Published in final edited form as:
Curr Opin Struct Biol. 2010 June ; 20(3): 295–304. doi:10.1016/j.sbi.2010.04.001.

Advances in RNA Secondary and Tertiary Structure Analysis by Chemical Probing

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Abstract

RNA is arguably the most versatile biological macromolecule due to its ability both to encode and to manipulate genetic information. The diverse roles of RNA depend on its ability to fold back on itself to form biologically functional structures that bind small molecules and large protein ligands, to change conformation, and to affect the cellular regulatory state. These features of RNA biology can be structurally interrogated using chemical mapping experiments. The usefulness and applications of RNA chemical probing technologies have expanded dramatically over the past five years due to several critical advances. These innovations include new sequence-independent RNA chemistries, algorithmic tools for high-throughput analysis of complex data sets composed of thousands of measurements, new approaches for interpreting chemical probing data for both secondary and tertiary structure prediction, facile methods for following time-dependent processes, and the willingness of individual research groups to tackle increasingly bold problems in RNA structural biology.

Introduction

In most cases, nascent RNA molecules exist in an unstructured or fully single-stranded form for only an instant. Within milliseconds, significant elements of an RNA fold to form higher-order structures. In many regions, the structure may initially consist primarily of simple base paired elements. Over the second to minute timeframe, some elements will continue to fold into compact elements stabilized by higher-order tertiary interactions. For regions that do not form either base pairing or tertiary interactions, this lack of structure may facilitate interactions with proteins, small regulatory RNAs, or the translation initiation or splice site selection machineries. RNA structure is also dynamic, meaning that a structure that plays an important functional role in one biological state may not persist in other regulatory regimes. A full understanding of RNA-mediated biology, regulation, and disease will require a deep understanding of the varied structures accessible to RNA. A powerful way to understand these structures, especially for large RNAs in solution, is by evaluating their conformations using chemical probing technologies.

Background

Chemical probing of RNA is a venerable field whose basic methods were worked out 25–30 years ago [1–3]. An RNA of interest is treated with a chemical reagent that “modifies” the

RNA in some way. The reagent can be a small organic molecule, a metal ion, or an RNase enzyme. Modification can be performed using purified components *in vitro* or in a complex biological environment *in situ* or *in vivo*. The experiment is performed such that reaction with the RNA is relatively limited and any two modification events are uncorrelated. Modification can result either in cleavage of the RNA or in formation of a covalent chemical adduct between the RNA and probe molecule. Chemical cleavage is usually detected by resolving end-labeled RNA fragments by size. Both classes of modification, cleavage and adduct formation, can be detected as a stop to primer extension mediated by a reverse transcriptase enzyme with sites of modification inferred from the length of the resulting cDNA fragments. There exist many excellent reviews describing conventional chemical probing experiments.

Structural Information Obtainable by Chemical Mapping

In general, four distinct types of information about RNA structure can be obtained from chemical probing experiments. The first group includes traditional chemical mapping reagents that form stable covalent adducts with one or more of the four RNA base groups (Fig. 1A). These base-specific reagents, which include dimethyl sulfate (DMS), kethoxal, diethyl pyrocarbonate (DEPC), CMCT and bisulfate yield structural information about the base stacking, hydrogen bonding, and electrostatic environment immediately adjacent to the base (sites of modification are shown in Fig. 2) [2,4]. Second, hydroxyl radicals, an OH group with an unpaired electron (•OH), can be generated in solution from hydrogen peroxide or molecular oxygen using an Fe(II)-EDTA catalyst. The hydroxyl radical cleaves the RNA backbone, likely by abstracting a proton from either the ribose C4′ or C5′, or both, positions [5] (in green, Figs. 1B & 2) providing information on the solvent accessibility of the backbone [5]. Third, two recently introduced experiments, in-line probing [6] and selective 2′-hydroxyl acylation analyzed by primer extension (SHAPE) [7,8], involve reaction with functional groups in the RNA backbone and ultimately measure local nucleotide flexibility or dynamics (Figs. 1C & 2). Finally, through use of bifunctional reagents or a group that is tethered to one part of an RNA and able to react with a second region, it is possible to use chemical mapping methods to obtain through-space information regarding RNA structure (Fig. 1D). Classic experiments that probe long-range structure include UV and chemically-induced cross-linking and site-directed cleavage and cross-linking.

Advance 1 -- Sequence-Independent Chemistries for RNA Structure Analysis

The traditional, base-selective chemical reagents have yielded many important insights regarding RNA structure-function relationships. However, in order to interrogate every nucleotide in an RNA, multiple reagents need to be used together. Some of the more useful reagents, like DMS, react at different functional groups depending on the nucleotide (in orange, Fig. 2). Methods that interrogate all nucleotides in an RNA simultaneously are especially useful and yield concise and highly informative experiments.

In-line probing has proven useful for developing secondary structure models for small RNAs and for quantifying ligand-induced conformational changes in RNA [6]. In-line probing is typically performed at pH 9 for 40 hours and measures local nucleotide flexibility by virtue of the ability of a nucleotide to undergo “spontaneous” cleavage that reflects base-catalyzed attack of the 2′-hydroxyl group on the adjacent phosphate diester to form a 2′,3′-cyclic phosphate product (Fig. 2) [6].

SHAPE chemistry interrogates local nucleotide dynamics in RNAs of virtually any size in a single experiment. A SHAPE experiment employs hydroxyl-selective electrophiles that react

with the 2′-hydroxyl group to form a 2′-*O*-ester adduct over periods of a few minutes [7,9,10], but that can be as short as 1 sec [11]. SHAPE measures local nucleotide dynamics because flexible nucleotides are better able to adopt conformations conducive to electrophilic attack by the 2′-hydroxyl group. Mechanistic studies show that SHAPE reactivities are largely independent of nucleotide type [12] and correlate strongly with the model-free order parameter calculated from NMR relaxation measurements on RNA [13], suggesting that this chemistry provides a quantitative measure of local nucleotide flexibility.

In addition to chemical probes, several RNases have been shown to react with single-stranded or unstructured regions in RNA, independent of the underlying sequence. *E. coli* RNase I was one of the first RNases to be characterized and reacts with RNA, independent of sequence. RNase I has proven highly useful in developing structural models for RNA regulatory elements [14]. RNase J1 from *B. subtilis* also cleaves single-stranded RNA regions with low sequence specificity and has been used to probe secondary structure and RNA-ribosome interactions [15].

These sequence-independent chemistries and RNase reagents make it possible to interrogate the local nucleotide environment at almost every position in RNA simultaneously. Used in conjunction with the hydroxyl radical reagent, which also reacts broadly with RNA (Fig. 2), these experiments afford an impressively comprehensive view of RNA secondary and tertiary interactions (Figs. 1B, C).

Advance 2 -- Software for High-Throughput RNA Structure Analysis

In principle, the experimental component of a chemical mapping experiment is straightforward. An RNA is incubated in an instructive cellular, *ex vivo*, or *in vitro* environment, allowed to react with a chemical or RNase probe, and the structural interrogation phase is complete. The results of a given experiment are then obtained by resolving and quantifying the fragments resulting from either RNA cleavage or primer extension at nucleotide resolution.

These steps have been traditionally accomplished by resolving nucleotide fragments in sequencing gels and estimating or individually quantifying band intensities. The steps required to read out the chemical probing information are time-consuming and labor-intensive. As a practical matter, individuals focusing on chemical probing analysis of RNA have spent more time and energy running sequencing gels and quantifying bands than actually designing experiments or interpreting their result. This bottleneck was addressed in a major way with the development of the Semi-Automated Footprinting Analysis (SAFA) program, which streamlines quantification of chemical mapping data as resolved in sequencing gels (Fig. 3A) [16]. A complete gel image, often containing thousands of reactivity measurements, can be quantified in 20–60 minutes. This represents roughly an order of magnitude improvement over manual methods.

In principle, gel electrophoresis could be replaced by any of the technologies now used in DNA sequencing. Commercial capillary electrophoresis instruments, widely used for routine DNA sequencing, yield single-nucleotide resolution read lengths of up to 1000 nts. Two software systems have been developed that make possible high-throughput analysis of RNA chemical mapping experiments, resolved by capillary electrophoresis. These are the Capillary Automated Footprinting Analysis (CAFA) [17] and ShapeFinder [18] programs. CAFA and ShapeFinder have different strengths. CAFA can be easier to set up and requires the use of only a single user-created fluorescently-labeled DNA primer. Good alignments between different experiments can be achieved for up to 250 nts in a single capillary electrophoresis read. CAFA has been optimized for repeated analysis of the same RNA,

which is important in biophysical studies of RNA folding [17,19]. ShapeFinder requires at least two fluorescently labeled DNA primers and involves greater effort in the initial set up and alignment phase, but is generally a good choice when analyzing long and novel RNAs (Fig. 3B). With ShapeFinder, read lengths of 400–650 nts are routinely achievable [20–22]. As data analysis software continues to improve, it will become possible to tackle biological problems involving RNA structure analysis in increasingly adventurous and comprehensive ways.

Advance 3 -- Secondary Structure Determination

An absolute prerequisite for understanding structure-function relationships involving RNA is an accurate understanding of the pattern of base pairing, or secondary structure. The most reliable way to establish an RNA secondary structure is by comparative sequence analysis. Unfortunately, this approach is applicable to only the small fraction of RNAs that have a large number of diverse, and alignable, homologs. RNA secondary structure can also be predicted from sequence alone based on free energy minimization, a field which continues to make strong advances [23,24].

Many features of RNA are difficult to account for in a secondary structure folding algorithm. Such features include folding kinetics, the role of higher-order interactions, and the requirement to develop accurate energy rules for all possible RNA structure motifs. For example, using one of the most accurate currently available dynamic programming algorithms, the prediction sensitivity for *E. coli* 16S ribosomal RNA is less than 50% (errors within the 5′ domain of the 16S RNA are shown in Fig. 4A).

A possible solution to the inability to predict RNA secondary structure accurately, especially for large RNAs, lies in incorporating experimental chemical mapping data into the prediction algorithm. A major advance in this field was the demonstration that, when chemical mapping data are rigorously incorporated into the dynamic programming algorithms widely used to predict RNA secondary structure, accuracies improve significantly [25]. In one example, if nucleotides that are reactive towards chemical reagents are prohibited from forming internal base pairs in a helix, the prediction accuracy for 16S RNA improved from 50 to 72% (Fig. 4B). At this level of accuracy, many structures are predicted correctly but significant errors remain. These residual errors likely reflect a number of features. Two critical issues are that most base-selective chemical agents appear to be sensitive to both solvent accessibility and electrostatic features [4,26] and it is difficult to place reactivity measurements obtained from multiple reagents on a uniform scale (see Fig. 2, left).

The SHAPE experiment is helpful here because reactivity measurements are obtained for almost every nucleotide in an RNA in a single experiment and these measurements can be quantitatively compared. 2′-Hydroxyl reactivity is roughly inversely proportional to the probability that nucleotide forms a canonical base pair [7,13] and it is therefore possible to convert SHAPE reactivities into pseudo-free energy change terms. These pseudo-free energy terms provide an experimentally-informed adjustment to the nearest neighbor model for RNA secondary structure folding [27]. This blended experimental chemical mapping and thermodynamic approach yields very high prediction accuracies for many RNAs. For example, the SHAPE-directed prediction for 16S ribosomal RNA includes up to 97% of the base pairs in the accepted structure [27] (Fig. 4C).

Advance 4 -- Time-Resolved RNA Structure Analysis

A full understanding of the structures of RNA and RNA-protein complexes requires knowledge of the mechanisms by which these systems fold and assemble as a function of

time. It is becoming increasingly clear that many RNAs fold by complex pathways and via energy landscapes that are commonly characterized as “rugged”, which means there exist many stable intermediate and non-functional states. A major set of advances have focused on developing efficient and information-rich approaches for performing chemical mapping as a function of time. The basic experiment involves initiating an RNA folding reaction, assessing structure at specific times by addition of a chemical probe, quenching the reagent (if needed), and quantifying the folding behavior as a function of time.

A method for time-resolved hydroxyl radical footprinting with very short (ms) time resolution was initially developed by exposing RNA to synchrotron radiation and controlling exposure time with a rapid mixing device [28]. More recently, the requirement for a synchrotron source has been eliminated by the development of “fast Fenton” methods [19,29]. Fast Fenton hydroxyl radical probing makes use of a rapid mixing and quench device and Fe(II)-EDTA-catalyzed reduction of oxygen to perform time-resolved footprinting in a conventional laboratory setting. Time-resolved hydroxyl radical footprinting provides a rich view of RNA folding on time scales spanning sub-second to many minutes [19,29] and for folding and assembly events involving small molecule-riboswitch interactions [30], large RNAs [31,32], and the ribosome [33,34].

The reaction of hydroxyl-selective electrophiles used in a SHAPE experiment can be tuned by varying the substituents near the reactive center [9,35]. Hydroxyl reactive electrophiles also undergo inactivation via the competing hydrolysis reaction with water. Benzoyl cyanide (BzCN) has a half-life in water of ~0.2 sec at 37 °C [11,36]. Thus, time-resolved analysis of local nucleotide flexibility can be followed in a simple way by initiating an RNA folding reaction and then obtaining ~1 sec snapshots of the RNA structure by mixing aliquots of the evolving reaction with pre-aliquoted BzCN reagent. Since the reagent undergoes auto-inactivation, a quench step is unnecessary if 1 sec time intervals are sufficient to monitor the dynamic processes of interest. This approach was used to develop a nucleotide-resolution model for folding of an RNase P RNA [11] and to show that a slow conformational change involving a single C2′-endo nucleotide represents the rate determining step for structural biogenesis of a 150-nt RNA domain [37].

Advance 5 -- Tertiary Structure Constraints

Many RNAs form both base-paired secondary structures and also complex higher-order, or tertiary, structure interactions. The chemical mapping reagents discussed thus far all react with RNA in ways that are sensitive to precise, very local, features of RNA structure (Fig. 1A–C). Tertiary interactions can be inferred from these local reactivity measurements in several ways. For example, most RNA nucleotides that form simple A-form helices or loop structures have roughly uniform and high solvent accessibility at the ribose backbone. Therefore, observation of nucleotides that are protected from hydroxyl radical-mediated cleavage can be taken as evidence of higher-order contacts [5].

Alternatively, if the tertiary structure of an RNA is known from high-resolution NMR or crystallography analysis, then comparison of the known structure and the observed reactivity pattern using a chemical mapping reagent can be used to infer whether a given tertiary interaction is formed in distinct RNA states or upon introducing mutations to the sequence. A number of recent studies have elegantly melded knowledge of higher-order structure with chemical mapping to follow folding pathways [38], monitor ligand binding [39,40], measure the thermodynamics and thermal stability of individual RNA structural elements [8,41], assess the effect of RNA mutations [42,43], evaluate the structure of a small RNA in complex with its biological partners [44], and explore RNA contacts within a crystal lattice [45].

A current challenge is to use chemical mapping information for *de novo* refinement of three-dimensional structures of RNA, especially for structures containing extensive and nontrivial long-range tertiary interactions. This kind of analysis requires knowledge of through-space connectivity information (Fig. 1D). Like single-site chemical modification, the use of chemical approaches to obtain through-space information for RNA is well-established. Classic methods include UV and chemical cross-linking and analysis of cleavage originating from a site-specific cleavage group. UV and chemical cross-linking experiments can, in principle, give a large amount of through-space connectivity information but tend to be very difficult to analyze; in contrast, RNAs containing site-directed cleavage groups tend to be laborious to create but analysis of the resulting through-space information is easier.

The ideal chemical mapping strategy for generating useful through-space connectivity information should (i) involve a simple approach for performing the initial chemical analysis, (ii) produce a large number of high-quality, short distance, through-space constraints, (iii) be straightforward to interpret, and (iv) be applicable to RNAs of arbitrary size and complexity. Although no current method fully meets all of these criteria, substantial progress has been made in developing broadly useful approaches for obtaining through-space information for RNA structure.

In multiplexed hydroxyl radical cleavage analysis (MOHCA) [46], RNAs are created in which nucleotides are randomly incorporated such that the probe nucleotide is ultimately linked to two reporter chemistries. An Fe(II)-EDTA moiety is tethered at the 2′-ribose position and is used to induce through-space cleavage of nearby (within ~25 Å) residues in the RNA. The site from which cleavage was initiated is identified by site-specific cleavage of the probe nucleotide using iodine-mediate cleavage of a 5′ phosphorothioate label (Fig. 5A). Sites of Fe(II)-EDTA mediated cleavage and the location of the probe nucleotide are identified by information-rich two-dimensional gel electrophoresis. This approach is straightforward to set up and yields a large number of high-quality, medium-distance constraints. The two-dimensional gel electrophoresis approach can be challenging to implement, especially as RNA size increases. MOHCA was used both to refine a model for the structure of the *Tetrahymena* group I intron P546 domain and to develop a model for a folding intermediate of this domain (Fig. 5A) [46].

The information-rich, but complex, cross-linking patterns obtained using conventional bifunctional cross-linking reagents can be read out by mass spectrometry in an approach termed MS3D [47,48]. Nitrogen mustard [bis(2-chloroethyl)methylamine] reacts preferentially at the N7 position of guanosine and N3 of adenosine and contains a relatively short spacer group (~9 Å, Fig. 5B). The RNA is digested with ribonuclease and the nucleotides that formed cross-links are detected by mass analysis and tandem sequencing by mass spectrometry. The initial cross-linking experiment is very easy to implement and MS3D yields a large number of short distance contacts. The final analysis of pairs of cross-linked nucleotides requires sophisticated instrumentation and considerable expertise. MS3D has been used to refine models for a mouse mammary tumor virus pseudoknot [47] and for an RNA involved in HIV-1 packaging [48] (Fig. 5B).

Site-directed cleavage can be accomplished by linking an Fe(II)-EDTA group to a molecule that binds selectively to a user-defined site in an RNA [49]. For example, MPE (methidiumpropyl-EDTA) binds selectively to a simple bulged RNA element [49,50]. An MPE binding site, and thus a tethered Fe(II)-EDTA group, can be placed at many sites by simply mutating the sequence of an RNA helix (Fig. 5C). Sequence-encoded, site-directed cleavage yields a large number of medium distance (25–35 Å) constraints that can be analyzed in a simple one-dimensional gel electrophoresis experiment. Site-directed cleavage requires that a new mutant RNA be created for each experiment and that careful controls be

performed to ensure that cleavage agent binding and sequence changes do not disrupt the native RNA structure. Sequence-encoded cleavage was used to refine the structure of $tRNA^{Asp}$ to roughly nucleotide resolution (4–6 Å RMSD) [49] (Fig. 5C).

To create useful three-dimensional models of an RNA, through-space information must be interpreted via a constraint-satisfaction algorithm. This “algorithm” can be computer-assisted manual modeling or can make use of computationally based automated methods. Recent approaches for refining three-dimensional RNA structures include adapting all-atom algorithms commonly employed for crystallographic and NMR refinements [48,51] and use of simplified approaches in which each RNA nucleotide is represented as a single sphere [52,53] or as three beads (corresponding to the phosphate, ribose and-based groups) [49,54].

Advance 6 – Recent Ambitious Experiments

The recent and ongoing confluence of advances in mapping chemistries, data analysis, and interest in understanding complex RNAs and RNPs have made it possible to address structure-function relationships in remarkably large systems. In some ways, new trends in RNA structure analysis contain an element of going back to the future. Among the first applications of chemical probing were analyses of the structure of *E. coli* 16S RNA in a free state and in intact 30S subunits [3]. Representative recent ambitious studies have returned to analysis of 16S RNA [34] and have tackled the group I intron [31] and an entire HIV-1 genome [20,55]. A current theme is that chemical mapping approaches may be entering a new era in which novel and biologically important inferences can be obtained, especially when large, nucleotide resolution, and complete data sets are obtained.

Time-resolved fast Fenton hydroxyl radical cleavage was used to obtain a global analysis of the folding of a group I ribozyme along a time trajectory from very early to late steps and to characterize nearly all transitions between the initial, intermediate, and final conformational states [31]. The results of this analysis emphasize the importance of early events in determining the flux through distinct folding pathways (Fig. 6A).

Synchrotron-mediated, time-resolved hydroxyl radical footprinting was used to follow assembly of the *E. coli* 16S ribosomal RNA (1542 nts) with all 30 small subunit proteins [34]. Observed kinetic profiles were used to infer RNA-RNA and RNA-protein interactions over time domains spanning four orders of magnitude. Some RNA nucleotides showed protection from hydroxyl radical cleavage in less than 20 ms whereas others were not protected until 1–3 minutes later. These data were interpreted in terms of a “chaotic” model for small subunit assembly involving concurrent nucleation events at many positions along the RNA, local refolding during assembly, and rapid appearance of native interactions throughout the RNA (Fig. 6B).

High-throughput SHAPE chemistry was used to analyze the overall architecture and develop a secondary structure model for an entire HIV-1 RNA genome (~9200 nts) using authentic viral RNA [55]. The SHAPE data support a model in which the genome contains more than 20 highly structured domains. Evolutionary comparisons and direct functional analysis indicate that many of these domains play important, and previously unrecognized, functions in viral replication (Fig. 6C).

Perspective

Chemical probing approaches for analyzing RNA structure are undergoing a renaissance in usefulness and impact. For many RNAs, including large structurally dynamic RNAs and conformational and functional intermediates, chemical mapping represents the best approach for obtaining structure information. In designing chemical probing experiments, researchers

should consider using a reagent that reacts broadly with all four nucleotides as a first choice. The mechanical and experimental elements of chemical probing experiments are becoming easier: it is now possible to analyze RNAs that are sufficiently long to fully represent most systems of interest. One should try to obtain sufficient equilibrium or kinetic data such that a particular system can be understood as completely as possible. At the present, *de novo* structure prediction of RNA secondary and tertiary structure is fraught with challenges. However, melding experimental information with computational approaches for structure analysis will likely make possible sophisticated understanding of the numerous roles of RNA structure in all areas of biology.

Acknowledgments

Many important contributions and insightful reviews, especially those published prior to 2005, could not be included due to space constraints. I am indebted to the colleagues who allowed images from their primary research work to be included in this review. The US National Institutes of Health and National Science Foundation support work in my laboratory focused on creating probing technologies for understanding the structural and functional biology of RNA.

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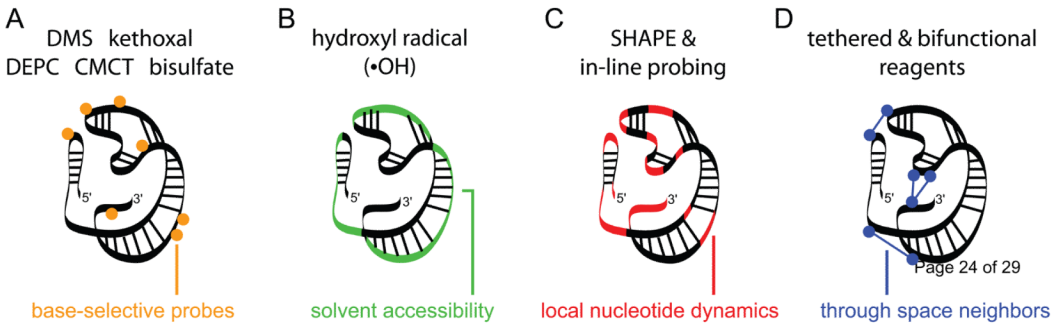

Figure 1.
Classes of RNA structure information obtained by chemical probing include (A) base selective data, (B) solvent accessibility information, (C) measurements of nucleotide dynamics, and (D) constraints on long-range interactions.

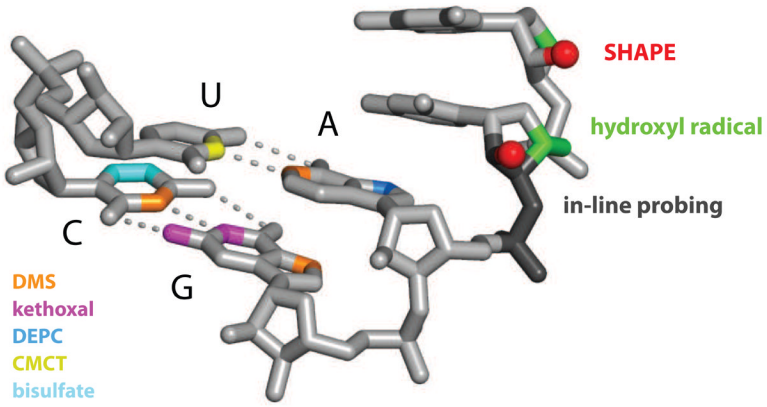

Figure 2.
Sites of RNA modification for base-selective (left) and sequence-independent (right) modification chemistries. DEPC and CMCT also react with guanosine (not shown).

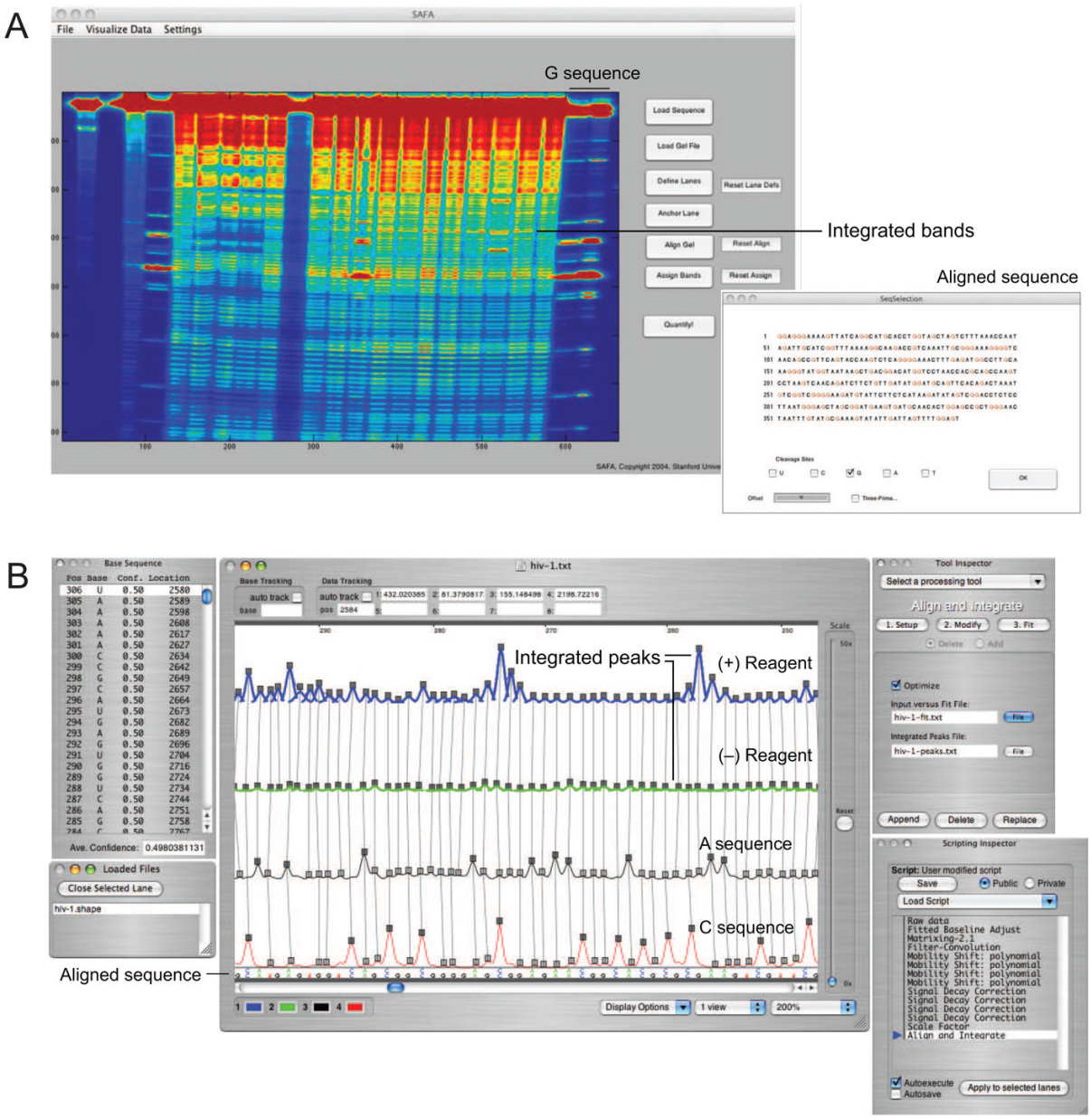

Figure 3.
Automated analysis of chemical probing experiments using the (A) SAFA and (B) ShapeFinder programs. Figures adapted from [18,56].

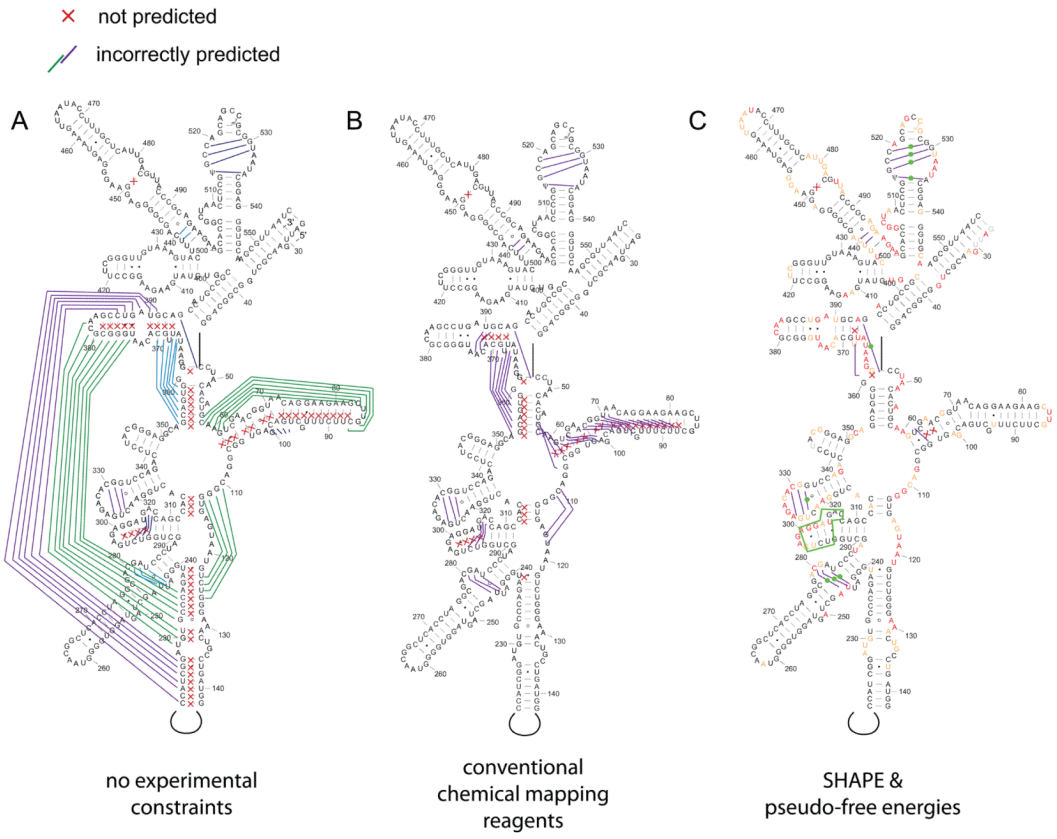

Figure 4.

Comparison of secondary structure prediction accuracy in the 5′ domain of *E. coli* 16S rRNA using free energy minimization (A) alone, (B) with conventional chemical modification reagents, and (C) with SHAPE-derived pseudo-free energy change terms. Missed base pairs are indicated by red x's; incorrectly predicted base pairs are represented by colored lines. Regions where experimental information supports local RNA refolding are indicated with green boxes and spheres in panel C. Figure adapted from [27].

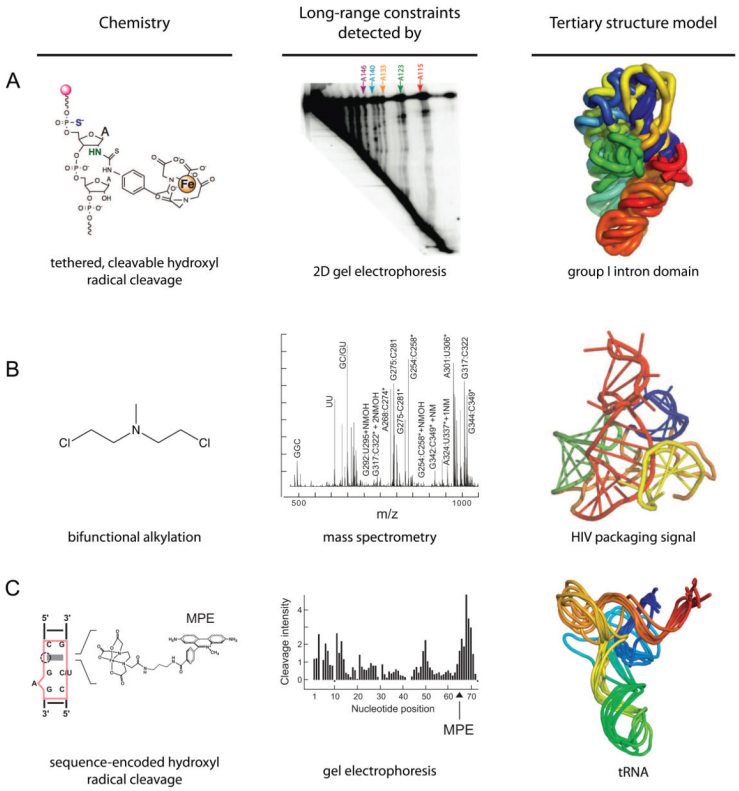

Figure 5.

Recent developments using chemical probing to obtain through-space constraints on RNA tertiary structure. (A) MOHCA, (B) MS3D, and (C) sequence-encoded cleavage. Figures are adapted from images in [46,48,49].

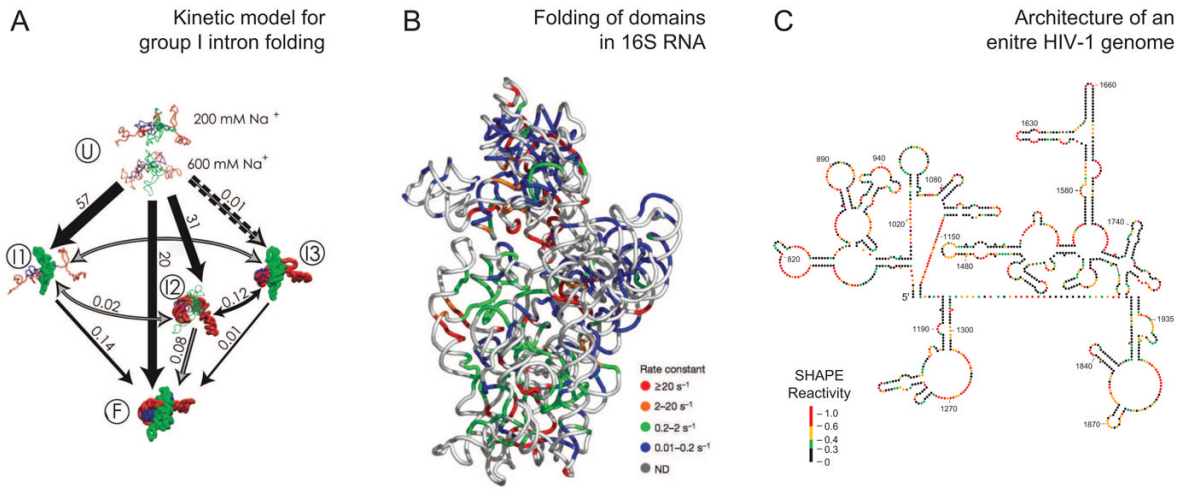

Figure 6.
Representative examples of the use of chemical probing technologies to address ambitious problems in biology. Adapted from [31,34,55].