RNA folding: beyond Watson–Crick pairs
Eric Westhof* and Valérie Fritsch

Introduction
In the history of nucleic acid structural biology, surprises have been recurrent. Often discoveries were not the result of serendipity, but rather of crystallization experiments planned to observe other structures. Thus, the first crystal structure of a base pair in 1963, a major achievement in those days, revealed the existence of Hoogsteen pairing [1]. From this time on, several other non-Watson–Crick base pairs were observed in crystal structures [2]. Watson–Crick base pairs were first observed at atomic resolution in the crystal structures of ApU [3] and GpC [4]: the crystals contained a full sugar–phosphate backbone together with neutral bases. Following this accomplishment, structural biologists focused their attention on Watson–Crick pairs, consigning Hoogsteen pairs to the formation of triple helices [5] and in corners of tRNA structures [6]. It is now accepted that nucleic acid bases possess three edges onto which hydrogen bonding can occur (Figure 1): the Watson–Crick edge, the Hoogsteen edge and, finally, the shallow groove edge.

Improvements in RNA synthesis, purification techniques, X-ray sources, data collection and refinement have led to high-resolution RNA crystal structures with careful analysis of disorder [7–10]. The purpose of the present overview is not exhaustivity; instead we illustrate the recurrence of some structural aspects that have furthered our understanding of RNA architecture and folding since the early days of tRNA structural studies. Table 1 summarizes several new structural characteristics observed in recent crystal structures, some of which are described in the text and illustrated in the figures. The P4–P6 domain of group I introns and the hepatitis delta virus (HDV) structures have been carefully reviewed ([11,12]) as have the hammerhead ribozymes [13]. A survey of the crystal structures of helical domains is also available [14]. Some RNA motifs have been described and discussed by Moore [15] and all observed base pairs have been itemized in a review [16]. The use of non-Watson–Crick pairs as a recognition element in RNA–peptide/protein complexes has been discussed recently [17,18].

The flourishing diversity of base pairing
Because base pairing is so diverse, and because almost any combination of bases is observed in various geometries, some definitions are useful to characterize and organize base pairs. Firstly the hydrogen-bonding sites on the nucleic acid bases are distributed on three main edges (Figure 1a): the Watson–Crick edge, which presents the usual Watson–Crick sites; the Hoogsteen edge and the shallow groove edge. Pairs involving the shallow groove edge of one base, called sheared, are now frequently observed (Figure 1c). In such sheared pairs, one base presents its Watson–Crick sites to the deep groove and the other base exposes its Watson–Crick sites to the shallow groove. Purine–purine as well as pyrimidine–pyrimidine sheared pairs are observed [19,20]. Secondly, the bases can approach each other so that the sugars are on the same side or on opposite sides of a line median to the hydrogen bonds: in the first case, the pairing is called cis in the second trans (Figure 1b). Accordingly, for clarity, the Watson–Crick pairs will be noted A•U and G•C: the wobble pairs with a standard geometry G•U, and the non-Watson–Crick pairs by a •• (Figures 1b,c). Finally, some pairs employ non-standard hydrogen-bonding patterns (Figure 2). Thus, so-called bifurcated (or more appropriately ‘chelated’ [21]) hydrogen bonds have been observed; recently, in high-resolution structures of the loop E of tRNA and, previously, in the lower resolution studies of tRNAs (G18Ψ55) [22]. The involvement of C–H bonds in some sort of hydrogen-bonding interaction cannot be dismissed owing to their frequent observation in high-resolution crystal structures [21] and to their surprising stability in long molecular dynamics simulations [23]. For example, the sheared G•A pair (Figure 1c) frequently exchanges with a sheared A•A pair in which the short distance between N7(A) and H–C2(A) indicates the presence of a C–H •• N hydrogen bond. Disconcertingly, some pairs are mediated via one or more inserted water molecules. More puzzling still is a recent example of a Hoogsteen-like A•C base pair with no direct hydrogen bond but only water-mediated hydrogen bonds between N4(C) and N7(A) [19]. The example shown in Figure 3 is rather surprising and unexpected. Indeed, a G•A pair with two hydrogen bonds between the Watson–Crick
sites of both bases and a distance between the C1′ atoms larger than those in Watson–Crick pairs (12.6 Å instead of 10.5 Å) occur in tRNA structures. In the water-mediated cis G•A pairs, however, there is only one direct hydrogen bond and the C1′...C1′ distance is even larger (14.8 Å) (Figure 3). The opening occurs in the shallow groove side, a fact that might be related to the presence of magnesium ions bound in the deep groove. In addition, the cis water-mediated G•A pair is sandwiched between two bifurcated pairs. Formation of triples in the deep groove has been well documented since the early work of Arnott [24] and the tRNA structures [6]. However, it now appears that triples in the deep groove exploit C–H...O hydrogen bonds (e.g., C1072•C1092 in Figure 4); in the P4–P6 structure [25], a homologous U259 •U107 base pair occurs in which the N3...H–N4 hydrogen bond has been replaced by N3–H...O4 while the C5–H...O2 hydrogen bond is maintained.

Hydrogen bonding in the shallow groove is common and versatile

Among the important findings from the recent RNA structures was the elucidation of the subtle and unforeseen roles of hydrogen bonding in the shallow groove of RNA helices. These roles were recently discussed extensively [16] and we present here a rapid overview. Hydrogen bonds in the shallow groove all involve the O2′ hydroxyl group, and adenine residues are the most frequent bases found to interact with the shallow groove edge of another base (for side-by-side pairs of the ‘AA-platform’ motif, see below) [26]. The adenine base interacts via its Watson–Crick sites (N1 and N6), Hoogsteen sites (N6 and N7) or shallow groove sites (N3 and C2–H) with the shallow groove sites (N3(R), N2(G), O2(Y) and the O2′ hydroxyl) of another base, which is often itself engaged in a Watson–Crick or Hoogsteen pair. The type of atom interacting with the hydroxyl group is different in

Figure 1

(a) The three hydrogen-bonding edges of a purine base (left) or pyrimidine base (right). In the shallow groove edge, the ribose hydroxyl O2′ hydrogen bond frequently participates in the hydrogen-bonded pair. (b) Examples of cis (left) and trans (right) base pairing. In cis pairing the glycosyl bonds are on the same side with respect to the hydrogen bonds linking the base pairs (or a median line between the two – rarely these – hydrogen bonds); in trans, the glycosyl bonds are on either side. The hydrogen bonding sites, either Watson–Crick or Hoogsteen, together with the cis or trans orientation, are related to the local orientation of the strands. Thus, in the two examples shown, with the bases in the usual anti conformation with respect to the sugar, the strands are locally parallel. A parallel orientation of strands, instead of the usual antiparallel orientation, can occur locally following a reversal of the sugar-phosphate backbone (as in the small spliceosome L. structure around the A•A pair) or globally because of the intricate folding of the strands (as for the invariant trans Watson–Crick R15•Y48 pair of tRNAs). (c) The standard wobble G•U (left) and sheared A•G (right) pairs.
The cis or trans pairs. Thus, in a cis Watson–Crick–shallow groove pair, the N1 nitrogen of adenine binds the hydroxyl group, whereas in a trans pair it is the N6 amino group that participates (Figure 5a). Similarly, in a trans shallow groove–shallow groove pair, the N1 atom binds the hydroxyl group, but in a cis pair it is the N3 atom (Figure 5b). With the bases in the anti conformation, these choices are related to the relative orientations of the paired strands (Figure 6). The sheared G•A pairs that close GNRA tetraloops belong to the family of trans Hoogsteen–shallow groove pairs. The surprising AA-plat-form motif [26], in which two consecutive nucleotides stay side-by-side in the same plane, involves a cis Hoogsteen–shallow groove contact between the 3′-base and the 5′-base. As in other sheared base pairs, the 3′-base of the AA platform is engaged in a Watson–Crick or Hoogsteen pairing. In fact, side-by-side platforms are not restricted to 5′-AA–3′ dinucleotides; 5′-GU–3′ platforms are observed in the sarcin loop [19] and in a complex formed between 23S rRNA and the ribosomal protein L11 [27] (Figure 7).

Interstrand or cross-strand stacking

In standard B-DNA structures, base stacking occurs mainly between bases on the same strand with the sequence having only a minor influence (intrastrand stacking). In RNA helices (or A-DNA helices), however, base stacking is strongly influenced by sequence: generally, in 5′-R–Y-3′ steps one observes intrastrand stacking, and in 5′-Y–R-3′ steps there is definite interstrand stacking. This tendency is accentuated in non-Watson–Crick pairs. A well-described example of that is of wobble G•U pairs, for which there is pronounced interstrand stacking between the guanine residues in tandem G•U pairs with the sequence order 5′-UG–3′ [28].

Table 1

<table>
<thead>
<tr>
<th>Name</th>
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References: [53,58, 25,26,42,43, 64], [60], [61], [63], [19,20], [82], [63], [64].

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display pronounced purine stacks; one of the best examples is loop E of 5S rRNAs [19]. The stabilizing effect of several layers of purine stacks is also seen in bent junctions. For example, in tRNAs the two bulging residues 59 and 60 stack on each other and on the two non-Watson–Crick trans pairs U8•A14/R15•Y48, stabilising the 90° interface between the two arms. In the recent nuclear magnetic resonance (NMR) structure of a complex between protein L30 and its regulatory RNA, the 130° bend between the two helical regions is stabilized by an interesting three-layer purine stack [29].

RNA–RNA recognition motifs

Whereas the Watson–Crick pairs between complementary bases are a necessity for forming the helical framework of a complex RNA, the non-Watson–Crick pairs are pivotal in RNA–RNA and RNA–protein recognition. The complementary Watson–Crick base pairs, with cis glycosyl bonds, form the only set of pairs that are isosteric in antiparallel helices. Thus, they promote the formation of helices with quasi-regular sugar–phosphate backbones that define the secondary structure. In single-stranded RNA molecules, stacking and base pairing drive the folding of the chain on itself through the formation of helical regions linked by non-helical elements, hairpin loops, internal bulges and multiple junctions. RNA tertiary structure will therefore comprise RNA–RNA interactions involving either two helices, two unpaired regions, or one unpaired region and a double-stranded helix [11,30].

The interactions between two helices are basically of two types: either two helices with a contiguous strand stack on each other or two distant helices position themselves so...
that hydrogen bonding between the sugar–phosphate backbones occurs in the shallow grooves. The second type of contact, observed as intermolecular crystal contacts [14], is beautifully illustrated intramolecularly in the P4–P6 structure [25]. An unpaired region belongs to either a single-stranded stretch (forming an internal loop or a bulge) or a hairpin loop closing a helix. Interactions between two unpaired regions can be mediated by standard Watson–Crick pairing leading to the formation of the tertiary motif called a pseudo-knot, if a single loop is involved [31] (with the possibility of co-axial stacking between the helices), or alternatively to loop–loop motifs. Interactions between an unpaired region and a double-stranded helix can lead to various types of motifs, and always involve non-Watson–Crick pairs. Hydrogen bonding of a single-stranded stretch, to sites either in the deep or the shallow groove of a double helix, leads to the formation of triples. Since the determination of the tRNA structure, those formed in the deep groove are well known [6] (Figure 4). As discussed above, the recent structures display a rich variety of triples formed in the shallow groove (Figures 5,6). Two RNA–RNA self-assembly motifs are known in which the unpaired region constitutes a terminal hairpin loop: GNRA tetraloops bind the shallow groove of an RNA helix [32,33], whereas GAAA tetraloops bind to a specific 11-nucleotide receptor [25,34]. Both motifs had been predicted on the basis of sequence analysis, coupled to molecular modelling, chemical probing, and in vitro selection studies [32,34]. The GNRA motif was first observed as an intermolecular contact in crystals of the hammerhead ribozyme [33], whereas the GAAA motif (Figure 8) links the two main helical domains of the P4–P6
structure [25]. Internal loops also form three-dimensional motifs, as their bases are engaged in non-Watson–Crick pairings leading to compact and helix-like regions which often bind magnesium ions. A well-described and analysed system is loop E of SS rRNA [35,36]. Likewise, the P4–P6 structure contains an adenine-rich loop that is organized around two magnesium ions and presents adenine residues for interacting with a helix [25].

Unpaired regions of the secondary structure are structured

In RNA, secondary structure is usually defined in terms of contiguous regions of Watson–Crick base pairs (including wobble G–U pairs) forming helices. Formally, the RNA folding problem is simpler than the protein folding problem [37,38]. Indeed, the energy content of the secondary structure is large compared with that of the tertiary structure, therefore, the energy of the interactions maintaining the three-dimensional architecture can be considered as a perturbation on the energy of the overall system. Experimentally, this hierarchical view of RNA folding is observed in UV melting of folded RNAs, where the cooperative melting of the tertiary structure is observed first before the broad and sequential melting of the secondary structure elements [39–41]. The melting of the tertiary structure also depends strongly on divalent ion concentrations, especially magnesium ions, implying that specific ion-binding sites are created during tertiary folding [37,40,44]. On the other hand, monovalent ions influence the stability of secondary structure elements [37,38,45]. The distinction between two-dimensional and three-dimensional structures is commonly observed during in vitro experiments (for a recent appraisal, see [46]). The hierarchy in RNA folding forms the basis of a modelling approach in which preformed RNA modules are assembled into complex architectures via defined tertiary contacts [30,32]. It is now clear that the single-stranded interhelical segments are rarely unpaired and instead form structured regions that tend to be helical-like and bind magnesium ions. This organization is beautifully illustrated by the loop E domain of SS rRNA. Solution data originally concluded that magnesium ions were necessary for the structuring of this loop [47,48]. Later, NMR evidence indicated the presence of several non-Watson–Crick pairs in the loop E of eukaryotic SS rRNA [49] and their presence was subsequently confirmed by high-resolution X-ray crystallography of the sarcin loop [19]. Thus, one can expect that most of the unpaired regions in the secondary structures of ribosomal RNAs or large catalytic RNAs are

Figure 5

Idealized drawings of shallow groove pairs. (a) Watson–Crick–shallow groove pairs and (b) shallow groove–shallow groove pairs, both in cis and trans. The strand direction is indicated as described in Figure 4. Note the systematic use of the hydroxyl group O2′–H. For more information and details see [16].
in fact structured and organized. It was suggested that the loop E motif, or the S motif, is an organizing motif of the structure of multihelix loops in 16S and 23S rRNAs [50]. In the recent crystal structure of the 70S ribosome [51], one such motif was indeed seen in the electron density.

Conclusions

Until very recently, the only known RNA structures were those of the RNA building blocks, some RNA fragments, and tRNA. The stereochemical rules, established several years ago on the basis of X-ray diffraction studies of nucleosides and nucleotides [52,53], are confirmed by the recent structures (see Figure 9). The bases adopt overwhelmingly the *anti* conformation with respect to the sugar. Although bases in the *syn* conformation are more frequent in NMR-derived structures than X-ray structures, their frequency is still very low and their presence rather exceptional. The sugar puckers are commonly in the C3′-*endo* conformation, even outside helical domains, and the C2′-*endo* pucker is restricted to tight turns or loops. The other torsional angles also adopt restricted conformations, with the torsion angles about the C3′–O3′ and C5′–O5′ bonds mainly in the *trans* region.

At the tertiary structure level, the tRNAs revealed several folding rules that are still valid. Firstly, neighbouring
helices in the secondary structure, with no unpaired residues on one strand, stack in a coaxial manner (e.g., as seen in helices 2 and 4 of the HDV ribozyme [54]). Secondly, non-Watson–Crick base pairs are used systematically for tertiary contacts between and within domains. Thus, hairpin loops frequently contain non-Watson–Crick base pairs at the interface between the helix and loop region (e.g., the trans Hoogsteen pair in the thymine loop, the bifurcated G•U pair in the UNCG tetraloop, or the 32...38 base pair in the anticodon loop [55]). At the junction between stacked helices, non-Watson–Crick pairs modulate the stacking and help formation of further tertiary contacts like base triples. For example, in the structures of tRNA, at the junction between the dihydrouridine and anticodon loop, a cis Watson–Crick like G•A pair (Figure 3) is frequently observed. Likewise, in the L11 complex, between the stacked helices B and C, there is a sheared G•A pair (Figure 1) [56]. Thirdly, loop–loop interactions rely on Watson–Crick pairs (e.g., G19–C56) as well as on more complex interactions like intercalation or non-Watson–Crick pairs (G18•Ψ55). Similarly, in the HDV ribozyme [54] there is a two-base-pair pseudo-knot mediated via two invariant Watson–Crick G = C pairs between a loop and a single-stranded region. Fourthly, the U-turn is a highly recurrent element of RNA structure and is of great importance in folding (e.g., two occurrences in the tRNA structure and almost one occurrence in any large structure). Fifthly, the 2′-hydroxyl group is systematically and astutely used in turns and close tertiary approach. Finally, C2′-endo sugar puckers appear in loops and non-helical regions of the sugar–phosphate backbone.

The new crystal structures, however, have disclosed new vistas on RNA stereochemistry and introduced new

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**Figure 7**

AA platforms are not restricted to AA dinucleotides. (a) A typical AA platform. (b) A 5′-G•U-3′ side-by-side contact (the arrangement is reconstructed as the coordinates are not available for this structure). Notice how the 3′-base forms a cis Watson–Crick pair while the 5′-base is poised to interact with its Watson–Crick site. In the recent crystal structure of the complex between thymidylate synthetase and its cognate RNA [66], the major identity elements in the anticodon loop, G38 and U66, form a platform with a N2(G38)•O4(U66) hydrogen bond which stacks on a flat surface of conserved

**Figure 8**

The specific receptor for the GAAA tetraloop the 11-nucleotide motif, as seen in the crystal structure of the P4–P6 domain. The AA platform (A225–A226) is sandwiched between a trans Hoogsteen pair (A248•U224) and a wobble pair (U247•G227). A248 forms a trans Watson–Crick A•A pair with A151, the apical base of the GAAA tetraloop. The bulging U249 base forms a cis Watson–Crick pair with A226 (see Figure 7).
unexpected rules of RNA folding. Firstly, some recognition and folding motifs are clearly used recurrently. In addition, like Russian dolls, three-dimensional motifs are assembled using frequent smaller stereochemical motifs. Thus, GNRA tetraloops exploit the celebrated U-turn motif. Similarly, a sheared G•A pair does not fit alone within a helix, because of the short distance between the O3′ atom of the guanine and the 5′-phosphate of the adenine [57]. Consequently, sheared G•A pairs occur in tandem (5′-GA-3′) or in conjunction with another non-Watson–Crick pair, such as a trans Hoogsteen base pair, 3′ to the guanine. The question remains open as to the number of recurrent motifs still to be discovered [15].

Secondly, several examples exist of intermolecular contacts that organize the crystalline packing, which are also used intramolecularly in a supramolecular fashion. A prominent example is the ribose zipper motif [25] for maintaining parallel stacking of helices; another example is given by members of the GNRA tetraloop family which bind to their respective receptors intramolecularly or intermolecularly. Thirdly, in the RNA structural world, the appearance of non-Watson–Crick pairs, in internal loops, junctions or hairpin loops, is now so prevalent that the words ‘mismatch’ and ‘mispair’ have lost their meaning. It now seems that one of the main functional and structural roles of RNA helices is to subtend a three-dimensional scaffold, critically maintained by non-Watson–Crick pairs, presenting recognition and binding motifs made of non-Watson–Crick pairs. In this respect, the pairs formed in the shallow groove, like the sheared pairs or the side-by-side bases in the AA-platform motif, are especially important as their Watson–Crick sites are available for RNA or protein binding. In these shallow groove pairs, except in the AA-platform motif, the O2′ hydroxyl group is implicated, emphasizing how functionally specific to RNA these motifs are. Interestingly, the only theoretical prediction of the side-by-side bases, an arrangement that does not use the hydroxyl group, was proposed for some DNA-specific sequences [58].

Presently, it is not clear how the appropriate functional equilibrium between Watson–Crick and non-Watson–Crick pairs is determined during evolution (natural or artificial). Divalent ions could be an important factor for the maintenance of the functional distribution of base pairs. Indeed, the recent crystal structures provide a wealth of new structural information and insight into divalent ion binding to RNA, especially for key magnesium ions. Although magnesium ions bind frequently to (and often link) the anionic phosphate oxygen atoms and the Hoogsteen sites of guanines, the non-Watson–Crick pairs, because of their effects on groove size and the sugar–phosphate backbone path, often mould ion-binding cavities. Thus, the balance between Watson–Crick and non-Watson–Crick pairs is strongly dependent on the concentration of divalent ions.

Nowadays, the structural ease with which RNA forms non-Watson–Crick pairs and the variety of these pairs is still a curse for RNA crystallographers, who often observe extended duplexes with several non-Watson–Crick pairs instead of the hoped for monomeric hairpin fold or motif. Thus, in continuation with its history, RNA crystallography...
persists in surprising us, keeping us away from despondency by the excitement and fascination of unexpected RNA structures.

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References


