Virus Adaptation and Treatment

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REVIEW

Mapping protein–RNA interactions

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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,

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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.

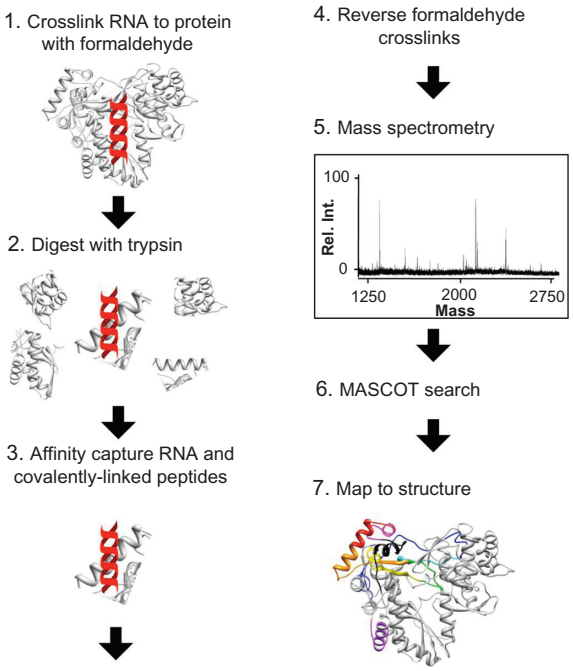

Figure 1 Overview of the reversible crosslinking affinity purification protocol.
Notes: Outline of seven steps in the general strategy used to map peptides within proteins that interact with ribonucleic acid. Nonstructural protein-5B (Protein Data Bank: 1QUV) is used to demonstrate the procedure schematically.
Abbreviation: RNA, ribonucleic acid.

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 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.

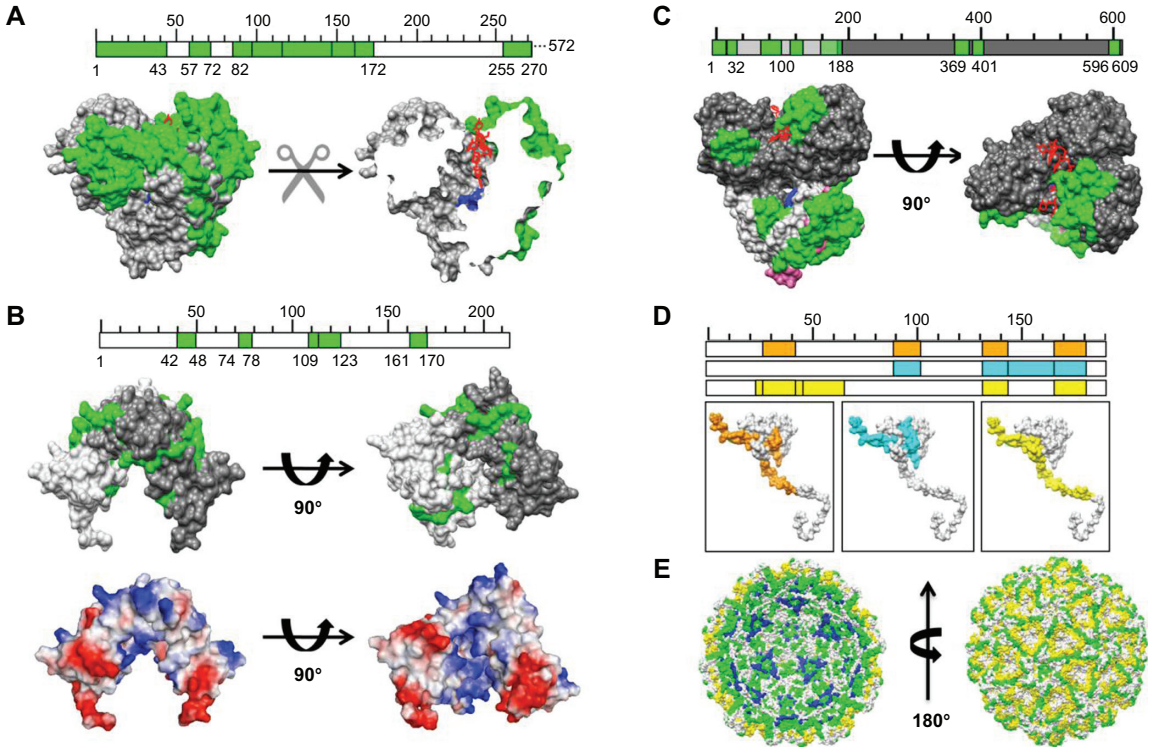

Figure 2 Reversible crosslinking affinity purification results within several different systems. (**A**) Peptides from nonstructural protein-5B found to interact with ribonucleic acid by the reversible crosslinking affinity purification assay.[12] The schematic represents the first 270 residues of the 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: 1ZH1). (**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 brome mosaic virus coat protein that interact with ribonucleic acid (Protein Data Bank: 1JS9). The linear polypeptides show regions of the brome mosaic virus capsid protein that contact ribonucleic acids. The space-filled model of a brome mosaic capsid monomer is shown in the rectangles. In both the schematics and the space-filled models, orange indentified locations of peptides that interact with a ribonucleic acid named SLD4 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 brome mosaic virus virion. Peptides from wild-type brome 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 brome 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

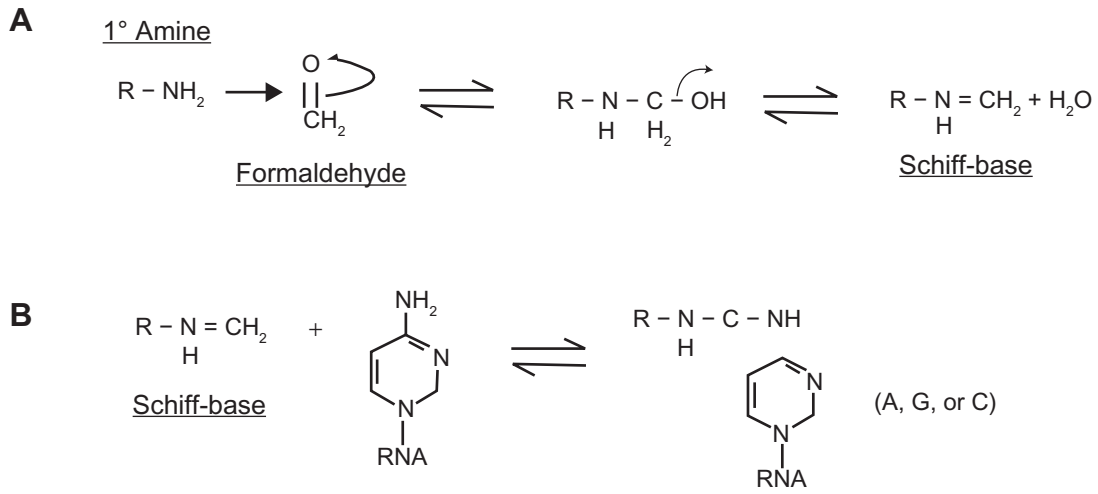

Figure 3 Chemical mechanism of formaldehyde crosslinking. (**A**) Formaldehyde first reacts with a primary amine to form methylol adducts, which rapidly dehydrate to form a Schiff base. (**B**) The resulting labile Schiff base can form crosslinks with several amino acids or ribonucleic acid bases as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

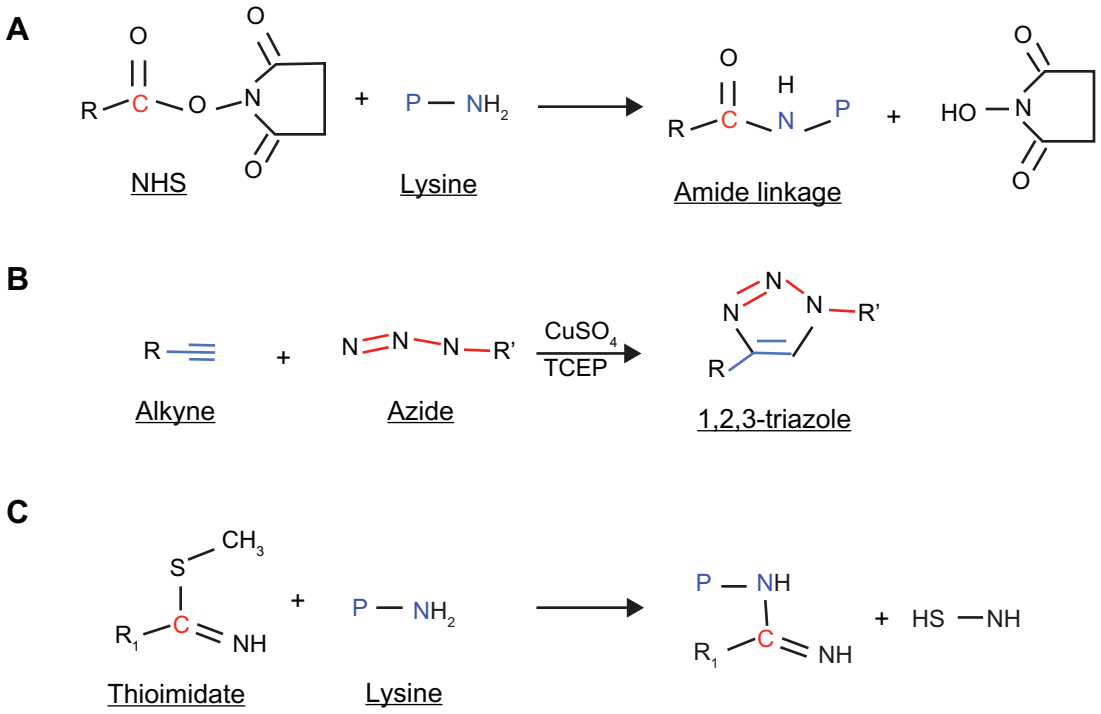

Figure 4 Schematics of conjugation chemistries reviewed. (**A**) N-hydroxysuccinimide reacts with primary amines in slightly alkaline conditions to form stable amide bonds. The lysine is shown as P–NH2 to denote that it is present in a peptide. (**B**) Azides and alkynes can form stable covalently attached 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 thioimidate reagents as described in the text.
Notes: For S-methylthioacetimidate, R_1 is CH_3- and results in a 41.05 Da mass increase per amino group modified. For S-methylthiopropionate, R_1 is CH_3CH_2- 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 thioimidates 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. Thioimidates are prepared

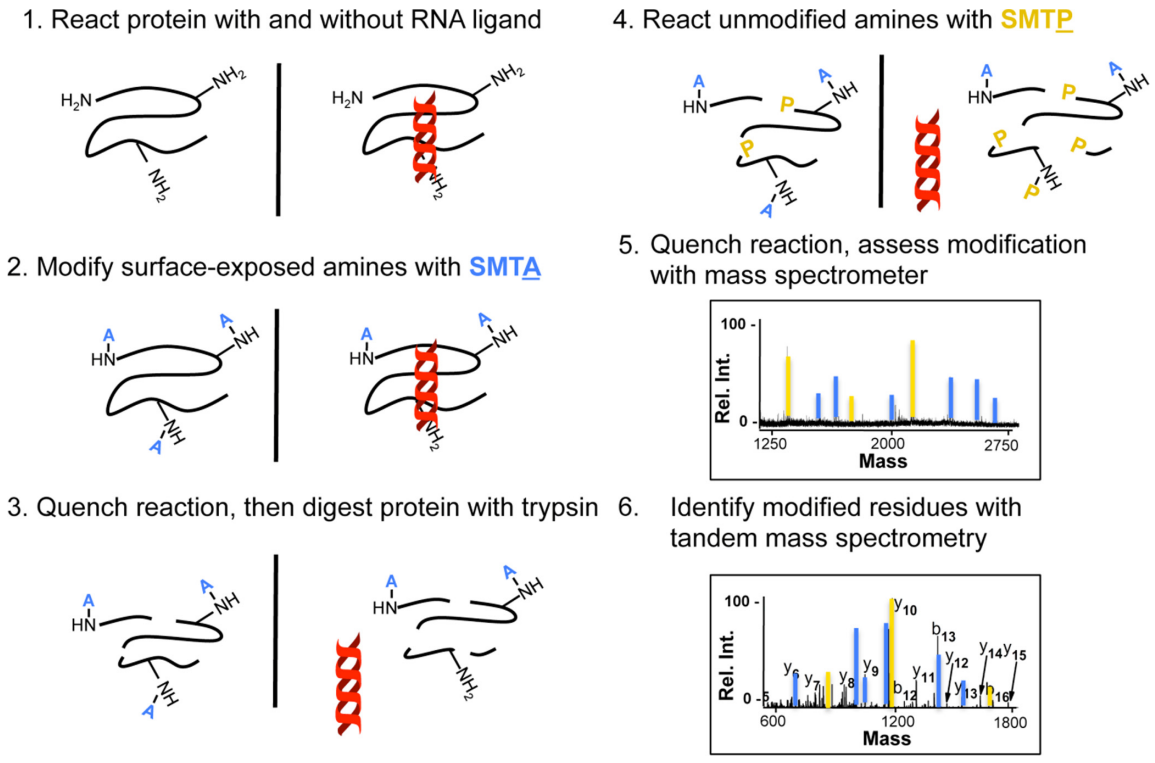

Figure 5 Amidination interference with thioimidates.
Notes: Outline of the general strategy used to map ribonucleic acid binding by modification interference and mass spectrometry. S-methylthioacetimidate modification results in a 41.05 Da mass increase and is depicted in blue, while S-methylthiopropionate 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 dinucelotide, 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 conformations[86] 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]

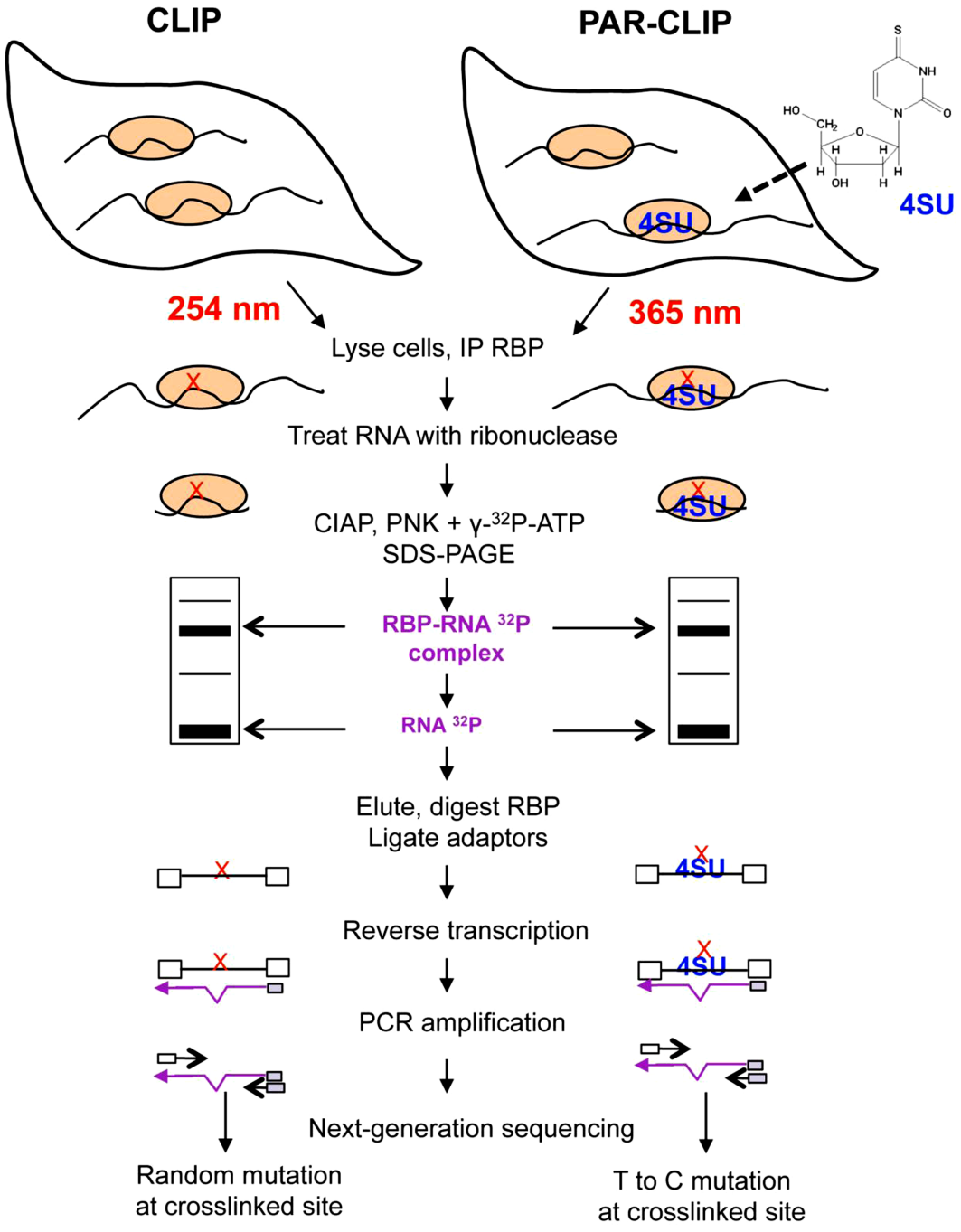

Figure 6 Flow diagram of crosslinking and immunoprecipitation and photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation, two techniques used to map where ribonucleic acid elements contact a protein of interest.

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.

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Disclosure

The authors report no conflicts of interest in this work.

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