REVIEW

From equilibrium to kinetic footprinting of RNA structure

Andrew M. MacMillan

Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7. E-mail: andrew.macmillan@ualberta.ca; Tel:/Fax: 780-492-3813

Received (in Cambridge, UK) 1st May 2001
First published as an Advance Article on the web 19th June 2001

Covering: 1975–2001

1 Introduction

RNA is a central molecule in the transmission, processing, and translation of genetic information. Although the critical role of RNA in cellular processes has long been recognized, the discoveries of autocatalytic[1] and catalytic processes[2,3] that are RNA-based have spurred even more interest in the chemistry and biology of RNA systems. The unique three dimensional structures adopted by various RNAs determine their activity and thus an understanding of structure is key to a full appreciation of their biological roles. Advances in high-resolution structure determination have provided insight into the organization and function of increasingly complex biochemical entities involved in RNA processing culminating in the recent structure of the large ribosomal subunit.[3] Despite these impressive achievements, structural analysis by X-ray or NMR techniques remains an arduous and sometimes capricious endeavour. There is thus a need for simple chemical probes to provide structural information in the absence of higher resolution data and to complement those data where they have been obtained.

Chemical tools for the analysis of nucleic acid structure were largely developed for use in characterization of DNA or protein–DNA complexes. Because RNA differs from DNA only by the presence of a 2′-hydroxy group, methodologies developed for the study of DNA systems have proven useful in the study of RNA structure and function as well. Many of these tools are reagents which react with some nucleic acid functionality in a specific fashion and render the phosphodiester backbone labile to cleavage. RNAs of different sizes, radio-labeled at the 5′ or 3′ ends with ^{32}P, may be separated at nucleotide resolution by denaturing polyacrylamide gel electrophoresis (PAGE) and then visualized by exposure of the gel to either X-ray film or another imaging surface. Nucleic acid fragments appear as bands in the gel; smaller fragments migrate faster than larger ones and the exact position in a particular sequence may be ascertained by comparison to a "ladder" of bands generated by partial alkaline hydrolysis, base-specific chemical sequencing, enzymatic sequencing, or base-specific enzymatic cleavage (the enzyme *RNase*T1, for example, cleaves RNA sequences only after guanosine residues). When a chemical reagent is used to induce cleavage of the RNA backbone, the presence of a band on a gel indicates that the targeted functionality was accessible to the modifying reagent while the absence of a band indicates that the functionality was inaccessible due to the formation of some kind of inter- or intra-molecular structure. Regions of decreased band intensity spanning one or more nucleotides are referred to as footprints and this type of experiment, known as protection footprinting, has been of immense power in the analysis of structure in nucleic acid systems (Fig. 1; *interference* footprinting involves pre-treatment of a nucleic acid with a modifying reagent in order to determine which modifications block structure formation).

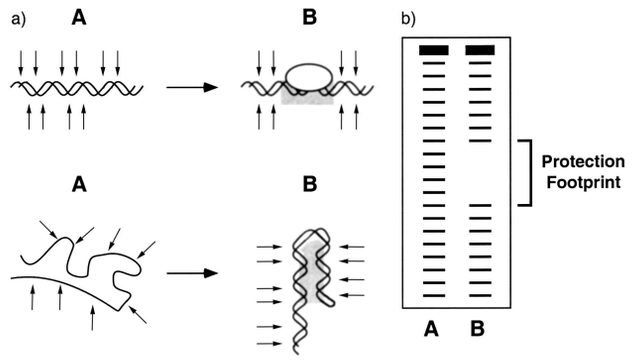

Fig. 1 Protection footprinting as a probe of nucleic acid structure. a) Formation of higher order structure in nucleic acids by protein–nucleic acid association (top) or folding of a nucleic acid (bottom). The structure of the complexed or folded molecule may be probed by treatment with a nucleic acid modifying agent which initiates strand cleavage. Arrows represent modification of the nucleic acid with such a reagent and shading denotes protection from modification; b) PAGE analysis of ^{32}P radio-labeled fragments obtained in the protection experiment. Comparison is made of the cleavage patterns obtained for the unbound or unstructured state (A) with the bound or structured state (B). Areas of reduced band intensity are referred to as footprints and represent the protection from chemical modification resulting from protein binding or higher order structure formation.

A large group of reagents have been used in protection footprinting experiments. Gilbert and co-workers showed that dimethyl sulfate (DMS), which reacts with N7 of guanosine and N3 of adenosine, can be used to detect contacts to these positions.[4] Diethyl pyrocarbonate† modification of DNA has been used as a probe of contacts to the N7 positions of G and A while osmium tetraoxide (OsO_4) and potassium permanganate ($KMnO_4$), which react with the 5,6 bond of the thymidine base, have been used as protection probes of changes in DNA helix parameters.[5–7]

Protection footprinting has typically been used to compare two different states in a system; for example, an unbound nucleic acid can be compared to a protein-bound nucleic acid or an unstructured nucleic acid can be compared to a structured or folded nucleic acid (Fig. 1). There have been a host of protection footprinting studies of protein–DNA systems ranging

† The IUPAC name for pyrocarbonic acid is oxydiformic acid.

DOI: 10.1039/b103013m

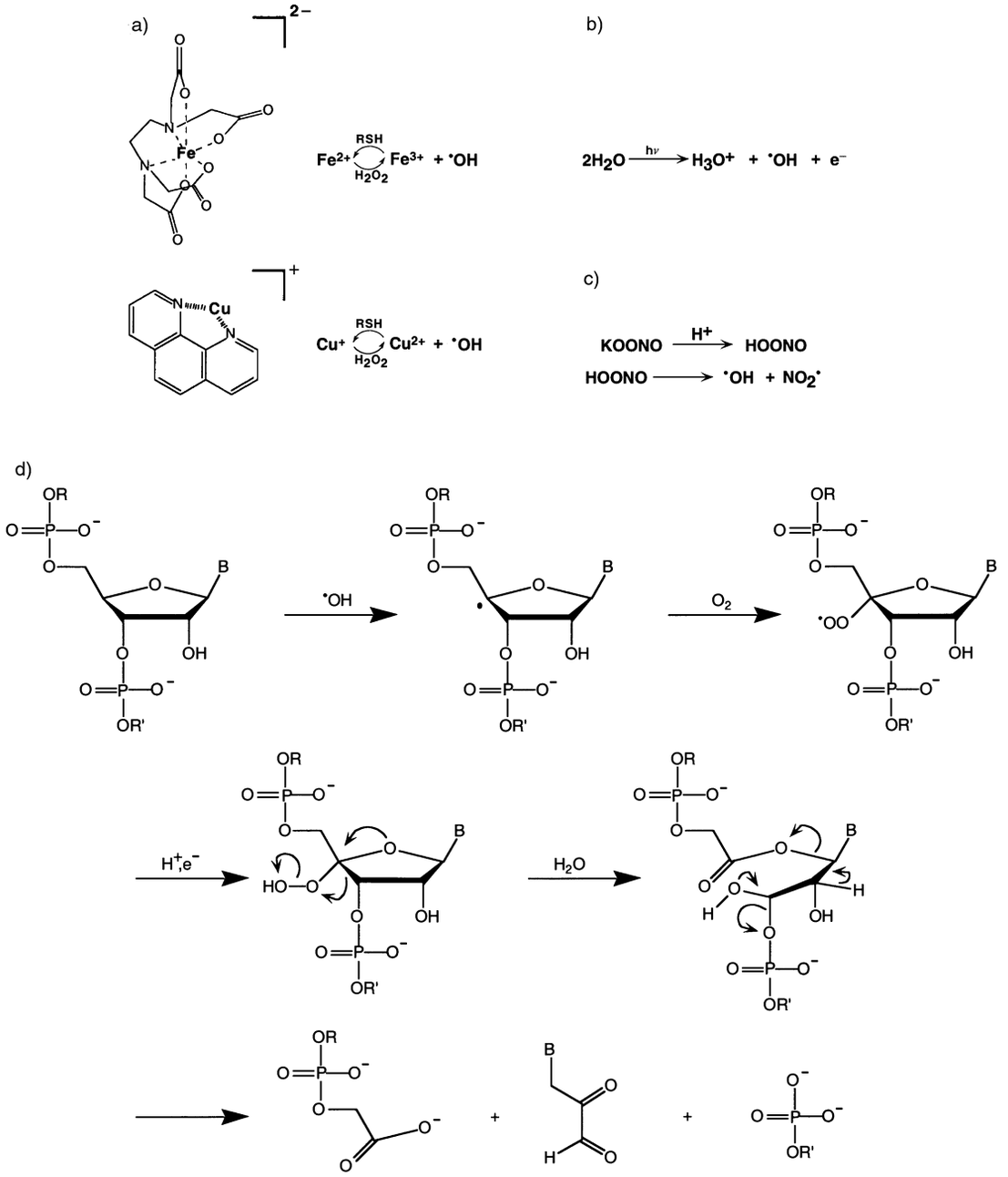

Fig. 2 Hydroxyl radicals as reagents for protection footprinting of nucleic acids. a) Radical generation from EDTA–Fe(II) and 1,10-phenanthroline–Cu(I) in the presence of H_2O_2 and a reducing agent; b) radiolysis of water by synchrotron X-ray irradiation to form hydroxyl radicals; c) thermal decomposition of peroxynitrous acid in neutral aqueous solution; d) one possible mechanism of RNA strand scission involving hydroxyl radicals generated by radiolysis of water under aerobic conditions. Hydrogen atom abstraction from C4′ is followed by a cascade of reactions leading to cleavage of the phosphodiester backbone.

from investigations of simple protein–DNA complexes to examinations of nucleosome–protein interaction.[8] The formation of higher order structure in DNA has been studied using protection footprinting techniques; for example, the G-quartet structure of telomeric DNA sequences, involving square-planar arrays of four hydrogen-bonded deoxyguanosine residues, was, in part, inferred from protection of a set of these residues from DMS modification in the presence of monovalent cations.[9] Studies such as these may be regarded as examples of equilibrium footprinting since they reflect observations of the thermodynamic equilibrium in a system. Recently, there has been considerable interest in the development of techniques to explore dynamic changes in RNA structure such as folding or conformational rearrangements. Kinetic protection footprinting techniques, as explored by a number of different laboratories, have proven useful for studies of RNA structural change in several systems.

2 Equilibrium and kinetic generation of hydroxyl radicals

Chemical footprinting with hydroxyl radicals has proven to be one of the most important techniques for probing nucleic acid structure. Diffusable hydroxyl radicals generated from a variety of transition metal complexes (Fig. 2a) are able to abstract hydrogen from the C1′, C2′, C4′, or C5′ positions on the deoxyribose or ribose ring initiating a cascade of reactions which culminate in scission of the phosphodiester backbone (Fig. 2d).[10] One important feature of hydroxyl radicals is that their reactivity is sequence independent and also independent of the single- or double-stranded nature of the nucleic acid target—thus, hydroxyl radicals serve as general probes of nucleic acid structure.

Hertzberg and Dervan were the first to apply Fenton chemistry to DNA footprinting using hydrogen peroxide in the presence of methidiumpropyl–EDTA–Fe(II).[11] This work was

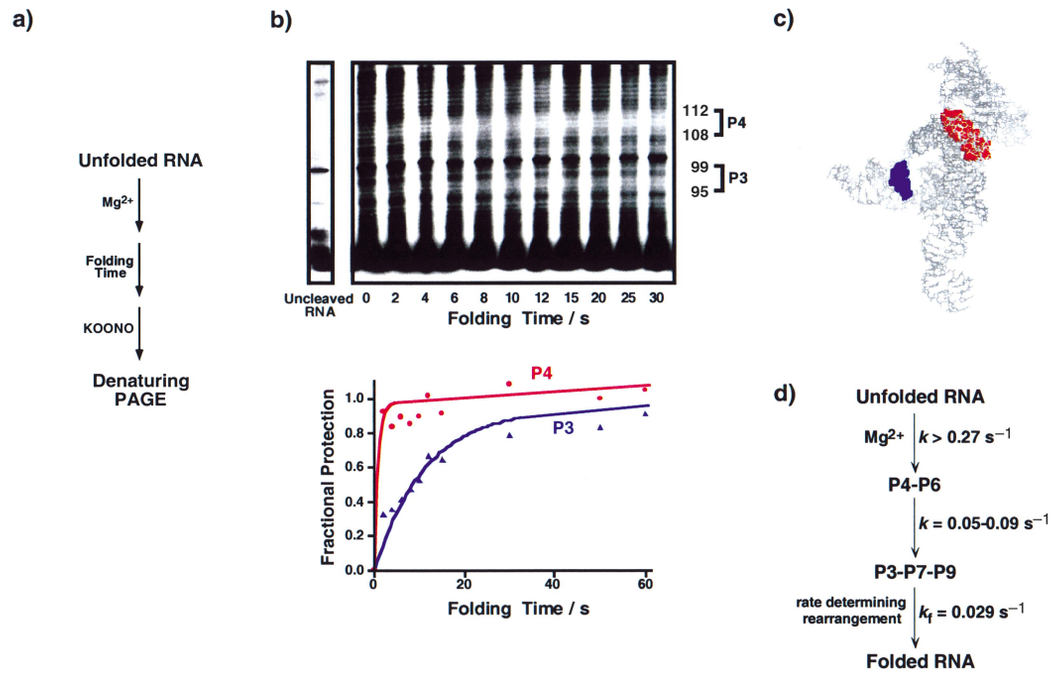

Fig. 3 Kinetic footprinting of the Group I ribozyme folding pathway using peroxynitrous acid.[19,20] a) Footprinting of ^{32}P end-labeled RNA was carried out at various times following the initiation of folding with Mg^{2+}; b) denaturing PAGE analysis of kinetic footprinting experiment highlighting the Watson–Crick base-paired P3 and P4 helical regions of the RNA (top) and graphical representation of fractional protection from radical induced cleavage of P3 and P4 as a function of time (bottom). To calculate the fractional peroxynitrous acid protection (footprint), background intensity was first subtracted and a correction for differences in lane loading was applied. Then the intensity of a region at a given time was divided by the intensity of the same region at time zero. The resulting fractional intensities were normalized from 0 to 1. Using data averaged from three separate experiments, plots of normalized fractional protection *versus* folding time were fitted to a first order exponential (assuming that the observed protections were first order processes); c) high resolution X-ray structure[24] of the Group I RNA showing location of P3 (blue) and P4 (red) regions; d) partial mechanism of folding of the Group I RNA.

extended by Tullius and Dombroski with the introduction of EDTA–Fe(II) as a general protection footprinting reagent[12] while Spassky and Sigman introduced 1,10-phenanthroline–Cu(I) as a complementary "chemical nuclease".[13] Subsequently both EDTA–Fe(II) and phenanthroline–Cu(I) have proven useful as probes of RNA structure.[14,15]

Hydrogen abstraction from deoxyribose or ribose is roughly diffusion limited and so the potential exists for the development of kinetic footprinting reagents based on this chemistry. A recent report showed that radical generation from EDTA–Fe(II) on the multi-second time-scale could be used for time-resolved studies of RNA structure formation in the hairpin ribozyme system.[16] With this notable exception, radical generation from transition metal complexes has been performed in the 30 second to minute time-scale and therefore has been most applicable to equilibrium footprinting experiments.

A major advance in the development of kinetic footprinting methodologies occurred with the application of synchrotron X-ray irradiation by Woodson and co-workers[17,18] to the generation of radicals for footprinting experiments (Fig. 2). Radicals generated within 50–100 ms by radiolysis of water were used to perform equilibrium footprinting studies on the *lac* repressor–DNA interaction and kinetic footprinting of the folding of a large RNA. Recently, it has been shown that peroxynitrous acid is useful for analyzing events with half-lives of greater than ~3 seconds.[19–21] The reagent is synthesized by oxidation of potassium peroxynitrite with peroxide followed by trapping with potassium hydroxide to yield the stable potassium salt.[22] Addition of potassium peroxynitrite to aqueous solutions buffered to pH 7 produces peroxynitrous acid which decomposes with a half-life of less than a second to generate hydroxyl radicals (Fig. 2).

3 Kinetic footprinting of RNA folding pathways

Structural and functional studies of RNA have been dominated in recent years by the characterization of ribozymes—RNA molecules capable of catalyzing biochemical transformations in the absence of protein cofactors. The Group I ribozyme, an RNA endonuclease composed solely of RNA, first studied by Cech and co-workers, has been used as a model system in the study of RNA structure formation and rearrangement because it has been extensively characterized biochemically and because high resolution X-ray structures of several large fragments have been determined.[23,24] Multiple RNA sequence comparisons were originally used to predict a number of Watson–Crick base paired (P) regions providing a two dimensional map of the structure of this 388 nucleotide ribozyme—the folding of the RNA into its active three dimensional structure is a magnesium ion dependent process producing a compact structure of tightly packed helical regions.

Kinetic footprinting of structure formation in a large radiolabeled RNA involves initiation of folding by addition of Mg^{2+} to a reaction followed at various times by treatment with rapidly formed hydroxyl radicals and subsequent analysis by denaturing PAGE. Kinetic footprinting based on either X-ray irradiation of water[17] or peroxynitrous acid treatment (Fig. 3)[19,20] confirmed a hierarchy of folding events for the Group I RNA which had initially been proposed by Zarrinkar and Williamson.[25] Most strikingly, sequences of the P4 and P3 helical regions were protected at very different rates (Fig. 3). This could be rationalized by the fact that the P3 helix is itself actually formed from nucleotides separated at quite some distance in the primary sequence and also by a consideration of the X-ray structure of the Group I RNA in which P4 was shown to be a buried core structure (Fig. 3).[24] Interestingly, kinetic footprinting with peroxynitrous acid did not reveal folding events taking place at the rate limiting step of folding as measured by an independent assay.[25] This reflects late rearrangements of an unknown nature which merit further investigation.

The folding path of the large M1 RNA, the catalytic subunit of the *E. coli* tRNA processing enzyme *RNase*P, has also been studied using peroxynitrous acid kinetic footprinting.[21] Similarities with the folding path of the Group I RNA were observed in that discrete sets of protection from radical induced cleavage were seen during the folding experiment. A two domain structure has previously been established for the M1 RNA.[26] The results of kinetic footprinting suggest that, in contrast to the Group I RNA, a single domain of M1 does not nucleate folding of the RNA. Rather protections are observed at the interface between domains midway through the folding process; these may be related to proposed misfolding events along the folding pathway.[27]

4 Photochemical radical production—novel kinetic footprinting reagents

Although reagents such as peroxynitrous acid, and more recently EDTA–Fe(II) have proven useful in kinetic footprinting experiments, they are limited in application by the half-life of hydroxyl radical generation. For example, the rate of decomposition of peroxynitrous acid means that it can only be used to study events which occur in the second time-scale range with half-lives greater than 3–4 seconds. It would be extremely useful to have access to novel reagents and methodologies which would allow the study of truly fast events such as those occurring in the micro-second to milli-second range. Currently the fastest events which may be measured are on the milli-second time-scale and require a synchrotron X-ray source. Photochemical generation of radicals by laser irradiation of an appropriate precursor in a stopped-flow flash photolysis experiment represents an attractive alternative to either synchrotron irradiation or the use of peroxynitrous acid. Advantages of such reagents are: they allow pre-mixing of all components of the footprinting reaction prior to irradiation, they allow for the precise control of the cleavage conditions by light, and they have potential for use in experiments such as kinetic probing of nucleic acid structure.

Although there have been a number of reports of photochemically induced cleavage of RNA and DNA involving both oxidative and hydrolytic cleavage pathways,[28] until recently, only hydrogen peroxide has been successfully used to photochemically footprint a nucleic acid using near molar concentrations of H_2O_2.[29] One common feature of organic or organometallic reagents that effect photochemical cleavage of nucleic acids is that they often bind to and interact with DNA and RNA.[28] Such interactions may alter the photochemistry of the precursor and perturb the structure of the DNA or RNA; in addition, sequence-specific binding to the polynucleotide results in sequence-specific cleavage upon photolysis. For example, irradiation of rhodium(III) complexes bound to DNA results in strand cleavage with observed products consistent with a sequence-specific radical based cleavage pathway.[30] Cobalt(III)–bleomycin complexes can also cleave DNA photochemically in a sequence-selective manner.[31] Both of these complexes are thus very useful as sequence-specific probes of nucleic acid structure. However, they have limited utility as footprinting reagents because of the requirement for sequence-independent cleavage in such experiments.

Several classes of organic compounds can act as photochemical sources of hydroxyl radicals (so-called photo-Fenton reagents). Examples include alkylperoxides[32] that undergo bond homolysis upon photo-irradiation yielding an alkoxyl radical and a hydroxyl radical, *N*-oxides,[33] and *N*-hydroxypyridines such as *N*-hydroxypyridin-2(1*H*)-one (N-HP)[34] and *N*-hydroxypyridine-2(1*H*)-thione (N-HPT)[35] that undergo N–O bond homolysis (Fig. 4). Little is known about either the interactions of these compounds with nucleic acids or the chemistry resulting from their irradiation in the presence of nucleic acids. N-HPT has an absorption maximum at 350 nm and is thus a reasonable choice for a photochemical source of radicals for use in experiments involving nucleic acids.

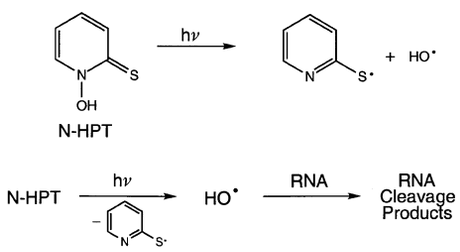

Fig. 4 Photochemical production of RNA reactive radicals from the caged hydroxyl radical precursor *N*-hydroxypyridine-2(1*H*)-thione (N-HPT).

Preliminary experiments with N-HPT have shown that it is an excellent candidate for a photochemical kinetic footprinting reagent.[36] Equilibrium experiments with unfolded and folded Group I RNA were performed and it was demonstrated that radicals produced by photolysis generate a footprint identical to that generated using peroxynitrous acid; this is important since it not only validates N-HPT as a radical source but indicates that RNA cleavage occurs in a sequence independent fashion. Even millimolar concentrations of N-HPT were shown not to interfere with either RNA folding or activity, another prerequisite for a candidate kinetic footprinting reagent.

5 Prospects

Equilibrium footprinting has been invaluable in the analysis of nucleic acid structure for both DNA and RNA. Recent methodological advances have resulted in the development of kinetic footprinting techniques which are suitable for analyzing events such as the folding pathways of complex RNAs. The application of stopped-flow laser flash photolysis to folding experiments using radical precursors such as N-HPT should facilitate a new generation of fast folding studies. It may be possible to introduce caged Mg^{2+} into a folding experiment in the presence of a radical source such as N-HPT.[37] Folding could then be initiated by a laser pulse at one wavelength while footprinting could be initiated by a second pulse at a different wavelength. This approach would get around the mixing problem and allow for even faster folding experiments.

Experiments described here have focused on kinetic footprinting of *in vitro* folding pathways of several large RNAs in the absence of cofactors other than divalent magnesium ion. RNA molecules fold and function in the cell in the presence of proteins with which they are complexed. There is evidence that association of proteins with RNA affects both the rate and mechanism of folding. It will be of interest then to perform kinetic footprinting experiments of RNA folding in the presence of specific or general protein cofactors. In addition, it may be possible to perform folding studies *in vivo* using light initiated footprinting techniques. Kinetic footprinting should yield valuable information about dynamics in several other RNA systems. Introns are removed from precursor mRNAs (pre-mRNAs) in eukaryotic cells by the spliceosome, a biochemical entity consisting of RNA and protein components. Spliceosome assembly on pre-mRNA substrates takes place over the course of 10–15 minutes and therefore should be amenable to footprinting studies with peroxynitrous acid. Transitions such as translocation of tRNAs on the ribosome take place rapidly and time-resolved studies in such a system will require fast radical generation such as is feasible using laser flash photolysis and N-HPT. Finally, kinetic experiments with N-HPT should be useful in the study of dynamics in other biological systems—there should be ready application of this methodology to studies of protein folding[38,39] based on the cleavage of exposed portions of the polypeptide backbone and mass spectrometric analysis.

6 Acknowledgements

I would like to thank Steven Chaulk (University of Toronto) and Oliver Kent (University of Alberta) and our collaborator John Pezacki (National Research Council, Canada) for their input and help with figures. Work in the author's laboratory was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC).

7 References

1 A. J. Zaug and T. R. Cech, *Science*, 1986, **231**, 470.
2 C. Guerrier-Takada, K. Gardiner, T. Marsh, N. Pace and S. Altman, *Cell (Cambridge, Mass.)*, 1983, **35**, 849.
3 N. Ban, P. Nissen, J. Hansen, P. B. Moore and T. A. Steitz, *Science*, 2000, **289**, 905.
4 W. Gilbert, A. Maxam and A. Mirabekov, *Alfred Benzon Symp.*, 1976, **9**, 139.
5 E. Bateman and M. R. Paule, *Mol. Cell. Biol.*, 1988, **8**, 1940.
6 M. J. McLean and M. J. Waring, *J. Mol. Recognit.*, 1988, **1**, 138.
7 J. A. Borowiec, L. Zhang, S. Sasse-Dwight and J. D. Gralla, *J. Mol. Biol.*, 1987, **196**, 101.
8 Q. Li and O. Wrange, *Methods*, 1997, **12**, 96.
9 J. R. Williamson, M. K. Raghuraman and T. R. Cech, *Cell*, 1989, **59**, 871.
10 W. K. Pogozelski and T. D. Tullius, *Chem. Rev.*, 1998, **98**, 1089.
11 R. P. Hertzberg and P. B. Dervan, *Biochemistry*, 1984, **23**, 3934.
12 T. D. Tullius and B. A. Dombroski, *Proc. Natl. Acad. Sci. U.S.A.*, 1986, **83**, 5469.
13 A. Spassky and D. S. Sigman, *Biochemistry*, 1985, **24**, 8050.
14 J. A. Latham and T. R. Cech, *Science*, 1989, **245**, 276.
15 C.-h. B. Chen and D. S. Sigman, *J. Am. Chem. Soc.*, 1988, **110**, 6570.
16 K. J. Hampel and J. M. Burke, *Methods*, 2001, **23**, 233.
17 B. Sclavi, M. Sullivan, M. R. Chance, M. Brenowitz and S. A. Woodson, *Science*, 1998, **279**, 1940.
18 B. Sclavi, S. A. Woodson, M. Sullivan, M. R. Chance and M. Brenowitz, *J. Mol. Biol.*, 1997, **266**, 144.
19 S. G. Chaulk and A. M. MacMillan, *Angew. Chem., Int. Ed.*, 2000, **39**, 521.
20 S. G. Chaulk and A. M. MacMillan, *Biochemistry*, 2000, **39**, 2.
21 O. Kent, S. G. Chaulk and A. M. MacMillan, *J. Mol. Biol.*, 2000, **304**, 699.
22 P. A. King, E. Jamison, D. Strahs, V. E. Anderson and M. Brenowitz, *Nucleic Acids Res.*, 1993, **21**, 2473.
23 J. H. Cate, A. R. Gooding, E. Podell, K. Zhou, B. L. Golden, C. E. Kundrot, T. R. Cech and J. A Doudna, *Science*, 1996, **273**, 1678.
24 B. L. Golden, A. R. Gooding, E. R. Podell and T. R. Cech, *Science*, 1998, **282**, 259.
25 P. P. Zarrinkar and J. R. Williamson, *Science*, 1994, **265**, 918.
26 A. Loria and T. Pan, *RNA*, 1996, **2**, 551.
27 T. Pan, X. Fang and T. Sosnick, *J. Mol. Biol.*, 1999, **286**, 721.
28 B. Armitage, *Chem. Rev.*, 1998, **98**, 1171.
29 R. B. MacGregor, *Anal. Biochem.*, 1992, **204**, 324.
30 C. S. Chow and J. K. Barton, *Methods Enzymol.*, 1992, **212**, 219.
31 I. Saito, T. Morii, H. Sugiyama, T. Matsuura, C. Meares and S. M. Hecht, *J. Am. Chem. Soc.*, 1989, **111**, 2307.
32 I. Saito, M. Takayama, T. Matsuura, S. Matsugo and S. Kawanishi, *J. Am. Chem. Soc.*, 1990, **112**, 883.
33 M. Sako, K. Nagai and Y. Maki, *J. Chem. Soc., Chem. Commun.*, 1993, 750.
34 B. M. Aveline, I. E. Kochevar and R. W. Redmond, *J. Am. Chem. Soc.*, 1996, **118**, 10124.
35 B. M. Aveline, I. E. Kochevar and R. W. Redmond, *J. Am. Chem. Soc.*, 1996, **118**, 10113.
36 S. G. Chaulk, J. P. Pezacki and A. M. MacMillan, *Biochemistry*, 2000, **39**, 10448.
37 J. H. Kaplan and G. C. Ellis-Davies, *Proc. Natl. Acad. Sci. U.S.A.*, 1988, **85**, 6571.
38 S. D. Maleknia, M. Brenowitz and M. R. Chance, *Anal. Chem.*, 1999, **71**, 3965.
39 K. Biemann, *Annu. Rev. Biochem.*, 1992, **61**, 977.