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Pseudoknots and the control of protein synthesis

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Current Opinion in Cell Biology 1990, **2**:1099–1103

Introduction

Pseudoknots are novel folds in the secondary structure of RNA that were first discussed in detail by Pleij *et al.* (*Nucleic Acids Res* 1985, 13:1717–1731). Any RNA hairpin loop which forms base pairs with sequences that are 3′ to the hairpin constitutes a pseudoknot (Fig. 1a). If a full turn of RNA helix were formed in the loop, the 3′ tail of the RNA would be brought through the center of the loop and tie a topological knot in the RNA; no RNA secondary structure has yet been found which would form an actual knot. In most known pseudoknots there is the possibility of coaxially stacking the two helical segments, leaving two single-stranded sections of RNA to cross the grooves of the two helical segments. Thus, a pseudoknot is one method of forming an extended, yet segmented, RNA helix.

Since the pseudoknot motif was first described in a tRNA-like structure at the 3′ terminus of some viral RNAs in

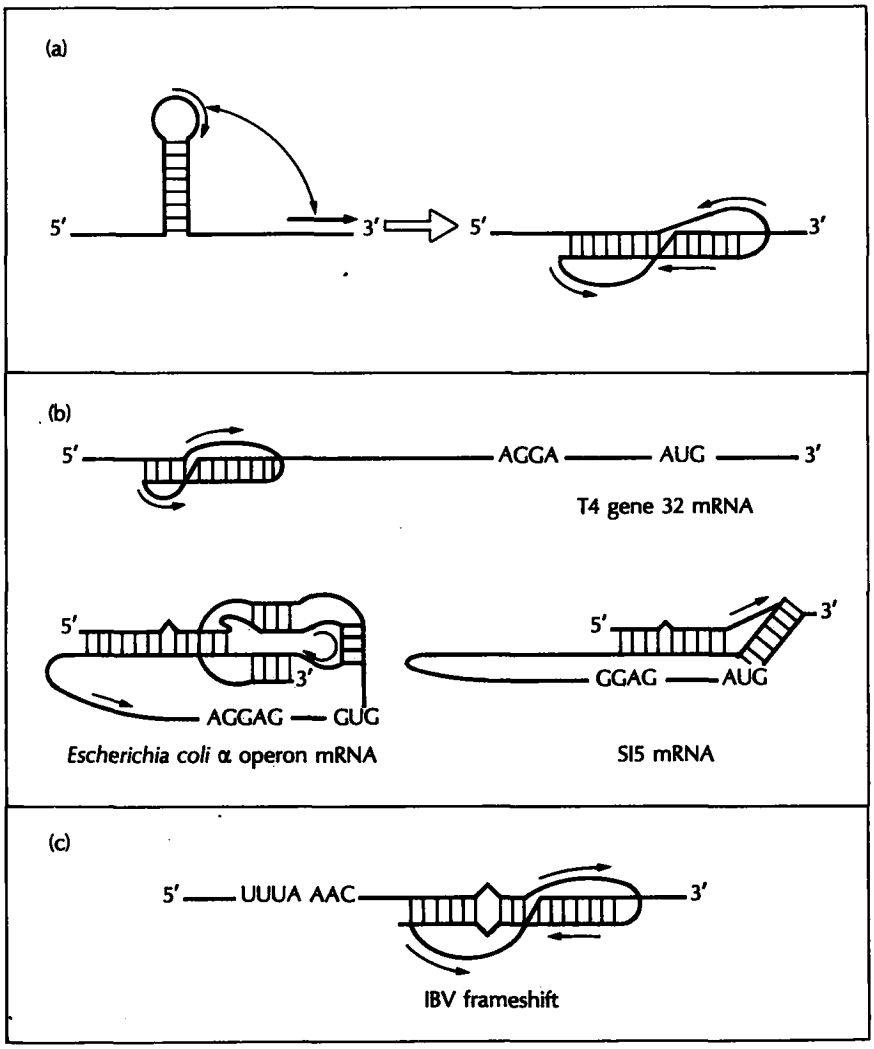

Fig. 1. A pseudoknot gallery: (a) The addition of base pairing between a hairpin loop and sequences 3′ to the loop forms a pseudoknot in which the two helical segments are coaxially stacked. Arrows in the right-hand diagram indicate the 5′- -3′ polarity of the backbone. (b) The structures of pseudoknots that act as targets for translational repressors: pseudoknots on phage T4 gene 32 mRNA and *Escherichia coli* α operon and S15 mRNAs. Shine-Dalgarno and initiation-codon sequences are indicated (the α operon initiates with GUG). (c) A frameshifting site found in the avian infectious bronchitis virus (IBV) coronavirus. The 'slippery' heptanucleotide sequence shown is located six nucleotides from the first pseudoknot base pair. The four pseudoknots shown in this figure are the only mRNA examples so far for which substantial experimental evidence supports their existence and functional importance.

Abbreviations

IBV—infections bronchitis virus; **RBS**—ribosome-binding site.

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plants, it has been detected in ribosomal RNAs, self-splicing introns, and other RNAs for which the unusual folding undoubtedly helps to define the three-dimensional structures of these molecules [1]. In the past 18 months, pseudoknots have been discovered in several mRNAs, where they alter the way in which ribosomes translate the mRNA: pseudoknots at the ribosome-binding site (RBS) can inhibit the initiation of translation when bound by a repressor protein, and pseudoknots within the coding region are sometimes required in order to induce a translational frameshift that may be necessary before a protein can be synthesized. In this article, I will describe these recently discovered mRNA pseudoknots, and offer some speculations as to why pseudoknots are particularly effective at interfering with the normal course of translation.

Pseudoknots modulate translational initiation

The first example of a pseudoknot which affects translation was discovered in the autogenously regulated T4 gene 32 mRNA. McPheeters *et al.* (*J Mol Biol* 1988, 201:517–535) showed that low concentrations of gp32 bind at a pseudoknot located far upstream of the RBS (Fig. 1b). As the concentration of gp32 is raised, additional gp32 copies bind cooperatively, until the mRNA is completely coated with gp32 between the pseudoknot and the RBS; at that point, competition between the bound protein and initiating ribosomes inhibits translation. Thus, the role of the pseudoknot seems to be to provide a high-affinity binding site for gp32; as the protein normally binds single-stranded DNA during T4 replication, one of the single-stranded linkers within the pseudoknot may adopt a conformation that is particularly suited to protein recognition [1].

Pseudoknots are more directly involved in modulating the initiation of translation in two recently reported cases of autogenously regulated ribosomal proteins in *Escherichia coli* (Fig. 1b). S4 binds to the leader sequence of the α-operon mRNA and represses the translation of all four ribosomal proteins in the operon (Thomas and Nomura, *J Mol Biol* 1987, 196:333–345). By using an extensive set of compensatory base changes to test a number of potential Watson–Crick base pairings, Tang and Draper were able to demonstrate conclusively that the pseudoknot shown in Fig. 1b is required for S4 recognition *in vitro* [2] and for translational repression *in vivo* [3]. In the case of this pseudoknot, its folding pattern is more complex then that depicted in Fig. 1a, because three segments of basepairing are formed within the loop of a very stable upstream hairpin. Nevertheless, the four helical segments can be arranged into a continuous, coaxial stack that is very similar to the basic pseudoknot shown in Fig. 1a.

The mRNA that encodes ribosomal protein S15 is monocistronic, and genetic experiments by Grunberg-Manago and colleagues [4] have demonstrated that it is autoregulated. Extensive 'structure-mapping' experiments using mRNAs prepared from a series of deletion mutants have strongly suggested that this mRNA also folds into a pseudoknot [5] (Fig. 1b). The similarity between the structures of the α and S15 mRNAs is striking: in both cases, the RBS is located on a long, single-stranded linker within the pseudoknot, and the initiation codon is at the beginning of the second helical segment.

Why a pseudoknot target for translational repressors?

Phillippe *et al.* [5] have postulated a simple mechanism for translational repression in the S15 mRNA. Based on their chemical modification studies, they suggest that the pseudoknot is unstable and in equilibrium with a competing hairpin formed by base pairing between sequences on either side of the initiation codon. As the ribosome must completely disrupt the second helical segment of the pseudoknot structure in order to allow tRNA binding to the second codon (if not to allow the formation of the fmet-tRNA–ribosome–mRNA complex), stabilization of the pseudoknot structure by S15 should repress translation. This mechanism is simply represented by the scheme:

$$\text{repressor.closed} \leftrightarrows \text{closed} \leftrightarrows \text{open} \xrightarrow{\text{ribosome}} \text{initiation}$$

where 'closed' refers to the complete pseudoknot and 'open' refers to the disrupted structure. In support of this mechanism, S15 binding appears to stabilize the pseudoknot folding, and mutations which disrupt the pseudoknot (shifting the closed–open equilibrium to the right) also abolish repression [5]. A weakness of this repression scheme is that it depends only on the pseudoknot structure rendering some part of the RBS inaccessible to initiating ribosomes, and should not be sensitive to the precise location of the RBS within the pseudoknot; thus, it provides no explanation as to why the RBS is similarly placed within the pseudoknots of the α and S15 RNAs. In addition, some mutations that disrupt helices in the α mRNA leader also have effects on both translational efficiency and repression which are not easily explained in terms of simple 'open' and 'closed' pseudoknot structures; S4 probably induces a more subtle conformational change in the mRNA than just stabilization of secondary structure [2].

Other repression mechanisms are possible. For instance, translational initiation takes place in two steps: a 'stand-by' ribosome–mRNA complex primarily involving the Shine–Dalgarno base pairing forms first, followed by a factor-mediated shift to the complete fmet-tRNA–ribosome–mRNA complex (Canonnaco *et al.*, *Eur J Biochem* 1989, 182:501–506). It is possible that the 'stand-by' complex forms on the intact pseudoknot; by analogy with the gp32 system, one can imagine that the pseudoknot even holds the Shine–Dalgarno sequence in a particularly favorable conformation for ribosome bind-

ing. Repression would then be a consequence of kinetic competition between bound ribosomes proceeding to form complete initiation complexes (K_{init}) and a repressor trapping the mRNA in some conformation that is unable to form the final initiation complex (K_r) (Fig. 2).

Kinetic competition could be a distinct advantage when a repressor binds mRNA with similar or weaker affinity than that of ribosomes, as in the case for S4. The way in which ribosomes bind to pseudoknots and the kind of competition that takes place between repressors and ribosomes are issues that will probably be resolved in the near future.

Pseudoknots induce ribosomal frameshifting

Brierly *et al.* [6] have shown that a frameshifting event in an open reading frame of the infectious bronchitis virus (IBV) coronavirus requires a pseudoknot structure downstream of the frameshift site (Fig. 1c). This is the only frameshift site for which evidence of pseudoknot involvement has emerged. The frameshift occurs at an AAC codon with 25–30% efficiency. Compensatory base changes were used to show that both helical segments of the pseudoknot are needed for efficient frameshifting: disruption of the first (5′) helix eliminates frameshifting (more than a 50-fold reduction in the efficiency of frameshifting), and disruption of the second helix reduces the efficiency by approximately 10-fold.

The frameshift site described by Brierly *et al.* [6] belongs to a larger class of −1 frameshifts occurring at adjacent 'slippery codons' with the form X XXY YYZ. (Slippery codons are sequences of four or more bases which allow a tRNA to shift reading frame while maintaining at least two anticodon-codon pairs.) Detailed experiments on the *gag–pol* frameshift in Rous sarcoma virus by Jacks *et al.* (*Cell* 1988, 55:447–458) showed that the two tRNAs bound in the P and A sites of a ribosome must simultaneously slip one nucleotide towards the 5′ end of the message. This 'simultaneous slippage' model has been refined in an excellent paper by Weiss *et al.* [7] to take into account recently discovered details concerning the movement of tRNA during transpeptidation and translocation (Moazed and Noller, *Cell* 1989, 342:142–148). The essence of the refined model is that the ribosome, in attempting to carry out translocation, must simultaneously let go of the tRNA anticodon in the P site and move the A-site anticodon over to the P site, in order to create an opportunity for slippage to occur.

In some but not all −1 frameshift sites, there is evidence that downstream secondary structure significantly enhances the frameshift efficiency. Those slippery heptanucleotide frameshift sites that have been investigated so far fall roughly into three classes. First, there are sites that are independent, or nearly independent, of any downstream structure (e.g. the *gag–pol* frameshift in human immunodeficiency virus; Wilson *et al.*, *Cell* 1988, 55:1159–1169). Although the overall effect of the downstream structure may be small, Weiss *et al.* [7] have shown that in one case a downstream hairpin A AAA AAC promotes frameshifting at the second of the two codons, whereas in its absence frameshifting occurs more frequently at the first codon (A AAA alone induces significant frameshifting). Thus, downstream elements probably influence the mechanism, if not the overall rate, of frameshifting in these cases. A second class consists of sites that are stimulated 10-fold or more by downstream hairpins; the RSV *gag–pol* frameshift is an example (Jacks *et al.*, 1988). Third, there are sites which require a downstream pseudoknot for efficient frameshifting. The only case for which a pseudoknot requirement has been conclusively demonstrated is the U UUA AAC sequence of IBV [6]. The assistance of a pseudoknot in this regard may be more the rule than the exception, as innovative computer searches [8,9] have found that all other known U UUA AAC frameshift sites are followed by sequences potentially forming pseudoknots, and potential pseudoknots tend to follow several other frameshifting heptanucleotides.

How do pseudoknots cause frameshifting?

It appears that some tandem slippery codons undergo simultaneous slippage fairly readily, while others require the assistance of a downstream hairpin or pseudoknot to attain high levels of frameshifting. How do these downstream structures help, and why are pseudoknots more effective than simple hairpins for some slippery sequences? In the model advanced by Weiss *et al.* [7], anything which slows the movement of a ribosome during translocation should give more opportunity for a −1 frameshifting to occur. Thus, the question becomes how do hairpins and pseudoknots force the ribosome to pause at translocation?

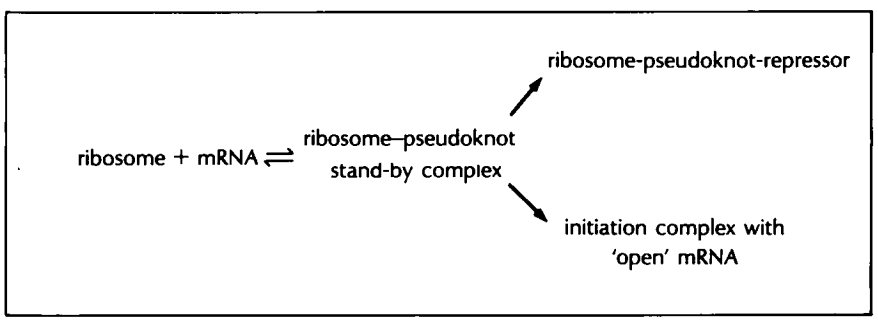

Fig. 2. Schematic of a possible mechanism for translational repression.

It should be kept in mind that those frameshifting sites first described in eukaryotic viral RNAs exhibit similar properties when transplanted into *E. coli* [7], and that there are examples of similar heptanucleotide downstream structure motifs functioning as efficient frameshift sites in normal *E. coli* genes (Flower and McHenry, *Proc Natl Acad Sci USA* 1990, 87:3713–3717; Sekine and Ohtsubo, *Proc Natl Acad Sci USA* 1989, 86:4609–4613). Thus, frameshifting must be enacted by the basic translational machinery that is conserved between prokaryotes and eukaryotes.

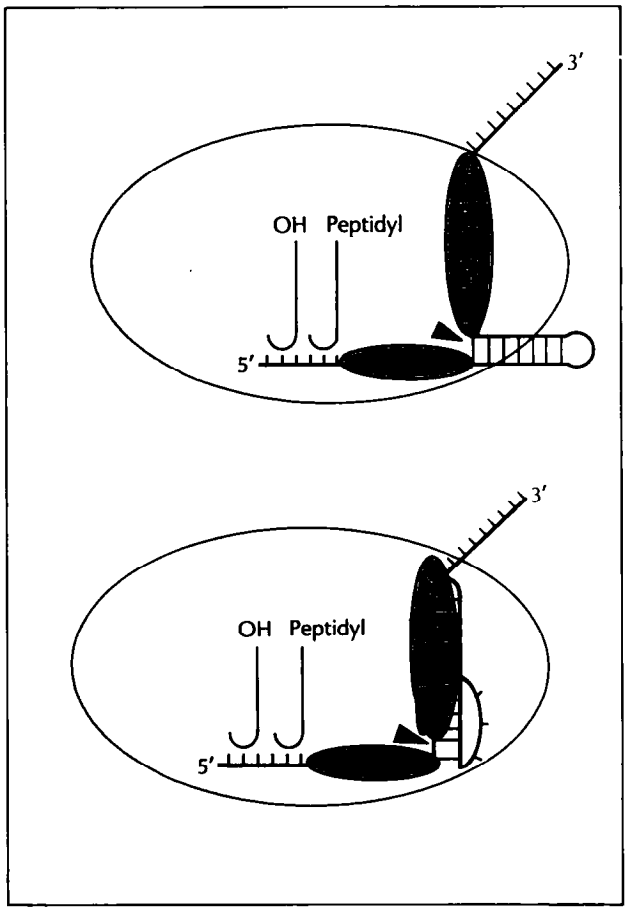

Fig. 3. A model for ribosome denaturation of mRNA secondary structure. Darker grey areas represent regions of ribosome interaction with the mRNA. The distances from the A site to a sharp bend in the mRNA (10 nucleotides) and from the bend to the 3′ end of ribosome contacts with mRNA (13 nucleotides) are taken from the nuclease digestion studies of Kang and Cantor (1985). These studies also show that 20 more nucleotides of mRNA are bound to the ribosome 5′ to the P site (not shown). The solid black ovals and triangle at the mRNA bend represent a hypothesized 'denaturase' activity of the ribosome which specifically binds single-stranded RNA (ovals) and may actively pull apart mRNA base pairs (triangle) to feed single-stranded mRNA into the ribosome-decoding site. Top, the 'denaturase' unwinds a simple hairpin; bottom, the 'denaturase' is unable to approach a pseudoknot in the same way as a hairpin.

Secondary structures of mRNA must be denatured by the ribosome before any mRNA sequence can be decoded, and this process probably slows translating ribosomes. One might also presume that more stable hairpins would promote longer pauses, and this seems to be supported by the fact that downstream structures which enhance frameshifting tend to be rich in G–C base pairs. However, there is no good correlation between frameshift efficiency and hairpin stability. For instance, mutations which stabilize the RSV *gag–pol* hairpin by 0.9 kcal, actually decrease the frameshifting rate twofold (Jacks *et al.*, 1988). Thermodynamic stability does not predict that pseudoknots should be more effective than hairpins at causing pauses in translocation; if anything, they are probably less stable than hairpins of the same number of base pairs (Puglisi *et al.*, *Nature* 1988, 331:283–286). Thus, an explanation of the effects of downstream secondary structures on translation must be sought in the kinetics of the denaturation process, rather than in the simple free energy of denaturation.

An obvious difference between simple hairpins and pseudoknots, as viewed by a translating ribosome, must be the geometries of the structures. The 5′ and 3′ single-strand tails of a hairpin are in close proximity, whereas the corresponding points on a pseudoknot are separated by the length of the two helical segments (compare for instance the structures in Fig. 1a). The way in which this difference might affect ribosome translocation is suggested in Fig. 3. Messenger RNA secondary structure is probably not melted until it is fewer than 10 bases away from the A site; this number is deduced from attenuation studies in which stalled ribosomes either denature (Alexieva *et al.*, *Proc Natl Acad Sci USA* 1988, 85:3057–3061) or allow formation of specific secondary structures (Stroynowski and Yanofsky, *Nature* 1982, 298:34–38). Kang and Cantor (*J Mol Biol* 1985, 181:241–251) have concluded from nuclease digestion studies that a sharp bend occurs in mRNA about 10 nucleotides 3′ to the A site. Fig. 2 presumes that the bend in the mRNA is a 'denaturase' which grabs the two single-stranded RNAs on either side of a helix and progressively pulls the helix apart to feed a single strand into the A site. The unusual geometry of a pseudoknot would allow less single-stranded RNA contact with the 'denaturase', and perhaps slow the ribosome-assisted unfolding of the pseudoknot. This is, of course, a rather speculative model; its essential feature is that the kinetics of translocation through pseudoknots may critically depend on the ability of the ribosomal apparatus to handle a secondary folding which is qualitatively different from the hairpins usually encountered.

Concluding remarks

Some retroviruses use the suppression of an amber stop codon between the *gag* and *pol* genes, rather than a frameshift, to express the *gag–pol* fusion. Ten Dam *et al.* [9] have noted that a potential pseudoknot structure is conserved eight nucleotides downstream of the amber codon in several of these viruses. The overall structure strongly resembles the IBV pseudoknot which induces a frameshift, and suggests that the encounter of a pseu-

doknot with the ribosomal machinery is able to induce misreading as well as frameshifting. Whether or not pseudoknots can induce the misreading of codons other than stop codons would be interesting to know. Thus, in a pleasing symmetry, pseudoknots are apparently able to modulate all aspects of ribosome function, i.e. initiation, elongation, and termination. Besides satisfying an interest in these pseudoknots as regulators of gene expression, investigations into the mechanisms by which these pseudoknots function will probably reveal new details of the translational machinery.

Annotated references and recommended reading

- • Of interest
- •• Of outstanding interest

1. • PLEIJ CWA: **Pseudoknots: a new motif in the RNA game.** *Trends Biochem Sci* 1990, 15:143–147.

A good introduction to the subject of pseudoknots, this recent short review provides a summary of pseudoknot types and geometries, and lists the known occurrences of pseudoknots in viral RNAs, mRNAs, and ribozymes.

2. •• TANG CK, DRAPER DE: **An unusual mRNA pseudoknot structure is recognized by a protein translational repressor.** *Cell* 1989, 57:531–536.

Compensatory base changes are used to demonstrate conclusively that a pseudoknot fold at the translational initiation site of α mRNA is recognized by a protein. This is a more complex example of pseudoknot folding than most others so far described.

3. • TANG CK, DRAPER DE: **Evidence for allosteric coupling between the ribosome and repressor binding sites of a translationally regulated mRNA.** *Biochemistry* 1990, 29:4434–4439.

This *in vivo* study extends the *in vitro* experiments on the α mRNA pseudoknot, and suggests that the pseudoknot mediates an allosteric interaction between a translational repressor and initiating ribosomes.

4. • PORTIER C, DONDON L, GRUNBERG-MANAGO M: **Translational autocontrol of the *Escherichia coli* ribosomal protein S15.** *J Mol Biol* 1990, 211:407–414.

Genetic studies establish that the S15 gene is translationally regulated and define the mRNA 'operator' sequence needed for this regulation.

5. • PHILIPPE C, PORTIER C, MOUGEL M, GRUNBERG- MANAGO M, EBEL J-P, EHRESMANN B, EHRESMANN C: **Target site of *Escherichia coli* ribosomal protein S15 on its messenger RNA. Conformation and interaction with the protein.** *J Mol Biol* 1990, 211:415–426.

Extensive 'structure mapping' studies on both the S15 mRNA and several mutants argue for a pseudoknot target site for the S15 repressor; the pseudoknot has some interesting parallels to the regulatory site of α mRNA.

6. •• BRIERLY I, DIGARD P, INGLIS SC: **Characterization of an efficient coronavirus ribosomal frameshifting signal: requirement for an RNA pseudoknot.** *Cell* 1989, 57:537–547.

An extensive study that defines a frameshifting site in the IBV coronavirus and uses compensatory mutations to demonstrate that a pseudoknot is necessary for frameshifting to occur. So far this is the only example of frameshifting for which there is conclusive evidence for pseudoknot involvement.

7. •• WEISS RB, DUNN DM, SHUH M, ATKINS JF, GESTELAND RF: ***E. coli* ribosomes re-phase on retroviral frameshift signals at rates ranging from 2 to 50 percent.** *New Biol* 1989, 1:159–169.

This paper is a useful introduction to the problem of dual slippery-codon frameshifts. Besides demonstrating that viral frameshift sites function in *E. coli*, this paper contains a wealth of quantitative data on frameshifting efficiencies and an excellent discussion of −1 frameshift mechanisms.

8. •• ABRAHAMS JP, VAN DEN BERG M, VAN BATENBURG E, PLEIJ C: **Prediction of RNA secondary structure, including pseudoknotting, by computer simulation.** *Nucleic Acids Res* 1990, 18:3035–3044.

Computer programs routinely used to find stable RNA secondary structures specifically exclude pseudoknots from their searches. This is a first attempt to include the possibility of pseudoknots in an easily accessible search program running on personal computers. The lack of thermodynamic parameters for pseudoknots, and the impossibility of searching through all possible pseudoknots in a reasonable amount of time, limit the predictive ability of the program, but it still seems to function well with known pseudoknot structures.

9. • TEN DAM EB, PLEIJ CWA, BOSCH L: **RNA pseudoknots; translational frameshifting and readthrough on viral RNAs.** *Virus Genes* 1990, 4:219–234.

These authors use a new computer program to look for potential pseudoknots near known viral frameshift sites, and also find that pseudoknots may be involved in the suppression of stop codons. This paper is a useful summary of the known −1 frameshift sites.