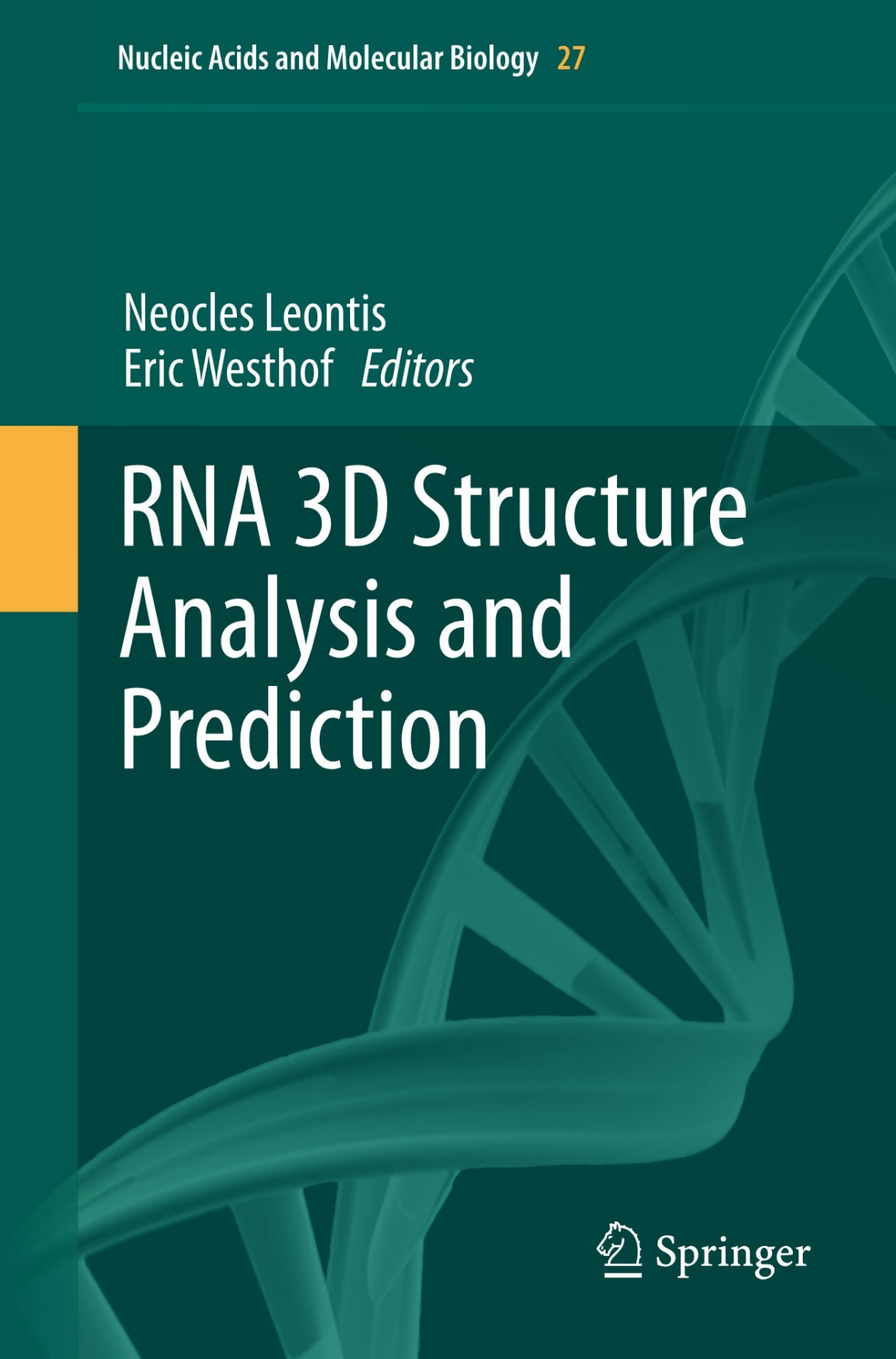
Nucleic Acids and Molecular Biology 27

Neocles Leontis
Eric Westhof *Editors*

RNA 3D Structure Analysis and Prediction



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RNA 3D Structure Analysis and Prediction

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Chapter 1
Introduction

Michael Levitt

I first encountered ribonucleic acid in October 1968 (see early history of Computational Structural Biology, Levitt 2001). I worked on RNA for a few years and published three out of my five first papers on RNA (Levitt 1969, 1972, 1973) before abandoning the system as being too simple and not nearly as interesting as protein folding. This was my first of several career-level mistakes. In 1976, I also refused to get involved in the analysis of DNA sequences when Bart Barrell brought me the DNA sequence of φX174 bacteriophage (Smith et al. 1977; Levitt 2001). What I find most surprising about these mistakes is that the decisions seemed very easy when I made them and regrets came much more slowly but lasted longer. In 2008, RNA caught my fancy again thanks to a HFSP International collaboration spearheaded by Michael Kiebler (Medical University of Vienna), and I have now come full circle with four of my five most recent papers involving RNA.

This background made the pleasure afforded me by the request to write this Introduction especially great both as a way to reflect on the past and also to look forward to the future. The first paper in the book entitled "Introduction to RNA Modeling" by Eric Westhof and Neocles Leontis provides a wonderful summary and a very useful table that summarizes the methods used to model RNA structure. This made me understand better why I moved from RNA to proteins almost 40 years ago: very little structural data was available for RNA then, whereas much more was available for proteins. With the determination of the atomic structure of the ribosome, this situation has changed: today a lot more is known about the structures that RNA adopts.

Comparing the history of structure predictions of protein with that of RNA can be very informative. Most methods used for both cases consist of the same choices. What is the best representation? What is the best method to generate and change structures? What is the best way to score the resulting structures so as to select those

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most native-like? Everyone wants detailed all-atom structures as they help determine function. The need to reduce computational complexity led to the first coarse-grained studies of protein folding in 1975, and such coarse graining (Levitt and Warshel 1975), in which several atoms are grouped into one interaction center, is now popular for RNA, being used for 5 of the 19 methods in the Westhof-Leontis Table (The Table). This immediately requires methods to add back atomic details, and such methods have matured enormously for proteins since the earliest methods by Ponder and Richards (1987), Holm and Sander (1991), and Levitt (1992). The latest version of Dunbrack's Scwrl method (Krivov et al. 2009) is able to place missing side chains with uncanny accuracy. Similar methods exist for RNA but are likely to undergo additional development.

The molecular representation is intimately connected to interatomic forces and, hence, the energy of the system. With all the atoms present, molecular mechanics or even quantum mechanical energy functions can be used. With coarse graining, such potentials can be derived from the chemical structures of the groups involved (e.g., do they stack, base-pair, etc.), paralleling what was done in the original protein course-graining work (Levitt and Warshel 1975). As more structural data is made available by structural biologists, statistical or knowledge-based potentials are a very useful alternative. Such potentials have a long history for proteins starting with Tanaka and Scheraga (1976) and extending to Summa and Levitt (2007). As the amount of protein structure grew exponentially, it became possible to use better representations and more atom types, extending from contact potentials between 20 amino acids (210 number) in 1976 to smooth, closely sampled distance-dependent functions for almost 200 atom types (over five million numbers). While knowledge-based energy functions are frustrating in their neglect of so much physics and even statistics (interactions are not independent but are assumed to be), they do work best at refining proteins (CASP7 to CASP9, Chopra et al. 2010). One can expect a continuous trend that leads to ever more complicated but better RNA knowledge-based functions.

Three physical methods are used to change molecular conformations: energy minimization (as used to refine my 1969 model of tRNA), molecular dynamics, and Monte Carlo random moves. The first two methods are thought to be more efficient for systems with many degrees of freedom, but they suffer from a massive drawback: the need for smooth differentiable energy functions. The Monte Carlo method has been very successfully used to model proteins by swapping a fragment of the main chain for a different, known native fragment and then keeping the result if it satisfies the Monte Carlo criterion (Simons et al. 1997). This process is clearly discontinuous. We have developed a new method called Natural Move Monte Carlo (Minary and Levitt 2010) that allows much more efficient sampling of both proteins and RNA. Surprisingly, more methods described in Table 2.1 use molecular dynamics instead of Monte Carlo to change conformation. This is expected to change in the future, except perhaps for refinement of detailed RNA structures or modeling of RNA dynamics. Fragment-based methods have also been very successful for RNA structure prediction. A major drawback is their dependence on what has already been seen and the impossibility of proper thermodynamic

sampling. Some of the problems associated with Monte Carlo moves have been solved in a very recent paper from our group (Sim et al. 2012).

Once one has an ensemble of putative structures, they need to be scored so as to pick out the best ones. Often such scoring is preceded by clustering, aimed at selecting representative structures from each energy basin. Clustering is a surprisingly tricky business, and we are pleased to have been able to develop a new method that seems to aid selection of near-native structures (Sim and Levitt 2011).

In conclusion, I am in complete agreement with the many groups who have contributed to the very impressive book: RNA structure prediction has clearly come of age and promises to make dramatic advances in the next few years. As such the publication of this book on RNA Structure Analysis and Modeling could not have been timed better!

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Chapter 2
Modeling RNA Molecules

Neocles Leontis and Eric Westhof

> Chercher plutôt la rigueur dans l'enchaînement de la pensée plutôt que la précision dans les résultats. Le modèle le plus crédible n'est pas nécessairement le plus réaliste, car il demande l'éxagération des traits caractéristiques par rapport aux traits contingents.
>
> —Abraham Moles, *Les sciences de l'imprécis*, Paris, Seuil (1990)
>
> Strive for rigor in the logical train of thought rather than in the precision of the results. The most enlightening scientific model is not necessarily the most realistic one, because it is necessary to exaggerate the characteristic features with respect to the contingent ones.
>
> —Translated by the authors

2.1 Introduction

A primary activity of scientific work is the construction of models to represent the nature and workings of phenomena we observe in the world around us. Models that represent the molecular components of living system in three dimensions (3D) and at atomic resolution are highly valued in molecular and structural biology. For example, the decipherment of the 3D structures of ribosomes, the complex protein-synthesizing nanomachines of the cell, represents a tremendous achievement, recently recognized with the Nobel Prize in Chemistry (http://nobelprize.org/nobel_prizes/chemistry/laureates/2009/). Nonetheless, this phenomenal success is

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tempered by the realization that even now, over 10 years after the first ribosome structures were solved, we still do not understand fully several aspects of their functioning. For all who have grappled with the complexities of ribosome structures, Richard Feynmann's pithy statement, "What I cannot create, I do not understand," rings especially true (Hawking 2001). This physics-based realization contrasts with another point of view of modeling. To paraphrase R. W. Hamming, who said, "The purpose of computing is insight, not numbers" (Hamming 1971), we should remember that the purpose of molecular modeling is functional insight, not detailed atomic models per se. Therefore, as we seek to improve our abilities to construct 3D models for molecules for which we do not yet have experimental atomic-resolution structures, we should bear in mind that it may not be necessary to achieve some arbitrary precision in the atomic coordinates to provide insight into biological function. Rather, we should think carefully to identify those predicted features that yield important insights (Table 2.1).

Thus, for those engaged in RNA modeling, critical questions to ponder include: *What* do biologists, who are trying to unravel the roles of RNA in complex biological processes (growth and development, learning and cognition, immune and stress responses, and disease), *really* need to know about the 3D structures of the RNA molecules they study, and in *what form* do they need it? In this context, how deep do we need to go into atomic details to gain useful insights? How can knowledge of RNA 3D structure be applied to infer RNA function? It is crucial to bear in mind that, historically, some imprecise models have been richer in biological insight than other, very precise ones. The famous, original 3D model for double-stranded DNA of Watson and Crick stands out in this respect.

With these fundamental issues as background, we turn to the reasons for renewed interest in RNA 3D modeling: New high-throughput experimental approaches, developed in the postgenomic era, have revealed the pervasive role of noncoding RNA molecules in all aspects of gene expression, from chromosome remodeling and regulation of epigenetic processes to transcription, splicing, mRNA transport and targeting, and translation and its regulation. Furthermore, while the number of protein-coding genes has changed little from the genome of the tiny 1,000-cell nematode *Caenorhabditis elegans* to that of our own species, *H. sapiens*, the number of ncRNAs has exploded and appears to scale with biological complexity (Taft et al. 2007). Evidence is building that many of these ncRNAs, like those involved in splicing and translation, which have been known for many years, function at least in part by forming complex 3D structures to interact specifically with proteins, other nucleic acids, and a wide range of small molecules.

2.2 Defining the Problem

For RNA molecules that form discrete 3D structures, the folding problem can be simply stated: What is the mapping from sequence space to three-dimensional space? As many biologically active RNA molecules are very long (up to thousands

Table 2.1 The various prediction programs described in the book with their main elements and outputs

Author	Program	Strategy	Output	CG	KB	MD	MC	2D	3D
Altman	RNABuilder (1)	Method: multiresolution, KB modeling with or without templates and fragment assembly	3D atomic structure model	x	x				x
Altman	NAST	Method: coarse-grained KB modeling with MD sampling	3D coarse-grained structure model	x	x	x			x
Bujnicki	ModeRNA	Input: sequence and 3D structure of template Method: homology and template-based modeling	3D atomic structure model						x
Bujnicki	SimRNA	Input: sequence Method: de novo coarse-grained modeling with Monte Carlo sampling with KB potential	Coarse-grained 3D model from which 3D atomic structure model is built using rebuild RNA	x	x		x		x
Chen	Vfold	Input: sequence and 2D structure Method: lattice-based coarse-grained modeling. MD refinement to obtain atomistic model	Free energies of pseudoknotted structures; coarse-grained 3D structures from which 3D atomic structure model is built	x		x			x
Das	FARNA/FARFAR	Input: sequence and knowledge-based potential Method: de novo modeling using KB potentials and MC sampling	3D atomic structure model		x		x		x
Dokholyan	DMD	Input: sequence and 2D and tertiary constraints Method: coarse-grained, multiscale conformational sampling by discrete MD	Full 3D atomic coordinates deduced from coarse-grained structure model. Folding thermodynamics	x		x			x
Major	MC-FOLD/MC-Sym	KB grammar for fragment assembly	3D atomic structure model		x				x
Santa Lucia	RNA123 (homology modeling module)	Input: sequence and 3D structure template Method: homology modeling with MD energy refinement	3D atomic structure model			x			x

(continued)

Table 2.1 (continued)

Author	Program	Strategy	Output	CG	KB	MD	MC	2D	3D
Shapiro	RNA2D3D	Input: 2D structure. Atomic modeling using fragment assembly and MD energy minimization	3D structures			x			x
Shapiro	MPGAfold	Genetic algorithm to optimize 2D structure	2D structures with pseudoknots					x	
Wang	G2G (global measurements to global structure)	Inputs: sequence and 2D structure; RDC and SAXS measurements method	Global structure of relative helical orientations					x	

Abbreviations: *CG* coarse graining, *KB* knowledge-based, *MD* molecular dynamics, *MC* Monte Carlo, *2D* two-dimensional, *3D* three-dimensional
References: RNABuilder (Flores et al. 2011); NAST (Jonikas et al. 2009); ModeRNA and SimRNA (Rother et al. 2011); Vfold (Cao and Chen 2011); Farna (Das and Baker 2007); FARFAR (Das et al. 2010); DMD (Ding et al. 2008); MC-FOLD/MC-Sym (Parisien and Major 2008); RNA2D3D (Martinez et al. 2008); MPGAfold (Shapiro et al. 2006); G2G (Wang et al. 2009)

of nucleotides), this question is relevant for those portions of RNA sequence that adopt stable architectures, required for their function during at least some period of time. In other words, given a sequence, produce a set of 3D coordinates for the nucleotides, that is biologically relevant and that satisfies the stereochemistry and physical chemistry of RNA molecules.

2.2.1 RNA Modeling Compared to Protein Modeling

In this regard, the parallels and contrasts between RNA and protein structure prediction and folding are apparent. Like proteins, RNA molecules are flexible linear polymers with astronomical conformational possibilities. Unlike proteins, RNA structures generally partition quite cleanly between secondary and tertiary hierarchical levels (Brion and Westhof 1997; Woodson 2010, 2011). Thus, as a rule, the first step in successful 3D modeling of RNA passes through a high-quality prediction of the main secondary structure elements. The state of the art in RNA secondary structure prediction is reviewed by Steger and coauthors in the third chapter of this volume. At the present state of our modeling efforts, the nature of the input data can play a decisive role at this stage of the process. Indeed, despite significant advances in 2D structure prediction, current methods still rely on theoretical approximations and an incomplete set of empirical energy parameters. Thus, working on a single RNA sequence may lead to incorrect evaluation of the importance or the role of one or more structural elements. The idiosyncrasies contained in single sequences can, however, often be ironed out by the use of multiple homologous sequences. Moreover, for RNA molecules, in contrast to proteins, one can obtain many additional experimental data containing much 3D information, using chemical or enzymatic probing and footprinting, small-angle X-ray scattering (SAXS), and cross-linking. The incorporation and computer use of such data changes the tractability of the problem. The chapters by Laederach, Wang and Fabris, and their coauthors (Chapters 15–17) address some of these issues and illustrate the challenges and power of integrating modern experimental data collection with modeling methods.

2.2.2 Defining the Inputs for RNA 3D Modeling

Inputs for the modeling of RNA 3D structure include, in addition to the sequence of the target RNA, the derived secondary structure and the sequences of available homologues, as well as all available experimental data. The database of known RNA 3D structures should also be considered an important resource for 3D modeling. This is especially the case for those approaches relying on a modular view of RNA architecture with the resulting assembly of RNA elements and modules (Jossinet et al. 2010; Westhof et al. 2011).

2.3 3D Modeling Methods and Approaches

A variety of modeling approaches are represented in the contributions to this volume. Some common themes emerge and will be summarized briefly with reference to specific chapters. As will become apparent to readers, promising approaches are rapidly adopted by multiple research groups, although specific implementations vary in ways that are usually not easy to discern. This volume focuses on methods that aim to achieve automaticity in 3D modeling, in the sense that they should require very little human intervention in the modeling process, beyond defining the inputs for the specific problem. The effort, rather, is focused "up front" on designing the algorithms and extracting and compiling relevant knowledge concerning RNA structure from structure databases for automated use by the implemented algorithms.

2.3.1 Homology Modeling

Automated methods generally address one or both of two distinct problems in biological structure prediction, namely, homology modeling and de novo prediction. Homology modeling concerns building atomically accurate 3D models of RNA molecules using at least one homologous 3D structure as template. RNA homology modeling draws on vast experience with protein homology modeling, and so considerable progress has been made already. The contributions of Altman, Bujnicki, and Santa Lucia focus, at least in part, on homology modeling and, between them, exhaustively address the issues involved.

2.3.2 De Novo Modeling

De novo prediction is necessary when no homologous 3D structure is known that can serve as a template for modeling. It is considerably more challenging than homology modeling, as it often requires generating a brand new 3D architecture from any known heretofore. As the goal is to do this without expert human input, the general approach is to generate large numbers of possible architectures and then to evaluate them, using what is already known about RNA structure. Automated, de novo 3D modeling approaches are therefore distinguished operationally by the kind of algorithm employed to generate potential 3D structural models, and also by the nature of the encapsulated knowledge concerning RNA structure that is used to score and rank models to arrive at a small set of predicted 3D structures, or in the favorable case, a single structure. The models generated by conformation-sampling algorithms are called "decoys" by practitioners. For the final output, most programs produce an all-atom predicted structure, which is generally quite "correct" in its

local, stereochemical detail, in the sense that bond lengths and angles are within allowed ranges and the model contains no unphysical nonbonded contacts. But this local precision, which most programs achieve routinely, should not mislead users of predicted 3D models into assuming the model is accurate on larger, biologically relevant length scales, ranging from structures of modular motifs to overall folds and architectures.

The contributions of Altman (Chapter 8), Bujnicki (Chapter 5), Chen (Chapter 10), Das (Chapter 4), Dokhalyan (Chapter 9), Santa Lucia (Chapter 6), and Shapiro and their coworkers (Chapter 7) address de novo 3D modeling and among them cover the major methods in use today. All of these methods deploy some kind of algorithm to sample conformation space and some kind of knowledge-based methods to score and rank proposed solutions to the 3D prediction problem. In addition, most approaches rely on some kind of reduced representation of the RNA structure ("coarse graining") to speed up the calculations and allow more thorough exploration of conformational space with available computer resources. Coarse graining is an art that requires striking the right balance between speed of calculation and sufficiently detailed representation of RNA structure to capture the molecular features that stabilize the active conformations. Other ways to speed up conformational sampling involve modification of the algorithms that propagate the dynamics, as represented by the discrete molecular dynamics (DMD) method reported by Dokhalyan and coworkers.

2.3.3 *Defining the Outputs of Different Modeling Approaches*

The outputs of modeling studies depend on the modeling approach and the aim of the study. Indeed, output data can be full atomic coordinates for every single nucleotide or, in the case of coarse-grained methods, coordinates for only a subset of atoms or even a single pseudoatom representing each nucleotide. The different outputs are directly related to the granularity of the modeling approach. Nonetheless, nominally atomic-resolution models, when poorly refined or badly assembled, may be no better or even worse than coarse-grained models, if the characteristic base-pairing and base-stacking interactions of the structures are not represented accurately.

2.3.4 *Precision of Models vs. Accuracy of Models*

There is no necessary correlation between precision and accuracy, and models with comparable precision can differ substantially in the accuracy with which they predict the important interactions between nucleotides that define the RNA 3D structure. Thus, low-precision models can be very accurate (e.g., the original Watson–Crick model for DNA) and highly precise ones can be partly or totally

inaccurate and thus misleading. Clearly, less-accurate models may not be at all pertinent for structural biology, while less-precise models can be very rich and enlightening. Still, these considerations should not be taken as license for not using in model building, whenever possible, high-resolution building blocks that are precise with respect to bond lengths and angles within nucleotides, and H-bond distances, van der Waals contacts, and relative orientations within base pairs and other interactions.

2.4 Databases for Extracting Knowledge

All of the precise structural data regarding RNA comes ultimately from atomic-resolution X-ray structures of nucleotides, oligonucleotides, and various biologically relevant structures, ranging in size from individual helical elements to the full ribosome. These data comprise all our basic knowledge of bond lengths, angles, and stereochemistry, as well as interaction preferences, including all types of base pairs and most stacking and base–backbone interactions. This information is used to build force fields and to infer rules for assembly of molecular moieties. These force fields and energetic rules are then used for producing and optimizing structures, sampling the conformational space, or simulating molecular dynamics. The quality and general value of the deduced force fields will strongly depend on the number and variety of structures available. In addition, the quality of the structures is of primary importance; it is directly related to the crystallographic resolution of the X-ray data and on the refinement process since a minor fraction of X-ray structures are obtained at true atomic resolution. One key parameter for compiling reference databases for knowledge extraction is the nonredundancy of the structures that are included in order to avoid bias in the deduced parameters. The chapter by Leontis and Zirbel (Chapter 14) addresses these issues and details a nonredundant database of structures extremely valuable for extracting knowledge about RNA as well as for benchmarking modeling strategies. In this respect, it is worth noting that less than 100 nonredundant RNA structures have been solved at 2-Å resolution or better.

2.5 Evaluating Models or "The Proof of the Pudding Is in the Eating"

As discussed above, 3D models are produced either to monitor our progress in the understanding and use of the physicochemical rules governing RNA architecture or to provide insight and help to experimentalists in the interpretation and meanings of biological data and in the design of new experiments. Although objectives may differ, in every case the models produced should be evaluated to assess their relevance to biological reality. Models that make testable predictions are

especially valuable and, as emphasized above, need not be particularly precise. Additional experiments devised on the basis of a given model will provide the relevant tests for evaluating it. Depending on the outcome, the model may be retained and perhaps "tweaked," or it may be rejected and radically revised, leading to new biological insight and further experimental tests. On the other hand, to assess the validity of force fields as well as other empirical assembly rules, precise numerical comparisons have to be performed in a systematic way. This highlights the need for discriminating and meaningful metrics to compare and evaluate predicted vs. experimental structures.

2.5.1 Metrics for Evaluating Models

The most common metric is the root mean square deviations (RMSDs) on corresponding atoms between the predicted and experimental models. RMSDs are easy to compute and yield a simple measure. However, to interpret RMSD values, some critical length scales in RNA structures should be kept in mind for comparison: First, stacking distance between bases is about 3.4 Å; second, successive P–P distance in RNA helices is about 7 Å. While RMSD values below 3.4 Å are of real value, RMSD values beyond 8 Å must be treated with caution. In addition, RMSDs, as generally calculated with rigid-body fitting, spread the errors between two sets of coordinates over the whole ensemble. Consequently, even correctly modeled regions will not superimpose properly and thus will also contribute to the overall RMSD value. Therefore, RMSD values should be supplemented with local structural comparisons, including, for example, the numbers of correct base stackings and of correct Watson–Crick base pairs and, especially for 3D architectures, the number of non-Watson–Crick pairs, correct both with respect to pairing partners and base-pair types (Leontis and Westhof 2001). For a summary of the types of non-Watson–Crick base pairs, see the Appendix of this volume. We stress the importance of predicting the correct non-WC pairings as well as the correct base stackings, both of which are key because there is no three-dimensional architecture without non-Watson–Crick pairs and additional stackings between pairs. While a simple mapping of the 2D structure into a 3D structure does lead to a three-dimensional fold, such a fold will lack the additional stackings or RNA–RNA contacts that are characteristic of the complete 3D architecture. In short, correct predictions imply correct choices of new base stackings between single-stranded nucleotides and helices as well as new long-range base-pair contacts. For these reasons, two new metrics particularly suitable to RNA were introduced: the deformation index and the deformation profile (Parisien et al. 2009). The deformation index monitors the fidelity of the interaction network and encompasses base-stacking and base-pairing interactions within the target structure. The deformation profile highlights dissimilarities between structures at the nucleotide scale for both intradomain and interdomain interactions. These tools demonstrate that there is little correlation between RMSD and interaction network fidelity. To improve force fields or modeling approaches, it is mandatory to assess the

origins of the errors. The deformation profile is a very useful tool for identifying the origins of incorrect modeling decisions.

2.5.2 *Necessity for Objective Evaluation of Modeling Efforts: RNA–CASP*

Structure prediction methods for proteins were boosted and consolidated by the CASP project (Critical Assessment of techniques for protein Structure Prediction), a systematic and worldwide evaluation of the predictions of new structures, prior to their publication (Kryshtafovych et al. 2005; Moult et al. 2009). CASP has proven extremely useful, productive, and constructive for benchmarking the progress made in the generation of new ideas and the objective assessment of the newly developed techniques. We believe that setting up a similar process will prove very healthy for the RNA structure-modeling field. To do so, several hurdles need to be overcome. In the case of RNA prediction, two levels would have to be distinguished, namely, the prediction of secondary structure and the modeling of 3D (tertiary) structure. The main issue, however, is how to establish efficient communication between research groups that determine RNA structures, whether at the secondary or tertiary structure levels, and research groups that predict RNA structures, so the latter can register their predictions before the structures are published. Clearly, despite the amazing advances in all aspects of the production of 3D RNA structures by X-ray crystallographic, NMR, or cryo-EM methods, the number of new structures produced per year remains rather low. The proposed process would follow these lines: (1) A structural group working on a new RNA structure (X-ray, NMR, chemical probing, cryoelectron microscopy, or mass spectroscopy) makes known their willingness to "play the game." (2) The group sends the sequence of the RNA under investigation to the coordinator. (3) The coordinator, without disclosing the identity of the experimental laboratory or the function and origin of the RNA, distributes the sequence to the theoreticians ready to tackle the challenge. Each theoretical group must agree not to disclose the sequence or distribute it further or to disclose its own progress or results in any fashion before publication of the structure by the experimental group. (4) The deadline for submitting structure predictions to the coordinator is agreed upon at the outset and generally will coincide with the date the experimental group submits their structures for publication. (5) During a special meeting, the coordinator discloses the theoretical results, and they are compared with the published experimental structures. (6) Special guidelines and rules for the comparisons will be agreed upon before the writing and publication of the analysis. Several laboratories dedicated to RNA bioinformatics around the world have expressed their keen interest to participate in such regularly held contests. The success and real progress generated by CASP in protein structure prediction should encourage us all to pursue this endeavor in the form of an ongoing RNA–CASP process. A first test of RNA–CASP was initiated at the end of 2010 and is now in the process of being published (Cruz et al. 2012).

2.6 Complications Limiting Modeling Approaches

Biological reality is complicated, and the applicability of physicochemical approaches based fundamentally on assumptions of thermodynamic equilibrium should always be properly evaluated as part of the theoretical modeling process. First, RNA molecules begin folding almost immediately as they are transcribed (cotranscriptional folding) so the issue of kinetic vs. equilibrium control in formation of biologically relevant structures is always a real one (Cruz and Westhof 2009). When the first structure to form is not the biologically relevant one, chaperone molecules are observed to play additional important roles. RNA molecules rarely act alone; on the contrary, they almost always act by binding to other RNA molecules or to proteins, and very frequently they bind to both types of macromolecules, if not also to small molecules.

An especially complicated problem is that of "induced fit," which occurs when the conformation adopted by an RNA molecule in isolation is not identical to that found in a complex with a small molecule ligand, antibiotic, or another RNA or a protein (Williamson 2000). Even small ligands, like hydrated magnesium ions, are difficult to treat in an appropriate fashion. Magnesium ions are especially difficult to treat when they bind, not as outer-sphere complexes (with a full share of coordinated water molecules), but instead as an inner-sphere complexes, with the loss of one or more water molecules and direct coordination to the RNA, generally in a state different from the original magnesium-free ion state (see Chapter 11 by P. Auffinger). Treating induced fit, at minimum, requires that the full dynamics of an RNA fragment be known in order to be able to select the proper conformation binding a given ligand. And it is not at all proven that the range of conformations accessible by the usual methods of molecular dynamics simulations, for example, actually covers the states obtained in the presence of the ligand or protein. Thus, one can study the dynamics of the A-site of the ribosome alone or in complex with an antibiotic (because crystal structures exist for all those different states), but the docking of an antibiotic to the A-site starting from an "empty state" (which is not the same as the state of the bound complex minus the ligand) has not been achieved yet (Moitessier et al. 2006).

2.7 Challenges for the Future: Dealing with Massive Data Streams and Connecting to Biology

Several main questions of great potential for biology continue to be actively pursued, and yet we have barely scratched the surface. One is the use of modeling predictions, firstly for searching noncoding RNAs in genomes and secondly for choosing among genomic regions those that are susceptible to fold into architectural domains or fragments (e.g., as riboswitches do). Another major question is the prediction of protein-binding sites along RNA sequences. Some consensus binding

sequences are known, but in most cases, only knowledge of the RNA 3D fold allows the full understanding of the binding surface and RNA–protein contacts.

2.8 Conclusion

For modeling to be relevant to twenty-first century biological research, data pipelines need to be developed, maintained, and intelligently monitored to deal with the massive data streams produced by modern high-throughput sequencing methods. This means aiming for full automaticity at all steps of the computations. In this way, one should be able to link computational predictions with the experimental high-throughput technologies being constantly developed and refined. The establishment of such links between experimental and computational high-throughput techniques will bring us closer to the establishment of complete "RNA structuromes" for a given microbial or multicellular organism (Underwood et al. 2010; Weeks et al. 2011).

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Chapter 3
Methods for Predicting RNA Secondary Structure

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Abstract The formation of RNA structure is a hierarchical process: the secondary structure builds up by thermodynamically favorable stacks of base pairs (helix formation) and unfavorable loops (non-Watson–Crick base pairs; hairpin, internal, and bulge loops; junctions). The tertiary structure folds on top of the thermodynamically optimal or close-to-optimal secondary structure by formation of pseudoknots, base triples, and/or stacking of helices. In this chapter, we will concentrate on available algorithms and tools for calculating RNA secondary structures as the basis for further prediction or experimental determination of higher order structures. We give an introduction to the thermodynamic RNA folding model and an overview of methods to predict thermodynamically optimal and suboptimal secondary structures (with and without pseudoknots) for a single RNA. Furthermore, we summarize methods that predict a common or consensus structure for a set of homologous RNAs; such methods take advantage of the fact that the structures of noncoding RNAs are more conserved and more critical for their biological function than their sequences.

3.1 Introduction

In this review, we will concentrate on software tools intended for prediction of secondary structure(s) of a given RNA sequence. The first such computational tool available was mfold (Zuker and Stiegler 1981); in the past 30 years, however, it was improved and refined several times (Zuker 2003). It is still commonly used, but it is now replaced by the UNAfold package (Markham and Zuker 2008), which includes several features not available in mfold. The two major alternative packages of

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comparable or even greater scope are the Vienna RNA (Hofacker 2003) and the RNAstructure (Reuter and Mathews 2010) packages. All rely on a simplifying thermodynamic model of nearest-neighbor interaction; we will briefly summarize this model in Sect. 3.2.1. In Sect. 3.2.2, we present some of the available tools.

Because all tools use the same basic thermodynamic model and associated thermodynamic parameters, they "know" about special features of certain loops: for example, parameters of thermodynamically extra-stable hairpin loops (for a review, see Varani 1995) or small internal loops with non-Watson–Crick base pairs are taken into account (e.g., see Xia et al. 1997), but no tool mentions such details in its output. More complex arrangements, for example, stacking of helices in multi-branched loops, are not taken into account, by and large, because of the increased computational complexity and the lack of relevant parameters. Furthermore, all of the abovementioned tools disregard pseudoknots, which are important structural features in many noncoding as well as messenger RNAs. Thus, we will turn to the prediction of pseudoknotted RNA structures in Sect. 3.3.

In those cases where a set of two or more homologous RNA sequences is available, comparative sequence analysis methods can be applied to predict a consensus structure common to all sequences in the set. Such approaches, which we review in Sect. 3.4, are based on the observation that in many cases, RNA secondary and tertiary structures are more conserved than primary sequence and are of greater importance for the biological function.

We apologize to all authors whose methods and tools we have not mentioned in this review for lack of space.

3.2 RNA Secondary Structure Prediction Based on Thermodynamics

3.2.1 Overview of RNA Secondary Structure Formation

A secondary structure of an RNA sequence R consists of base stacks and loops. It is defined—at least in the context of this chapter—as

$$R = r_1, r_2, \ldots, r_N,$$

with the indices $1 \leq i \leq N$ numbering the nucleotides $r_i \in \{\mathrm{A}, \mathrm{U}, \mathrm{G}, \mathrm{C}\}$ in the $5' \rightarrow 3'$ direction. Base pairs are denoted by $r_i{:}r_j$ or, for short, $i{:}j$ with $1 \leq i{<}j \leq N$. Allowed base pairs are *cis*-Watson–Crick (WC; A:U, U:A, G:C, C:G) and wobble pairs (G:U, U:G). Formation of base pairs belonging to a given secondary structure is restricted by

$$j \geq 4 + i, \tag{3.1}$$

which gives the minimum size of a hairpin loop, and the order of two base pairs i:j and k:l has to satisfy

$$i = k \quad \text{and} \quad j = l, \tag{3.2}$$

or

$$i < j < k < l, \tag{3.3}$$

or

$$i < k < l < j. \tag{3.4}$$

Condition (3.2) allows for neighboring base pairs but disallows any triple strand formation; a base triple j:k:l would force $i = k$ and $j \neq l$. Condition (3.3) allows for formation of several hairpin loops in a structure. Condition (3.4) explicitly disallows "tertiary" interactions; such interactions do, in fact, occur in many RNAs, for example, in pseudoknots (see Sect. 3.3).

Structure formation—from an unfolded, random coil structure, C, into the folded structure, S—is a standard equilibrium reaction with a temperature-dependent equilibrium constant, K:

$$\begin{aligned} &\mathrm{C} \rightleftharpoons \mathrm{S}, \\ &K = \frac{[\mathrm{S}]}{[\mathrm{C}]}, \\ &\Delta G_T^0 = -RT \ln K = \Delta \mathrm{H}^0 - T \cdot \Delta S^0. \end{aligned}$$

At the denaturation temperature $T_\mathrm{m} = \Delta H^0 / \Delta S^0$ (melting temperature or midpoint of transition), the folded structure S has the same concentration as the unfolded structure ($K = 1; \Delta G^0_{T_\mathrm{m}} = 0$). This is only true if the structure S denatures in an all-or-none transition. In most cases, however, structural rearrangements and/or partial denaturation take place prior to complete denaturation, as temperature is increased.

The number of possible secondary structures of a single sequence grows exponentially ($\approx 1.8^N$) with the sequence length N (Waterman 1995). Accordingly, all possible structures S_i of a single sequence coexist in solution with concentrations dependent on their free energies $\Delta G^0(\mathrm{S}_i)$; that is, each structure is present as a fraction given by (3.5):

$$f_{\mathrm{S}_i} = \exp\left(\frac{-\Delta G_T^0(\mathrm{S}_i)}{RT}\right)/Q. \tag{3.5}$$

The partition function, Q, for the ensemble of all possible structures, is given by (3.6):

$$Q = \sum_{\text{all structures } \mathrm{S}_i} \exp\left(\frac{-\Delta G_T^0(\mathrm{S}_i)}{RT}\right). \tag{3.6}$$