

Mapping protein–RNA interactions

Robert Vaughan
William Running
Rongsu Qi
C Cheng Kao

The Biochemistry Interdisciplinary
Program, Indiana University,
Bloomington, IN, USA

Abstract: There is a significant need to develop approaches for rapid and accurate mapping of protein–ribonucleic acid (RNA) interactions, especially to complement structure-based methods. Approaches using mass spectrometry to map regions in proteins that contact RNA have now been established. These include a reversible crosslinking affinity purification method, residue-specific modification interference assay, and photoactivatable crosslinking and mass spectrometry. Novel methods to identify nucleotides within RNA that contact proteins using photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation are also available. In combination, these methods should generate results that will lead to more specific hypotheses concerning the biological properties of protein–RNA interactions. This review summarizes some recent advances in select assays useful for mapping protein–RNA interactions.

Keywords: hepatitis C virus, positive-strand RNA virus, reversible crosslinking, RNA binding, mass spectrometry

Introduction

Protein–ribonucleic acid (RNA) interactions are central to the infection process of numerous viruses, including those that cause multiple forms of hepatitis, encephalitis, and respiratory disease. Analysis of protein–RNA interactions will also improve our understanding of basic molecular processes, such as RNA encapsidation, regulation of gene expression, and viral evasion of innate immune responses. The information gained from analyses of protein–RNA interactions should inform treatment strategies, since altering such interactions would be detrimental to the pathogen.

Advances in the structural characterizations of protein–RNA interactions have accelerated in the past decade, with structure elucidation of the ribosome and portions of the spliceosome as major accomplishments.^{1–3} However, atomic resolution of protein–RNA complexes remains challenging. An illustration of this is that only short fragments of the encapsidated RNA within viral particles have been resolved by X-ray crystallography.^{4,5} There is also a significant underrepresentation of protein–RNA complexes in the Protein Data Bank; of the approximately 79,000 entries in the Protein Data Bank (as of February 2012), only 1200 are in complex with RNA (1.5%). Approximately 10% of *Saccharomyces cerevisiae* genes have been annotated to be RNA-binding proteins, but experimental examination showed that this number is an underestimation.^{6,7} A similar situation likely exists for the human proteome. Therefore, as more RNA-binding proteins are identified, there will be a need for rapid and sensitive approaches to map protein–RNA interactions. For the proteins with known structures,

Correspondence: C Cheng Kao
The Biochemistry Interdisciplinary
Program, Indiana University,
Bloomington, IN 47405, USA
Tel +1 812 855 7583
Fax +1 812 856 5710
Email ckao@indiana.edu

the mapping results could be analyzed to inform hypotheses about how the interactions affect conformational changes and the function of the proteins.

This review summarizes several approaches to mapping protein–RNA interactions using crosslinking and mass spectrometry (MS). These approaches are highly adaptable and can be used in combination with bioinformatic analyses and molecular modeling. Approaches such as reversible crosslinking affinity purification (RCAP) and modification interference have been successfully applied to purified virions, innate immune receptors, and several hepatitis C virus (HCV) nonstructural proteins to better understand how these proteins interact with RNA.^{8–12} Recent advances have also been made using photoactivatable crosslinking and DNA sequencing to identify the positions within RNAs that contact proteins.^{13–15} The goal of this review is to offer a general guide to the use of these approaches for probing protein–RNA interactions.

Mapping protein contact with RNA RCAP assay

Reversible crosslinking has been widely used to study protein–nucleic acid interactions, especially *in vivo* using chromatin immunoprecipitation.^{16,17} Chromatin immunoprecipitation is a way to determine whether specific proteins interact with certain genomic regions within a cell and it has been instructive for protein–RNA interactions. In chromatin immunoprecipitation, proteins associated with the chromatin are covalently attached by formaldehyde crosslinking. Then the DNA is sheared by sonication or micrococcal nucleases and selectively immunoprecipitated using an antibody specific to the protein of interest. Then, the covalent linkage is reversed and the associated DNA is identified by polymerase chain reaction, sequencing, or microarray analysis.¹⁸ While this data provides valuable information on the nucleic acids that contact a specific protein, it also has the potential to be coupled with highly sensitive MS to better define the regions of the proteins that contact nucleic acids.

The RCAP method (ie, reversible crosslinking, affinity purification, and peptide fingerprinting) was originally developed as an extension of chromatin immunoprecipitation to be used *in vitro* to map the regions within a protein that bind RNA *in vitro*,¹² but has since been extended to map regions that bind DNA and phosphorothioate-containing DNA.⁹ Specifically, it is used to selectively pull down peptides within an RNA-binding protein that interact directly with RNA. A general schema for the RCAP assay is depicted in Figure 1. Briefly, a protein is first crosslinked to RNA with

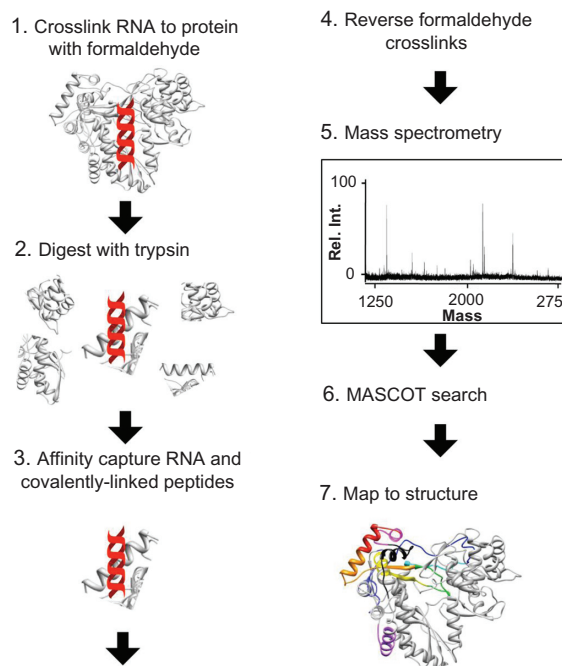


Figure 1 Overview of the reversible crosslinking affinity purification protocol.

Notes: Outline of seven steps in the general strategy used to map peptides within proteins that interact with ribonucleic acid. Nonstructural protein-5B (Protein Data Bank: 1QUV) is used to demonstrate the procedure schematically.

Abbreviation: RNA, ribonucleic acid.

formaldehyde and then digested with a protease (especially trypsin). Next, RNA–peptide conjugates are affinity purified and extensively washed to remove any uncrosslinked peptides. Lastly, RNA–peptide crosslinks are reversed and analyzed by MS. A matrix-assisted laser desorption/ionization time-of-flight MS has typically been used for all of these studies due to ease, availability, and sensitivity; however, any MS capable of analyzing peptides would be sufficient. Confirmation of the peptide assignment is performed by tandem MS (MS/MS), usually by means of collision-induced dissociation. Mascot or other search engines have been used to aid in identifying peptides within the spectra, or theoretical digests can be performed using engines such as ProteinProspector to make peptide assignments.^{19,20}

Nonstructural protein-5B (NS5B), the RNA-dependent RNA polymerase of HCV, was used to provide proof of concept for the RCAP assay. NS5B is an important drug target for HCV and the focus of extensive mechanistic and structural studies.^{21–24} Despite having 88 crystal structures present in the Protein Data Bank, only one is in complex with a short rU5 RNA.^{22,25,26} While this crystal structure provides valuable insight into how NS5B binds template RNA, additional structural interactions with RNA – including the RNA synthesized from the template RNA – have not been determined.^{12,27}

The RCAP assay identified several RNA-binding peptides that mapped to the template channel of NS5B (Figure 2A).¹² Recombinant NS5B mutants with one or more amino acid substitution in the predicted RNA contact sites were all found to have reduced RNA synthesis *in vitro*, at least 50% less when compared to the wild-type protein.¹² In addition, the mutants were also affected for the replication of subgenomic HCV replicons in human hepatocytes.¹²

The RCAP assay has also been used to characterize protein–RNA contacts between the HCV nonstructural protein-5A (NS5A) and poly-U RNA.⁸ NS5A is especially interesting since it is an RNA-binding protein that possesses no known enzymatic activity. NS5A contains three domains, with only domain I being able to form a sufficiently ordered structure to produce crystals. The remaining two domains of NS5A contain regions that are intrinsically disordered.

As is the case of other proteins with intrinsic disorder, NS5A interacts with a large number of both HCV and cellular protein partners,^{28–31} and at least one consequence of their interaction is the modulation of host innate immune responses.^{8,32} Using the RCAP assay, NS5A was shown to directly interact with RNA in domains I and II (Figure 2B), which is in good agreement with RNA pull-downs using NS5A deletion mutants.⁸ Since the structure of domain I has been solved, the peptides that were found to contact RNA could be superimposed upon the electrostatic charges of domain I, and an excellent correlation was observed between the two properties (Figure 2B).³³ It was noted that nine of the 14 peptides purified by the RCAP assay mapped directly within the disordered regions in domain II.^{8,34} The RCAP mapping approach could be used to help elucidate ligand interaction with proteins that contain intrinsically disordered regions.

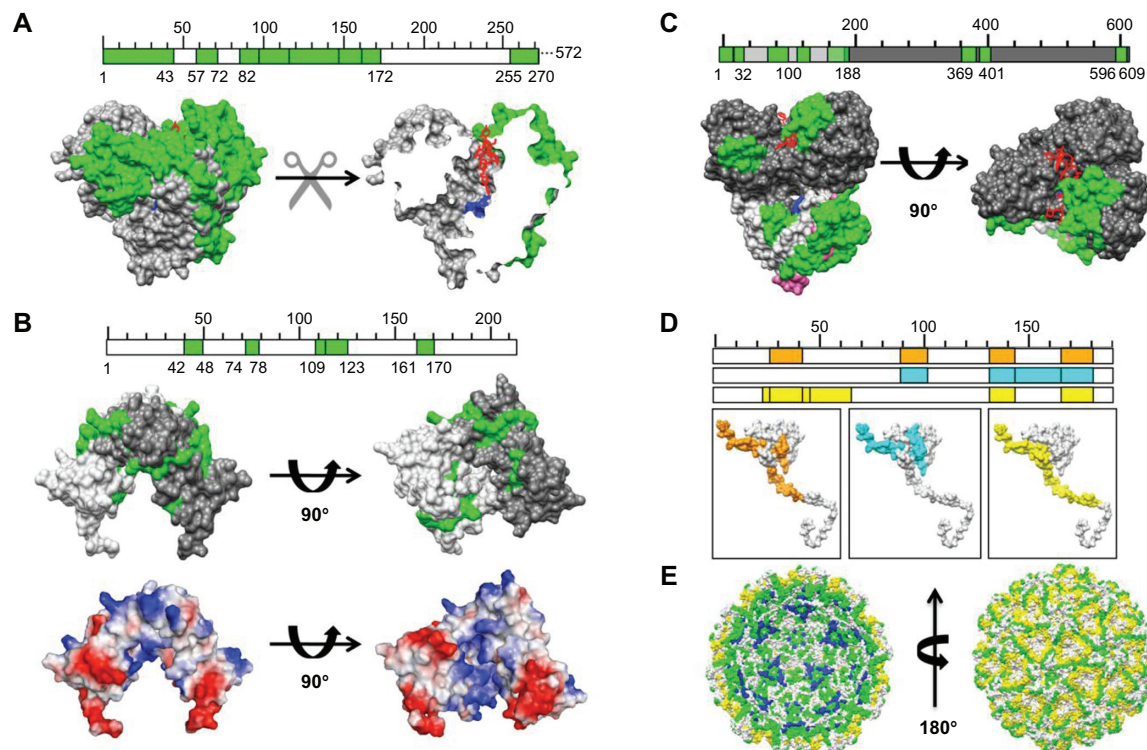


Figure 2 Reversible crosslinking affinity purification results within several different systems. **(A)** Peptides from nonstructural protein-5B found to interact with ribonucleic acid by the reversible crosslinking affinity purification assay.¹² The schematic represents the first 270 residues of the nonstructural protein-5B polypeptide, where ribonucleic acid-binding peptides were identified in green. The space-filled model shows the locations of the peptides in the structure of the nonstructural protein-5B protein (Protein Data Bank: 1NB7). The active site residues are blue, and the location of ribonucleic acids within the template channel is colored red. The right panel in the space-filled model has part of the polymerase removed to better illustrate the template channel. **(B)** Upper: peptides from domain I of nonstructural protein-5A found to interact with poly-U ribonucleic acid.⁸ Lower: electrostatic potential of nonstructural protein-5A (Protein Data Bank: 1ZHI). **(C)** Peptides within nonstructural protein-3 found to interact with ribonucleic acid (Protein Data Bank: 308R). The helicase domain is colored grey, the protease domain white. Peptides purified with the reversible crosslinking affinity purification assay are green, the active site blue, the bound ribonucleic acid red, and the 4A peptide cofactor pink. **(D)** Peptides found within the bromo mosaic virus coat protein that interact with ribonucleic acid (Protein Data Bank: 1JS9). The linear polypeptides show regions of the bromo mosaic virus capsid protein that contact ribonucleic acids. The space-filled model of a bromo mosaic capsid monomer is shown in the rectangles. In both the schematics and the space-filled models, orange identified locations of peptides that interact with a ribonucleic acid named SL4 that was previously shown to direct ribonucleic acid-dependent ribonucleic acid synthesis.¹¹ Blue surfaces are those that interact with a ribonucleic acid motif that regulates translation (middle box), and yellow are those found to interact within the virion.¹¹ **(E)** Changes in ribonucleic acid contacts within the bromo mosaic virus virion. Peptides from wild-type bromo mosaic virus that are coprecipitated with the virion ribonucleic acids are colored in blue. The peptides that precipitated with the virion ribonucleic acid from the R142A mutant, a ribonucleic acid-binding mutant, are colored yellow. Overlapping peptides are colored in green.

Note: Oligomer structures of the bromo mosaic virus capsid protein (Protein Data Bank: 1JS9) were generated using VIPERdb oligomer generator.⁹³

RNA binding by nonstructural protein-3 (NS3), a bifunctional protease and helicase, was also characterized using RCAP. The presence of the protease domain has been shown to affect adenosine triphosphatase and helicase activities within the helicase domain; however, direct binding between the protease domain and RNA has not been observed,^{35–37} but in vitro selection has identified RNA aptamers that can bind the protease.³⁸ Using the RCAP approach, RNA was shown to bind directly to the protease domain of NS3 (Figure 2C, left). The location of the peptides purified from the helicase domain are in good agreement with the crystal structure available for the NS3 helicase domain in complex with a short DNA,³⁹ as well as the structure of full length NS3 in complex with a short poly-U (Figure 2C, right).⁴⁰ RNA contacts within the protease domain were confirmed both by complementary MS-based approaches as well as functional effects on protease activity (data not shown).

The RCAP assay could also be used for higher order molecular assemblies.¹¹ Brome mosaic virus is a positive-sense RNA virus that encapsidates its RNAs in a T = 3 icosahedral particle. The RCAP assay showed that the brome mosaic virus capsid interacted with RNA motifs differently when dissociated capsid subunits were bound to the regulatory RNA motifs and to the intact viral particles (Figure 2D and E). Using the RNA contact sites identified in the peptides, mutations in the capsid protein were found to have distinct effects on brome mosaic virus RNA translation and encapsidation.¹¹

Analysis of the interaction of the above proteins and RNA strongly suggests that the RCAP assay could be generally used for the biochemical study of protein–RNA interactions.

Note, however, that the protocol could be manipulated so that the specific ribonuclear complex can be examined. The sections below provide guidance to the processes used.

Crosslinking agents

Crosslinking reagents have been used to stabilize protein–protein and protein–nucleic acid interactions for both structural studies as well as to demonstrate an interaction between potential binding partners.⁴¹ In the RCAP assay, covalent attachment of peptides to RNA is a necessary step to allow for stringent washes of the RNA to completely remove any unbound peptides. This is important since contamination by unbound peptides could significantly impact interpretation of the MS results. Formaldehyde is preferred as the crosslinking agent for the properties described below.

Formaldehyde is used in the RCAP assay because of its reversibility and the short distance between crosslinks.^{17,42} Due to the size of formaldehyde, crosslinks are only formed between groups that are within 2 Å of each other. It reacts with the exocyclic amines of nucleic acid bases (adenine, guanine, and cytosine) and the side chains of lysine, cysteine, trypsin, histidine, with the peptide amino termini being the most reactive in peptide deoxynucleoside or trinucleotide crosslinks.^{42,43} Furthermore, residues that are exposed on the surface are more reactive than those buried, as demonstrated by an increase in the extent of protein modification in the presence of denaturants such as urea and dithiothreitol.^{16,17} Formaldehyde induces the formation of a methylene bridge via dehydration and the formation of a Schiff base (Figure 3A and B).⁴² At a final concentration of 0.1%, a sufficient amount of crosslinking occurs within 10 minutes, which is then

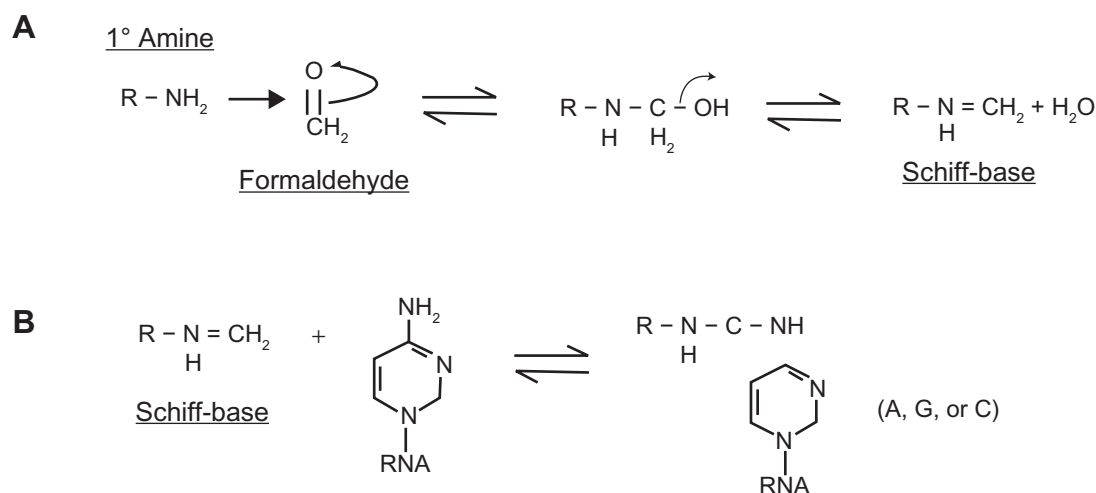


Figure 3 Chemical mechanism of formaldehyde crosslinking. **(A)** Formaldehyde first reacts with a primary amine to form methylol adducts, which rapidly dehydrate to form a Schiff base. **(B)** The resulting labile Schiff base can form crosslinks with several amino acids or ribonucleic acid bases as shown.

Abbreviations: A, adenosine; G, guanine; C, cytosine; RNA, ribonucleic acid.

followed by addition of 200 mM glycine to quench further reactions. Notably, buffers that contain primary amines, such as tris(hydroxymethyl)aminomethane, need to be avoided since they could be directly involved in the coupling reaction and thus reduce crosslinking efficiencies.⁴⁴ The pH of the reaction can be tailored to an extent to suit a specific protein, but acidic buffers (below pH 6.0) should be avoided as they inhibit the formation of a Schiff base. Under these conditions, formaldehyde crosslinking to DNA is 50–150 times more efficient than ultraviolet crosslinking.^{44,45}

Since formaldehyde preferentially targets lysine, this is an advantage in studies of protein–RNA interactions. Lysines often interact with the negative charge on the phosphate backbone in RNA. Furthermore, lysines have the greatest propensity to be present at the surface interface of a protein that binds RNA.⁴⁶ While conjugation of lysine to RNA will block cleavage by trypsin, the lack of a cleavage at a particular lysine can provide additional confidence to the assignment of RNA contact sites in the protein. If necessary, proteases with other cleavage specificities can be used.

Formaldehyde crosslinks can be reversed by either heating the samples in an aqueous buffer containing high salt, a low pH buffer, or both.¹⁷ Reversibility of the crosslink can simplify data acquisition and allow higher quality analysis of the peptides in positive-mode MS due to the removal of the highly negatively-charged RNAs. Chemical or photoreactive crosslinks that are difficult to reverse can affect the identification of peptides.⁴¹ In addition, reversible crosslinkers such as dithiobis(succinimidyl propionate) are less convenient for rapid mapping using MS due to their large masses.^{41,47}

Note that the efficiency of reversing formaldehyde crosslinks needs to be balanced with undesired modifications of the peptides. In general, slightly acidic buffers (~pH 5.5) and heating times of no longer than 1 hour in 200 mM sodium chloride yield favorable results. Salt must be removed prior to MS analysis as the presence of monovalent ions can interfere with the ionization of the sample. This can be combined with a concentration step using a c18 ZipTip® (Millipore Corporation, Billerica, MA) and eluting of the samples in 80% acetonitrile/0.1% trifluoroacetic acid.

Affinity capture of RNA

Affinity capture of RNA is a critical step in the RCAP assay. The original study characterizing template binding of NS5B used a biotin-containing RNA that allows for the purification of RNA by streptavidin agarose (New England Biolabs, Beverly, MA).¹² The high affinity interaction between biotin and streptavidin is useful,⁴⁸ but streptavidin exists as a

homotetramer and can contribute to contaminating peaks.¹² Therefore, alternative conjugation chemistries to eliminate the use of other proteins in the affinity capture reaction are desirable.

One strategy is to chemically synthesize the RNA with an amine and a carbon spacer at a terminus of the RNA. The RNA could then be conjugated to N-hydroxysuccinimide (NHS) Sepharose® (Sigma-Aldrich Corporation, St Louis, MO) (Figure 4A). The exocyclic amines already present within RNA bases are not accessible to the bulky NHS group. The total reaction efficiency of NHS is between 60%–80% over a 30-minute time period;^{49–51} and using this conjugation chemistry, RCAP spectra were found to contain less contaminating peaks.^{9,10} Conjugation takes place efficiently in conditions of 100 mM sodium borohydride and pH 8.5 for 5 hours, followed by a 1-hour quenching reaction with 200 mM glycine. The resin should be washed to remove undesired materials and quantified for RNA coupling by ultraviolet spectroscopy prior to incubation with the desired protein(s). Should the RNA have a stable secondary structure, it may be desirable to heat the RNA resin to 100°C for 3 minutes in the protein-binding buffer and rapidly cool it on ice prior to its use in the RCAP assay.

Conjugation of the RNA to resin is unnecessary if the RNAs are of a length that can be selectively precipitated. Lithium chloride is particularly useful due to its high specificity for precipitating RNA, but not DNA, protein, or carbohydrates.^{52,53} Precipitation with 3 M lithium chloride was used to map the peptides that are in contact with encapsidated RNA (approximately 900–3000 base pairs) from the brome mosaic virus capsid.⁵⁴ The pellet can be gently washed with 70% ethanol to remove undesired salts. Robust signals were observed in the presence of formaldehyde and were absent in control experiments.¹¹ In the authors' experience, selective precipitation of RNA with lithium chloride can be used for any protein–RNA complexes where the RNA is longer than 60 nucleotides. This method obviates any manipulation of the RNAs and should be widely applicable.

Many RNA-binding proteins can interact with two or more RNAs. Examples of this include viral RNA-dependent RNA polymerases that will interact with both the template and nascent RNAs,⁵⁵ and RNA helicases that will interact with substrate or product RNAs. Therefore, the selective capture of one or more RNA will be needed to better characterize the enzymatic reactions. This could be achieved by the selective incorporation of an affinity tag to only one of the RNAs. For example, it should be possible to incorporate alkyne-labeled nucleotides or oligonucleotide primers into

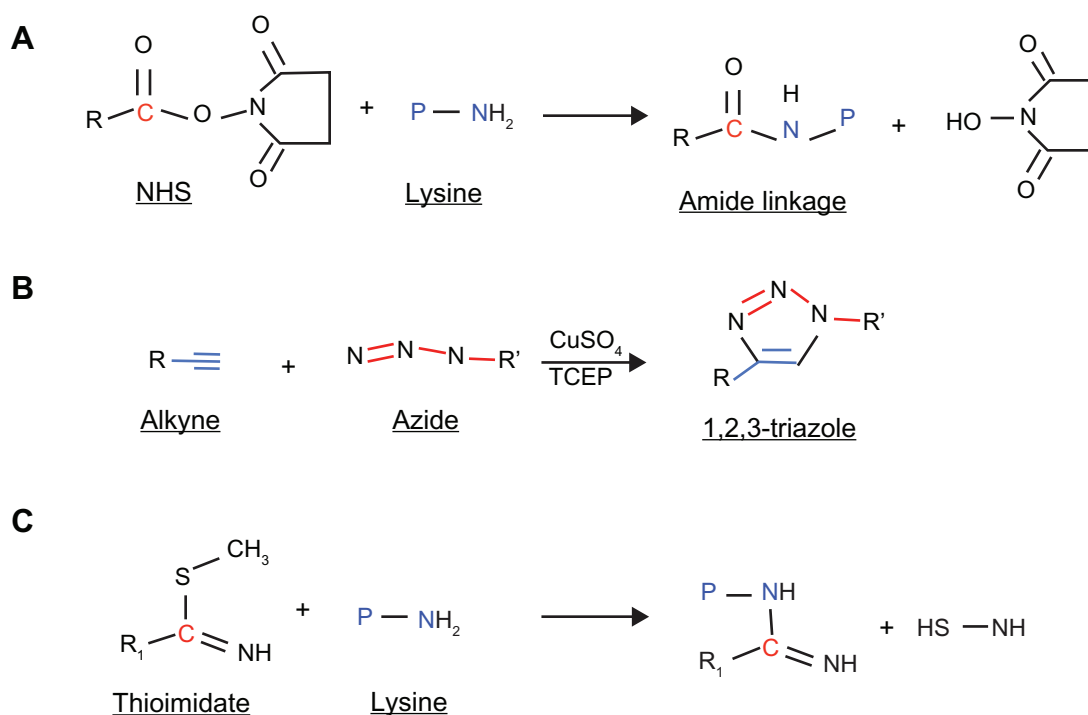


Figure 4 Schematics of conjugation chemistries reviewed. **(A)** N-hydroxysuccinimide reacts with primary amines in slightly alkaline conditions to form stable amide bonds. The lysine is shown as P–NH₂ to denote that it is present in a peptide. **(B)** Azides and alkynes can form stable covalently attached complexes via cycloaddition in the presence of copper(I), which is reduced from copper(II) by tris(2-carboxyethyl)phosphine. The bonds from the alkyne and the azide that contribute to the 1,2,3-triazole formation are, respectively, in blue and red. **(C)** Modification chemistry of protein or peptide amino groups by the thioimide reagents as described in the text.

Notes: For S-methylthioacetimidate, R₁ is CH₃– and results in a 41.05 Da mass increase per amino group modified. For S-methylthiopropionate, R₁ is CH₃CH₂– and results in a 55.08 Da mass increase.

Abbreviations: NHS, N-hydroxysuccinimide; TCEP, tris(2-carboxyethyl)phosphine.

the nascent RNA of viral RNA-dependent RNA polymerases and then to selectively capture the nascent RNA using cycloaddition (click chemistry) (Figure 4B).^{56,57} This reaction occurs both rapidly and with high specificity between azide and alkyne groups in the presence of the catalyst, copper(I), which is made by addition of copper(II) sulfate and the reducing agent, tris(2-carboxyethyl)phosphine, to the reaction mixture.⁵⁷ Furthermore, click chemistry adds the possibility of performing such analyses *in vitro*, since such moieties are not present in biological samples.⁵⁷ Azide-containing resin can be purchased commercially or synthesized easily using NHS-agarose and azide-poly(ethylene glycol)-4-amine (Click Chemistry Tools Inc, Macon, GA). The selective coupling of the RNAs by RCAP can be used to address issues about the mechanism of action of various RNA enzymes.

Modification interference assay

While the RCAP assay relies on capturing the peptides that contact RNAs, complementary approaches have been established to determine whether RNAs can prevent the specific modifications of residues in a protein. Assays based on interference of protein modification have been successfully

used to map the interaction in ribosomal proteins and between the HCV NS5B protein and the template RNA.^{58–60} Since this approach cannot distinguish between ligand interactions and a conformational change in the protein caused by ligand binding, it is recommended for use as a complement to the RCAP assay. In addition, modifications need to be monitored throughout the entire protein and necessitate more sophisticated MS, sometimes with the use of more than one protease, to achieve as close to complete sequence coverage as possible.

Chemical modification of amino acid residues has a long history in biochemistry, and a broad palette of reagents is available.⁶¹ The use of MS and liquid chromatography MS (LC-MS) methods to localize reactive sites and quantify extents of modification has spurred reinvestigation of this field.^{61,62} Because of their preferential involvement in the interfacial surface of protein–nucleic acid interactions, targeting positively-charged residues is an ideal strategy for mapping protein–RNA interactions.⁶³ Lysine, arginine, and histidine residues can be modified with reagents, but the tendency of some reagents to alter both the size and charge of these residues must be taken into consideration.⁶²

Arginine-specific modification with phenylglyoxal or its derivatives (eg, hydroxyphenylglyoxal) will alter both the size and charge of the residue, potentially affecting protein structure.⁶⁴ Additionally, arginine residue reactivity with phenylglyoxylate is a complex function of their surface exposure and local environment in a protein's tertiary structure.⁶⁵ Specifically, in an MS-based study of chemical labeling of basic residues in hen's egg lysozyme, an inverse correlation between arginine surface accessibility and phenylglyoxal reactivity was observed. The most reactive residues, R5 and R125, had the lowest surface accessibility and were in close proximity to hydrogen bond-accepting groups that could function as intramolecular catalysts for phenylglyoxal derivative formation.⁶⁵ Modification of lysine residues with acid anhydrides either neutralizes (acetic anhydride) or reverses (succinic anhydride) the side chain charge and can eliminate hydrogen bond donors.⁶⁶ While such reagents have been successfully used to map solvent accessibility, these changes in charge and size can affect protein structure and could potentially lead to incorrect conclusions.

Reacting lysine residues with imidoesters converts primary amines to amidines, adding a less bulky mass

tag without altering the charge (Figure 4C).^{67,68} Primary amines on lysines and the N-terminus specifically react with S-methylthioacetimidate, causing a 41 Da mass increase while retaining the native positive charge.⁶⁷ A general schematic for this assay is depicted in Figure 5. Amidination interference has been used previously as a mass tag to differentially modify surface-exposed lysine side chains of several standard proteins such as hemoglobin, ubiquitin, and ribosomal proteins.^{67,69} Preservation of native protein structure after amidination has been demonstrated in monomeric proteins by the use of circular dichroism and spectrophotometric measurement of apparent melting temperature values,^{70,71} and by the close agreement between predicted and observed extents of modification in bacterial ribosomal proteins.^{59,69} Solvent accessibility is monitored both by a mass shift in the peptide as well as by a missed trypsin cleavage site. Lysine amidination with thioimides can be performed between pH 6.0–9.0.⁷² Under typical reaction conditions, equal volumes of a solution of the protein–RNA complex of interest and a freshly made 200 mM solution of S-methylthioacetimidate in 250 mM tris(hydroxymethyl)aminomethane are mixed and allowed to react for 1 hour. Thioimides are prepared

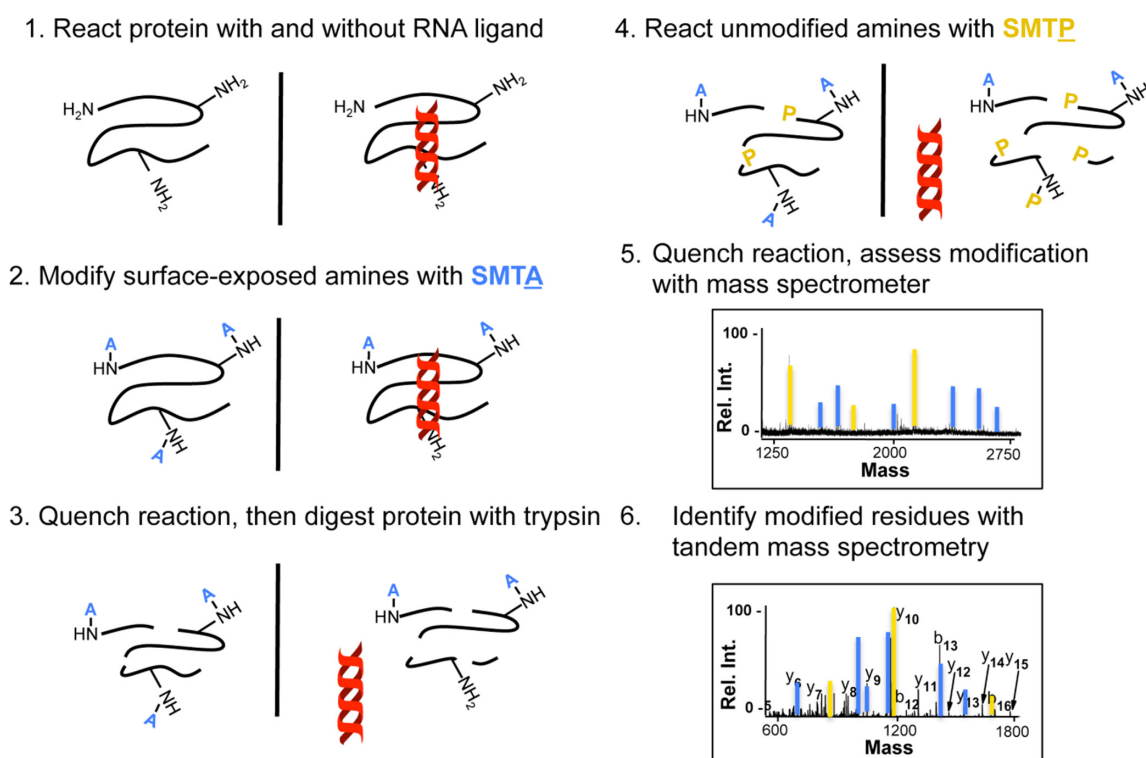


Figure 5 Amidination interference with thioimides.

Notes: Outline of the general strategy used to map ribonucleic acid binding by modification interference and mass spectrometry. S-methylthioacetimidate modification results in a 41.05 Da mass increase and is depicted in blue, while S-methylthiopropionate modification results in a 55.08 Da mass increase and is depicted in gold. Ligand binding inhibits S-methylthioacetimidate modification, while S-methylthiopropionate is used to react with any unmodified primary amines to normalize ionization efficiencies.

Abbreviations: RNA, ribonucleic acid; SMTA, S-methylthioacetimidate; SMTP, S-methylthiopropionate.

as hydroiodide or hydrochloride salts and require higher buffer concentration (250–500 mM) in reagent preparation.⁶⁷ Buffers other than the free base form of tris(hydroxymethyl) aminomethane can be used to obtain other pH values. The modification reaction can be quenched by the addition of acid, which also helps to hasten the hydrolytic decomposition of S-methylthioacetimidate. However, to prevent denaturation of the target protein, glutamate should be considered for use as a quenching reagent. Trypsin digestion can be performed after exchanging the buffer to contain 100 mM ammonium bicarbonate prior to MS analysis.

For improved quantification of the extent of S-methylthioacetimidate modification, it may be useful to react the digested peptides with S-methylthiopropionate to react with the previously unreacted lysines and the N-terminal amines. S-methylthiopropionate has the same reactivity as S-methylthioacetimidate but increases a protein or peptide's mass by 55 Da per modification. The 14 Da difference between the two mass tags insures that all peptides in a sample have the same ionization efficiency, allowing the extent of protection of a lysine to be quantified.⁷³ The production of S-methylthioacetimidate and S-methylthiopropionate by chemical synthesis is described in Lauber and Reily.⁶⁰

Identify crosslink(s) within a peptide

More sophisticated MS techniques can be used to confirm the identities of crosslinked peptides, the nucleotides they are conjugated to, and the site of attachment within the protein sequence. To date, most studies of crosslinked protein–polynucleotide species have used either the natural photoreactivity of nucleotide bases or photoreactive base analogs, such as 5-bromouracil, 4-thiouracil, or 8-azidopurines, to generate photocrosslinked peptide–polynucleotide conjugates.^{74,75} While these crosslinks are not easily reversible, the chemical properties of photocrosslinked species that have been analyzed in depth should be shared by RCAP-generated protein–RNA conjugates.

After crosslink formation, the protein component of the conjugate is digested with a protease. Photocrosslinked peptide–nucleotide conjugates can be selectively purified away from uncrosslinked peptides by taking advantage of the presence of the RNA component's phosphodiester backbone. Anion exchange chromatography, immobilized metal ion affinity chromatography, or size exclusion chromatography have all been used to generate samples enriched in peptide–RNA conjugates.^{76–79} In most cases, the polynucleotide component is then degraded with nucleases such as ribonuclease T1 or ribonuclease A. MS of these

peptide–nucleotide conjugates can then be obtained in either positive or negative ion mode using matrix-assisted laser desorption/ionization or electrospray ionization sources. Conditions used to obtain good quality spectra depend on the relative size of each component.^{75,80} When the oligonucleotide comprises the greater portion of the conjugate, LC-MS in ammonium acetate buffers and negative mode ionization may yield better results. Conversely, mononucleotide, dinucleotide, or trinucleotide–peptide conjugates require LC-MS in formic acid-containing buffers for positive mode ionization. MS/MS localize the site of attachment of the nucleotide and can be generated using either postsource decay in matrix-assisted laser desorption/ionization instruments^{78,80,81} or collision-induced dissociation in ion trap or triple quadrupole instruments.^{79,80,82,83} Recent results indicate that the use of electron capture dissociation or electron transfer dissociation can provide MS/MS results that are superior to collision-induced dissociation.⁸⁴ Krivos and Limbach observed that subjecting the +3 or +4 charge states of mononucleotide or dinucleotide–peptide conjugates to either electron capture dissociation or electron transfer dissociation provided optimal peptide backbone fragmentation and minimal interference from dissociation of the phosphodiester bonds.⁸⁴ Concise summaries of the challenges, procedural strategies, and representative data for the study of peptide–polynucleotide photocrosslinks can be found in two papers from the Urlaub lab.^{77,79}

The application of this procedure to positive ion mode time-of-flight MS allowed the identification of peptides from the components of the U1 small nuclear ribonucleoprotein spliceosomal complex or in vitro reconstituted subcomplexes.⁷⁸ These data also allowed the composition (although not the sequences) of the dinucleotide, trinucleotide, and pentanucleotides crosslinked to the peptides to be determined. Negative ion mode spectral data were used to identify a peptide from another spliceosome protein p14/SF3b14a attached to a tetranucleotide from the U2 small nuclear RNA.⁷⁷ Photocrosslinked complexes from the U1 small nucleotide ribonucleoprotein were analyzed using an inline nano LC-MS to detect peptide–nucleotide conjugates. The identities of peptides from protein 61K crosslinked to an adenosine-uridine dinucleotide and residues 173–180 of protein 70K crosslinked to a trinucleotide of AUC were confirmed by its peptide fragment ion series observed in positive ion mode collision-induced dissociation MS/MS.⁷⁹ Nucleotide fragment ion series in the same spectra demonstrated that the peptides were photocrosslinked to RNA at the U residues.

When considering the adaptation of procedures developed for photocrosslinked protein–RNA complexes to RCAP products, it is important to note that formaldehyde crosslinked trinucleotide–amino acid and octapeptide–nucleoside conjugates were stable enough to be separated by high performance LC in typical LC-MS solvent systems (0.1% formic acid in water or acetonitrile) for analysis by electrospray ionization MS in both negative and positive ion mode.⁴³ Fragmentation spectra of conjugates of formaldehyde-crosslinked material contained primarily data on the peptide sequence. The MS/MS spectra of both trinucleotide–amino acid conjugates and nucleotide–tripeptide conjugates showed preferential cleavages of the base-to-amino acid methylene bonds. Even without the application of advanced MS techniques, the reversibility of formaldehyde crosslinking holds forth the possibility of parallel analysis of a conjugate with and without hydrolytic heat treatment, providing mass information for both the total complex and each component.

Mapping the site of interaction in RNA

Identification of the RNA sequences recognized by RNA-binding proteins is important to providing information on how RNA-binding proteins bind specifically to an RNA sequence or structure.⁸⁵ While short oligonucleotides can be identified by MS approaches, as described above, it is a challenge to identify longer RNAs. A variety of methods to map RNA contact sites in RNA–protein complexes have been developed using methods that combine modifications in RNA with polymerase chain reaction, microarray, or DNA sequencing.¹³ For example, selective 2′-hydroxyl acylation analyzed by primer extension chemistry can identify RNA structures involved in protein–RNA interactions at single nucleotide resolution.⁸⁶ It takes advantage of the ability of electrophiles, such as N-methylisatoic anhydride to react selectively with flexible RNA nucleotides at the 2′-hydroxyl group on the ribose, generating an adduct that can stop primer extension by reverse transcriptase. This method is useful in mapping RNA conformations⁸⁶ as affected by both intra and intermolecular interactions. As with the modification interference assay, it may be difficult to distinguish the changes in N-methylisatoic anhydride modification as a result of a conformational change in the RNA or as a result of direct protein–RNA contacts. Furthermore, reverse transcription has a propensity to terminate in structured regions of RNA, sometimes making it a challenge to interpret whether the termination was due to crosslinks with a protein.

The crosslinking and immunoprecipitation (CLIP) assay can identify the RNAs that contact the proteins in cells (Figure 6, left panel). In CLIP, cells or tissues are irradiated at 254 nm to induce covalent bonds between proteins and the target RNA. Immunoprecipitation of the protein along with the covalently-linked RNA can then be monitored by analysis of the RNAs using approaches such as RNA microarray, reverse transcription, and DNA sequencing.¹³ Disadvantages of CLIP include the relatively low efficiency of crosslink formation and low-resolution map of the position the RNA contacts to the protein. High throughput sequencing of RNA isolated by CLIP, which couples CLIP to next-generation sequencing, is a more powerful approach because crosslinks can increase the frequency of nucleotide substitutions in the complementary DNA. The position where RNA-binding proteins interact with RNA can then be determined using next-generation sequencing by the detection of an increased mutation rate at the crosslinking site(s). Other modifications of CLIP have also been developed to better separate crosslinking events from noncrosslinked backgrounds.¹³

Photoactivatable ribonucleoside-enhanced CLIP (PAR-CLIP) is a method recently developed by Hafner et al,^{14,15} which uses photoactivatable nucleosides to enable crosslinking at a higher wavelength than what is typically used in CLIP methods. This allows only modified nucleotides to crosslink to proteins (Figure 6, right panel). The crosslinking efficiency of the photoactivatable nucleosides to bound proteins at 365 nm can be more than a log higher than that with natural bases at 254 nm. In PAR-CLIP, the cells are grown in medium containing 4-thiouridine or 6-thioguanine, which can be taken up by cells and incorporated into cellular RNA without causing detectable cytotoxicity or change in gene expression. Live cells are crosslinked at 365 nm, lysed, and immunoprecipitated with antibodies against RNA-binding proteins or an epitope-tagged version of the RNA-binding protein. Similar to CLIP, the complex is then treated with ribonuclease to digest the RNA that is not bound to RNA-binding proteins and, thus, not protected from digestion. The RNA can be radioactively labeled by a polynucleotide kinase reaction and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. The band visualized by autoradiography with the correct mobility as the protein–RNA complex is cut and the complex is eluted from the gel. Subsequently, the protein is digested with proteinase K and adaptors are ligated to the RNA fragments to allow reverse transcription and amplification of the complementary DNA for deep sequencing.^{13–15}

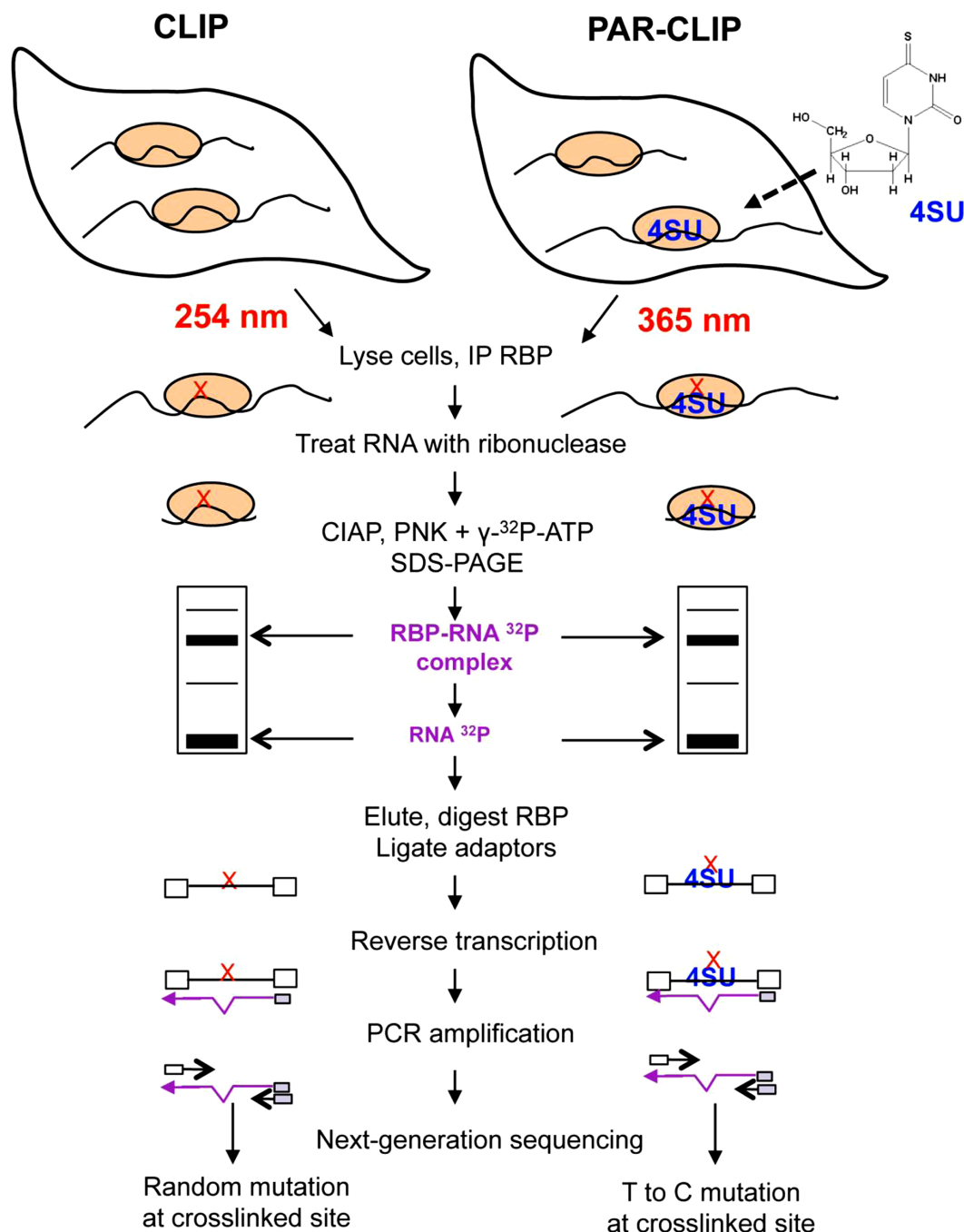


Figure 6 Flow diagram of crosslinking and immunoprecipitation and photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation, two techniques used to map where ribonucleic acid elements contact a protein of interest.

Note: The main difference between the two techniques is the use of modified nucleotides in photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation, which allows increased efficiency in protein crosslinking to ribonucleic acid.

Abbreviations: 4SU, 4-thiouridine; ATP, adenosine triphosphate; C, cytosine; CIAP, calf intestinal alkaline phosphatase; CLIP, crosslinking and immunoprecipitation; IP, immunoprecipitate; PAR-CLIP, photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation; PCR, polymerase chain reaction; PNK, polynucleotide kinase; RBP, ribonucleic acid-binding protein; RNA, ribonucleic acid; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; T, thymine.

Importantly, for PAR-CLIP using 4-thiouridine, a characteristic thymine to cytosine transition in the complementary DNA corresponding to the crosslinking residue can be used to pinpoint crosslinking residues. This mutation is caused by the change of hydrogen bonding of crosslinked 4-thiouridine,

resulting in a mismatch during the reverse transcription step. The use of 6-thioguanine can result in a characteristic guanine to adenine mutation, although the crosslinking efficiency and the corresponding mutation rate are lower than that of RNAs with 4-thiouridine.^{13–15}

PAR-CLIP provides a way to precisely map RNA elements that contact a protein of interest. It has been successfully applied in transcriptome-wide analysis of the targets of several RNA-binding proteins and ribonucleoprotein particles, both in cell culture^{14,87,88} and in live organisms such as *Caenorhabditis elegans*.⁸⁹ It was also used to identify viral and cellular microRNA targetome in Kaposi's sarcoma-associated herpesvirus and Epstein–Barr virus infected human lymphoma and immune cell lines.^{90–92} However, one factor to consider is the rate of conversion of the nucleoside analogs to nucleotides, as these kinases could be limiting in some cells. Nonetheless, when combined with methods such as RCAP, modification interference, and LC-MS, PAR-CLIP will provide information on the RNA sequences/structures that interact with proteins.¹³ Given that 4-thiouridine-5'-triphosphate is commercially available, PAR-CLIP should be directly applicable to protein–RNA contacts in biochemical reactions. In the context of viral–host interactions, or viral protein–RNA interactions, the information gained from PAR-CLIP should generate testable hypotheses that will reveal new insights for the viral infection process.

Summary

This review summarized the recent advances in mapping protein–RNA interactions by RCAP, MS, and PAR-CLIP. While this list is not exhaustive, it provides a framework applicable to any system and potentially bridge the gap in the current lack of structural information about protein–RNA interactions.

Acknowledgments

This work was funded by the National Institute of Allergy and Infectious Diseases grant 1RO1AI073335 and 1RO1AI090280 to CK.

Disclosure

The authors report no conflicts of interest in this work.

References

- Liu S, Ghalei H, Luhrmann R, Wahl MC. Structural basis for the dual U4 and U4atac snRNA-binding specificity of spliceosomal protein hPrp31. *RNA*. 2011;17(9):1655–1663.
- Leung AK, Nagai K, Li J. Structure of the spliceosomal U4 snRNP core domain and its implication for snRNP biogenesis. *Nature*. 2011;473(7348):536–539.
- Schuwirth BS, Borovinskaya MA, Hau CW, et al. Structures of the bacterial ribosome at 3.5 Å resolution. *Science*. 2005;310(5749):827–834.
- Fisher AJ, Johnson JE. Ordered duplex RNA controls capsid architecture in an icosahedral animal virus. *Nature*. 1993;361(6408):176–179.
- Wery JP, Reddy VS, Hosur MV, Johnson JE. The refined three-dimensional structure of an insect virus at 2.8 Å resolution. *J Mol Biol*. 1994;235(2):565–586.
- Scherrer T, Mittal N, Janga SC, Gerber AP. A screen for RNA-binding proteins in yeast indicates dual functions for many enzymes. *PLoS One*. 2010;5(11):e15499.
- Tsvetanova NG, Klass DM, Salzman J, Brown PO. Proteome-wide search reveals unexpected RNA-binding proteins in *Saccharomyces cerevisiae*. *PLoS One*. 2010;5(9):e12671.
- Hwang J, Huang L, Cordek DG, et al. Hepatitis C virus nonstructural protein 5A: biochemical characterization of a novel structural class of RNA-binding proteins. *J Virol*. 2010;84(24):12480–12491.
- Ranjith-Kumar CT, Duffy KE, Jordan JL, et al. Single-stranded oligonucleotides can inhibit cytokine production induced by human toll-like receptor 3. *Mol Cell Biol*. 2008;28(14):4507–4519.
- Ranjith-Kumar CT, Murali A, Dong W, et al. Agonist and antagonist recognition by RIG-I, a cytoplasmic innate immunity receptor. *J Biol Chem*. 2009;284(2):1155–1165.
- Yi G, Vaughan RC, Yarbrough I, Dharmiah S, Kao CC. RNA binding by the brome mosaic virus capsid protein and the regulation of viral RNA accumulation. *J Mol Biol*. 2009;391(2):314–326.
- Kim YC, Russell WK, Ranjith-Kumar CT, Thomson M, Russell DH, Kao CC. Functional analysis of RNA binding by the hepatitis C virus RNA-dependent RNA polymerase. *J Biol Chem*. 2005;280(45):38011–38019.
- Ascano M, Hafner M, Cekan P, Gerstberger S, Tuschl T. Identification of RNA-protein interaction networks using PAR-CLIP. *Wiley Interdiscip Rev RNA*. 2012;3(2):159–177.
- Hafner M, Landthaler M, Burger L, et al. Transcriptome-wide identification of RNA-binding protein and microRNA target sites by PAR-CLIP. *Cell*. 2010;141(1):129–141.
- Hafner M, Landthaler M, Burger L, et al. PAR-CLIP – a method to identify transcriptome-wide the binding sites of RNA binding proteins. *J Vis Exp*. 2010;41:2034.
- Orlando V, Strutt H, Paro R. Analysis of chromatin structure by in vivo formaldehyde cross-linking. *Methods*. 1997;11(2):205–214.
- Niranjanakumari S, Lasda E, Brazas R, Garcia-Blanco MA. Reversible cross-linking combined with immunoprecipitation to study RNA-protein interactions in vivo. *Methods*. 2002;26(2):182–190.
- Collas P. The current state of chromatin immunoprecipitation. *Mol Biotechnol*. 2010;45(1):87–100.
- Perkins DN, Pappin DJ, Creasy DM, Cottrell JS. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis*. 1999;20(18):3551–3567.
- Clauser KR, Baker P, Burlingame AL. Role of accurate mass measurement (+/- 10 ppm) in protein identification strategies employing MS or MS/MS and database searching. *Anal Chem*. 1999;71(14):2871–2882.
- Poenisch M, Bartenschlager R. New insights into structure and replication of the hepatitis C virus and clinical implications. *Semin Liver Dis*. 2010;30(4):333–347.
- Chinnaswamy S, Cai H, Kao C. An update on small molecule inhibitors of the HCV NS5B polymerase: effects on RNA synthesis in vitro and in cultured cells, and potential resistance in viral quasispecies. *Virus Adaptation and Treatment*. 2010;2(1):73–89.
- Chinnaswamy S, Murali A, Li P, Fujisaki K, Kao CC. Regulation of de novo-initiated RNA synthesis in hepatitis C virus RNA-dependent RNA polymerase by intermolecular interactions. *J Virol*. 2010;84(12):5923–5935.
- Chinnaswamy S, Yarbrough I, Palaninathan S, et al. A locking mechanism regulates RNA synthesis and host protein interaction by the hepatitis C virus polymerase. *J Biol Chem*. 2008;283(29):20535–20546.
- O'Farrell D, Trowbridge R, Rowlands D, Jager J. Substrate complexes of hepatitis C virus RNA polymerase (HC-J4): structural evidence for nucleotide import and de-novo initiation. *J Mol Biol*. 2003;326(4):1025–1035.
- Lee JH, Nam IY, Myung H. Nonstructural protein 5B of hepatitis C virus. *Mol Cells*. 2006;21(3):330–336.

27. Kao CC, Yang X, Kline A, Wang QM, Barket D, Heinz BA. Template requirements for RNA synthesis by a recombinant hepatitis C virus RNA-dependent RNA polymerase. *J Virol*. 2000;74(23):11121–11128.
28. Lan KH, Sheu ML, Hwang SJ, et al. HCV NS5A interacts with p53 and inhibits p53-mediated apoptosis. *Oncogene*. 2002;21(31):4801–4811.
29. Wang J, Tong W, Zhang X, et al. Hepatitis C virus non-structural protein NS5A interacts with FKBP38 and inhibits apoptosis in Huh7 hepatoma cells. *FEBS Lett*. 2006;580(18):4392–4400.
30. Shirota Y, Luo H, Qin W, et al. Hepatitis C virus (HCV) NS5A binds RNA-dependent RNA polymerase (RdRP) NS5B and modulates RNA-dependent RNA polymerase activity. *J Biol Chem*. 2002;277(13):11149–11155.
31. Ahn J, Chung KS, Kim DU, et al. Systematic identification of hepatocellular proteins interacting with NS5A of the hepatitis C virus. *J Biochem Mol Biol*. 2004;37(6):741–748.
32. Fridell RA, Wang C, Sun JH, et al. Genotypic and phenotypic analysis of variants resistant to hepatitis C virus nonstructural protein 5A replication complex inhibitor BMS-790052 in humans: in vitro and in vivo correlations. *Hepatology*. 2011;54(6):1924–1935.
33. Tellinghuisen TL, Marcotrigiano J, Rice CM. Structure of the zinc-binding domain of an essential component of the hepatitis C virus replicase. *Nature*. 2005;435(7040):374–379.
34. Li X, Romero P, Rani M, Dunker AK, Obradovic Z. Predicting protein disorder for N-, C-, and internal regions. *Genome Inform Ser Workshop Genome Inform*. 1999;10:30–40.
35. Frick DN, Rypma RS, Lam AM, Gu B. The nonstructural protein 3 protease/helicase requires an intact protease domain to unwind duplex RNA efficiently. *J Biol Chem*. 2004;279(2):1269–1280.
36. Gu B, Pruss CM, Gates AT, Khandekar SS. The RNA-unwinding activity of hepatitis C virus non-structural protein 3 (NS3) is positively modulated by its protease domain. *Protein Pept Lett*. 2005;12(4):315–321.
37. Zhang C, Cai Z, Kim YC, et al. Stimulation of hepatitis C virus (HCV) nonstructural protein 3 (NS3) helicase activity by the NS3 protease domain and by HCV RNA-dependent RNA polymerase. *J Virol*. 2005;79(14):8687–8697.
38. Hwang J, Fauzi H, Fukuda K, et al. The RNA aptamer-binding site of hepatitis C virus NS3 protease. *Biochem Biophys Res Commun*. 2000;279(2):557–562.
39. Kim JL, Morgenstern KA, Griffith JP, et al. Hepatitis C virus NS3 RNA helicase domain with a bound oligonucleotide: the crystal structure provides insights into the mode of unwinding. *Structure*. 1998;6(1):89–100.
40. Appleby TC, Anderson R, Fedorova O, et al. Visualizing ATP-dependent RNA translocation by the NS3 helicase from HCV. *J Mol Biol*. 2011;405(5):1139–1153.
41. Yakovlev AA. Crosslinkers and their utilization for studies of intermolecular interactions. *Neurochem J*. 2009;3(2):139–144.
42. Metz B, Kersten GF, Hoogerhout P, et al. Identification of formaldehyde-induced modifications in proteins: reactions with model peptides. *J Biol Chem*. 2004;279(8):6235–6243.
43. Lu K, Ye W, Zhou L, et al. Structural characterization of formaldehyde-induced cross-links between amino acids and deoxynucleosides and their oligomers. *J Am Chem Soc*. 2010;132(10):3388–3399.
44. Toth J, Biggin MD. The specificity of protein-DNA crosslinking by formaldehyde: in vitro and in drosophila embryos. *Nucleic Acids Res*. 2000;28(2):e4.
45. Zhang L, Zhang K, Prandl R, Schoffl F. Detecting DNA-binding of proteins in vivo by UV-crosslinking and immunoprecipitation. *Biochem Biophys Res Commun*. 2004;322(3):705–711.
46. Jones S, Daley DT, Luscombe NM, Berman HM, Thornton JM. Protein-RNA interactions: a structural analysis. *Nucleic Acids Res*. 2001;29(4):943–954.
47. Smith AL, Friedman DB, Yu H, Carnahan RH, Reynolds AB. ReCLIP (reversible cross-link immuno-precipitation): an efficient method for interrogation of labile protein complexes. *PLoS One*. 2011;6(1):e16206.
48. Piran U, Riordan WJ. Dissociation rate constant of the biotin-streptavidin complex. *J Immunol Methods*. 1990;133(1):141–143.
49. Hnatowich DJ, Winnard P Jr, Virzi F, et al. Technetium-99 m labeling of DNA oligonucleotides. *J Nucl Med*. 1995;36(12):2306–2314.
50. Kalkhof S, Sinz A. Chances and pitfalls of chemical cross-linking with amine-reactive N-hydroxysuccinimide esters. *Anal Bioanal Chem*. 2008;392(1–2):305–312.
51. Morpurgo M, Bayer EA, Wilchek M. N-hydroxysuccinimide carbonates and carbamates are useful reactive reagents for coupling ligands to lysines on proteins. *J Biochem Biophys Methods*. 1999;38(1):17–28.
52. Barlow JJ, Mathias AP, Williamson R, Gammack DB. A simple method for the quantitative isolation of undegraded high molecular weight ribonucleic acid. *Biochem Biophys Res Commun*. 1963;13:61–66.
53. Cathala G, Savouret JF, Mendez B, et al. A method for isolation of intact, translationally active ribonucleic acid. *DNA*. 1983;2(4):329–335.
54. Auffray C, Rougeon F. Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. *Eur J Biochem*. 1980;107(2):303–314.
55. Ferrer-Orta C, Arias A, Escarmis C, Verdager N. A comparison of viral RNA-dependent RNA polymerases. *Curr Opin Struct Biol*. 2006;16(1):27–34.
56. Kolb HC, Finn MG, Sharpless KB. Click chemistry: diverse chemical function from a few good reactions. *Angew Chem Int Ed Engl*. 2001;40(11):2004–2021.
57. Speers AE, Cravatt BF. Profiling enzyme activities in vivo using click chemistry methods. *Chem Biol*. 2004;11(4):535–546.
58. Deval J, D'Abramo CM, Zhao Z, et al. High resolution footprinting of the hepatitis C virus polymerase NS5B in complex with RNA. *J Biol Chem*. 2007;282(23):16907–16916.
59. Running WE, Reilly JP. Ribosomal proteins of *Deinococcus radiodurans*: their solvent accessibility and reactivity. *J Proteome Res*. 2009;8(3):1228–1246.
60. Lauber MA, Reilly JP. Structural analysis of a prokaryotic ribosome using a novel amidinating cross-linker and mass spectrometry. *J Proteome Res*. 2011;10(8):3604–3616.
61. Lundblad RL. *Chemical Reagents for Protein Modification*, 3rd ed. Boca Raton, FL: CRC Press; 2005.
62. Mendoza VL, Vachet RW. Probing protein structure by amino acid-specific covalent labeling and mass spectrometry. *Mass Spectrom Rev*. 2009;28(5):785–815.
63. Kannan N, Schneider TD, Vishveshwara S. Logos for amino-acid preferences in different backbone packing density regions of protein structural classes. *Acta Crystallogr D Biol Crystallogr*. 2000;56(Pt 9):1156–1165.
64. Carven GJ, Stern LJ. Probing the ligand-induced conformational change in HLA-DR1 by selective chemical modification and mass spectrometric mapping. *Biochemistry*. 2005;44(42):13625–13637.
65. Suckau D, Mak M, Przybylski M. Protein surface topology-probing by selective chemical modification and mass spectrometric peptide mapping. *Proc Natl Acad Sci U S A*. 1992;89(12):5630–5634.
66. Habeeb AF, Cassidy HG, Singer SJ. Molecular structural effects produced in proteins by reaction with succinic anhydride. *Biochim Biophys Acta*. 1958;29(3):587–593.
67. Janecki DJ, Beardsley RL, Reilly JP. Probing protein tertiary structure with amidination. *Anal Chem*. 2005;77(22):7274–7281.
68. Inman JK, Perham RN, DuBois GC, Appella E. Amidination. *Methods Enzymol*. 1983;91:559–569.
69. Liu X, Reilly JP. Correlating the chemical modification of *Escherichia coli* ribosomal proteins with crystal structure data. *J Proteome Res*. 2009;8(10):4466–4478.
70. Reynolds JH. Acetimidation of bovine pancreatic ribonuclease A. *Biochemistry*. 1968;7(9):3131–3135.
71. Fazili KM, Mir MM, Qasim MA. Changes in protein stability upon chemical modification of lysine residues of bovine serum albumin by different reagents. *Biochem Mol Biol Int*. 1993;31(5):807–816.

72. Thumm M, Hoenes J, Pfeleiderer G. S-methylthioacetimidate is a new reagent for the amidination of proteins at low pH. *Biochim Biophys Acta*. 1987;923(2):263–267.
73. Beardsley RL, Reilly JP. Quantitation using enhanced signal tags: a technique for comparative proteomics. *J Proteome Res*. 2003;2(1):15–21.
74. Meisenheimer KM, Koch TH. Photocross-linking of nucleic acids to associated proteins. *Crit Rev Biochem Mol Biol*. 1997;32(2):101–140.
75. Steen H, Jensen ON. Analysis of protein-nucleic acid interactions by photochemical cross-linking and mass spectrometry. *Mass Spectrom Rev*. 2002;21(3):163–182.
76. Urlaub H, Hartmuth K, Luhrmann R. A two-tracked approach to analyze RNA-protein crosslinking sites in native, nonlabeled small nuclear ribonucleoprotein particles. *Methods*. 2002;26(2):170–181.
77. Kuhn-Holsken E, Dybkov O, Sander B, Luhrmann R, Urlaub H. Improved identification of enriched peptide RNA cross-links from ribonucleoprotein particles (RNPs) by mass spectrometry. *Nucleic Acids Res*. 2007;35(15):e95.
78. Kuhn-Holsken E, Lenz C, Sander B, Luhrmann R, Urlaub H. Complete MALDI-ToF MS analysis of cross-linked peptide-RNA oligonucleotides derived from nonlabeled UV-irradiated ribonucleoprotein particles. *RNA*. 2005;11(12):1915–1930.
79. Lenz C, Kuhn-Holsken E, Urlaub H. Detection of protein-RNA crosslinks by NanoLC-ESI-MS/MS using precursor ion scanning and multiple reaction monitoring (MRM) experiments. *J Am Soc Mass Spectrom*. 2007;18(5):869–881.
80. Jensen ON, Kulkarni S, Aldrich JV, Barofsky DF. Characterization of peptide-oligonucleotide heteroconjugates by mass spectrometry. *Nucleic Acids Res*. 1996;24(19):3866–3872.
81. Geyer H, Geyer R, Pingoud V. A novel strategy for the identification of protein-DNA contacts by photocrosslinking and mass spectrometry. *Nucleic Acids Res*. 2004;32(16):e132.
82. Golden MC, Resing KA, Collins BD, Willis MC, Koch TH. Mass spectral characterization of a protein-nucleic acid photocrosslink. *Protein Sci*. 1999;8(12):2806–2812.
83. Martin Richter F, Hsiao HH, Plessmann U, Urlaub H. Enrichment of protein-RNA crosslinks from crude UV-irradiated mixtures for MS analysis by on-line chromatography using titanium dioxide columns. *Biopolymers*. 2009;91(4):297–309.
84. Krivos KL, Limbach PA. Sequence analysis of peptide:oligonucleotide heteroconjugates by electron capture dissociation and electron transfer dissociation. *J Am Soc Mass Spectrom*. 2010;21(8):1387–1397.
85. Licatalosi DD, Darnell RB. RNA processing and its regulation: global insights into biological networks. *Nat Rev Genet*. 2010;11(1):75–87.
86. Weeks KM, Mauer DM. Exploring RNA structural codes with SHAPE chemistry. *Acc Chem Res*. 2011;44(12):1280–1291.
87. Skalsky RL, Corcoran DL, Gottwein E, et al. The viral and cellular microRNA targetome in lymphoblastoid cell lines. *PLoS Pathog*. 2012;8(1):e1002484.
88. Malterer G, Dolken L, Haas J. The miRNA-targetome of KSHV and EBV in human B-cells. *RNA Biol*. 2011;8(1):30–34.
89. Lebedeva S, Jens M, Theil K, et al. Transcriptome-wide analysis of regulatory interactions of the RNA-binding protein HuR. *Mol Cell*. 2011;43(3):340–352.
90. Jungkamp AC, Stoeckius M, Mecnas D, et al. In vivo and transcriptome-wide identification of RNA binding protein target sites. *Mol Cell*. 2011;44(5):828–840.
91. Hoell JI, Larsson E, Runge S, et al. RNA targets of wild-type and mutant FET family proteins. *Nat Struct Mol Biol*. 2011;18(12):1428–1431.
92. Gottwein E, Corcoran DL, Mukherjee N, et al. Viral microRNA targetome of KSHV-infected primary effusion lymphoma cell lines. *Cell Host Microbe*. 2011;10(5):515–526.
93. Shepherd CM, Borelli IA, Lander G, et al. VIPERdb: a relational database for structural virology. *Nucleic Acids Res*. 2006;34(Database issue):D386–D389.

Virus Adaptation and Treatment

Publish your work in this journal

Virus Adaptation and Treatment is an international, peer-reviewed open access journal focusing on the study of virology, viral adaptation and the development and use of antiviral drugs and vaccines to achieve improved outcomes in infection control and treatment. The journal welcomes original research, basic science, clinical & epidemiological

Submit your manuscript here: <http://www.dovepress.com/virus-adaptation-and-treatment-journal>

studies, reviews & evaluations, expert opinion and commentary, case reports and extended reports. The manuscript management system is completely online and includes a very quick and fair peer-review system, which is all easy to use. Visit <http://www.dovepress.com/testimonials.php> to read real quotes from published authors.

Dovepress