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RNA pseudoknots

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RNA pseudoknots result from Watson–Crick base pairing involving a stretch of bases located between paired strands and a distal single-stranded region. Recently, significant advances in our understanding of their structural and functional aspects have been accomplished. At the structural level, modelling and NMR studies have shown that a defined subset of pseudoknots may be considered as tertiary motifs in RNA foldings. At the functional level, there is evidence that the realm of functions encompassed by RNA pseudoknots extends from the control of translation in prokaryotes, retroviruses and coronaviruses to the control of catalytic activity in ribozymes and the control of replication in some plant viruses.

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Introduction

The possibility of pseudoknot formation has been suggested ever since the early thinking on the folding of single-stranded RNA molecules [1]. It was not until the experimental and theoretical work of Pleij and his collaborators [2], however, that the existence of pseudoknots in RNA molecules became recognized. Recently, a variety of biological functions have been attributed to pseudoknotting in RNA single strands. In the first part of this review, we will examine the problems related to the definition of pseudoknots and will try to convey the message that pseudoknots with at least some approximate co-axial stacking between two adjacent helices are of particular value for understanding and modelling RNA three-dimensional foldings. In the second part, we will discuss the various functional aspects of pseudoknotting and the advances made during the past year. Finally, we will comment on the difficulties in predicting and recognizing pseudoknots in RNA sequences.

Structural features

In the folding of a single-stranded RNA molecule, there are only three ways in which two base-paired segments can be related to each other: two consecutive hairpins; two helices separated by an internal bulge; and, pseudoknots [1]. The first two motifs can be represented as two-dimensional graphs without self-intersections whereas pseudoknots cannot, as they are fundamentally three-dimensional structures in which the four base-paired strands alternate along the sequence of the RNA molecule. This general definition of pseudoknotting

is illustrated in Fig. 1. In other words, pseudoknots can be most generally defined as standard Watson–Crick base pairing involving a stretch of bases located between paired strands and an outlying partner (e.g. Watson–Crick interactions between two hairpin loops or between a loop and a bulge) [2,3,4]. In an important piece of work, Pleij and his collaborators [2] demonstrated that because of the special geometry of RNA helices, it is possible for the two helical stems in the pseudoknot to be co-axially stacked. This arrangement constitutes a very important subset of pseudoknots for biological functions.

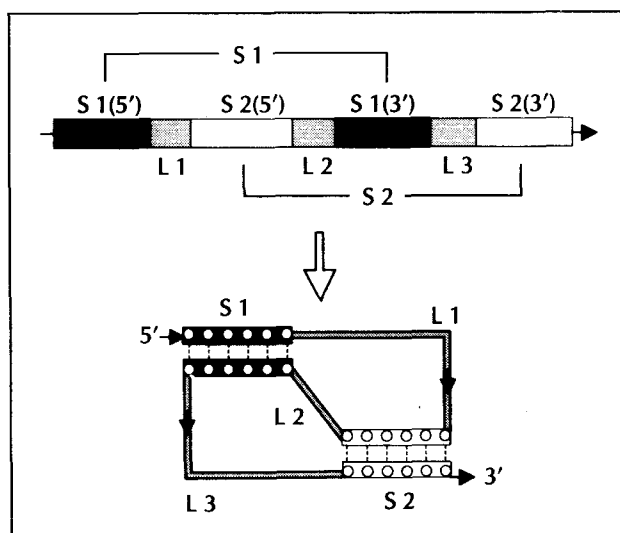


Fig. 1. Definition of a pseudoknot at the level of the nucleic acid sequence and secondary structure. RNA segments that will form double-stranded helices are labeled S1 (in black) and S2 (in white); the connecting RNA segments are labeled L1, L2 and L3 (in grey).

Abbreviations

BMV—brome mosaic virus; HIV—human immunodeficiency virus; IBV—infectious bronchitis coronavirus; MMTV—mouse mammary tumor virus; MuLV—Moloney murine leukemia virus; TYMV—turnip yellow mosaic virus.

The 'classic' pseudoknot structures can be divided into three types, each of which is obtained after severing one connecting loop in a hypothetical closed chain (Fig. 2) [3•,5,6•]. In type (i), there is no loop bridging the deep groove; in type (ii), there is no segment connecting the ends of the whole helical stem; and, in type (iii), there is no loop over the shallow groove. Type (ii) is the best described classic pseudoknot, in which the first loop (in the 5' to 3' direction) crosses the deep groove and the second one crosses the shallow groove. This motif was first modelled by Dumas and coworkers [7] on the basis of enzymatic and chemical probing for the tRNA-like 3' end of turnip yellow mosaic RNA virus. Recently, NMR studies have led to a model consistent with the previously proposed stacked co-axial helices in the pseudoknot [4•]. The other two types are not so well characterized. An example of a type (iii) pseudoknot has been modelled

for a tertiary peripheral base pairing in introns of subgroup IA [3•].

The definition portrayed in Fig. 1 is general and does not set any constraint on either the lengths of the connecting segments or the helices. A full turn of RNA double helix requires 11 bp. When each of the helices forming a pseudoknot makes a full turn, a topologically knotted structure is obtained. In all proposed pseudoknot structures, however, the helical stems are shorter than a full helical turn. Further, the minimum length of the connecting segments bridging the two grooves depends clearly on the length of S1 and S2, i.e. on the number of base pairs that each contains. Thermodynamic studies have been carried out only on a pseudoknot of type (ii) which contained 3 bp in helix S1 and 5 bp in helix S2 [4•,8]. In the presence of Mg^{2+} , a stable pseudoknot structure is obtained

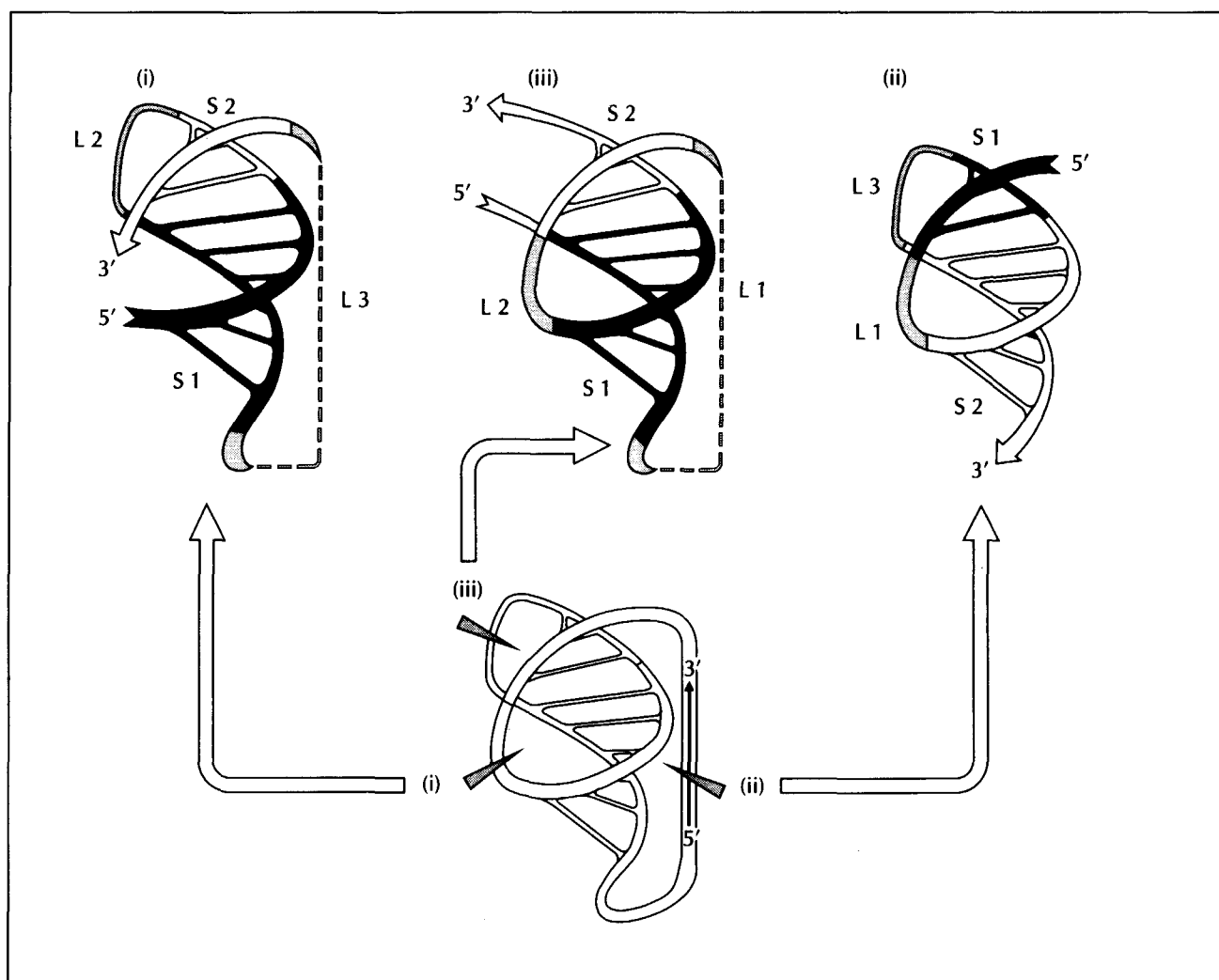


Fig. 2. The three types of 'classic' pseudoknots with co-axial stacking of the two base-paired helices and with single-stranded segments crossing the RNA grooves. RNA segments forming double-stranded helices are labelled S1 and S2; the connecting RNA segments are labeled L1, L2 and L3 (same colour code as in Fig. 1). Right-handed RNA helices (similar to A-form DNA helices) are characterized by a deep but narrow groove (equivalent to the major groove of B-DNA) and a shallow but wide groove (equivalent to the minor groove of B-DNA). In the central drawing of a hypothetical circular RNA, the connecting segment (i) crosses the deep groove whereas segment (iii) crosses the shallow groove. Segment (ii) bridges the whole length of the co-axial helices. The type (ii) pseudoknot, obtained when the loop L2 is absent, is most common and is denoted H by Pleij [2]. Types (i) and (iii) are obtained when loops L1 or L3, respectively, are absent, and are rare. Adapted from [3•].

when L1 is equal or larger than three nucleotides and L3 is equal or larger than four nucleotides [8]. With six base pairs in S2, however, one could expect that a single nucleotide in L1 is sufficient to obtain a stable pseudoknot structure.

When the connecting segments contain a large number of nucleotides, they may be structured and partially base-paired. Several situations can be envisaged. If L1 or L3 is large, a type (ii) pseudoknot becomes equivalent to either a type (i) or type (iii), respectively. Types (i) and (iii) could still be described as motifs even if the segment crossing the whole helical stem is long and structured. Where there is no short loop over either groove, however, the notion of structural motif is obviously lost. Figure 3 illustrates schematically another type of RNA tertiary motif, which also is the outcome of the particular geometry of RNA helices and which exhibits stacking of two helices. It results from the formation of base triples between a single strand and a double-stranded helix (either in the deep groove or in the shallow groove of the helix) and can lead to a segment of small triple helix. A comparison of Figs 2 and 3 highlights the three-dimensional relationships between the triple-helix motif and the classic pseudoknot structures. The double helix in the triple-helix motif and the 'main' hairpin in the pseudoknot motif might both be considered as 'preformed' structural elements controlling efficient interactions between nucleotides widely spaced in the RNA sequence [3•].

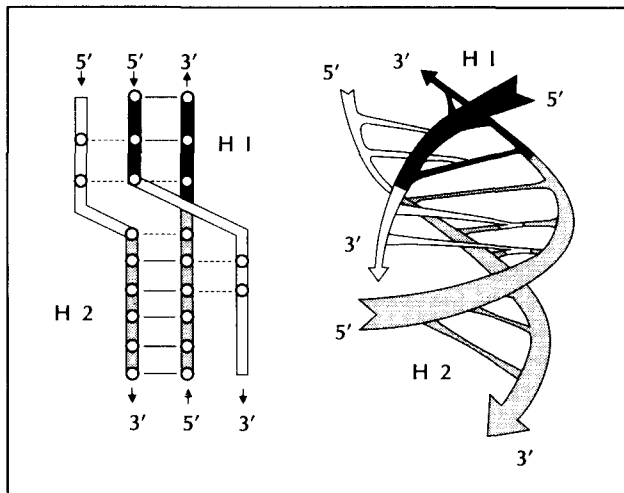


Fig. 3. Two-dimensional and three-dimensional diagrams illustrating how a pseudoknot can be related to a triple helix. Instead of crossing the deep groove, the single-stranded 3' end of helix H1 (in black) interacts with the side of the bases exposed in the deep groove of helix H2 (in grey). Similarly, instead of crossing the shallow groove, the single-stranded 5' end of helix H2 (in grey) interacts with the shallow groove of helix H1 (in black). A three-dimensional example of such a double triple-helix motif has been proposed in the core of the catalytic introns of group I [25].

Functional significance of RNA pseudoknots

The formation of classic pseudoknots leads to a compact structure, the stability of which can be affected by

the size of the loops and the number of base pairs. Thermodynamic data on these aspects are still scarce. Recent work has indicated that pseudoknots are only marginally more stable than their constituent hairpins (by less than 2 kcal mol^{-1}) [4•,9••]. If upheld by further evidence, this observation suggests a role for pseudoknots as conformational switches or control elements in several biological functions [10].

In molecules that lack an overall three-dimensional fold, pseudoknots fold locally and their positions along the sequence reflect their function [11•]. For example, pseudoknots that are folded at the 5' end of mRNAs tend to be involved in translational control whereas those at the 3' end maintain intact signals for replication. In molecules with catalytic and mechanistic activities, pseudoknots are located at the core of the tertiary fold and involve nucleotides that are far apart in the sequence.

5'-End pseudoknots: translational control

Pseudoknots appear to adopt two roles in the control of mRNA translation: either specific recognition of a pseudoknot by some protein is required for control, as described for the 5' end of mRNAs in some prokaryotic systems [10,12]; or, the presence of a folded pseudoknot is necessary with no requirement on the nucleotide sequence [13••–15••]. In several viruses, the expression of replicases is controlled either by ribosomal frameshifting [13••–15••,16,17] or by in-frame read-through of stop codons [18••]. In both cases, pseudoknot formation is necessary, as was shown for frameshifting in infectious bronchitis coronavirus (IBV) [13••], in the double-stranded RNA virus of *Saccharomyces cerevisiae*, subtype 1 or LA [14••,17], or in the mouse mammary tumor virus (MMTV) [15••], and for read-through in Moloney murine leukemia virus (MuLV) [18••]. The requirements appear, however, more strict for read-through than for frameshifting. In IBV, the nature of the nucleotides in the loops crossing the grooves had no influence on frameshifting [13••]. Further, the insertion of three residues in the loop crossing the deep groove, or deletion in the loop crossing the shallow groove (down to the minimal length of six residues) does not affect the frameshifting process [13••]. In contrast, the nature of the nucleotides and the length of both loops are crucial for read-through [18••]. Nevertheless, the correct position of the pseudoknot in the 3' direction with respect to the slip site in ribosomal frameshifting, and with respect to the AUG codon in read-through is an absolute requirement [13••,18••]. Moreover, Brierley *et al.* [13••] demonstrated that the replacement of the pseudoknot by a constructed stem-loop structure with the same length and composition is unable to direct frameshifting. Recently, it was demonstrated that a pseudoknot located 3' to the slip site as well as an optimal heptameric shift site were both necessary for efficient ribosomal frameshifting in the double-stranded RNA virus of yeast [14••,17] and in MMTV [15••]. Single mutants with disrupted S1 or S2 had low frameshifting efficiencies

whereas double mutants able to form a pseudoknot but with sequences different from those of the wild type had restored frameshifting efficiencies [14••,15••]. The folding of the frameshifting region into a pseudoknotted three-dimensional structure is, thus, more important than the primary structure.

The presence of three pseudoknots in 16S rRNA has been suggested on the basis of comparative sequence analysis (reviewed in [2]). The first pseudoknot lies between the 5' end and the region around nucleotide 915. The second is located at a phylogenetically conserved tertiary interaction between positions 570 and 866, also in the 5' domain [2]. The third pseudoknot involves the 530 stem-loop structure [19], which is known to be important for the binding of tRNA to the ribosomal A site [20], and was recently shown to be essential for ribosomal function [21••]. Interestingly, some mismatches such as A-C or C-A in the third pseudoknot were lethal, whereas the wobble base pairs (G-U, U-G) conferred resistance to streptomycin, an antibiotic that perturbs the control of translational accuracy [21••]. The same 530 region interacts with protein S12 [22]. Mutations in S12 lead to streptomycin resistance and give rise to hyperaccurate ribosomes [21••]. These observations are particularly interesting in view of the suggested conformational switch that involves two pseudoknots, one at the 5' end and one in the region around nucleotide 915 [23]. The equilibrium between the two states would be controlled by S12 and S4 (a protein which has effects opposite to those of S12 with respect to translational accuracy) [23]. Clearly, further experiments are required to prove this model. There has, however, been suggestion of an alternative conformational switch between the pseudoknot at position 915 and a new one that involves the anti-Shine-Dalgarno sequence [24].

Core pseudoknots: control of catalytic activity

Like the pseudoknot at the 5' end of the 16S rRNA, the pseudoknots at the core of the catalytic RNAs of group I introns [25] and ribonuclease P [26,27,28•] are highly conserved. A pseudoknot in the satellite RNA of barley yellow dwarf virus, which belongs to the 'hammerhead' class, has been characterized [29••]. It has been suggested that pseudoknotting stabilizes the structure required for the self-cleavage of the hepatitis delta virus RNA [30]. Secondary structures for genomic and antigenomic sequences that share a common stem-loop axehead motif could be developed without a pseudoknot [31••], indicating that pseudoknotting might not be needed for the catalytic action. In support of this, a *trans*-active ribozyme was engineered from the normally *cis*-active molecule [31••,32••]. Though not capable of catalytic activity in the absence of protein [33], the mitochondrial-RNA-processing RNase could, as the RNA component of RNase P, possess a cage-shaped structure centered around a pseudoknot [26]. The existence of two pseudoknots in eubacterial ribonuclease P RNA is attested by phylogenetic and mutational analyses [28•]. The second pseudoknot (which base pairs nucleotides

82–85 with 276–279, using *Escherichia coli* nomenclature) is absent in *Bacillus subtilis* and it was suggested, on the basis of molecular models, that an additional helix present in *Bacillus* (and absent in *E. coli*) could hold an equivalent architectural function [28•]. Although these two pseudoknots impose topological constraints on the three-dimensional fold, neither is considered absolutely essential for catalytic activity *in vitro* [26]. Similarly, in the self-splicing introns of group I, a new pseudoknot, P11, was shown to be not essential for self-splicing activity *in vitro*, however, mutants with disrupted P11 required a higher concentration of MgCl₂ for self-splicing to occur [3•]. By contrast, mutants that strengthen base pairing in P11 self-splice more efficiently than the wild type at high temperature [3•]. This peripheral pseudoknot, which is present only in the subgroup IA, assists therefore the formation and the maintenance of an active conformation without itself being necessary for catalysis. Two similar pseudoknots, involving one terminal loop and a distal section of the molecule, have already been proposed for intron-exon interactions in group II introns [34,35]. The ribozyme in the satellite RNA of barley yellow dwarf virus [29••] undergoes self-cleavage at a low rate which was attributed to the presence of a pseudoknot. Base substitutions that prevented folding of the pseudoknot increased the self-cleavage rate up to 400-fold, whereas compensatory mutations reduced the self-cleavage rate in proportion to the helical stability of the helix responsible for pseudoknot formation [29••].

3'-End pseudoknots: replication control

Pseudoknotting underlies the tRNA-like motifs at the 3' end of several groups of plant viral RNA genomes (for a review, see [11•]). This structural similarity is paralleled in biological function as the tRNA-like motifs are recognized by many tRNA-specific enzymes such as aminoacyl-tRNA synthetases, nucleotidyl transferase, or RNase P [11•]. The tRNA-like structure has been shown to be necessary for the initiation of replication of a positive-sense RNA virus, brome mosaic virus (BMV) [11•]. A telomeric function of the tRNA-like structure of BMV was also demonstrated *in vivo* [36], in agreement with the 'genomic tag model' associated with such 3'-terminal tRNA-like motifs [37]. The dependence of replication on aminoacylation appears to be variable. The turnip yellow mosaic virus (TYMV) depends on valylation for replication [38] whereas BMV, which is normally tyrosylated, does not [39••]. On the basis of this observation, transcripts bearing mutations in the tRNA-like domain on one of the three genomic RNAs of BMV (which lead to decreased tyrosylation function *in vitro*) are able to interfere *in trans* with the replication of the other viral RNAs when inoculated in barley protoplasts [39••]. A mutational analysis of the pseudoknot in TYMV concluded that mutations in the loop bridging the shallow groove has stronger effects on valylation efficiency than those in the segment crossing the deep groove [9••]. Recently, the stretch of three pseudoknots preceding the tRNA-like structure in tobacco mosaic virus was shown to act as the functional equivalent of a poly(A) tail, stabi-

lizing a reporter mRNA and increasing gene expression up to 100-fold [40••].

Searching for pseudoknots in RNA sequences

The formation of pseudoknots is a three-dimensional process and is central to the RNA folding pathway. Yet, at present, pseudoknot formation is incompatible with the dynamic programming method used in the present algorithms for predicting RNA secondary structures [41,42]. Nevertheless, two programs for the prediction of pseudoknot formation have been developed recently [5,43]. Both simulate the RNA fold by a sequential selection around the most stable stems [5,43] and, further, by sequential addition of folding domains (200–400 bp) during RNA synthesis [43]. An implicit assumption in the prediction of secondary structures is that all structural elements co-exist in the final folded form; however, the possibility of alternative or transient pairings during the folding pathway or the function of the RNA should be kept in mind.

Generally, it is difficult to prove the existence of pseudoknots merely on the basis of phylogenetic comparisons of only a small number of sequences. For example, a phylogenetically conserved sequence for pseudoknots does not necessarily mean that it is an important structural signal for the *bicoid* mRNA [44], or the RNA template in telomerase [45,46], or the RNA in the signal recognition particle [47]. In these instances, systematically directed mutagenesis (with double mutants) coupled to chemical probing and functional tests would be required in order to reach consistent and biologically relevant structures. In contrast, with a large number of sequences, phylogenetic comparisons can be the most powerful approach for determining both the two-dimensional structure and the possible pseudoknots, which are then considered as those Watson–Crick pairings that are not contained in the secondary pairings [19,25,27,35]. Achieving these goals requires rigorous investigations on the frequency of compensatory base changes and the frequency of the underlying mutational events in the helical stems. Additionally, it is necessary to develop a new method, such as that described in [25], with which to distinguish those coordinated changes that result from authentic molecular constraints from those that arise from historical contingencies.

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