Leveraging cross-link modification events in CLIP-seq for motif discovery

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ABSTRACT

High-throughput protein–RNA interaction data generated by CLIP-seq has provided an unprecedented depth of access to the activities of RNA-binding proteins (RBPs), the key players in co- and post-transcriptional regulation of gene expression. Motif discovery forms part of the necessary follow-up data analysis for CLIP-seq, both to refine the exact locations of RBP binding sites, and to characterize them. The specific properties of RBP binding sites, and the CLIP-seq methods, provide additional information not usually present in the classic motif discovery problem: the binding site structure, and cross-linking induced events in reads. We show that CLIP-seq data contains clear secondary structure signals, as well as technology- and RBP-specific cross-link signals. We introduce Zagros, a motif discovery algorithm specifically designed to leverage this information and explore its impact on the quality of recovered motifs. Our results indicate that using both secondary structure and cross-link modifications can greatly improve motif discovery on CLIP-seq data. Further, the motifs we recover provide insight into the balance between sequence- and structure-specificity struck by RBP binding.

INTRODUCTION

During the past decade, an increasing amount of attention has been drawn toward the complex mechanisms of post-transcriptional regulation of gene expression, heavily driven by interactions between RNA and RNA-binding proteins (RBPs) (1–3). It is now clear that post-transcriptional regulation is a major modulator of phenotype, with important roles in practically all biological processes, and implications for many human diseases (4–6). Approaches such as RNA-seq have uncovered complex relationships between RBPs and their binding specificities (7), underscoring the need for a greater understanding of their activities. Despite substantial progress, much remains to be learned about post-transcriptional regulation. One of the prime movers of recent and continuing genome-wide insights is CLIP-seq (cross-linking with immunoprecipitation followed by high-throughput sequencing), which allows a high-resolution investigation of the binding sites for a given RBP. Three variants of CLIP-seq have been developed: high-throughput sequencing CLIP, termed HITS-CLIP (3); Photoactivatable-ribonucleoside-enhanced CLIP, termed PAR-CLIP (8); and individual nucleotide resolution CLIP-seq, termed iCLIP (9). Each of these CLIP-seq variants allows target mRNAs to be identified, but importantly the resolution is high enough to localize binding to within a small window. Beyond this, statistical models are needed to exactly localize the binding site. Even with iCLIP, which inherently gives single-nucleotide resolution for the cross-link location, background noise and sequencing artifacts will mean that not every locus identified by the assay is a bona fide binding site, nor will the localization always be perfect, as cross-linking biases are well known (10,11).

In addressing the problem of simultaneous binding site characterization and localization, much of the analysis methodology has been borrowed from the field of transcription factors, for example from the analysis of sequences identified in ChIP-seq experiments. Binding sites for RBPs though are different from transcription factor binding sites: they tend to be shorter, and have characteristic secondary structures (12). Most existing motif-discovery methods concentrate exclusively on primary sequence, but the shorter length of RBP binding sites, coupled with the abundance of highly similar non-binding sites and the proclivity toward low sequence specificity in some RBPs present difficulties for such approaches. Despite mounting evidence that RNA secondary structure plays a role in RBP binding site selection, few motif discovery tools consider it (13,14).

The CLIP-seq experimental procedure causes sequencing reads to exhibit characteristic substitutions and deletions relative to the reference genome at the cross-link location. We call these substitutions and deletions diagnostic events (DEs). (8,10,15,16). Although the cross-link location is not...
necessarily within the binding site, it is generally in close proximity. These properties of CLIP-seq reads have been employed to localize the cross-linking location (17,18), but their distribution relative to the binding site has not previously been exploited for motif-discovery in a probabilistic model, and tools using them have been restricted to individual variants of CLIP-seq.

Here, we describe the Zagros algorithm for simultaneous motif characterization and binding site localization from CLIP-seq data that uses a model specifically designed for CLIP-seq derived RNA binding sites. Our method models sequence, secondary structure and technology-specific cross-linking events using a formalization where we treat the locations of motif occurrences as missing data. Zagros is the first motif-discovery method that is able to exploit all of the additional information inherent in CLIP-seq data of any variety, and we demonstrate that this extra information has utility in successfully recovering RBP motifs in CLIP-seq data. Zagros source code licensed under the GNU General Public License (version 3) is freely available for download from http://smithlabresearch.org.

Using our method, we show that motifs enriched in CLIP-seq datasets represent a trade-off between sequence and structure specificity, suggesting that RBPs with highly specific sequence motifs require less structural constraints on their binding sites to achieve their specificity.

MATERIALS AND METHODS

Data sets

To investigate the properties of CLIP-seq, the sequence and secondary structure preference of a range of RBPs, and to evaluate our proposed method we collected a set of public data derived from 20 studies, covering iCLIP (9,10,19–21), HITS-CLIP (22–32) and PAR-CLIP (8,33–35). This collection constitutes data profiling 40 RBPs (36 human and 4 mouse). A complete description is provided in supplementary materials. Sequence data was mapped to hg19 and mm9 using Novoalign (Novocraft, http://www.novocraft.com). Definitions of genes, exons and UTRs were taken from RefSeq (36).

Defining sets of target 3'UTRs

To investigate the structure preferences of hexamers in RBP targets, for each CLIP-seq dataset we defined a set of target 3' UTRs. To do this, we binned CLIP-seq reads in 1nt bins (iCLIP) or 20nt bins (PAR-CLIP, HITS-CLIP), and retained only those bins that could be uniquely assigned to a single transcript. For each 3' UTR we found the bin with the largest number of reads. We then ranked 3' UTRs by the count of reads in the bin with the most reads, and selected the top 1000 3' UTRs as our target set for each RBP. The non-target set is simply any 3' UTR region (as defined by refseq) that is not contained in the target set.

Calculating secondary structure

In our model we represent secondary structure using base pairing probabilities using McCaskill's algorithm (37). These probabilities are then input either with just the sequences, or with both the sequences and the cross-link modification events, and are considered part of the data. Our model includes parameters for the base-pair-probability of each position within the motif; these parameters are learned from the data using expectation maximization.

Simulated data

Each simulated dataset was produced by randomly selecting 500 segments, each of length 50 bp, from human 3' UTRs (RefSeq), and planting within each a motif occurrence from a randomly generated position weight matrix with an information content of approximately 0.5 bits per column. Structure was imposed by taking a short segment upstream of the placed motif and planting its reverse complement downstream of the motif, forming a hairpin loop. The offset of diagnostic events from the start of the planted motif was simulated (once per dataset) as a uniform random variable on the range $-8$ to $+8$. The number of diagnostic events placed for a motif occurrence, where diagnostic events were present, was sampled from the empirical distribution observed in the same 50 bp window of the same 3' UTR in CLIP-seq datasets. A more detailed description of the simulation procedure is given in supplementary methods. In total we simulated 2200 datasets. Of these, 1100 have no particular structure imposed on the motif occurrence, but have a DE-fraction ranging from 0 (no sequences with diagnostic events) to 1 (all sequences having diagnostic events), with a step of 0.1 and 100 simulations for each level. The remaining 1100 have no diagnostic events, but a structure-fraction ranging from 0 (no sequences have motif occurrences with any particular structure imposed on them) to 1 (the motif occurrence in every sequence is forced to adopt a ssRNA conformation), with a step size of 0.1 and 100 simulations for each level.

Recovered PWMs were compared to the simulated PWM by calculating the Kullback–Leibler divergence of the recovered PWM from the simulated one. We estimated a false discovery threshold for KLD by randomly shuffling the recovered and simulated motifs, and considered a recovered motif to match the simulated motif if $P < 0.05$.

CLIP-seq derived data

To evaluate the performance of Zagros on recovering previously reported RBP binding motifs from CLIP-seq data, we defined a set of CLIP-seq target sequences for each dataset. As with the simulated data, these sequences were each 50bp in length, and we selected 500 sequences per datasets. To select these sequences, we binned CLIP-seq reads for each dataset into 50 bp bins, and selected the top 500 bins with the greatest number of CLIP reads.

Sequence vs. structure specificity

We ran zagros using both structure and diagnostic events. We used a motif length of 6 for all datasets to avoid any biases in counting oligomer occurrences that would be introduced by varying lengths. We retained only those datasets
where the recovered motif contained a match to the previously reported consensus. Base-pair probabilities for each recovered motif are the average across all positions in the motif.

RESULTS AND DISCUSSION

CLIP-seq data encodes RBP-specific sequence and structure signals

Prior to developing our model, we sought to investigate the interaction of sequence and secondary structure in RBP targets identified from CLIP-seq data. Since structure is inherently a property of sequence, our purpose here was to determine to what extent the local sequence of the binding site directly informs its structure. If structure can trivially be inferred from the sequence of the motif, then explicitly modeling it is unlikely to be beneficial in motif-discovery. Conversely, if the interaction between sequence and structure in CLIP-seq targets is identifiably different from non-targets, then leveraging this may improve motif-discovery.

Earlier work has shown that matches to a known consensus sequences for an RBP in target genes derived from RIP-Chip show different RNA structure to those in non-target genes (13). We extended this by determining the structural bias of all hexamers in CLIP-seq target sets, while remaining agnostic of what the RBP binding motif was. To do this, we selected a set of target 3' UTRs for each RBP, and counted the number of times each hexamer occurred in the target set in single- or double-stranded conformation, and also for non-targets. We computed an odds-ratio for all hexamers: the odds of the hexamer being single-stranded in target 3' UTRs (target structure) against the odds of it being single-stranded in non-target 3' UTRs (background structure). In each dataset, for each hexamer/ratio, we calculated a P-value using Fisher’s exact test, where our null hypothesis is that the odds-ratio does not significantly deviate from 1. Figure 1A shows the results for three example RBPs, HuR, IGF2BP1, and TIAL1, with hexamers that show the most strongly significant deviation from their background structure being close matches to previously reported sequence motifs for these proteins (8,20,38–40). To determine whether this trend was present in a range of CLIP-seq datasets, we calculated P-values in this fashion for all hexamers across all CLIP-seq datasets. From these, we determined the frequency of P-values (Figure 1B). As a control, we also did this using randomly selected 3' UTRs. We noted an enrichment of significant P-values when using CLIP-derived target 3' UTRs which was not present with randomly selected 3' UTRs, suggesting that targets identified by CLIP-seq are enriched for structural motifs, as well as sequence motifs.

CLIP-seq diagnostic events follow RBP- and technology-specific patterns

CLIP-seq has several stages, from UV irradiation to high-throughput sequencing of the short isolated RNA segments. Of the three CLIP-seq variants, only iCLIP was designed to have single-nucleotide resolution, achieved through read truncation at the cross-link location (9). Still, single-nucleotide resolution has been achieved in PAR-CLIP and HITS-CLIP by exploiting characteristic deletions and mutations caused by cross-linking (17,18). The reverse transcriptase used in HITS-CLIP sometimes skips the crosslinked RNA nucleotide bound to the protein residue, resulting in a nucleotide deletion, while in PAR-CLIP, 4sU
labeled transcripts sometimes induce T → C conversions in the sequencing step, identifiable as mismatches to the reference genome when mapping. We refer to these deletions, mutations and truncations collectively as ‘diagnostic events’ (DEs). The relative prevalence of each type of event varies by technology (see Figure 2A).

HITS-CLIP and iCLIP have similar mapping rates, however only 8–20% of the mapped reads in HITS-CLIP have a deletion at the cross-link site, whereas for iCLIP around 99% of mapped reads appear to have been truncated at the cross-link site. More specifically, in 99% of the reads, we did not find any deletions, which can either mean that reverse-transcriptase has read through the cross-link location with no deletion or it has been halted at the cross-link location. Only in the latter case the read in fact contains the diagnostic event. Sugimoto et al. (10) estimated that 82% of the reads are truncated at the cross-link location, but for simplicity, Zagros considers all of the reads without deletions to have a diagnostic event at the truncation site. For more information on diagnostic events on iCLIP data refer to supplementary information, Section 2.9. PAR-CLIP has a higher percentage of reads containing diagnostic events than HITS-CLIP, but suffers lower mapping rates than HITS-CLIP and iCLIP. Since DEs for iCLIP are identified as the 5’ mapping location, in the absence of a deletion, each read can have at most one DE. For HITS-CLIP and PAR-CLIP, it is possible that sequencing errors or even multiple actual cross-links could lead to multiple potential DEs per sequence. Fortunately for our analyses, usually only one DE can be found (Figure 2B). In order to study the distribution of diagnostic events around binding sites, we searched for occurrences of simple consensus sequences, defined from literature (see supplementary materials), around CLIP sites identified for each RBP in our data. We observed that diagnostic events are often enriched at a particular distance from the consensus match, and follow a characteristic distribution. We noted that distance from the start of the consensus match to the most likely position of a diagnostic event is specific to the RBP (Figure 2C). Efforts to model CLIP-seq diagnostic events must account for the variability imparted by RBP and technology.

A probabilistic model of sequence, structure and diagnostic events

Our model of sequence and structure builds upon the widely used classic mixture model introduced by Lawrence and Reilley (41) with many notable extensions (42). Let S be a set of n sequences such that each member $S_i \in S$ has the same length m. Define the set $X = \{X_1, \ldots, X_n\}$ of motif occurrence indicators in correspondence with S so that $X_{ij} = 1$ exactly when a motif occurrence starts at position $j$ in $S_i$. We use the “zero-or-one occurrence per sequence” assumption (ZOOPS), so $\|X_i\|_1 \in \{0, 1\}$, as used in the well-known MEME program (43). We augment this data with secondary structure indicators $T = \{T_1, \ldots, T_n\}$, with $T_{ij}$ indicating that position $j$ of $S_i$ has a paired structural state, which implicitly assumes a single underlying secondary structure for each sequence. In explaining our model, we assume a fixed motif width $w$ and use the notation $S_i(\{X\})$ to denote the $w$ consecutive positions of $S_i$ beginning at the unique position $j$ such that $X_{ij} = 1$; if $\|X_i\|_1 = 0$, then $S_i(\{X\})$ is empty. We also define $S_i(\{\overline{X}\})$ as the concatenated positions of $S_i$ not in $S_i(\{X\})$.

We augment the usual motif model $M = (\{M_i\})_{i=1}^w$ and background model $f$ to account for secondary structure: $f$ and the $M_i$ are multinomial distributions over $\{A, C, G, U\} \times \{\text{paired}, \text{unpaired}\}$, as explained in detail in supplementary methods. Then

$$\Pr(S, T, X | M, f) = \prod_{i=1}^n \Pr(S_i, T_i, X_i | M, f) \times \prod_{i=1}^n \Pr(S_i(\{X\}), T_i(\{X\}) | f, X, O_i),$$

where $O_i = 1$ if sequence $i$ contains a motif occurrence and 0 otherwise. When dealing only with sequence and structure, the prior $\Pr(X | M, f, O)$ is uniform, and can be disregarded. For any sequence $s$ having structural indicators $t_i$, $\Pr(s, t | M) = \prod_{k=1}^w M_k(s_k, t_k)$ and

$$\Pr(s, t | f) = \prod_{i=1}^w f(s_k, t_k).$$

Next we make an additional augmentation to the model to account for cross-linking. We assume each sequence contains one cross-link location. We estimate the probabilities of where the cross linking is located using diagnostic events as follows:

$$\Pr(C_i = l | O_i = 1, D) = \frac{D_{ij} + \epsilon}{\sum_{j=1}^m D_{ij} + \epsilon},$$

where $D_{ij}$ is the count of diagnostic events at location $j$ in sequence $i$ (we treat $D$ as a fixed model parameter), $C_i$ is the location of the cross-link in sequence $i$, and $\epsilon$ is a pseudo-count to avoid zero-counts.

We bring information about diagnostic events in via the prior on motif occurrences, $\Pr(X | M, f, O, D, g)$, where $g = \{g_1, g_2\}$ models the distance between cross-link site and motif occurrences, as

$$\Pr(X | O, D, g) = \prod_{i=1}^n \prod_{j=1}^{m-w+1} \Pr(X_{ij} = 1 | O_i = 1, D, g) X_{ij},$$

where

$$\Pr(X_{ij} = 1 | O_i = 1, D, g) = \sum_{l=1}^m \Pr(C_i = l | O_i = 1, D) \times [g_1(1 - g_2)^{(j-l+g_2)}]^{K}_.$$
density of diagnostic events moving away from what we believe to be true binding sites in many of the higher quality data sets. This also makes sense conceptually in iCLIP, since the rate parameter can be viewed as the probability that the truncation will mistakenly occur one base too soon or too late.

Our exploration of CLIP-seq data highlighted the fact that cross-linking is often quite noisy. To balance the impact of CLIP-seq cross-link events against sequence and structure information, we include the parameter $K$ – a tuning parameter that modulates the impact of diagnostic events on the algorithm. Low values (near 0) reduce the impact of diagnostic events, while higher values increase it. For all results reported here, we fixed this at 1.1 (see supplementary methods). Hence, in using a restricted partition function algorithm, we need only recompute expected values for $T$ using a restricted partition function algorithm (44), but doing so would be computationally prohibitive.

The contribution of diagnostic events, given the motif locations is independent of the sequences $S$ and secondary structure indicators $T$, as well as the model parameters $M$ and $f$. This makes it easy to decompose the estimation procedure into steps involving $S$ and $T$ and other steps involving $D$. This has intuitive appeal: if every observed diagnostic event corresponded to a functional binding event, and if every functional binding event resulted in a diagnostic event, then we should be able to estimate the binding site indicators $X$ using data from $D$ alone, only involving the parameters $g$.

Use of RNA secondary structure and diagnostic events improves motif discovery in simulated data

To examine the extent to which our expanded model improves motif recovery, we produced a set of simulated datasets. For each simulation we generated a random position weight matrix of length six, and used this to plant occurrences into 500 sequences of length 50nt, that were randomly selected from human 3’ UTRs. For equal proportions of the datasets, we fixed the secondary structure of the occurrence to be single-stranded in 100% of the occurrences, 90% of the occurrences and so on down to 0% of the occurrences (we call this the structure-fraction of the dataset). We also simulated diagnostic events with distance geometrically distributed around some offset from the motif occurrence. This offset is fixed for each dataset, but varies uniformly at random in all dataset on the range ± 8nt. Similar to the structure, for equal proportions of the datasets, we planted these diagnostic events for 100% of the motif occurrences, 90% of the occurrences and so on down to 0% of the occurrences (we call this the DE-fraction of the dataset). Further details of the simulation process are given in ‘Materials and Methods’ section.

For each simulated dataset we ran Zagros in four different ways: with just the simulated sequences; with the sequences and base-pair probabilities (RNA secondary structure); with the sequences and diagnostic event locations; and with the sequences, structure, and diagnostic events. Although Zagros can be run in these four different modes, when we refer simply to Zagros, we mean the version us-
occurrences with diagnostic events increases (Figure 3B). It
deviations in performance are observed as the fraction of motif
is possible to increase the impact of diagnostic events by in-
structural information is provided also increases, while it re-
For each fraction of occurrences with planted motifs, we simulated 500 random datasets. Error-bars are 95% confidence interval from 1000 bootstrap samples. (B) As in panel (A), but as a function of the fraction of planted motif occurrences that have one or more diagnostic events. Zagros was run either with just sequence data, or sequence and diagnostic events data. (C) Comparison with other methods.

Zagros uncovers a potential link between sequence- and structure-specificity in RBP binding sites

We applied Zagros to investigate the relationship between structure and sequence specificity of RBP binding. We determined the sequence specificity of the motif reported by Zagros as the frequency with which the consensus sequence from the motif occurred in either human or mouse exons, depending on the organism for which the CLIP experiment

Zagros recovers previously validated motifs from CLIP-seq data

We compared the performance of Zagros to DME, MDSCAN and MEME on a set of datasets derived from a range of CLIP-seq experiments comprised of multiple replicates. We selected a subset of 19 RBPs from this data for which a sequence preference had previously been reported (excluding miRNA-associated RBPs, such as Ago2; list of selected datasets and details of sequence selection provided in supplementary materials). We report results for those replicates where at least one of the tested methods was able to recover the expected motif. When one of the algorithms designed for transcription factor binding sites finds the reverse compliment of a motif, we count that as a success, since they could easily be modified to be strand specific. Figure 4 shows twelve example datasets and the top-scoring motif recovered for each dataset; the previously described binding site for each is also shown. Note that in all the examples, the match to the previously described site is obtained by Zagros using all three of sequence, structure and diagnostic events.

Although the other tested programs sometimes recover the expected motif, Zagros achieves the most consistent recovery. In some cases, it is clear that just the addition of structure is sufficient, such as with HuR. In other cases, such as for the IGF2BP proteins and hnRNPC, Zagros achieves a close match to the expected motif only through the use of both structure and diagnostic events. In general, the combination of both extra pieces of information gives the best result. Logos for all tested datasets are provided in Supplementary Figures S1–S5.
was conducted. Consensus sequences that occur more frequently are considered to have low-specificity, while those that occurred relatively rarely were considered to have high-specificity. We then ordered RBPs by their mean specificity and plotted the mean base-pair probabilities recovered by Zagros for each (Figure 5). There was a negative correlation (Spearman's correlation coefficient: $-0.36$) between sequence specificity and mean base-pair probability. In their seminal work, Schneider et al. (45) found that binding sites tend to contain enough information for them to be recognized, but not more than is required. RBP binding sites tend to be short and degenerate, leading to generally low sequence-based information. It is reasonable to assume then that other features, such as secondary structure, contribute the necessary information to overcome the deficit and allow the sites to be recognized. Importantly though, the work of Schneider et al. indicates that (owing to random drift), the additional information contributed will not be more than is required for recognition. Our results are supportive of this.
Our proposed model brings together the sequence and structure of RBP binding sites, and augments this with technical information from the CLIP-seq assay (cross-linking induced diagnostic events). Our model, when fit using the expectation maximization algorithm, showed much improved performance in simulated datasets with structural specificity and informative diagnostic events. Moreover, we demonstrated that this approach recovers meaningful motifs from CLIP-seq datasets.

Finally, our finding of a correlation between the sequence-specificity of motifs recovered for RRM-containing RBPs (as measured by the frequency that the consensus occurs in exonic sequences) and the strength of mean base-pair probability for the motif support the hypothesis that sequence and structure function in concert to achieve RBP binding specificity.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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