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RNA Crosslinking Methods

Michael E. Harris and Eric L. Christian

Center for RNA Molecular Biology, Department of Biochemistry, Case Western Reserve University School of Medicine, Cleveland, Ohio, USA

Abstract

RNA–RNA crosslinking provides a rapid means of obtaining evidence for the proximity of functional groups in structurally complex RNAs and ribonucleoproteins. Such evidence can be used to provide a physical context for interpreting structural information from other biochemical and biophysical methods and for the design of further experiments. The identification of crosslinks that accurately reflect the native conformation of the RNA of interest is strongly dependent on the position of the crosslinking agent, the conditions of the crosslinking reaction, and the method for mapping the crosslink position. Here, we provide an overview of protocols and experimental considerations for RNA–RNA cross-linking with the most commonly used long- and short-range photoaffinity reagents. Specifically, we describe the merits and strategies for random and site-specific incorporation of these reagents into RNA, the crosslinking reaction and isolation of crosslinked products, the mapping crosslinked sites, and assessment of the crosslinking data.

1. Introduction

RNA–RNA crosslinking is a well-established and widely used method for obtaining secondary and tertiary structural information from structurally complex RNAs and ribonucleoproteins (RNPs) when classical methods of structural analysis such as X-ray crystallography and NMR are not practical (Branch *et al.*, 1985; Butcher and Burke, 1994; Datta and Weiner, 1992; Downs and Cech, 1990; Favre *et al.*, 1998; Harris *et al.*, 1994; Ofengand *et al.*, 1979; Sun *et al.*, 1998; Wassarman and Steitz, 1992; Yaniv *et al.*, 1969; Zwieb and Brimacombe, 1980; Zwieb *et al.*, 1978). Crosslinking is generally achieved using ultraviolet (UV) light to induce the formation of a covalent bond between unmodified RNAs or between RNA and a photoaffinity reagent incorporated randomly or at specific positions in the RNA structure (Elad, 1976). Due to the conformational flexibility and promiscuous chemical reactivity of most photoaffinity reagents, the conformational heterogeneity of RNAs in solution, the inherent ambiguities in mapping the site of crosslinking and other factors (see below), the resolution of this method can be highly variable (typically between 5 and 30 Å) (Huggins *et al.*, 2005; Sergiev *et al.*, 2001; Whirl-Carrillo *et al.*, 2002). While this variability generally precludes the use of crosslinking to demonstrate specific intra- or intermolecular interactions, this method nevertheless provides highly valuable evidence for the proximity of functional groups at the time of UV induction.

The power of crosslinking lies in the ability to rapidly identify potential regions of intra- and intermolecular interaction that can be tested by site-specific modification and subsequent kinetic and thermodynamic studies. In addition, crosslinking can generate a range of distance constraints useful for the development of three-dimensional models of complex RNA structures (Chen *et al.*, 1998; Harris *et al.*, 1994, 1997; Malhotra and Harvey, 1994; Mueller *et al.*, 1997; Pinard *et al.*, 2001). Moreover, the methods used for this approach are relatively straightforward, can be applied under a wide range of experimental conditions, and require relatively little material for analysis. Crosslinking, therefore, is not optimally used as a stand-alone approach but rather as a key analytical tool that can provide an explicit

physical context for designing new structure–function experiments and for interpreting structural information when used in conjunction with other biochemical and biophysical methods.

Herein, we describe general experimental protocols for RNA–RNA crosslinking with the most common long- and short-range photoaffinity reagents, as well as strategies for random and site-specific incorporation of these reagents to initially survey and subsequently target regions of potential intra- or intermolecular interaction. We will initially describe the merits and incorporation of individual photoaffinity reagents into RNA (Section 2), followed by methods for the crosslinking reaction and isolation of cross-linked products (Section 3), mapping crosslinked sites (Section 4), and finally suggestions for assessing the validity of individual results (Section 5). The description is designed to be sufficiently general in order to be useful as a guideline for an experimentalist at the graduate level who is considering application of photocrosslinking of RNA in their research. However, a basic understanding of techniques for handling nucleic acids is assumed. Specific reaction conditions will be based on our studies with RNase P RNA; however, such conditions are similar to that in other *in vitro* studies of RNA biochemistry. Indeed, some or all of the methods described in this chapter have been successfully applied in analysis of structural and catalytic RNAs as well as the major cellular RNPs, including the ribosome, and the spliceosome (Christian *et al.*, 1998; Druzina and Cooperman, 2004; Harris *et al.*, 1994, 1997; Huggins *et al.*, 2005; Juzumiene and Wollenzien, 2001; Juzumiene *et al.*, 2001; Kim and Abelson, 1996; Leung and Koslowsky, 1999; Maroney *et al.*, 1996; Montpetit *et al.*, 1998; Newman *et al.*, 1995; Pinard *et al.*, 1999; Pisarev *et al.*, 2008; Podar *et al.*, 1998; Rinke-Appel *et al.*, 2002; Ryan *et al.*, 2004; Sergiev *et al.*, 1997; Wassarman and Steitz, 1992; Yu and Steitz, 1997). Crosslinking is thus likely to remain a very useful tool in deconstructing the structure and function of RNAs and RNPs for many years to come.

2. Synthesis of Modified RNA Crosslinking Substrates

In the absence of detailed structural information, a useful crosslinking strategy is to begin with a broad survey of potential regions of intra- and intermolecular contact followed by site-specific positioning of crosslinking agents, mutagenesis, and biochemical analysis to systematically derive structure–function relationships. Nonspecific crosslinking approaches using UV light or psoralin have been used to establish distance constraints in a wide variety of experimental systems involving large structural RNAs and RNP complexes (Behlen *et al.*, 1992; Branch *et al.*, 1985; Brandt and Gualerzi, 1992; Downs and Cech, 1990; Noah *et al.*, 2000; Sawa and Abelson, 1992; Sun *et al.*, 1998; Wassarman and Steitz, 1992; Zwieb *et al.*, 1978). The relatively strict geometric constraints needed for bond formation by these simple and direct methods often produce more accurate structural information than other crosslinking methods when such data have been subsequently evaluated in light of crystal structures (Sergiev *et al.*, 2001; Whirl-Carrillo *et al.*, 2002). However, the number of crosslinks produced using UV light or psoralin is generally too small to be used to broadly survey potential regions of intra- and intermolecular interaction or proximity.

Photoaffinity reagents are generally much more sensitive to UV induction and have fewer geometric constraints for bond formation when incorporated into RNA. Thus, they will tend to produce a much larger number of cross-links and consequently more information. There is, of course, the potential for a tradeoff between the number of crosslinks observed and accuracy since the increased ability to react may increase the probability of producing misleading crosslinking results, particularly for the longer range photoaffinity reagents (>9 Å). However, such a tradeoff is reasonable in an initial survey of an RNA structure, or when there is an insufficient level crosslinking from which to design experiments.

Long-range photoaffinity reagents placed site specifically at various positions throughout an RNA structure provide a practical means of generating a relatively large set of potential intra- or intermolecular interactions or distance constraints that can be subsequently refined using site-specific analysis of more geometrically restricted photoaffinity reagents or mutagenesis. Accordingly, we will initially describe methods for synthesis and incorporation of azidophenacyl (APA) derivatives, which are useful, highly reactive photoaffinity reagents for long-range RNA crosslinking. This section will be followed by methods for random and site-specific incorporation of the thionucleotide photoagents 6-thioguanosine (6sG) and 4-thiouridine (4sU), which are some of the most convenient and broadly applied short-range (~3 Å) photoaffinity reagents in RNA crosslinking studies.

2.1. Long-range photoaffinity crosslinking with azidophenacyl modifications

APA is an arylazide photoagent (Hixson and Hixson, 1975; Hixson *et al.*, 1980) that positions an azido moiety *ca.* 9 Å from its point of attachment to the RNA and can be used to identify general features of an RNA structure such as position and orientation of RNA helices (Chen *et al.*, 1998; Harris *et al.*, 1994, 1997; Nolan *et al.*, 1993). APA photoagents like their thionucleotide counterparts have the important chemical property of being reactive over a wide range of experimental conditions, but are inert until activated with UV light. Moreover, photocrosslinking with these reagents is very sensitive. Most applications use radiochemical detection, which requires only picomole or even femtomole amounts of material.

APA is conveniently attached to the 5'- or 3'-ends of RNA, and may also be placed at different positions in a molecule by developing a series of circularly permuted RNAs as described in the following section. Attachment of APA to the 5'-end of an RNA involves reaction with a 5'-guanosine monophosphorothioate (GMPS) (Burgin and Pace, 1990; Murray and Atkinson, 1968). Efficiencies of 5'-GMPS incorporation of 70–90% can be achieved with GMPS:GTP ratios between 10:1 and 40:1 in otherwise standard *in vitro* RNA transcription reactions. GMPS is available commercially (Amersham), but can also be made by chemical phosphorylation (Behrman, 2000). Gel-purified 5'-GMPS RNAs are subsequently reacted with azidophenacyl bromide (Fluka/Pierce) by resuspending the 5'-GMPS RNA in 100 µl of 40% methanol; 20 mM sodium bicarbonate, pH 9.0; 0.1% SDS; and 5 mM azidophenacyl bromide and incubating for 1 h at room temperature. Reactions are then brought to 200 µl by the addition of a solution containing 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA. The residual uncoupled photoagent is then removed by phenol extraction and the RNA recovered by ethanol precipitation using standard methods.

Attachment of APA to the 3'-end of an RNA can be accomplished by chemically modifying the terminal ribose to contain a primary amine. The amine is subsequently modified with a bifunctional reagent containing both the azido group for crosslinking and a hydroxysuccinimidyl group for reacting with the amine (Oh and Pace, 1994). This modification is accomplished by initially oxidizing the 3'-*cis*-diol of 5–10 µg RNA in 50–100 µl of 3 mM sodium periodate, 100 mM sodium acetate, pH 5.4, for 1 h at room temperature to create the 3'-dialdehyde. The RNA is subsequently recovered by ethanol precipitation, resuspended in 100 µl of 20 mM imidazole, pH 8.0, 5 mM NaCNBH₃, 1 mM ethylene diamine and incubated at 37°C for 1 h. NaBH₄ is then added to 5 mM and the incubation continued for another 10 min. Following reaction with NaBH₄, RNAs are precipitated twice with ethanol and reacted with 10 mM photoagent [*N*-hydroxysuccinimidyl-4-azidobenzoate (Pierce)] in 50 mM HEPES/NaOH, pH 9.0, at room temperature for 1 h in the dark before being recovered by ethanol precipitation. Reactions from this point forward should all be performed in amber tubes and significant care should be taken to minimize exposure to ambient light to prevent preactivation of APA.

As noted above, APA photoaffinity reagents may be positioned at different specific sites within a folded structure through the use of circularly permuted RNAs (cpRNAs) that ideally reposition of the 5'- and 3'-ends of the molecule without significantly altering the primary sequence or the overall three-dimensional structure. The above criteria of maintaining the overall three-dimensional structure obviously limit the positions on an individual molecule where the rearranged 5'- and 3'-ends can be placed. That said, structural RNAs are often sufficiently stabilized by internal interactions to tolerate discontinuities at many positions in the polynucleotide backbone (Guerrier-Takada and Altman, 1992; Pan *et al.*, 1991; Reich *et al.*, 1986; van der Horst *et al.*, 1991; Waugh and Pace, 1993). Thus, with sufficient care cpRNAs can often be constructed to position APA throughout the molecule of interest. In cases where functional cpRNAs cannot be obtained, meaningful crosslinking analysis may still be possible both within or between fragments or independent folding domains of the RNA (Leonov *et al.*, 1999). Crosslinking has also been done from oligonucleotides annealed to a folded RNA target. However, this method necessarily disrupts local secondary and has often produced results in conflict with high-resolution structural data (Sergiev *et al.*, 2001).

Templates for circularly permuted RNAs are generated by PCR amplification of tandem genes that are generally separated by a sufficient length of added sequence to connect the original 5'- and 3'-ends of the molecule without perturbing overall folding or biological activity. Forward and reverse primers that define the 5'- and 3'-ends of the cpRNA and contain promoter sequences and restriction enzyme sites for cloning are directed in the appropriate direction in the upstream and downstream genes to yield a single gene product upon amplification. In choosing the position the 5'-end of the cpRNA it is important to take into account the sequence requirements of the polymerase used during *in vitro* transcription. T7 RNA polymerase optimally requires two consecutive G residues at the 5'-end; however, sufficient amounts of RNA can be obtained from transcripts beginning with GU, GC, and GA (Milligan and Uhlenbeck, 1989). SP6 polymerase, which has a less stringent sequence requirement for initiation than T7, can be used when the efficiency of T7 RNA polymerase proves insufficient. Also note that since the 3'-end of the cpRNA is formed by runoff transcription, restriction sites that cut at a distance from their recognition site (e.g., *FokI* or *BbsI*) must be designed into the downstream PCR primer to avoid loss of RNA coding sequences.

Transcripts of cpRNAs are generated by standard *in vitro* transcription methods. In our bacterial RNase P studies, 2–4 µg of linearized template DNA was combined at room temperature with 40 units of phage T7 RNA polymerase in a 100 µl reaction containing 40 mM Tris-HCl, pH 8.0, 1 mM spermidine, 5 mM dithiothreitol, 0.1% Triton X-100, 20 mM magnesium chloride, and 1 mM NTPs and incubated for 5–12 h. RNAs are gel-purified by electrophoresis through 4% polyacrylamide/8 M urea gels, visualized by UV shadow and passively eluted into 0.3 M sodium acetate, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1% sodium dodecyl sulfate overnight. As noted above, efficient 5'-labeling with GMPS will require altering the above conditions with GMPS:GTP ratios between 10:1 and 40:1. Also, note for GMPS-containing RNAs that 1 mM dithiothreitol should be included in the elution buffer to inhibit formation of disulfide.

The importance of establishing that cpRNAs reflect the native structure cannot be overstated since without such evidence crosslinking studies cannot be interpreted. For ribozymes, this can be achieved in the comparison of the kinetic parameters k_{cat} and K_m of cpRNAs with their native counterparts (Chen *et al.*, 1998; Harris *et al.*, 1994, 1997). Other structural RNAs can be compared on the basis of diagnostic metal ion cleavage, chemical modification, or their binding kinetics to other molecules. cpRNAs altered in catalytic

function or some other diagnostic feature are likely to contain structural distortions that limit their experimental utility and thus must be excluded from subsequent structural studies.

2.2. Short-range crosslinking using thionucleosides

Although a wide variety of chemical and photoreagents are available for short-range (~3 Å) RNA–RNA crosslinking, the thionucleotides 6sG and 4sU are preferred due to their simple molecular structure, relative stability, and high reactivity (Christian *et al.*, 1998; Dubreuil *et al.*, 1991; Favre *et al.*, 1998; Sergiev *et al.*, 1997; Sontheimer, 1994). In thionucleotide photoagents, the crosslinking thio moiety is attached directly to the nucleotide base and can thus be used to refine the distance constraints between positions that appear to be proximal by long-range crosslinking or other phylogenetic or biochemical data (Favre *et al.*, 1998). Importantly, 6sG and 4sU differ from their corresponding “parent” nucleoside by only a single atomic substitution, the replacement of a nucleobase oxygen by sulfur, thus reducing the potential for significant perturbation of RNA structure. Exposure of these thionucleotides to UV light produces a sulfur radical that can react efficiently with functional groups that are in proximity. Crosslinking reactions involving these reagents can be very efficient, making it easier to isolate sufficient quantities of crosslinked species for mapping of crosslinked nucleotides and for assessing the extent to which the observed crosslinks reflect the biologically relevant structure.

6sG and 4sU can serve as effective structural probes by either random incorporation or site-specific substitution. Random incorporation of these analogs during *in vitro* transcription provides a rapid means of surveying potential contacts over an entire RNA transcript and can be used as an alternative, or preferably, a complementary strategy to the broad structural probing using APA and cpRNAs. While this approach can generate a large number of crosslinks, it can be difficult to separate individual crosslinked species on polyacrylamide gels and thus pinpoint the site of crosslinking. This difficulty can often be reduced or eliminated by a reduction in the concentration of added thionucleotide in the transcription mixture or by selective deletion or mutation of nucleotide positions contributing to the crosslinking signal (Christian and Harris, 1999).

Site-specific incorporation by transcription or RNA ligation (see below) eliminates both the ambiguity of the source of crosslinking and generally increases the experimental signal. This latter feature is due to the fact that essentially all RNAs in the reaction mixture will contain a photoagent agent at a single position rather than diluted throughout the molecule, allowing for the total crosslinking signal to be distributed among a smaller number of crosslinked species. Site-specific analysis, however, is inherently more labor intensive since each position must be examined independently and generally requires the synthesis of a distinct site-specifically modified RNA in sufficient yield.

Random incorporation of thionucleotides by transcription is achieved by supplementing standard reaction conditions with the thionucleotide triphosphate of interest. In practice, sufficient levels of thionucleotide incorporation often require a reduction in the concentration of the corresponding unmodified nucleotide triphosphate. The relative concentrations of thio- and unmodified nucleotide triphosphate required to produce optimal crosslink will of course vary for individual RNAs and must be determined empirically. Optimally, crosslinking substrates should contain no more than one photoagent per molecule. The shifted absorption maxima for 4sU (330 nm) and 6sG (~340 nm) are generally well separated from the average absorption maximum for RNA (~260 nm) and can be used to quantify levels of incorporation. In our studies, 4sU-containing pre-tRNA crosslinking substrates used to identify potential regions of intermolecular interaction with RNase P RNA are transcribed under the following conditions. Linearized plasmid template (2 µg) is diluted at room temperature into transcription buffer containing 40 mM Tris–HCl,

pH 7.9, 6 mM MgCl₂, 2 mM spermidine, and 10 mM dithiothreitol, 1 mM each ATP, CTP, GTP, and 0.1 mM each UTP and s⁴UTP (USB) in a total volume of 100 µl and incubated overnight at 37 °C. These reactions are terminated by the addition of 100 µl of 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, and purified on polyacrylamide gels as described above. Transcripts (20 pmol) are then often subsequently 5'-end-labeled for binding and crosslinking studies with 150 µCi of [γ -³²P] ATP (New England Nuclear) and T4 polynucleotide kinase (Life Sciences).

Site-specific incorporation of 6sG and 4sU can be achieved by priming transcription of circularly permuted constructs with the monophosphate form of s⁶G (s⁶GMP) (Christian *et al.*, 1998) or dinucleotide primers (e.g., s⁴UpG) (Milligan and Uhlenbeck, 1989). The advantage of this approach is the ability to use the same set of cpRNAs to compare crosslinking reagents with different levels of geometric constraint. The main drawbacks of this approach are that s⁶GMP is not commercially available and must be synthesized by chemical phosphorylation of s⁶G (Behrman, 2000) and that priming with dinucleotide primers can be very inefficient. An alternative and more broadly used approach involves site-specific incorporation of 6sG or 4sU into a short oligonucleotide fragment by chemical synthesis and its subsequent joining to remaining sequences of a functional RNA by oligonucleotide-directed RNA ligation (Moore and Sharp, 1992). This approach allows the incorporation of a far greater range of modified nucleotides than can be incorporated by transcriptional initiation and avoids the potential structural complications of crosslinking adjacent to a gap in the polynucleotide backbone. The main drawback of this approach is that oligonucleotide-directed ligation can be inefficient, and thus it may be difficult to obtain sufficient material to map weaker crosslinks or to study biochemically.

Oligonucleotide-directed RNA ligation in our studies is done essentially as described by Moore and Sharp (Moore and Sharp, 1992). In general, synthetic RNA fragments containing the modified nucleotide of interest are joined to a single transcript containing the remaining sequences of the RNA being studied. In large molecules such as RNase P RNA, this is done using cpRNAs beginning immediately downstream of the 3'-end of the synthetic RNA fragment and ending immediately upstream of the fragment's 5'-end. In practice, we obtained significantly greater yields ligating only one of the junctions between the modified oligonucleotide and RNA transcript and that the remaining gap, when carefully positioned, rarely altered the kinetic features of the ribozyme. Ligation of two or more junctions is possible but often requires reoptimization of the reaction conditions. In our studies, synthetic RNA fragments (Dharmacon) are 5'-³²P-end-labeled with 150 µCi of [γ -³²P] ATP (New England Nuclear) and T4 polynucleotide kinase (Life Sciences) and purified on 22.5% polyacrylamide gels as described above. For the ligation reaction equal amounts (10 pmol) of the end-labeled oligonucleotide, RNA transcript, and a DNA oligonucleotide complementary to the synthetic RNA and the first 20 nucleotides of the transcript are combined in a total volume of 6.8 µl (in distilled water), heated to 60 °C for 2 min and then immediately frozen on dry ice for at least 2 min. Samples are subsequently thawed on wet ice and supplemented with 1 µl 10×T4 ligase buffer (New England Biolabs), 1 µl 50% 8000 g/mol polyethylene glycol (Fluka), 0.5 µl (200 units) RNasin (Promega), and 0.7 µl (280 units) T4 DNA ligase (New England Biolabs). Samples are then incubated for 2 h at 30 °C, combined with an equal volume (10 µl) of gel loading buffer, heated to 90 °C for 2 min, and then purified on polyacrylamide gels as described above. In our experience, 6sG- and 4sU-modified RNAs were more sensitive to preactivation by ambient light than those modified with APA, which again can be minimized by using dark amber tubes.

3. Generation of Crosslinked RNAs

3.1. General considerations of reaction conditions

The central difficulty in crosslinking studies is not the ability to generate crosslinked molecules, but rather the generation of crosslinks that reflect the native conformation or structure of interest. This difficulty stems from the strong tendency of RNA to adopt multiple conformations in solution as well as the sensitivity of RNA structure to mono- and divalent metal ion concentration and temperature (Brion and Westhof, 1997; Pyle and Green, 1995; Treiber and Williamson, 2001; Uhlenbeck, 1995). Non-native intra- or intermolecular interactions can be very stable, sometimes more stable than their biologically relevant counterparts, and can dominate a population of molecules even under optimal experimental conditions. And unfortunately, the efficient photocrosslinking agents so often necessary to generate sufficient crosslinking signal are sure to dutifully report the presence of both correctly folded and misfolded structures.

Ensuring that the presence of a mixed population of RNA structural isomers will not complicate the results is (or at least should be) built upon the often time consuming process of determining how to optimally fold the RNA prior to reaction. Such conditions are best derived from a detailed understanding of the influence of the individual parameters of the experimental system (e.g., mono- and divalent ion concentrations and identity, temperature, pH, and macromolecular concentration) on biological activity. In the course of establishing folding conditions, it is also useful to examine the effect of these parameters on the level of crosslinking observed in the reaction since gaining the highest efficiency possible is important for subsequent identification of crosslinked nucleotides and analysis of the retention of biological activity of the purified crosslinked species. The development of reaction conditions for crosslinking should thus be viewed as a reiterative process of testing and validation.

3.2. Crosslinking photoactivation

In contrast to the considerable experimental preparation note above, the crosslinking reaction itself is a quick and simple process. This procedure will be illustrated here using an example of pre-tRNA crosslinking to bacterial RNase P RNA. However, the conditions used are largely applicable as an outline for RNA crosslinking in general. Purified 5'-³²P-end-labeled pre-tRNA containing 4sU and unlabeled bacterial RNase P RNA transcripts are resuspended separately in reaction buffer, in this case (2 M ammonium acetate; 50 mM Tris-HCl, pH 8.0) for refolding. The RNA-containing solutions are heated to 90 °C for 1 min in a programmable heating block (MJ Research) and then cooled to room temperature using a standard water bath over a period of approximately 20 min. Divalent metal ions, in this example 25 mM CaCl₂, are then added and the RNAs incubated at 37 °C for 15–30 min to insure that as much of the RNA as possible has attained the native, folded form. Equal volumes of substrate and enzyme RNA are subsequently mixed and incubated for 2 min. In this instance Ca²⁺ is used to replace the optimal metal ion for the reaction, Mg²⁺, in order to slow the rate of catalysis and permit the assessment of the binding affinity of the substrate (Smith *et al.*, 1992).

Intermolecular crosslinking reactions (100–200 µl) are done under conditions of enzyme excess ([E]/[S] = 10, [E] ≥ *K*_d, often 100 nM photoagent-containing pre-tRNA and 1 µM RNase P ribozyme) in order to insure that the majority of the photoagent-modified substrate is bound to the ribozyme. For intermolecular interactions it is also important to demonstrate that the formation of crosslinks are dependent on the presence of the interacting RNA species (here the ribozyme) and occur in a concentration-dependent manner over a broad range of concentrations. The observation of the same crosslinked species at both high and

low concentrations of the ribozyme provides evidence that the crosslinks are intermolecular in nature and reflect the structure of high affinity complexes.

We found that optimal crosslinking in our experimental system occurred at 4 °C, and thus the following crosslinking apparatus was assembled on a standard laboratory ice bucket. The most efficient levels of crosslinking were observed when 10–20 µl aliquots were transferred to a parafilm-covered aluminum block and irradiated as separated droplets rather than a single pool of liquid. Aluminum blocks can be obtained from a standard dry-bath incubator and are precooled in ice for at least 1 h prior to the experiment. Parafilm and samples should be placed on the block just before irradiation to minimize dilution or contamination by condensation. The top of the block should be set 2–3 cm below the surface of the ice bucket. A standard ~3 mm thick glass plate is set on top of the bucket over the samples to help filter out shorter wavelengths (<300 nm) of UV light that can damage the RNA sample and produce photoagent-independent crosslinks. In our studies samples were irradiated for 5–15 min at 366 nm at a distance of 3 cm using a model UVP Model UVGL-58 ultraviolet lamp from UVP, Upland, CA. Note, however, that while 6sG is also activated by 366 nm light, the APA-derived crosslinking agents are activated at 302 nm (UVP Model UVM-57). Aliquots are recovered from the block, diluted to 200 µl with 10 mM Tris–HCl, pH 8.0, 0.5 mM EDTA, 0.3 M sodium acetate, then extracted twice with 50%/50% phenol/chloroform and once with chloroform alone and precipitated by addition of three volumes of ethanol.

We observed that significantly greater crosslinking can be achieved by increasing time or lamp intensity. Such efforts, however, must be balanced against damage to the RNA structure and the understanding that the increase in crosslinking yield can also vary significantly from position to position. The conditions above were also chosen in part to slow the rate of reaction in studies of substrate binding to offset the relatively long periods of time, kinetically speaking, required to obtain sufficient levels of crosslinked material. More powerful lamps (e.g., UV Products, B-100AP, 365 nm) generally only reduce the time required by several fold.

Progress in increasing efficiency without damaging the RNA has come from the recent use of nanosecond pulse laser technology to form RNA–RNA crosslinks in bacterial ribosomes (Shapkina *et al.*, 2004). Crosslinks obtained with one (22 ns, 248 nm) pulse showed about fourfold greater yield than that obtained with transilluminator irradiation. Importantly, such crosslinking could be achieved with relatively low levels of UV-induced strand breakage. In contrast to low intensity UV light which leads to the absorption of single photons by bases to induce formation of the S₁ and T₁ excited states (Cadet and Vigny, 1990; Daniels, 1976; Fisher and Johns, 1976), the higher energy of the pulse laser is thought to allow the absorption of two photons to form the higher activation states of H_T and H_S (Budowsky *et al.*, 1986; Cadet and Vigny, 1990). Importantly, bases in H_T and H_S states crosslink more efficiently and may have expanded specificity relative to the excited states produced with low intensity UV (Budowsky *et al.*, 1986). Indeed, new crosslinks could be observed in a system that has been analyzed for decades by UV crosslinking (Shapkina *et al.*, 2004). Pulse laser crosslinking has not as yet been characterized using the photoagents above. However, it is not difficult to imagine the experimental possibilities and potential impact of site-specific photoagents being activated by pulse laser in a rapid quench or stop flow apparatus to monitor changes in secondary and tertiary structure as a function of time. For a group of methods that has not seen much change in recent years, this seems fertile ground for future work.

3.3. Isolation of crosslinked products

Identification and isolation of crosslinked RNAs is accomplished by taking advantage of the significantly reduced mobility of the crosslinked RNAs relative to uncrosslinked RNA on

denaturing polyacrylamide gels. Cross-linked RNAs are thus purified on low percentage denaturing polyacryl-amide gels (4%, 19:1 acrylamide, bis) and recovered as described above. Gels should contain an adjacent lane in which the photoagent is omitted from the reaction to demonstrate that the formation of the more slowly migrating species depends on presence of the crosslinking reagent and not from adventitious crosslinking due to UV light. An additional lane should contain samples that are not irradiated since crosslinking can occur during sample workup that may not necessarily reflect the functional structure. Intermolecularly crosslinked RNAs such as that between RNase P RNA and its pre-tRNA substrate form Y-branched structures that usually migrate slower than lariat forms, and thus can be easily separated from a strong background level of intramolecular crosslinking. That said, intra- and intermolecular crosslinks formed independent of the photoagent should not necessarily be discarded as they may contain useful structural information.

Once a valid intra- or intermolecular crosslink has been identified, the next step is to isolate sufficient quantities of the individual crosslinked species for mapping. A general rule of thumb is that picomole amounts of material are optimal for primer extension mapping and functional studies used for validation. Thus it is necessary to scale the crosslinking reaction up accordingly. For primer extension analysis it is important that the radioactivity of the RNA crosslink be significantly less than the signal generated by primer extension sequencing. This can be achieved by the addition of a small quantity of radiolabeled RNA to the crosslinking reaction. In contrast, direct sequencing of the crosslinked RNA by alkaline hydrolysis (see below) is best done with RNAs with the highest specific activity possible. In this latter case, 5'-³²P-end-labeling can be done prior to the crosslinking reactions or to the purified crosslinked species itself. Note, however, that conditions required for the removal of the nonradioactive 5'-terminal phosphate prior to 5'-³²P-end-labeling can lead to nicks in the backbone of the RNA crosslink and reduce the quality of the sequencing data. Breaks introduced in the backbone of RNAs labeled prior to the crosslinking reaction, however, will generally not produce crosslinks that comigrate with their full-length counterparts and will be eliminated from the experimental background during purification.

Large-scale crosslinking reactions can be loaded into a continuous well across the entire top of a 35 × 45 cm × 0.4 mm thick sequencing gel and are typically run at 100 W and ~50 °C for sufficient time to separate adjacent crosslinked species by at least 1 cm to avoid cross-contamination when individual bands are physically excised from the gel. Electrophoresis conditions for preparative scale reactions often differ from those used for the initial identification of the crosslink species and may need to be reoptimized to achieve the best degree of separation between crosslinked bands or from uncrosslinked RNA. Crosslinked RNAs are purified from gels as described above with the exception that the scale in terms of both the volumes used for elution and subsequent extraction with phenol and chloroform are much larger. In practice, preparative scale phenol/chloroform extractions are more easily accomplished using 15 ml organic resistant polypropylene tubes (Falcon) and a standard clinical centrifuge. Following extraction we have found that addition of 0.01 µg/µl glycogen as a carrier greatly improves recovery of the relatively low concentration of crosslinked RNAs from larger volumes and does not interfere in subsequent primer extension mapping, alkaline hydrolysis sequencing, or monitoring the crosslinked species for biological activity. More detailed considerations for isolating large crosslinked RNAs (rRNA) has been reviewed by Wollenzien and coworkers (Juzumiene *et al.*, 2001).

4. Mapping of Crosslinked Nucleotides

4.1. Alkaline hydrolysis mapping

As noted above, sites of crosslinking are generally mapped by alkaline hydrolysis or primer extension mapping. In alkaline hydrolysis mapping 5'- or 3'-³²P-labeled crosslinked RNAs

are treated with base to randomly cleave the phosphate backbone at a level of approximately one site per molecule. Reactions typically involve treating crosslinked RNAs with 0.1 M NaOH in 8 M urea at 80 °C for 1–10 min and terminating the reaction by the addition of a molar equivalent of HCl or an excess of buffer at neutral pH. When these RNA fragments are separated on polyacrylamide gels a gap is revealed in the sequencing ladder. The sequencing ladder below the gap accurately reflects the length of RNA fragments from the ³²P-labeled 5'-or 3'-end up to the point of the crosslink. Labeled RNAs containing a break in the backbone distal to the site of crosslinking, however, will be significantly larger in molecular weight due to covalent attachment of added sequence, and accordingly will migrate much more slowly in the sequencing gel. The first band below the gap thus reflects the break in the RNA backbone immediately 5'to the site of crosslinking. The position of the crosslink can be identified by simply counting the number of bands from the labeled end of the molecule or by running the alkaline ladder adjacent to lanes of standard dideoxy sequencing. This approach is simple, quick and generally very accurate (Sergiev *et al.*, 2001; Whirl-Carrillo *et al.*, 2002). The main disadvantage is that in larger RNAs the site of crosslinking can be distant from the labeled end of the molecule (>150–200 nts) and thus more difficult to resolve on standard sequencing gels. Alternative, more complex, yet very accurate methods, however, have been developed in the mapping of crosslinks in ribosomal RNA of using RNase H and RNA fingerprinting (Mitchell *et al.*, 1990).

4.2. Primer extension mapping

In primer extension sequencing 5'-³²P-labeled primers are annealed to different positions within the crosslinked RNA and extended with reverse transcriptase. Because reverse transcriptase is unable to extend through the site of crosslinking, the last nucleotide incorporated by reverse transcriptase is interpreted as being immediately upstream of the site the crosslink itself. The site of crosslinking is thus derived from the terminal position of the longest primer extension product when compared alongside a standard sequencing reaction of unmodified RNA on polyacrylamide gels. The advantage of this approach is the ability to place primers throughout an RNA structure to optimize resolution of the position of blocked primer extension products. The disadvantage of this approach is that normal RNA structural features can block primer extension to yield misleading results. Indeed, the comparison of crosslinking methods with high-resolution structures has led to the observation that primer extension is the least reliable of the current mapping methods despite its widespread use (Sergiev *et al.*, 2001; Whirl-Carrillo *et al.*, 2002). That said, the majority of interactions or constraints predicted by primer extension are likely to be correct and thus effort should not be placed in avoiding this very useful approach but rather in optimizing reaction conditions and careful interpretation of the results. Indeed, the resolution of an individual distance constraint is often more dependent on how the crosslink is mapped than the photoagent itself (Sergiev *et al.*, 2001; Whirl-Carrillo *et al.*, 2002).

Conditions that have yielded consistently low background levels in primer extension sequencing in our experiments are as follows. Typically, 0.2 pmol of 5'-³²P end-labeled sequencing primer is annealed to 0.05–0.2 pmol of crosslinked material in a total volume of 5 μ l at 65 °C for 3 min in 50 mM Tris–HCl, pH 8.3, 15 mM NaCl, and 10 mM DTT and set immediately on dry ice. Samples are then thawed on ice and MgCl₂ (1 μ l) is added to a final concentration of 6 mM, followed by the addition of each of the four deoxynucleotides (dATP, dCTP, dGTP, dTTP) to a final concentration of 400 nM. Reactions (8 μ l) are initiated by the addition of 2 units (2 μ l) of AMV reverse transcriptase (Boehringer Mannheim) and then incubated at 47 °C for 5 min. Reactions are then quenched by the addition of an equal volume of 0.5 M NaCl, 20 mM EDTA, and 5 μ g of glycogen (Boehringer Mannheim) and precipitated in 2.5 volumes of ethanol. Primer extension reactions are then resuspended in 2 μ l dH₂O and denatured in the presence of an equal

volume of gel loading buffer (95% formamide, 150 mM Tris-HCl, pH 8.0, 15 mM EDTA, 1 mg/ml each bromophenol blue, xylene cyanol FF) for 3 min at 95 °C. Samples are allowed to cool on ice before loading (2 μ l) onto a 6% (19:1) polyacrylamide gel adjacent to standard dideoxy sequencing reaction of uncrosslinked RNA.

5. Assessing the Validity of Crosslinking Data

Determining the structural relevance of a crosslink should include consideration of the following criteria. First, the number and distribution of observed crosslinks should be consistent with that normally observed with a given crosslinking agent. Crosslinking with long-range structure probes such as APA usually involves several adjacent nucleotides in 1–4 distinct regions of the target RNA while the number of nucleotides and crosslinked regions of RNA is significantly reduced in short-range structural probes such as 6sG and 4sU. The general suspicion of a structurally heterogeneous population of RNA should thus be raised when the number or distribution of observed crosslinks exceeds the general guidelines noted above. This should be initially be addressed by a re-examination of renatur-ing conditions prior to the crosslinking reaction, followed changes in the placement of the crosslinking reagent itself.

Second, the efficiency of crosslinking provides correlative rather than direct evidence for structural proximity or conformational stability. The strong geometrical and chemical requirements for bond formation dictate that the relative proximity of two crosslinked sites is not strictly linked to the level of crosslinking that is actually observed. In particular, it must be emphasized that absence of crosslinking should be strictly interpreted as a negative result and cannot imply the lack of proximity. Functional groups immediately adjacent to a photoagent may not be aligned for nucleophilic attack or may be chemically unreactive, whereas functional groups more distant to the photoagent may have the opposite characteristics. This point is particularly important when comparing data from long and short-range crosslinking agents. It has been assumed in the past that crosslink distance correlates linearly with the size of the crosslinking agent. However, this correlation was not observed when long and short-range crosslinking studies were compared in the context of established crystallographic structures (Sergiev *et al.*, 2001; Whirl-Carrillo *et al.*, 2002). While the absence of correlation may be partially due to experimental error (e.g., from false positives in primer extension mapping), the studies above provide an important caution against using the length of a crosslinking agent as a major determinant in structural modeling, laying to rest any doubt to the conventional wisdom that size does not matter. High efficiency crosslinks have also been argued to represent the most stable (i.e., native) structure in the population. Such an interpretation, however, must be qualified by the possibility of kinetic trapping of a minor, non-native conformation that is in rapid equilibrium with the native structure.

Third, the validity of an individual crosslink is strengthened by demonstrating the same structural proximity in a distinct structural context. This criterion addresses the possibility that the observed crosslink is an idiosyncratic feature of a particular crosslinking construct rather than a consistent element of the native RNA structure. The most direct approach to addressing this concern is to determine whether the same nucleotides or regions of RNA structure become crosslinked regardless of which of the nucleotides or regions of RNA in question contains the crosslinking agent (Chen *et al.*, 1998; Harris *et al.*, 1997). The demonstration of reciprocal crosslinks from different photoagents or under different experimental conditions provides further support that the observed results are not due to the perturbation of the native structure. Generality of the crosslinking results can also be established by reproducing the crosslink in a homologous RNA (Chen *et al.*, 1998; Christian *et al.*, 1998; Harris *et al.*, 1994, 1997; Noah *et al.*, 2000). Preferably, the RNAs being

compared should differ somewhat with respect to their primary sequence and secondary structure while retaining similar properties of three-dimensional folding and biological function. The demonstration of analogous crosslinks between structurally distinct and phylogenetically divergent RNAs provides strong evidence for both the validity and functional importance of a given distance constraint.

Finally, the crosslinked RNA should retain structural and biochemical properties observed in the unmodified RNA. Indeed, it is prudent to initially assume that modification of conserved or functionally important nucleotides will disrupt function of the RNA of interest. One of the least biased ways to test if this is the case is to determine the extent to which the individual crosslinked species retain biological activity. Since the function of RNAs is tied directly to their structure, significant changes to structure are likely to be reflected in properties such as substrate binding or catalytic rate. Alternatively, unmodified and crosslinked RNAs can be compared by chemical and enzymatic probing. Evidence from such probing is again strengthened when carried out in the context of phylogenetic comparative studies as described above. Ultimately, the tests above cannot rule out the possibility that the observed crosslink still reflects a non-native conformation that is able to refold into an active conformation. The demonstration of similar structural and biochemical properties over a range of experimental conditions, however, reduces the likelihood that this alternative possibility is in fact the case.

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