGGCUCCAAAAAAC 57
HAstV-8              ---GCAGGUUUAGAAGGCUUCCUCCAAAGGGUUAAGUCAAAAAACAAGGCCCCAAAAAAC 57
HAstV-3              ---GCUGGUUUGGAAGGCUUUCUCCAGAGGGUCAAAUCAAAAAAUAAGGCUCCAAAAAAC 57
OAstV                -GAGCAUGGACUAAUGCCUUUCACUCAGCG--UAGGAAGCGUGUCCAGCAGCCAAAAAAC 57
TAstV                UUUUCAGGAAUUGAGAAGUUAG---AAGAUCAUGUGGUCAGUGGAGAGUGUCAAAAAAAC 57
ANV                  ---GAUUGUGGCGAGACUUUCGUUGAAAGGCAAGACUUCCACGUUUGUAAGUCAAAAAAC 57
                            *     *    **                                 *******
                                                                          slippery
                                                                          sequence

HAstV-1 [W]          UA-CAAAGGGCCCCAGAAGACCAAGGGGCCCAAAACUACCACUCAUUAGAUGCAUGGAAA 116
HAstV-1 [L]          UA-CAAAGGGCCCCAGAAGACCAAGGGGCCCAAAAUUACCACUCAUUAGAUGCGUGGAAA 116
HAstV-2              UA-CAAAGGGCCCCAGAAGACCAAGGGGCCCAAAAUUAUCACUCAUUAGAUGCAUGGAAA 116
HAstV-5              UA-CAAAGGGCCCCAGAAGACCAAGGGGCCCAAAACUACCACUCAUUAGAUGCAUGGAAA 116
HAstV-8              UA-CAAAGGGCCCCAGAAGACCAAGGGGCCCAAAACUACCACUCAUUAGAUGCAUGGAAG 116
HAstV-3              UA-CAAAGGGCCCCAGAAGACCAGGGGGCCCAAAACUACCACUCACUAGAUGCAUGGCAG 116
OAstV                UC-CAAAGGGGCCCUGAAGACCCGGGCCCCGAAGAGUGCAA---AUUAGACUACUGGGAG 113
TAstV                UAAUAGAGGGGCCUGUGACAACAAAGGCCCCUACCCCCGUACCAGAUUGGCUUAAAAUAU 117
ANV                  UA--AAUGAGCCCCCUUCGG---GGGGCUACACACCUGUCCCUGACCAUCUUAGGUGGAA 112
                     *   *  * **             *                                *
                     spacer  5'-stem  loop  3'-stem

HAstV-1 [W]          UUGUUGCUAGAGCC-UCCGCGG--- 137
HAstV-1 [L]          UUGUUGCUAGAGCC-UCCGCGG--- 137
HAstV-2              UCAUUGCUAGAACC-UCCACGU--- 137
HAstV-5              UCUCUGCUAGAACC-UCCGCGG--- 137
HAstV-8              UCGCUACUAGAACC-UCCGCGG--- 137
HAstV-3              CUACUACUGGAGGC-CCCACGG--- 137
OAstV                CAGCUUGUUGAACCAUCUAAGGAA- 137
TAstV                UUGCAUGGGAAGAUGACAUA----- 137
ANV                  CAACUGGCAAAUCUAUAUGGAACCU 137
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Fig. 3. (A). Sequence alignment of the frameshift regions of various human and animal astroviruses. The alignment was performed using the ClustalW program within the facilities of the European Bioinformatics Institute (http://www.ebi.ac.uk/index.html). Asterisks indicate fully conserved residues. The slippery sequence AAAAAAC is such a fully conserved motif. The sequences studied were from HAstV-1 [W] (Willcocks *et al.*, 1994), HAstV-1 [L] (Lewis *et al.*, 1994), HAstV-2 (Jiang *et al.*, 1993), HAstV-3 (Oh and Schreier, 2001), HAstV-5 (Lewis *et al.*, 1994), HAst-8 (Mendez-Toss *et al.*, 2000), OAstV (Jonassen *et al.*, 2000), TAstV (Koci *et al.*, 2000) and ANV (Imada *et al.*, 2000).

the stem-loop stimulatory RNAs for the various serotypes and strains is indicated. All of the astroviruses employ the slippery sequence AAAAAAC and have the potential to form a hairpin precisely six nucleotides downstream of this sequence. Within the human astroviruses (HAstV-serotype 1, 2, 3, 5, 8) there is very little sequence variation. Indeed, in the region encompassing the slippery sequence and downstream hairpin, they are identical except for HAstV-3, where the ultimate loop nucleotide is G, not A. For the mammalian astrovirus of sheep (OAstV; Jonassen *et al.*, 2001), several changes are seen within the frameshift region, but they are all predicted to be silent with respect to the ability of the signal to promote frameshifting. Most of the changes are single nucleotide substitutions within the spacer and loop regions. There is also phylogenetic support for the formation of the hairpin structure in OAstV.

Two sequence differences are observed within the stem region, but together they maintain base-pairing, generating a G-C base pair at the equivalent position of the HAstV-1 C-G pair. When the comparison is extended to avian astroviruses there is increased sequence divergence. The frameshift region of turkey astrovirus (TAstV) is still recognisably similar to that of HAstV, despite the potential for an eight base-pair stem (as opposed to seven in HAstV) and a more variable loop sequence (Fig. 3b). The TAstV signal retains the G-rich 5'-arm of the stem and a 10 nucleotide loop as seen in most HAstV. However, the frameshift signal of avian nephritis virus (ANV), a proposed

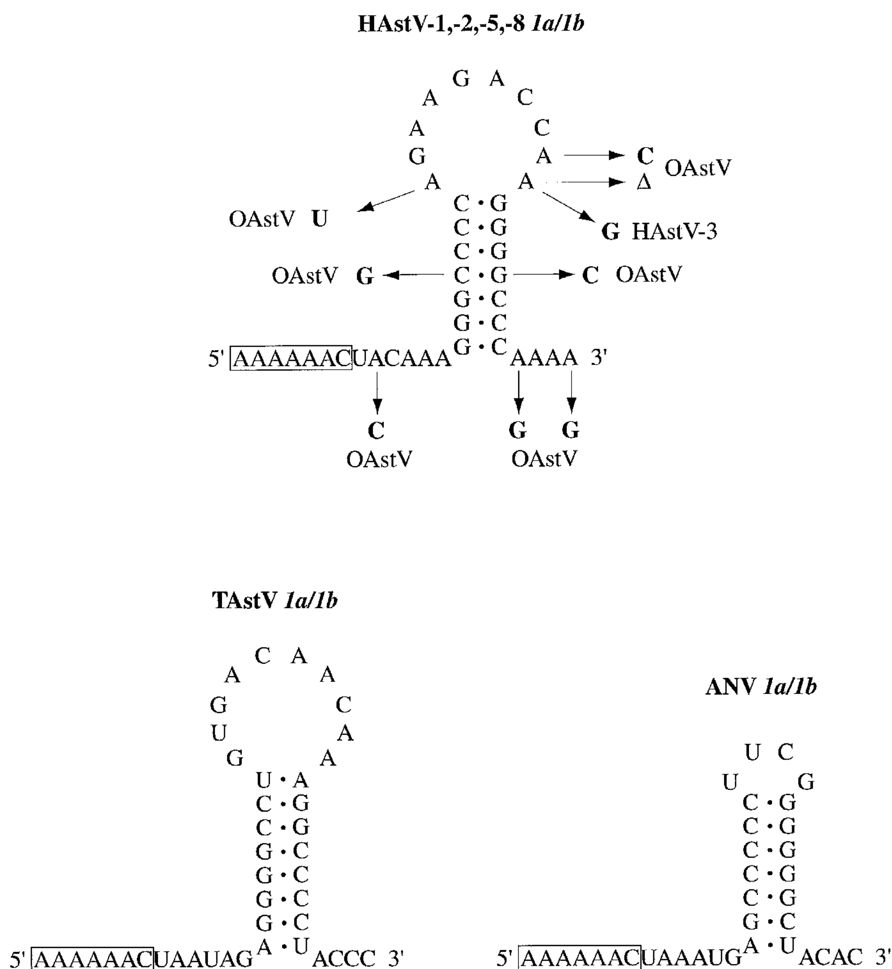


Fig. 3. (B). Secondary structure predictions of the stimulatory RNAs from the frameshift signals of HAstV-1, -2, -5, -8, TAstV and ANV. No sequence differences are present between HAstV -1, -2, -5 and -8 in the region studied.

astrovirus of chickens (Imada *et al.*, 2000), shows quite notable differences (Fig. 3b). The most obvious feature is the reduced loop size, to only four nucleotides, but also the stem has a C-rich 5'-arm as opposed to the G-rich stretch seen in other astroviruses. One interesting feature of the avian astroviruses is that the 1a termination codon (UAA) is located immediately downstream of the slippery sequence, whereas it is present after the frameshift signal in the HAstVs. There is no experimental evidence, however, to suggest that having a termination codon immediately downstream of the slippery sequence modifies the efficiency of the process (Brierley *et al.*, 1992).

The conservation of the slippery sequence in all astrovirus genomes sequenced to date suggests a preference for the use of lysyl- and asparaginyl-tRNAs in the frameshift process. There is considerable interest in whether the tRNAs involved in frameshifting are canonical tRNAs or special "shifty" tRNAs, more prone to frameshift than their "normal" counterparts (Jacks *et al.*, 1988a). In this respect, no novel tRNAs have been described as yet, but it has been noted that all the A-site tRNAs that function in frameshifting are decoded by tRNAs with a highly modified base in the anticodon loop (see Hatfield *et al.*, 1992 and references therein). In $tRNA^{Asn}$ (decoding AAC and AAU), the wobble base is queuosine (Q), in $tRNA^{Phe}$ (UUC, UUU), wyebutoxine (Y) is present just 3' of the anticodon, in $tRNA^{Lys}$ (AAA, AAG), the wobble base is 5-methylcarboxymethyl-2-thiouridine (mcm^5s^2U) (eukaryotes), and in $tRNA^{Leu}$ (UUA) 2-methyl-5-formylcytidine is present at the wobble position. Thus the tRNAs that decode the astrovirus frameshift signal ($tRNA^{Lys}$, $tRNA^{Asn}$) each have a modified wobble base. Hatfield *et al.* (1992) have raised the possibility that hypomodified variants (with the modification removed) of these tRNAs may exist which could act as specific "shifty" tRNAs, since such variants would have a considerably less bulky anticodon and be more free to move around at the decoding site. Support for this hypothesis comes from the observation that purified $tRNA^{Phe}$ populations devoid of the Y modification can stimulate frameshifting at certain slippery sequences in RRL (Carlson *et al.*, 1999, 2001). In contrast, the frameshift capacity of $tRNA^{Asn}$ appears to be uninfluenced by the absence of the Q modification in either prokaryotic (Brierley *et al.*, 1997) or eukaryotic cells (Marczinke *et al.*, 2000); thus, a simple lack of a bulky modification is not in itself sufficient to stimulate frameshifting. At present, therefore, the role of modified or hypomodified bases in frameshifting is uncertain. In some retroviruses, the relative proportions of hyper- and hypo-modified tRNAs varies in response to virus infection (Hatfield *et al.*, 1989). The possibility exists therefore that frameshift efficiencies could change during infection as a consequence of virus-induced changes in cellular tRNA modification levels. This could potentially allow the regulation of frameshifting (and hence downstream product levels) during the virus life cycle, although there is no evidence for this as yet.

Models for the ribosomal frameshifting process

Despite considerable study, the mechanism of programmed –1 ribosomal frameshifting has remained elusive. The central theory of frameshifting is that a specific ribosomal

pause occurs upon encounter of the stimulatory RNA. In its simplest form, pausing will increase the time at which ribosomes are held over the slippery sequence, allowing more time for the tRNAs to realign in the –1 frame (Jacks *et al.*, 1988a). There is good experimental evidence that pausing occurs at frameshift signals. Polypeptide intermediates corresponding to ribosomes paused at RNA pseudoknots have been detected at the frameshift sites of IBV (Somogyi *et al.*, 1993) and of the yeast double-stranded RNA virus L-A (Lopinski *et al.*, 2000), and footprinting studies of elongating ribosomes have defined the site of pausing at the L-A signal (Tu *et al.*, 1992; Lopinski *et al.*, 2000) and more recently at the IBV and simian retrovirus-1 (SRV-1) signals (Kontos *et al.*, 2001). One of the great virtues of the pausing model is its ability to accommodate the variety of stimulatory RNAs that are present at -1 frameshifting signals, including stem-loops. Notwithstanding the range of secondary and tertiary features presented to the ribosome, as long as pausing occurs, frameshifting results. Unfortunately, the idea that a pause alone is sufficient to induce frameshifting is highly questionable. Simple provision of a roadblock to ribosomes in the form of stable RNA hairpins (Brierley *et al.*, 1991; Somogyi *et al.*, 1993), a tRNA (Chen *et al.*, 1995) or even different kinds of RNA pseudoknots (Napthine *et al.*, 1999; Liphardt *et al.*, 1999) is not sufficient to bring about frameshifting. Furthermore, non-frameshifting pseudoknots and stem-loops exist that can still pause ribosomes (Tu *et al.*, 1992; Somogyi *et al.*, 1993; Lopinski *et al.*, 2000; Kontos *et al.*, 2001). These experiments, of course, do not rule out a contribution of pausing to the mechanism of frameshifting since there are no documented examples where frameshifting has occurred in the absence of a detectable pause.

Regardless of whether or not pausing is the sole mediator of frameshifting, a contributor to the process, or simply a by-product of the stimulatory RNA-ribosome interaction, it is a matter of considerable interest how it is brought about. One possibility is that the stimulatory RNAs are especially resistant to unwinding by a ribosome-associated, hypothetical RNA helicase responsible for unwinding mRNA structures ahead of the decoding centre. RNA pseudoknots have been shown to possess unusual structural features which may be refractory to standard helix unwinding, for example triple helical regions formed between pseudoknot stem 1 and loop 2 regions (Le *et al.*, 1998; Su *et al.*, 1999; Michiels *et al.*, 2001). Modelling studies predict that such triplexes are likely to be one of the first features of the pseudoknot to be encountered by the ribosome (Giedroc *et al.*, 2000), where it could function to stabilise stem 1 and increase the time taken to unwind the structure. As noted several years ago (Draper, 1990) and re-iterated recently (Michiels *et al.*, 2001), another potential barrier to unwinding is the unusual topology of the pseudoknot at the beginning of stem 1, where in addition to the two base-paired strands, loop 2 adds a third strand in close proximity (Fig. 2). Perhaps the ribosome does not deal effectively with this kind of topological arrangement. In one form or another, it has been speculated that the resistance to unwinding creates a strain in the mRNA that can only be relieved by tRNA movement, i.e. by frameshifting. Although there is no direct experimental evidence to support this hypothesis, it is a compelling one.

When frameshifter pseudoknots were first described, it was proposed that they could act as binding sites for cellular or viral proteins responsible for promoting or

regulating the frameshift process (Jacks *et al.*, 1988a; Brierley et., 1989). Up to date, however, no such pseudoknot binding proteins have been unearthed. Certainly, if such factors exist, they are not easily titratable. The addition of a large molar excess of the SRV-1 pseudoknot to an *in vitro* translation reaction programmed with an SRV-1 frameshift reporter mRNA has no effect on frameshifting (ten Dam *et al.*, 1994), and the same is true for the IBV pseudoknot (Brierley and Inglis, unpublished observations). Furthermore, several laboratories have reported a failure to identify frameshifter pseudoknot binding factors in searches using traditional band-shift and UV crosslinking methods. These experiments do not rule out the involvement of a protein or protein complex that is tightly associated with the translation apparatus, perhaps even an integral ribosomal component, since the pseudoknot may be recognised only in the context of the elongating ribosome.

Astrovirus frameshifting: stem-loop versus pseudoknot?

The question of whether the stimulatory RNA of astroviruses is a stem-loop or a pseudoknot is an important one from the perspective of models for ribosomal frameshifting which, as seen above, are mainly tailored towards pseudoknot-containing sites and rely on pseudoknot-specific features. One way in which this can be rationalised is to propose that the stem-loop stimulators are in fact pseudoknots that have not been identified yet. Most studies of frameshifter stem-loops have been carried out on regions subcloned from the context of the natural mRNA, and it is possible that some long-range interactions have been overlooked. This is certainly true for astroviruses and needs to be addressed, although the present experimental and phylogenetic evidence supports the idea that the astrovirus signal is indeed a stem-loop. The availability of infectious astrovirus cDNA clones (Geigenmüller *et al.*, 1997; Imada *et al.*, 2000) should allow an inspection of the precise sequence requirements for astrovirus frameshifting in the context of the complete genome. A second possibility is that the stem-loops themselves possess hitherto uncharacterised, novel structural features that can promote frameshifting. A high resolution structure of a frameshifter stem-loop would be informative in this regard. Of course, it may be that our models of frameshifting are incomplete. Mutational analysis of such sites has already provided hints that the traditional combination of slippery sequence and hairpin may not be the sole defining feature of the signal and that other elements may contribute. Kim *et al.* (2001) have recently measured the frameshift efficiencies evoked *in vitro* by a series of human immunodeficiency virus type 1 (HIV-1) *gag/pol* - human T-cell lymphotropic virus type II (HTLV-II) *gag/pro* chimeras. They defined four elements, namely the slippery sequence, spacer, stem-loop *and* a region upstream of the slippery sequence and combined these in various ways to create a range of hybrid sites. Surprisingly, it was found that the regions flanking the slippery sequence and stem-loop could influence frameshifting quite dramatically, possibly by modulating stem-loop unfolding kinetics. It is not yet known whether the frameshifting efficiencies of the human and animal astroviruses differ. Certainly the spacer and flanking nucleotide sequences vary, and

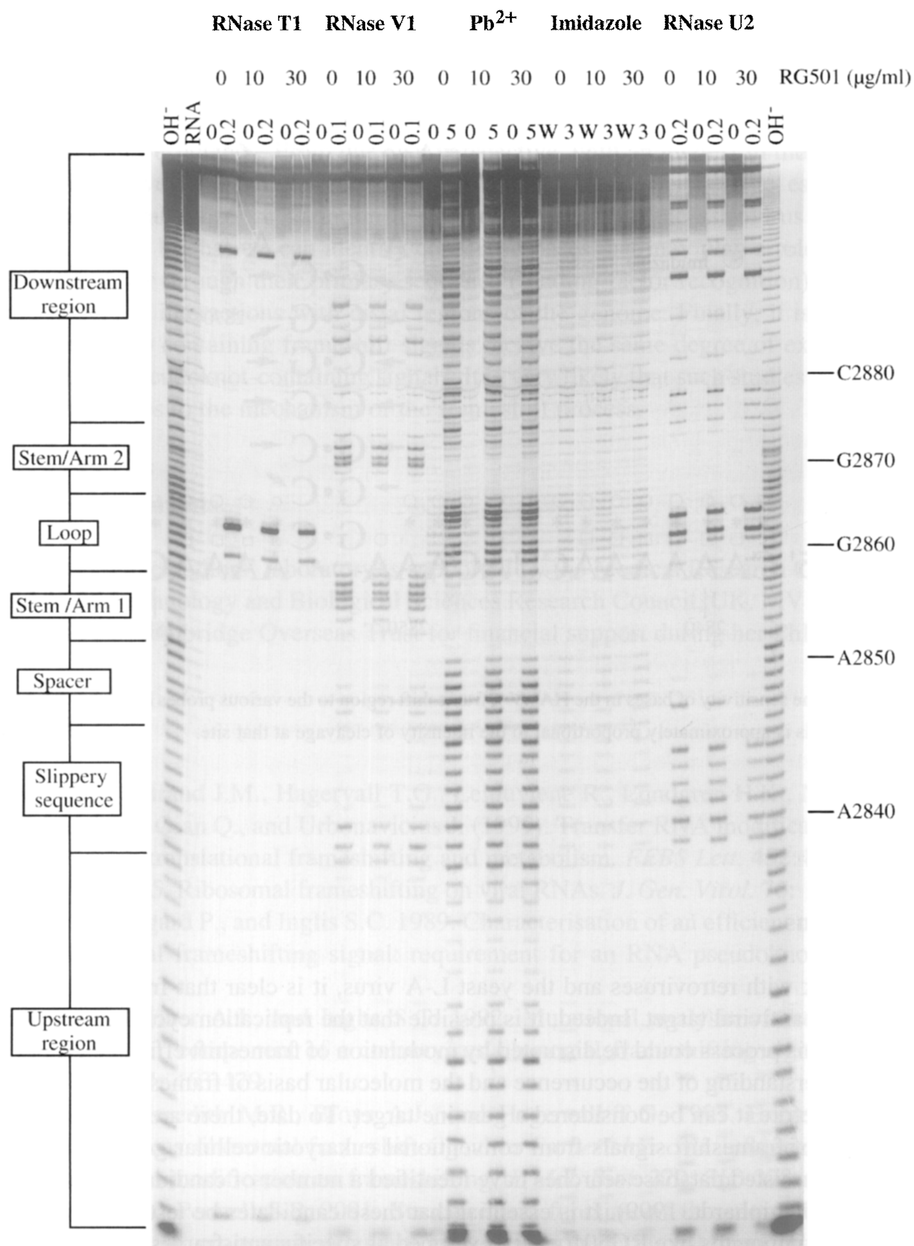
on the basis of the studies discussed above, this could affect the level of frameshifting. Another poorly studied area is how the translational environment influences frameshifting at stem-loop-containing sites. Experiments have revealed that under certain conditions, the HAstV-1 frameshift signal can produce frameshift efficiencies in the region of 25%, but the molecular basis for this enhanced frameshifting is not fully understood (Lewis and Matsui, 1996). Animal virus frameshift signals have largely been studied in *in vitro* translation systems, where the ribosomal load on the mRNA is thought to be low. To date, only a few groups have studied the process *in vivo,* where translation takes place on polysomes and the ribosomal load is probably higher. Under these circumstances, one would predict a reduced efficiency of frameshifting, since refolding of the stimulatory RNA unwound by the first ribosome in the polysome may not occur before the next ribosome is encountered. This topic is discussed in more detail elsewhere (Lewis and Matsui, 1996).

Frameshifting as a target for antiviral intervention

For most viruses that employ frameshifting during the replication cycle, its exact role is uncertain, but it is reasonably well understood for the retroviruses where it allows replicative enzymes (as part of the Pol polyprotein) to be synthesised as a C-terminal extension of the structural proteins (Gag). The product of ribosomal frameshifting, the Gag-Pol polyprotein, is incorporated into virions and this is an essential step in the virus life cycle, since the reverse transcriptase enzyme (encoded in *pol*) is required for subsequent events. There is growing evidence that modulation of frameshift efficiency can reduce retroviral infectivity, either by eliminating the replicase from virions, or by influencing particle assembly (Karacostas *et al.*, 1993; Hung *et al.*, 1998; Shehu-Xhilaga *et al.*, 2001). The same is true for the yeast double-stranded RNA virus L-A, which has Gag and Pol homologues (Dinman and Wickner, 1992). Modulation of frameshift efficiency may also diminish the infectivity of positive-strand RNA viruses, since it would alter the stoichiometry of non-structural proteins within the infected cell. Various antibiotics have been found to influence frameshifting efficiency at the L-A signal, and this has led to the working hypothesis that they could be used as antiviral drugs (Dinman *et al.*, 1997, 1998). High-throughput screening has also been employed in the search for candidate anti-frameshift drugs active against the HIV-1 stem-loop signal (Hung *et al.*, 1998). One such compound, RG501 {1,4-bis-[N-(3-N,N-dimethylpropyl) amidino] benzene tetrahydrochloride} was found to stimulate frameshifting at the HIV-1 signal about two-fold and inhibited HIV-1 replication in tissue culture. The drug was also able to stimulate frameshifting at the stem-loop-containing signals of HIV-2, simian immunodeficiency virus type 1 and HTLV I *gag/pro*, but not HTLV-1 *pro/pol*, which is thought to contain a pseudoknot. Recently, we have demonstrated that RG501 also stimulates frameshifting at the HAstV-1 signal *in vitro,* but not at pseudoknot containing sites (Vidakovic, Hamirally and Brierley; unpublished observations). It has been speculated (Hung *et al.*, 1998) that RG501 acts by binding to the loop region of hairpins (perhaps by intercalation), stabilising the structure and

promoting frameshifting by increasing ribosomal pausing. We have sought evidence that RG501 binds to the HAstV-1 stimulatory stem-loop by probing the secondary structure of the HAstV-1 frameshift signal in the presence and absence of RG501, and the results are shown in Fig. 4. In this experiment, a short (109 nucleotides) transcript containing the HAstV-1 frameshift region was prepared, end-labelled with ^{33}P-γ-ATP and treated with limiting quantities of enzymatic or chemical structure-specific probes. The single-strand specific enzymatic probes employed were RNase T1, which cleaves 3' of unpaired G residues and RNase U2, which cleaves 3' of single-stranded A or G, with a preference for A. To probe double-stranded regions, RNase V1 was employed, which cleaves in helical regions. RNase V1 is not base-specific but cleaves RNA that is in helical conformation, whether base-paired (a minimum of 4-6bp are required) or single stranded and stacked. We also used the single-strand-specific chemical probes imidazole (Vlassov *et al.*, 1995) and lead acetate (Krzyzosiak *et al.*, 1988; Kolchanov *et al.*, 1996). In reactions with RG501, the compound was preincubated with the RNA for 10 minutes at 30°C prior to structure probing. The structure probing reactions were analysed on a 15% denaturing polyacrylamide gel and bands visualised by autoradiography (Fig. 4a). A summary of the structure probing data is shown in Fig. 4b. The reaction conditions are such that only a proportion of the input RNA is cleaved and the remainder appears as a major band at the top of the gel. The 5'-cleavage fragments migrate at a faster rate and are identified by their specific reactivities and by comparison to a marker ladder of bands differing by single base increments prepared by limited alkaline hydrolysis of the input RNA. The outcome of this experiment was unambiguous in that the regions of the frameshift signal proposed to be single- or double-stranded behaved exactly as predicted, confirming that when the HAstV-1 signal is expressed on a short transcript it folds into a stem-loop. However, we were unable to find any differences in the structure probing pattern when the RNA had been preincubated with RG501, even though the concentrations of RG501 were chosen to mimic those that had an effect on frameshifting *in vitro*. A related approach has been used to demonstrate the binding of Hoechst 33258 dye to the TAR RNA of HIV-1 (Dassonneville *et al.*, 1997), but in our case, we were unable to find any differences. It is possible that the RG501 did bind to the astrovirus transcript but the resulting complexes were not sufficiently altered in conformation to affect cleavage by the various enzymatic and chemical probes.

Fig. 4. (A). Secondary structure probing of the HAst-1 [W] ribosomal frameshifting signal in the presence and absence of compound RG501. A short transcript of 109 nucleotides derived from plasmid pAV1 (Marczinke *et al.*, 1994) was 5' end-labelled with γ-[^{33}P]-ATP and subjected to limited digestion by enzymatic and chemical probes (according to Napthine *et al.*, 1999) in the presence and absence of varying quantities of RG501. Sites of cleavage were identified by comparison with a ladder of bands created by limited alkaline hydrolysis of the RNA (OH^-) and the position of known RNase U2 and T1 cuts, determined empirically. Products were analysed on a 15% acrylamide/7M urea gel. Enzymatic structure probing was with RNases U2, V1 and T1. Uniquely cleaved nucleotides were identified by their absence in untreated control lanes (0). The number of units of enzyme added to each reaction (final volume 50μl) is indicated. Chemical structure



probing was with 2M imidazole (I, hours of reaction) or lead acetate (Pb^{2+}; mM concentration in reaction). The water lane (W) in the imidazole panel represents RNA which was dissolved in water, incubated for 3 hours and processed in parallel to the imidazole-treated samples. RNA represents an aliquot of the purified RNA loaded directly onto the gel without incubation in a reaction buffer.

Fig. 4. (B). The sensitivity of bases in the HAstV-1 frameshift region to the various probes is shown. The size of the symbols is approximately proportional to the intensity of cleavage at that site.

Conclusions

From work with retroviruses and the yeast L-A virus, it is clear that frameshifting is a potential antiviral target. Indeed, it is possible that the replication cycle of any virus that uses this process could be disrupted by modulation of frameshift efficiencies, but a better understanding of the occurrence and the molecular basis of frameshifting will be required before it can be considered a genuine target. To date, there are no confirmed examples of frameshift signals from conventional eukaryotic cellular genes, although computer-assisted database searches have identified a number of candidates (Hammell *et al.*, 1999; Liphardt, 1999). It is essential that these candidates be tested rigorously; although compounds like RG501 were developed as specific anti-frameshifting agents, the occurrence of cellular frameshifting signals would potentially preclude the use of such agents. For all positive-stranded RNA viruses, the exact role of frameshifting is unknown. Presumably, the frameshift allows the required ratio of viral proteins to be produced, but it may also serve to downregulate levels of viral replicases, which may be toxic in high amounts. Regarding astrovirus frameshifting, there is still much to

learn. The key issue will be to determine the sequence requirements for frameshifting within the context of the complete virus genome, especially regarding the stimulatory RNA and whether it can form a pseudoknot. Infectious cDNA clones will be invaluable in this analysis and will also allow the study of the effect on virus replication of varying the frameshift efficiency. From our own perspective, with an interest in the frameshift mechanism, we are currently sequencing astrovirus clinical isolates to establish the level of natural sequence variation that is tolerated within the astrovirus frameshift region. It may be that we can identify conserved bases that may play a role in frameshifting, either through their primary sequence (binding factor recognition) or in long range, tertiary interactions with distal regions of the genome. Finally, it is important that stem-loop containing frameshift signals receive the same degree of experimental attention as pseudoknot-containing signals. It is very likely that such studies will reveal important clues to the mechanism of the frameshift process.

Acknowledgements

Research in the authors' laboratory is supported by the Medical Research Council UK and the Biotechnology and Biological Sciences Research Council, UK. MV would like to thank the Cambridge Overseas Trust for financial support during her PhD studies.

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