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# Mapping nucleic acid structure by hydroxyl radical cleavage

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Hydroxyl radical footprinting is a widely used method for following the folding of RNA molecules in solution. This method has the unique ability to provide experimental information on the solvent accessibility of each nucleotide in an RNA molecule, so that the folding of all domains of the RNA species can be followed simultaneously at single-nucleotide resolution. In recent work, hydroxyl radical footprinting has been used, often in combination with other global measures of structure, to work out detailed folding pathways and three-dimensional structures for increasingly large and complicated RNA molecules. These include synthetic ribozymes, and group I and group II ribozymes, from yeast, the *Azoarcus* cyanobacterium and *Tetrahymena thermophila*. Advances have been made in methods for analysis of hydroxyl radical data, so that the large datasets that result from kinetic folding experiments can be analyzed in a semi-automated and quantitative manner.

## Addresses

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## Introduction

This year marks the 20th anniversary of the first use of the hydroxyl radical as a high-resolution tool for the structural study of nucleic acids [1]. Although some early experiments used the hydroxyl radical to characterize the structures of DNA molecules of unusual shape (such as bent A-tract DNA [2,3] or the four-stranded Holliday junction recombination intermediate [4]), its main use has been as a high-resolution footprinting agent for protein–DNA complexes [5]. But in recent years, the hydroxyl radical also has become one of the most widely used chemical probes for assessing the folded structure of nucleic acids, particularly RNA.

The characteristic chemistry of the hydroxyl radical with a nucleic acid is to cause a strand break in a DNA or RNA

molecule that follows initial abstraction of a hydrogen atom from a backbone (deoxy)ribose residue somewhere in the chain. Because the hydroxyl radical is an extremely reactive and non-discriminating free radical, the site of attack has no dependence on the base sequence of the nucleic acid. Instead, the susceptibility of a particular nucleotide to hydrogen abstraction is governed by its accessibility to solvent [6]. This property makes the hydroxyl radical footprinting method the experimental equivalent of a calculated solvent-accessible surface area of a nucleic acid molecule, and makes it possible to use hydroxyl radical footprinting results as rigorous constraints on three-dimensional models of folded nucleic acid molecules.

The most common method for generating the hydroxyl radical ( $\bullet\text{OH}$ ) for footprinting makes use of the Fenton reaction of iron(II) EDTA with hydrogen peroxide [7]:

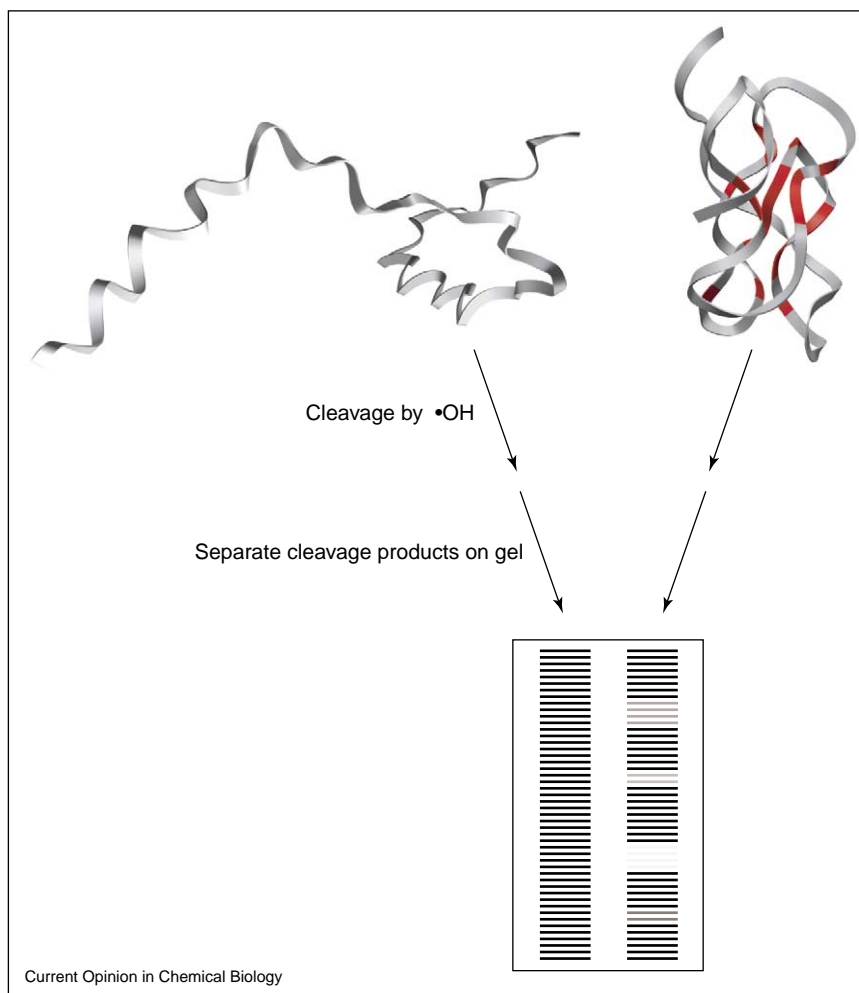


In recent years, alternative ways to produce a flux of hydroxyl radicals for footprinting have been developed, including homolytic dissociation of peroxyxynitrous acid [8] and synchrotron radiolysis of water [9]. Each of these methods has its strengths, and each appears to generate a similar cleavage pattern.

Latham and Cech were the first to show that the hydroxyl radical cleavage pattern of a folded RNA molecule directly reveals which segments of the RNA backbone are on the outside and which are on the inside of the molecule [10]. Folded RNA is thus capable of ‘autofootprinting’ itself (a term introduced by Seeman [11] to describe the cleavage pattern of a DNA junction), because one part of the RNA chain can fold over and protect another part of the chain from attack by the hydroxyl radical (Figure 1).

Much emphasis has been placed on the elaborate, protein enzyme-like, folds of catalytically active ribozymes. However, it must also be appreciated that all natural RNA molecules (messenger RNA, transfer RNA, ribosomal RNA, and so on) fold into three-dimensional structures. Hydroxyl radical footprinting experiments provided some of the first information on the tertiary fold and shape of the iron-responsive element of ferritin mRNA [12]. Hydroxyl radical cleavage experiments also were used to delineate the footprint of a repressor protein bound to the ferritin message [12]. Subsequent NMR studies of fragments of the iron-responsive element of ferritin mRNA [13] were consistent with the hydroxyl radical

Figure 1



Hydroxyl radical footprinting of RNA. Top left, unfolded RNA. Top right, the same RNA folded in its native tertiary structure. Places where folding brings distant regions of the RNA chain together, thus decreasing solvent accessibility of the backbone, were calculated and are colored red. After hydroxyl radical cleavage and denaturing gel electrophoresis, the gel patterns differ. The unfolded RNA molecule is cleaved uniformly, giving rise to a homogeneous ladder of bands on the gel (left, bottom). The gel pattern for the folded RNA (right, bottom), in contrast, shows several region where strand cleavage is inhibited, corresponding to the sites of low solvent accessibility in the folded structure (right, top).

footprinting results. In other pioneering experiments, hydroxyl radical footprinting uncovered RNA–RNA and RNA–protein interactions that occur when the 30S and 50S subunits associate to form the ribosome [14,15]. An important point is that the structural conclusions from these hydroxyl footprinting studies later were confirmed in detail by the X-ray structure of the complete 70S ribosome [16].

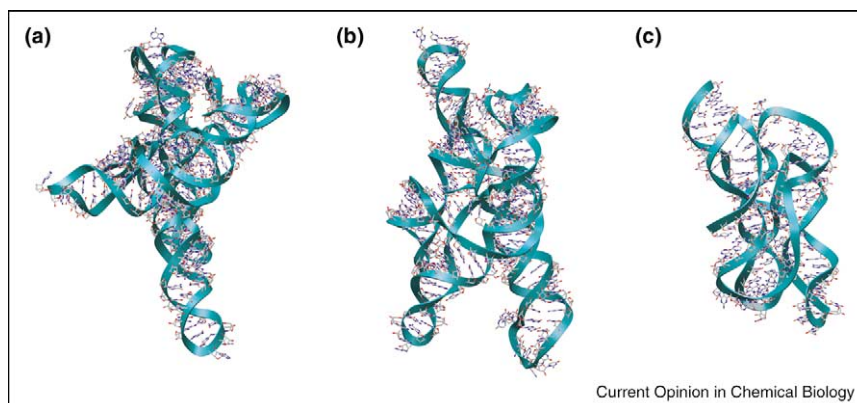
This review covers recent sophisticated uses of hydroxyl radical footprinting to characterize folding pathways in diverse RNA molecules (Figure 2), to validate the X-ray crystal structure of a ribozyme, and to give the first view of the folded structure of a synthetic ribozyme. Also discussed are a new two-dimensional gel method that revealed the effect of a hydroxyl radical-induced lesion on

the shape of a DNA molecule (Figure 3), and technological advances in analyzing the hydroxyl radical cleavage pattern that promise to make the method even more useful in the future. Other recent work has employed the hydroxyl radical to assay for the correct folding of increasingly elaborate multistrand DNA junctions [17], and to assess the structural effect of DNA mismatches and bulges [18].

### Group II ribozyme folding

Hydroxyl radical footprinting has proven to be of notable value in defining the folding pathway of an RNA molecule. Typically, RNA is in an unfolded state in conditions of low ionic strength. A classic experiment is to monitor an experimental observable as a function of increasing concentration of  $Mg^{2+}$ , which promotes RNA folding (at least

Figure 2



A rogues' gallery of ribozymes. These three-dimensional structures illustrate the types of folded RNA molecules that are being studied by hydroxyl radical footprinting. **(a)** The L-21 Scal group I ribozyme from *Tetrahymena thermophila* (PDB 1X8W). **(b)** The *Azoarcus* group I ribozyme (PDB 1U6B). **(c)** A model of the class I ligase ribozyme (PDB 1QXI).

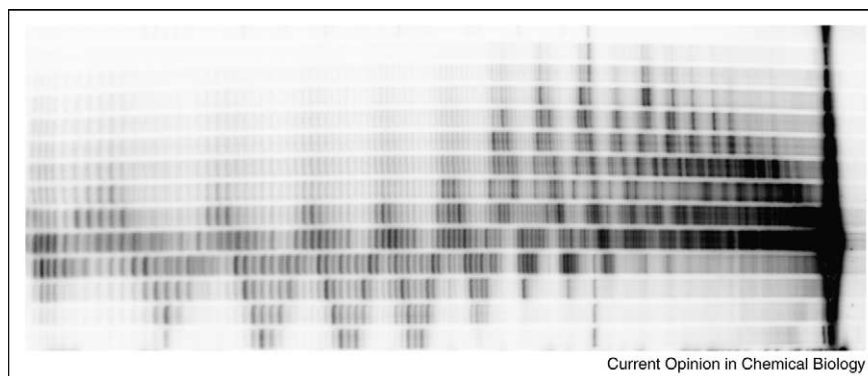
in part) by neutralizing backbone negative charges that interfere with close approach of non-contiguous regions of the RNA chain. The hydroxyl radical offers a unique perspective on the RNA folding process, because of the ability of this experiment to simultaneously monitor the change in solvent exposure of nearly every nucleotide individually in the RNA molecule as it folds. Hierarchical folding of the multiple domains of a complex RNA molecule can thereby be revealed.

Recent work has focused on the 618 nt ribozyme D135 that is derived from the self-splicing group II intron ai5 $\gamma$  from the yeast mitochondrion [19 $\bullet$ ]. This is the largest RNA molecule for which the folding pathway has been characterized. D135 contains three major domains (domains 1, 3 and 5) derived from the six domains of

the full-length group II intron. The surprising result from previous hydroxyl radical footprinting experiments [20] was that no significant difference was observed for the Mg<sup>2+</sup>-dependence of the folding of the various local regions of the ribozyme, indicating a lack of hierarchical folding of this large RNA molecule, in striking contrast to the behavior of other, smaller, ribozymes [21].

In recent work [19 $\bullet$ ], results from a variety of methods, including hydroxyl radical footprinting, were used to investigate folding and unfolding of the D135 group II ribozyme. The combination of these methods afforded global, local and functional viewpoints from which to observe folding. For example, sedimentation velocity measurements provided information on the overall degree of compaction *versus* Mg<sup>2+</sup> concentration, and

Figure 3



DNA gapped by reaction with the hydroxyl radical has a fixed bend in its backbone. This figure shows a phosphorimage of a denaturing gel on which was run the DNA fractions recovered from a native gel that resolved the different shapes adopted by a library of singly gapped DNA duplexes. Please see the original reference [32 $\bullet$ ] for more details of the experiment. This gel image reveals a sinusoidal modulation (with 10-nt periodicity) of the native gel mobilities of the collection of singly gapped DNAs, which (not coincidentally) resembles the helical structure of DNA. The gel image offers clear evidence that a single-nucleoside strand gap imparts a directed bend in the DNA helix axis. Adapted from [32 $\bullet$ ] with permission. Copyright 2003, the National Academy of Sciences.

produced values for  $K_{Mg}$  and the Hill (cooperativity) coefficient similar to those derived from previous footprinting experiments [20]. Global and local views of folding thus were found to correspond.

Unfolding experiments were conducted by treating the folded ribozyme with increasing concentrations of urea. Global unfolding was monitored by circular dichroism spectroscopy, whereas hydroxyl radical footprinting was used to observe how local regions of the intron unfolded. An advantage of the hydroxyl radical is that it can be used in the presence of denaturants, like urea, so nucleotide-resolution unfolding experiments are feasible. Unfolding of the ribozyme was found to be highly cooperative, with footprinting experiments revealing little domain hierarchy in loss of protection with increasing urea concentration.

From the results of these and other experiments, the authors concluded that there are no kinetic traps [21] in the folding pathway of this group II ribozyme, despite its large size and structural complexity. The data were consistent with a two-state folding mechanism, in remarkable contrast to the more complicated, hierarchical folding pathways observed previously for other, smaller, RNAs. Because tertiary interactions between distant sites, which are observed clearly by hydroxyl radical footprinting, are required for folding, the apparent two-state folding behavior is even more surprising.

### **Tetrahymena group I ribozyme folding**

The L-21 Sca1 ribozyme (Figure 2a), derived from the *Tetrahymena thermophila* group I self-splicing intron, is the best-studied ribozyme folding system. Time-resolved synchrotron hydroxyl radical footprinting was pioneered in this system [22] and used to resolve the time-dependent hierarchy of folding of the different domains of this ribozyme.

Early work focused on the magnesium dependence of folding. In two recent papers [23<sup>••</sup>,24], folding as a function of monovalent ion concentration was studied. Hydroxyl radical footprinting experiments [23<sup>••</sup>] showed that a similar overall structure forms in 1.5 M NaCl and in 10 mM MgCl<sub>2</sub>. One difference noted was a lesser extent of footprinting protection when the ribozyme was folded in sodium compared with magnesium. The rate of folding, as studied by time-resolved synchrotron hydroxyl radical footprinting, was found to be 50- to 250-fold faster in sodium. These ion valency-dependent differences in folding suggest that RNA might be more dynamic in a solution containing exclusively monovalent ions. The slower formation of native tertiary contacts when the ribozyme is folded in magnesium suggests that divalent ions inhibit the formation of these contacts, perhaps by stabilizing misfolded structures. A major departure from earlier folding results for magnesium was that folding of the P4-P6 domain in sodium ion is not concerted.

Nearly all hydroxyl radical footprinting experiments, including RNA folding studies, employ *inhibition* of strand cleavage (protection) as the key experimental observable. In recent folding studies of the *Tetrahymena* group I intron [23<sup>••</sup>], the unusual occurrence of *enhanced reactivity* of adenine 122 in the folded ribozyme was noted. This phenomenon was used as a probe of the conformation of J5/5a, a key 'hinge' region of the ribozyme. The X-ray crystal structure of the ribozyme shows that the RNA backbone is distorted around A122 in the folded ribozyme, explaining the folding-associated hyperreactivity to hydroxyl radical. Folding in divalent and monovalent ions gave different kinetic patterns for this phenomenon. In sodium, the rate of appearance of A122 hyperreactivity was fast (consistent with the fast overall folding rate in monovalent ion solution). However, the change in reactivity of A122 upon folding was much smaller for sodium compared with magnesium. It was concluded that monovalent ions might not be capable of stabilizing the full structure of the hinge, whereas divalent ions specifically stabilize the J5/5a hinge.

### **Azoarcus group I ribozyme folding**

The smallest known self-splicing group I ribozyme is the intron in the tRNA<sup>Ile</sup> gene of the *Azoarcus* cyanobacterium (Figure 2b). The intron interrupts the anticodon loop of the tRNA. Self-splicing removes the intron and generates the mature tRNA molecule. Several recent studies have focused on the folding pathway and structure of this model ribozyme, both with and without the tRNA exons attached to the intron.

Folding of the exon-free 195 nt *Azoarcus* intron was studied as a function of magnesium ion concentration, using four experimental approaches [25<sup>•</sup>]. Two of these methods, RNase T1 digestion to assess secondary structure, and native gel electrophoresis to study the acquisition of native overall shape, each exhibited a transition midpoint of a few hundred micromolar magnesium. The other two methods, hydroxyl radical footprinting to reveal tertiary contacts, and a direct assay of self-splicing activity, also showed similar transition midpoints, but this time the magnesium midpoint occurred at a few millimolar magnesium ion concentration. All tertiary interactions exhibited similar transition midpoints, evidence against hierarchical folding of the various domains of the ribozyme. Time-resolved synchrotron hydroxyl radical footprinting showed that the *Azoarcus* group I intron acquires all of its tertiary structure within 100 msec, rapid compared with the folding of the *Tetrahymena* group I intron, and comparable in rate to the initial fast folding of the P4-P6 domain of the *Tetrahymena* ribozyme.

The interesting conclusion of this work is that catalytic activity accompanies attainment of the final folded tertiary structure of the ribozyme. In contrast to some other ribozymes, enzymatic activity does not require a higher magnesium concentration than is necessary for tertiary

folding, indicating that there may not be low-affinity metal-binding sites associated with the splicing reaction for this ribozyme. This work represents another example of the synergistic coupling of a variety of functional and global structural assays with hydroxyl radical footprinting, to correlate high-resolution local folding information with global shape and function.

The above study focused on folding of the *Azoarcus* intron alone [25<sup>•</sup>]. A more recent paper investigated whether the presence of the tRNA exon attached to the intron affects folding of the complete 284 nt *Azoarcus* group I pre-tRNA<sup>Ile</sup> [26<sup>••</sup>]. Hydroxyl radical footprinting demonstrated that the intron and the tRNA segments of the pre-tRNA form separate modular domains that have tertiary structures similar to the folds they adopt in isolation. An interesting difference was noted in the hierarchy and cooperativity of folding of the various domains of the intron. In contrast to the results cited above for the exon-less intron [25<sup>•</sup>], for which there was little difference in the magnesium concentration midpoint for formation of the various tertiary contacts, for the complete pre-tRNA there was a range of magnesium dependence and cooperativity for formation of tertiary interactions. The authors suggested that metastable intermediates may be forming during folding of the pre-tRNA that are not present when the intron alone folds. The sensitivity and comprehensive nature of the hydroxyl radical footprinting experiment makes possible such detailed insights into the folding pathway.

A three-dimensional structural model for the *Azoarcus* group I intron was proposed on the basis of phylogenetics, structural homology with other RNAs and footprinting [25<sup>•</sup>]. The advent of a landmark X-ray structure of the *Azoarcus* intron [27] gives the opportunity to compare [28<sup>•</sup>] the crystal structure with the model. The RNA molecule that was crystallized was designed to imitate the pre-2S splicing intermediate, with a short segment of the 5' exon base-paired, and a bit of the 3' exon covalently attached and base-paired, to the intron.

The X-ray structure was validated by hydroxyl radical footprinting, to assess the similarity of the solid-state structure with the structure of the ribozyme in solution [29<sup>•</sup>]. An excellent correlation was found between the experimental hydroxyl radical protections, and ribose solvent accessibility calculated from the X-ray structure. While the footprint/phylogenetic model [25<sup>•</sup>] is similar overall to the crystal structure model, the two structural models do differ in detail. The rmsd (normalized to 100 residues) between the two structures was found to be 3.8 Å, showing that the overall folds are quite similar. The main difference between the two is the angle between the P4-P6 and the P3-P9.0 domains, which is more acute in the footprint/phylogenetic model. According to the paper that analyzed the X-ray structure [28<sup>•</sup>], the acute angle in

the footprint/phylogenetic model was based on a footprint observed in the P6a helix [25<sup>•</sup>]. This footprint was not seen when the RNA molecule that was crystallized was footprinted in solution by hydroxyl radical [28<sup>•</sup>]. Supporting the footprinting result was the observation that P6a was solvent-exposed in the X-ray structure. However, the P6a footprint is very clear in primary gel data published by the authors of the footprint/phylogenetic model [29]. It will be interesting to see how this difference is resolved. Perhaps the intron adopts different conformations with and without its exon substrate bound, and this is reflected by a change in a tertiary contact that is apparent in the hydroxyl radical footprint.

### Modeling the three-dimensional structure of the class I ligase ribozyme

The discovery of natural ribozymes [30], such as the self-splicing introns discussed above, led to efforts to produce synthetic ribozymes by selection from random RNA libraries. A recent paper [31<sup>•</sup>] discussed the use of hydroxyl radical footprinting data to produce a three-dimensional structural model of the class I ligase ribozyme (Figure 2c), a synthetic ribozyme that catalyzes a reaction similar to that of RNA polymerase. At the outset of this work, a detailed secondary-structure map was available for this 118 nt-long ribozyme. Hydroxyl radical footprinting experiments revealed that a substantial fraction (more than 15%) of the ribozyme's nucleotides are protected when the ribozyme adopts its folded structure. Complete folding occurred at a magnesium ion concentration less than 1 mM. This contrasts with the much higher magnesium concentration (40–50 mM) required for ligase activity, indicating that additional low-affinity metal ion binding sites must be occupied for catalysis to occur.

Several three-dimensional models were constructed on the basis of elements of secondary structure (for example, two pseudoknots) that were known to be present in the structure. These models were then tested for consistency with the hydroxyl radical footprinting data, which indicated that one model was highly favored. A key feature of the structural model is the configuration of a four-way junction that is formed by the crossing of stacked helices P4-P5 and P6-P7. The original secondary-structure diagram depicted P5 near P6, and P4 near P7. The footprint-based three-dimensional model flips the orientation of the arms of the junction to juxtapose P5 with P7, and P4 with P6. This prediction of the model was tested directly by a cross-linking experiment, which showed that P5 and P7 indeed are near in space to each other.

### Structural effect of a hydroxyl radical-induced gap on duplex DNA

How the strand gap that is produced by the hydroxyl radical cleavage reaction affects the structure of a nucleic acid molecule has been addressed only recently [32<sup>•</sup>]. This question is important for understanding how DNA

repair proteins might recognize sites of oxidative damage in genomic DNA. In these experiments, a novel two-dimensional gel electrophoresis method was developed to investigate the change in shape experienced by a duplex DNA molecule in which a hydroxyl radical-induced lesion had been introduced. The first electrophoretic dimension involved a native gel, which separates DNA molecules by shape. It was found that DNA treated with hydroxyl radical runs with a pronounced 'smear' on such a gel, showing that the collection of lesioned DNA molecules possesses a variety of shapes. Slices of the native gel containing DNA molecules having different mobilities (and thus shapes) were excised, and the DNA from each slice was electrophoresed on a denaturing gel to reveal which DNA lesions were associated with which native gel mobility. The remarkable result (Figure 3) is a virtual image of the helical structure of DNA captured in the electrophoretic pattern. This pattern was interpreted to show that a site of hydroxyl radical-induced oxidative damage imparts a directed bend (and not a freely-rotating swivel) on the axis of the DNA double helix, a structural anomaly that may be recognized by repair proteins.

### Advances in analysis of hydroxyl radical footprinting patterns

Much quantitative information is embodied in a hydroxyl radical cleavage pattern. This is both a blessing and a curse. Although a distinct advantage of this experiment is that every nucleotide in an RNA or DNA molecule gives a signal, quantitative analysis of the intensities of so many closely spaced electrophoretic bands can be a problem. One early attempt at quantitation of gel images of hydroxyl radical cleavage patterns [33] involved curve fitting to deconvolute closely spaced bands, which allowed accurate determination of the integral of each band. Recent work has resulted in a semi-automated [34<sup>•</sup>] method for deconvolution and peak fitting of hydroxyl radical cleavage patterns. This work was motivated by the need to compare several lanes-worth of cleavage data produced by time-resolved synchrotron footprinting experiments on RNA folding. The key development reported in this paper is a new automated method for objectively choosing bands to act as standards for normalization of one lane to another, which in the past has been difficult, especially for RNA folding studies.

Another recent paper tackled the problem of quantitation of hydroxyl radical footprinting experiments performed on supercoiled DNA [35<sup>•</sup>]. The problem here is that once a single cut is made in the backbone of a supercoiled DNA molecule, supercoiling tension is released and the DNA molecule is in a fundamentally different conformational state. Therefore, for accurate analysis, only the first 'hit' on a supercoiled DNA molecule should be considered. The authors present a rigorous mathematical analysis of this problem, and provide a procedure for extracting the cleavage pattern of supercoiled DNA from

the experimental pattern, which is a combination of cleavage data for supercoiled and nicked DNA species.

### Conclusions

The work discussed in this review illustrates how hydroxyl radical footprinting is being used for the quantitative structural study of increasingly complex nucleic acid molecules, exemplified here by natural ribozymes. Footprinting patterns of folded RNA molecules are used in a similar way to NOEs in NMR spectroscopy — the observation of an autofootprint (along with secondary structure information, from other methods) provides strong evidence that two distant segments of an RNA molecule are near in space in the native structure [31<sup>•</sup>], and thus provides a rigorous constraint on the three-dimensional fold of the molecule. The addition of a time-dependent variant of hydroxyl radical footprinting, synchrotron footprinting [9], makes the method even more versatile. Again there is an analogy in NMR spectroscopy, hydrogen exchange, which is used to follow folding kinetics (usually of proteins), and similarly provides solvent accessibility information throughout the molecule as it folds.

Hydroxyl radical footprinting differs from spectroscopic methods in that there is no fundamental physical or quantum mechanical theory through which experimental results can be rigorously understood and calculated. Quantitative treatment of footprinting patterns is the first step [33,34<sup>•</sup>], but to move to the next level in interpretation a detailed chemical model is necessary to describe the footprinting phenomenon. This is evident in the literature in the number of recent papers that still cite the 4' hydrogen of RNA, or the minor groove of DNA, as the site of attack of hydroxyl radical, even though evidence has now been presented that the 5' deoxyribose hydrogens are the predominant site of hydroxyl radical attack in DNA [6]. While such experiments have not yet been published for RNA, work underway in our laboratory is aimed at determining which ribose hydrogens in RNA react with the hydroxyl radical, and how local secondary and tertiary structure affects this reactivity. This work, and work from other laboratories, will make hydroxyl radical footprinting an even more valuable method for visualizing the three-dimensional shapes of the ever-expanding varieties of RNA molecules that are now being recognized for their central place in biology.

### Acknowledgements

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