Video Article

A Novel Saturation Mutagenesis Approach: Single Step Characterization of Regulatory Protein Binding Sites in RNA Using Phosphorothioates

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Abstract

Gene regulation plays an important role in development. Numerous DNA- and RNA-binding proteins bind their target sequences with high specificity to control gene expression. These regulatory proteins control gene expression either at the level of DNA (transcription) or at the level of RNA (pre-mRNA splicing, polyadenylation, mRNA transport, decay, and translation). Identification of regulatory sequences helps understand not only how a gene is switched on or off, but also which downstream genes are regulated by a particular regulatory protein. Here, we describe a one-step approach that allows saturation mutagenesis of a protein binding site in RNA. It involves doping DNA template with non-wild-type nucleotides within the binding site, synthesis of separate RNAs with each phosphorothioate nucleotide, and isolation of the bound fraction following incubation with protein. Interference from non-wild-type nucleotides results in their preferential exclusion from the protein-bound fraction. This is monitored by gel electrophoresis following selective chemical cleavage with iodine of phosphodiester bonds containing phosphorothioates (phosphorothioate mutagenesis or PTM). This single-step saturation mutagenesis approach is applicable to the characterization of any protein binding site in RNA.

Video Link

The video component of this article can be found at https://www.jove.com/video/57816/

Introduction

Gene regulation plays an important role in biology. Genes can be regulated at the level of transcription, pre-mRNA splicing, 3' end formation, RNA export, translation, mRNA localization, decay, post-translational modification/stability, *etc.* Both DNA- and RNA-binding proteins play key roles in gene regulation. While molecular genetic analyses have identified numerous regulatory proteins, only a small subset of them have been characterized fully for their cellular functions or binding sites *in vivo*. Phylogenetic sequence analysis and mutagenesis offer complementary approaches to characterize DNA- or RNA-protein interactions.

RNA-binding proteins are important in developmental processes, including sexual differentiation. The *Drosophila* protein Sex-lethal (SXL) or the master sex-switch protein is absent in males, but present in females. It recognizes uridine-rich sequences or pyrimidine-tracts adjacent to specific splice sites in downstream pre-mRNA targets (*transformer*, *Sex-lethal*, and *male-specific lethal2*) in somatic cells[1,2,3,4]. In addition, it regulates polyadenylation site switching by binding to uridine-rich polyadenylation enhancer sequences in the *enhancer of rudimentary* (*e(r)*) transcript[5,6]. SXL likely regulates additional targets in the female germline that remain to be identified[1,7,8,9,10,11,12,13].

Typically, characterization of a binding site involves mutagenesis, for example, by deletion or substitution of single or multiple nucleotides. Each mutant binding site, relative to the wild-type RNA sequence, is then analyzed using a series of protein concentrations to determine its binding affinity (K_d or equilibrium dissociation constant) for the protein of interest; K_d is the protein concentration required to obtain 50% RNA binding. This labor-intensive process of detailed mutagenesis involves generation and analysis of numerous mutants — three non-wild type nucleotides for each position in the binding site. Thus, there is a need for an alternative approach for faster, simpler, and inexpensive saturation mutagenesis of protein binding sites in RNA.

Here, we describe a one-step approach that allows saturation mutagenesis of a protein binding site in RNA. It involves doping DNA template with non-wild-type nucleotides within the binding site, synthesis of separate RNAs with each phosphorothioate nucleotide, and isolation of the bound fraction following incubation with protein. Interference from non-wild-type nucleotides results in their preferential exclusion from the protein-bound fraction. This is monitored by gel electrophoresis following selective chemical cleavage with iodine of phosphodiester bonds containing phosphorothioates (phosphorothioate mutagenesis or PTM). This single-step saturation mutagenesis approach is applicable to the characterization of any protein binding site in RNA.

Protocol

NOTE: **Figure 1** provides an overview of phosphorothioate mutagenesis and summarizes key steps in the process.

1. Generation of a Library of Mutants — Doping DNA Template with Non-wild Type Nucleotides

1. Synthesize T7 Primer (5'-GTAATACGACTCACTATAG-3') by chemical synthesis on a DNA synthesizer.
2. Synthesize a doped oligonucleotide (complementary strand) by chemical synthesis on a DNA synthesizer corresponding to the protein binding site. Use an appropriate mixture of phosphoramidites during chemical synthesis for each site of doping (X below) with a ratio of 90% A as the wild-type nucleotide and 10% T as the non-wild type nucleotide (see Representative Results for more detail on this ratio).
   Note: Here, the sequence of the doped oligonucleotide is 5'-GTTCACTACACTXGAXAXAXAXCAXCXAXAXGXTGCC*CTATAGTGAGTCGTATTAC*-3'. The underlined sequence is the reverse complement of SXL protein binding site, plus additional nucleotides outside the binding site that provide useful controls confirming that not every change in the RNA affects binding, as well as being loading controls for comparison and normalization of nucleotides within the binding site. The SXL-binding site sequence UUUUUGUUGUUUUUUUU, which is present in *transformer* pre-mRNA[14,15,16,17], was used to devise the proposed methodology. The sequence in italics is complementary to the T7 primer sequence and is the T7 promoter for *in vitro* transcription.

2. Synthesis of RNA

1. **Synthesize RNA in a 20 µL transcription reaction, as previously described[18].**
   1. Mix T7 transcription buffer and 1 µM T7 oligonucleotide, 1 µM doped oligonucleotide, 10 mM dithiothreitol (DTT), 2 mM GTP, 1 mM each ATP, CTP, and UTP (guanosine, adenosine, cytidine, and uridine triphosphate), and 2 U/µL T7 RNA polymerase.
   2. Add, in two separate microcentrifuge tubes, 0.167 mM α-thio ATP or 0.05 mM α-thio UTP to incorporate phosphorothioates (see schematics in **Figure 2**) into RNAs.
      NOTE: For alternative protocols, add 0.2 mM α-thio CTP or 0.2 mM α-thio GTP to an appropriate transcription reaction containing a doped oligonucleotide to test the two remaining nucleotide substitutions.
   3. Incubate the RNA synthesis reaction mixture for 2 h at 37 °C.
2. Add 2.5 µL heat-labile alkaline phosphatase and 2.5 µL 10xphosphatase buffer to the RNA sample. Incubate this 25 µL reaction for 10–30 min at 37 °C to remove 5' phosphates.
3. Inactivate the alkaline phosphatase enzyme by heating at 80 °C for 2–5 min.
4. Radiolabel the 5' end of dephosphorylated RNA (5 pmole) using 1 µL T4 polynucleotide kinase and 1 µL γ-^{32}P ATP in a 10 µL reaction volume. Incubate the reaction mix for 30–60 min at 37 °C.
5. Inactivate the T4 polynucleotide kinase enzyme by heating at 65 °C for 20–30 min.
6. Gel purify the RNA by electrophoresis in a 10% denaturing polyacrylamide gel. Locate RNA on the gel by autoradiography. Excise the gel slice containing radiolabeled RNA, crush in a microcentrifuge tube by pressing against the walls with a pipette tip, and soak in proteinase K (PK) buffer (100 mM Tris, pH 7.5, 12.5 mM EDTA, 150 mM NaCl, 1% Sodium dodecyl sulfate). Rotate the tube at room temperature from 2 h to overnight.
7. Centrifuge the gel slurry, discard the gel and collect the buffer solution.
8. Extract the solution twice with equal volume of phenol-chloroform and once with chloroform and collect the aqueous phase.
9. Add to the aqueous phase 0.1 volume of 3M Sodium Acetate, pH 5.2, carrier tRNA or glycogen, and 2.5 volume of ethanol. Keep the sample at -80 °C for 1 h.
10. Centrifuge the sample for 5–10 min in a high speed microcentrifuge at 16,873 x *g*. Remove the buffer/ethanol solution carefully without disturbing the RNA pellet.
11. Wash the pellet with 70% ethanol and centrifuge for 2–5 min. Remove ethanol carefully. Dry the pellet in air.
12. Resuspend the pellet in 20–50 µL diethyl pyrocarbonate (DEPC)-treated water. Store at -20 °C until use.
    NOTE: Perform all steps with radiolabeled RNA using appropriate precautions and a plexiglass shield to protect from radioactivity. Presently, spin columns are more commonly used and more convenient to remove unincorporated radioactivity, as an alternative to gel purification of RNA.

3. Protein Binding Reaction and Separation of Bound RNA

1. Express the recombinant protein in and purify from *E. coli*.
2. Estimate recombinant protein concentration by spectrophotometry or by separating in an SDS-polyacrylamide gel next to a known protein standard, bovine serum albumin (BSA). Visualize the protein of interest and the BSA standard by staining the gel with Coomassie Brilliant Blue R-250. Quantitate the protein in comparison to known quantities of BSA dilutions on the same gel.
   NOTE: Store recombinant protein at -80 °C until use and dilute in 20 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), pH 8.0, 1 mM dithiothreitol (DTT), 0.2 mM Ethylenediaminetetraacetic acid (EDTA), 0.05% NP-40, 20% glycerol. Use of 0.5–1.0 mM protease inhibitor phenylmethane sulfonyl fluoride (PMSF) is optional.
3. Perform RNA-binding reaction (20–100 µL) in 10 mM Tris-HCl, pH 7.5, 1 mM DTT, 50 mM KCl, 0.5 units/µL RNase inhibitor, 0.09 µg/µL acetylated bovine serum albumin, 1 mM EDTA, 0.15 µg/µL tRNA, 5'-end radiolabeled RNA, and 6 µL of appropriate concentration (a concentration at which ~50% of RNA binds to protein) of the protein.
   NOTE: This binding buffer works for three RNA–binding proteins (SXL, U2AF[65], and PTB), but must be standardized for a protein of interest. Estimate protein concentration empirically, using various dilutions for a given protein preparation, that is required to obtain approximately 50% RNA binding. This condition or K_d represents the most sensitive part of the RNA-binding curve.

4. Incubate the protein binding reaction for 20–30 min at 25 °C (or on ice).
5. **Separate the protein-bound RNA fraction from the unbound fraction using one of two approaches:**
    1. Nitrocellulose filter binding
        1. Apply the binding reaction (20–100 µL) onto a nitrocellulose filter connected to a vacuum manifold at room temperature.
        NOTE: Only the RNA-protein complex is retained on the filter and unbound RNA flows through the filter. The filter binding approach allows higher recovery of the bound RNA and is faster and simpler, compared to the gel mobility shift assay. By placing a DEAE membrane underneath the nitrocellulose filter it is also possible to collect the unbound RNA fraction or free RNA for comparison with the bound fraction.
        2. Cut the portion of the nitrocellulose filter containing the retained radioactive RNA into smaller pieces to fit into a microcentrifuge tube, soak in sufficient PK buffer (300–500 µL containing 10–20 µg PK) to immerse the filter pieces, and elute RNA from the filter for 2–3 h or overnight.
        3. Extract with phenol-chloroform, chloroform, and collect aqueous phase. Add sodium acetate and ethanol and, after incubation in freezer, centrifuge, wash, dry and resuspend RNA in DEPC-treated water. Follow these steps as detailed in steps 2.10 to 2.14 above.

    2. Gel mobility shift
        1. Prepare and polymerize a 5% native polyacrylamide gel (60:1 Acrylamide:bis-acrylamide) in 0.5x TBE (standard Tris-Borate-EDTA buffer) before starting the binding reaction, as an alternative to the filter binding method.
        2. Pre-run the gel for 15 min at 250 V in a cold room (4 °C).
        3. Load each binding reaction (above) into separate wells of the above pre-run gel.
        NOTE: The protein storage buffer provides sufficient glycerol for sample loading into wells.
        4. Separate the protein-bound RNA by gel electrophoresis in a cold room at 250 V for 1 to 2 h, depending on the RNA size and specific protein.
        5. Locate the position of the RNA-protein complex on the gel by autoradiography. Excise the gel slice containing the RNA-protein complex. Elute the RNA from the gel slice by crushing the gel slice and soaking in the PK buffer.
        6. Extract with phenol-chloroform, chloroform, and collect aqueous phase. add Sodium acetate and ethanol and, after incubation in freezer, centrifuge, wash, air dry, and resuspend RNA in DEPC-treated water. Follow these steps as detailed in steps 2.10 to 2.14 above.

4. Analysis of Iodine-cleaved Phosphorothioate Products for Detection of Mutant Nucleotide Positions

1. Add 1 mM iodine in a 20 µL DEPC-treated water containing up to 10 µg carrier tRNA to cleave RNAs (bound and total RNAs) at the sites of phosphorothioate incorporation. Incubate at room temperature for 5 min.
NOTE: Further details on iodine cleavage with slightly different conditions — 7% (v/v) iodoethanol, heating at 95 °C for 3 min — can be found in Gish & Eckstein 1988[19].
2. Precipitate cleaved RNA by adding sodium acetate/ethanol, as described above. Resuspend in a loading dye for denaturing gels. Heat and load the sample to separate RNA fragments by electrophoresis in a 15–20% denaturing polyacrylamide gel.
3. Expose the polyacrylamide gel to an X-ray film.
4. Detect bands in the bound fraction versus total pool using autoradiography.
NOTE: Perform all steps with iodine in an exhaust hood. For RNA separation shown here, a wedge-shaped gel is employed, which is achieved by doubling the thickness of the both spacers at the bottom of the gel plates by inserting an extra inch-long spacer at the bottom of the gel. This shape allows more uniform or closer spacing between shorter RNA fragments. Alternatively, a phosphorimager can be used rather than X-ray films for detection and quantitation of radioactivity.

Representative Results

Principle of saturation mutagenesis using doping:

For an appropriate molar ratio of wild-type and other nucleotides, use an equal mixture of all four nucleotides if only one position is to be analyzed. However, if multiple positions are analyzed simultaneously, the ratio of non-wild type to wild- type nucleotides must be adjusted, *i.e.,* reduced. Otherwise, in addition to single substitutions, which is desired, there will also be templates with multiple non-wild type nucleotides in a molecule, precluding analysis of the effect of single-nucleotide substitutions. Thus, as a rule of thumb, use a ratio of non-wild type to wild-type nucleotides of 1/n, where n is the number of positions to be analyzed. Here, we analyzed 10 positions simultaneously and used a mixture containing 90% of the wild-type nucleotide and 10% of the non-wild type nucleotide for doping the DNA template. Separate transcription reactions are done, in which each transcription reaction is performed with one of the four phosphorothioates. Thus, each reaction monitors a specific nucleotide at the doped positions.

Principle of partitioning due to interference:

In the mutagenesis approach presented here, RNAs have on an average fewer than one phosphorothioate residue per molecule. During the process of binding, RNA molecules partition between the protein-bound fraction and the unbound fraction. Since a particular nucleotide is linked to a phosphorothioate linkage, cleavage of the phosphorothioate backbone linkage is a readout of the presence of a specific nucleotide at that position. It is expected that at any given position, when a nucleotide is changed it may have either no effect on binding or it may inhibit binding, partially or completely. If presence of a specific nucleotide has no effect on binding it will partition equally between the protein-bound and unbound fractions. However, if a specific nucleotide at a given position interferes with protein binding it will be preferentially excluded from the protein-bound fraction. The degree of interference can be quantitatively monitored for each of the positions in the same reaction following gel electrophoresis. This concept is illustrated in a schematic (**Figure 3**). In total RNA fraction (lane T), band intensity is approximately equal for all doped positions (bands 1, 3–7). In protein-bound RNA fraction (lane B), at positions 1, 4, and 7, the nucleotide has no effect on binding. However, at positions 3 and 6, it interferes with binding and thus is excluded from the bound fraction. At position 5, interference is partial. Thus, comparisons of four paired lanes, T and B for each nucleotide, allow for analysis of all four nucleotides. It should be noted that wild-type nucleotide for a given position reflects the effect, if any, of sulfur in the RNA backbone on protein binding.

The accompanying autoradiograph (**Figure 4**) shows two pairs (α-thio A and α-thio U) of lanes from a denaturing gel (T for Total pool and B for Bound fraction). Several observations can be made by comparing the intensities of bands at each position between each pair of lanes (T and B). First, the majority of the signal is at the top of the gel, *i.e.,* uncleaved product, for both α-thio A lane and α-thio U lane. Second, for α-thio A pair lanes, several bands (iodine cleavage products) are identical between the bound and the total RNA fractions for example, bands above 1; relevant bands within the binding site for the α-thio A lane are numbered for reference. Third, bands at the bottom of the gel are more closely spaced (*e.g.,* bands below 6) than is usual for a sequencing gel. Fourth, several bands are present in the total RNA fraction but absent or significantly reduced in the bound RNA fraction (*e.g.,* bands 1, 2, 3, and 5). Fifth, for the α-thio U lane pair, while most bands are comparable between the total and bound lanes, some bands are relatively less intense in the bound fraction (*e.g.,* bands 7 and 8).

These observations provide evidence for successful development of this new method and lead to the following conclusions: First, for both α-thio A pair lanes and α-thio U pair lanes, because most of the RNA is uncleaved, it provides evidence that only a tiny fraction of the RNA contains modified or phosphorothioate residues. This is important because it ascertains that RNA molecules contain no more than one phosphorothioate residue. Second, identical or similar intensities for several bands outside of the binding site between the two lanes, for both α-thio A and α-thio U, demonstrate that loading is comparable for both lanes, allowing easy comparison between lanes. Third, more closely spaced bands are achieved, for α-thio A and α-thio U, by the wedge-shape of the gel (thinner at the top and thicker at the bottom), thus allowing analysis of longer sequence reads and offering higher resolution. Typically, shorter fragments are more widely spaced in a gel with uniform thickness. Fourth, disappearance or reduced intensity of certain bands in the bound fraction indicates that the non-wild-type nucleotide is preferentially excluded from the bound fraction (α-thio A). Relative intensities of different bands within the bound lane also offer quantitative information about the extent of interference from non-wild type residues at different locations. In other words, the mutant or non-wild type nucleotides at specific positions interfere with protein binding. Finally, testing the effect of backbone sulfur substitution (phosphorothioate) at one of the non-bridging oxygens (α-thio U), shows that three positions show minor effects from backbone sulfurs (*e.g.,* 6, 7 and the one below 7). Our combined results show that several positions within the binding site show preferential disappearance or reduced intensities of specific bands in the bound fraction. This is due to base rather than backbone substitution, indicating protein interactions with specific bases in the binding site (α-thio A). Meanwhile, incorporation of α-thio C shows an interference pattern comparable to α-thio A. However, only 3–4 α-thio G substitutions show small but detectable interference centered around, for example, positions numbered 2 and 3 (data not shown). This method has been used to reveal differences in how the splicing repressor SXL and the general splicing factor U2 snRNP Auxiliary Factor (U2AF^{65}) bind to an identical pre-mRNA splicing signal sequence (polypyrimidine-tract) in distinct manner[15].

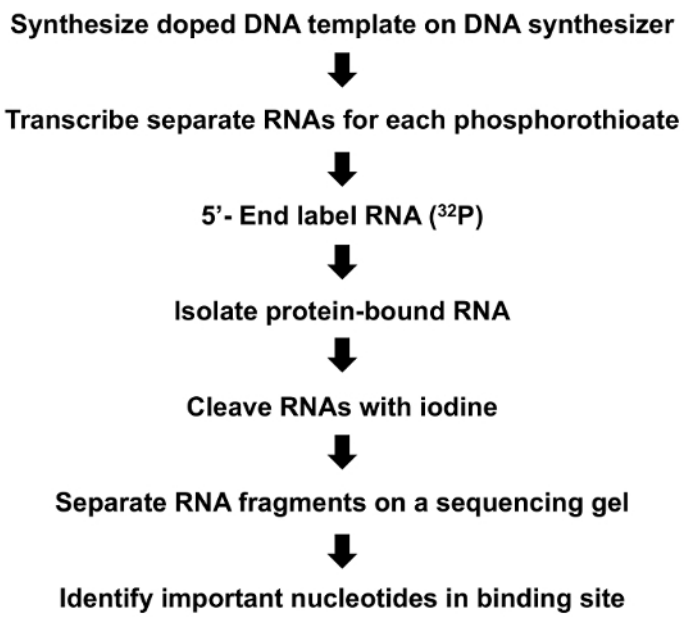

Figure 1: Flow chart of key steps in phosphorothioate mutagenesis (PTM). Please click here to view a larger version of this figure.

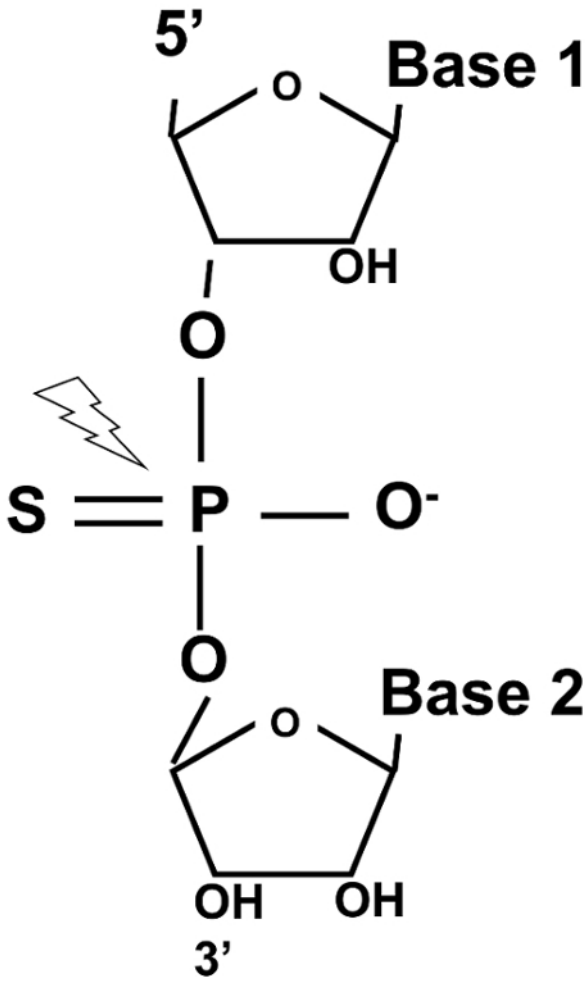

Figure 2: Schematic of a phosphorothioate linkage between two nucleotides, which can be chemically cleaved by iodine. Sulfur replaces one of the non-bridging phosphate oxygens. Please click here to view a larger version of this figure.

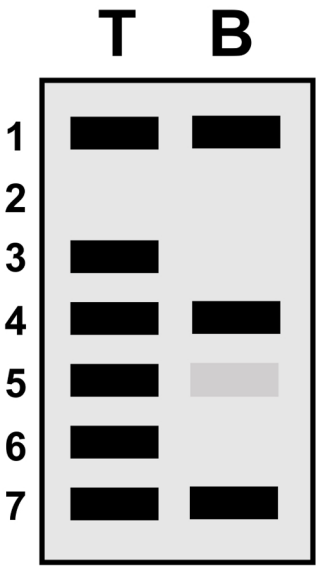

Figure 3: Principle of partitioning due to interference. Hypothetical RNA contains representative α-thio nucleotides at six positions, including a protein-binding site. Following protein binding, RNA is cleaved at sites of phosphorothioate incorporation by iodine. Positions 1–7 within and around the binding site are arbitrarily numbered for reference in the text. Position 2 or missing band represents no phosphorothioate incorporation or non-doped nucleotide. Lane T is total RNA and lane B is protein-bound RNA. Please click here to view a larger version of this figure.

Figure 4: Phosphorothioate mutagenesis (PTM) approach allows saturation mutagenesis of a polypyrimidine-tract/3' splice site present in the *transformer* pre-mRNA. Lane T is total RNA and lane B is protein-bound RNA. α-thio A represents RNA synthesized in the presence of α-thio ATP and identifies doped positions with α-thio adenosines in the sequence. Three strong bands correspond to adenosines in the sequence at those positions. α-thio U represents RNA synthesized in the presence of α-thio UTP and identifies all uridines, including doped positions, in the sequence. Vertical line on the left marks the SXL binding site. Positions 1–8 within the binding site are numbered for reference purposes and for ease of description in the results section. Please click here to view a larger version of this figure.

Discussion

Mutagenesis has long been used to characterize protein binding sites. First, a series of mutants can be constructed and individually tested in binding assays to analyze their effects on binding affinity. While a standard mutagenesis approach offers a way to analyze several sequences, multiple steps involved in the standard approach, such as constructing mutants and performing a series of binding reactions for each mutant, is laborious and time consuming and may not allow saturation mutagenesis, especially for longer sequences. Second, a sequence can be randomized and the pool used for multiple cycles of binding and polymerase chain reaction (PCR) amplification. These sequences that bind protein will have to be cloned and sequenced to identify and validate a binding site from the consensus sequence. Nonetheless, this iterative binding and amplification option involves multiple steps and is laborious in identifying residues that are important within the binding site. Moreover, repeated sequence amplifications inherent to PCR may introduce sequence bias. Third, sequences selected from the random pool can be sequenced using a faster option of high throughput sequencing, although it is relatively expensive. Therefore, to overcome these limitations of multiple steps, laborious and/or expensive methods, we devised a faster, inexpensive, and most importantly a single-step method, described here, to accomplish saturation mutagenesis of a binding site (PTM).

The key steps in this approach include identification or tagging of the non-wild-type nucleotide(s). We used phosphorothioate nucleotides, in which one of the oxygens in the phosphate backbone is substituted with sulfur, as described (**Figure 2**). The advantage is that iodine can be used to chemically cleave the backbone of the phosphorothioate nucleotide. Thus, iodine cleavage of the RNA substrate containing a phosphorothioate backbone generates a sequencing-type ladder, where the site of cleavage is a proxy or tag for the presence of one of the four bases at that position. A comparison of the unbound and total fractions identifies residues that are preferentially excluded from the bound fraction and thus are important for protein binding. In contrast, residues that are not important for binding remain equally distributed between the two fractions. This approach can be used to define binding sites for any RNA-binding protein.

In summary, this one-step saturation mutagenesis approach, combining doping, phosphorothioates, and iodine cleavage, offers a powerful means for the characterization of any binding site in RNA. However, this approach requires that the binding site is already known prior to mutagenesis. Furthermore, protein–binding conditions need to optimized for each protein. The following precautions need to be emphasized. Precautions for RNA handling must be exercised, such as wearing gloves, preparation of solutions and buffers in DEPC-treated water, autoclaving pipette tips and tubes, and dedicating equipment for RNA use only to avoid RNAses. Similarly, care involving radioactive shields, radioactive monitoring while using radioactive material is essential, and use of exhaust hood while using hazardous chemicals are necessary.

Disclosures

The author declares no competing financial interests.

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