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Structure Determination of Group II Introns

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Abstract

Group II introns are self-splicing catalytic RNAs that are able to excise themselves from pre-mRNAs using a mechanism identical to that utilized by the spliceosome. Both structural and phylogenetic data support the hypothesis that group II introns and the spliceosome share a common ancestor. Structures of group II introns have given insight into the active site required for the catalysis of RNA splicing. This review outlines crucial aspects of the structure determination of group II introns such as sample preparation and data processing. Given that group II introns are large RNAs that must be synthesized through *in vitro* transcription, there are special considerations that must be taken into account in terms of purification and crystallization, as compared to the isolation of large intact ribonucleoprotein complexes such as the ribosome. We specifically focus on the methodology used to determine the structure of the eukaryotic group II intron lariat from the brown algae *Pylaiella littoralis*. The techniques described in this review can also be applied for the structure determination of other large RNAs.

1. Introduction

The origin of non-coding introns has been an outstanding question in the field of evolutionary biology for many decades. Recent structural studies have shed light on this problem using x-ray crystallography and cryo-EM techniques on the group II intron [1–3] and the spliceosome [4, 5], respectively. This work revealed that the self-splicing group II intron and the spliceosome share a conserved active site architecture indicating a shared ancestry. Group II introns are self-splicing ribozymes that can catalyze their excision from pre-mRNAs without the need for any protein cofactor and are largely found in bacteria, fungi and plants. In contrast, the spliceosome is found in fungi, plants and higher eukaryotes, such as mammals. This phylogenetic distribution suggests that the spliceosome evolved from a prokaryotic group II intron ancestor. In addition, the structural homology between the two splicing systems indicates that the spliceosome is also a ribozyme with the active site solely comprised of highly conserved RNA elements [4, 5]. Despite their mechanistic and structural similarities, there are large differences in the methodology required for the structure determination of group II introns versus the spliceosome. This

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review will focus on the methodology used to determine the crystal structure of the group II intron lariat from the eukaryote *Pylaiella littoralis*. The techniques described herein can also be applied to the x-ray structure determination of other large RNAs.

2. Methods

2.1. Selection of candidate species for structure determination

Group II introns are found in many species of bacteria and fungi and can be classified into multiple phylogenetic subclasses that correlate with defined consensus structures [6]. Group II intron secondary structures can be broadly classified into three main classes called the IIA, IIB, and IIC introns. Bacterial group IIC introns are considered to be the most primitive and are highly dependent upon the encoded maturase protein to splice via the lariat pathway [7, 8]. IIA and IIB introns are largely found in eukaryotes and are therefore thought to be closer relatives to the spliceosome. Bacteria also contain “hybrid” introns that exhibit consensus structures containing features of both IIA and IIB introns, thus representing intermediate evolutionary forms [6]. Even though all three classes of group II introns have well-defined consensus structures, there can still be large deviations in size between specific introns in a class. This is due to the fact that nonconserved, solvent-exposed loops of group II introns can tolerate large insertions without affecting the ribozyme activity. As a result, some group II introns gain random insertions in these regions over the course of evolution. It is essential to select a group II intron candidate for structure determination that lacks these large insertions and conforms as much as possible to the consensus secondary structure.

Following secondary structure screening, this initial batch of group II introns are tested for *in vitro* splicing activity under different ionic conditions. *In vitro* splicing assays are done in the presence of 10 to 100 mM magnesium chloride and monovalent ions varied 100 to 500 mM. The identity of the monovalent ion can also have highly variable effects upon splicing activity in different group II introns. The intron exhibiting the highest levels of splicing activity (as evidenced by lariat formation) at the lowest magnesium concentration is selected for structure determination. High activity under low magnesium is preferable as nucleic acid screens require variation of magnesium concentration to sample crystallization space. For example, a group II intron having an obligate requirement for high magnesium (>30 mM) would greatly reduce the chances of obtaining hits in a crystallization screen.

2.2. Construct variation for crystallization

It was first reported by Scott et al. (1995) that the successful crystallization and structure determination of the hammerhead ribozyme was highly dependent on the screening of multiple constructs rather than a large number of crystallization conditions [9]. The engineering of tetraloops into structured RNAs to favor the formation of crystal contacts has been successfully employed for the group I intron [10], the group II intron domain V and VI construct [11, 12], the U4 snRNP core domain [13], and RNase P [14].

The U1A-binding aptamer has also been engineered into constructs to provide a platform for binding the small, positively charged 10kDa spliceosomal U1A protein [15, 16]. This favors the formation of high-resolution lattice contacts through RNA-protein interactions. The

attached protein provides an opportunity to form unique crystal contacts through the more diverse chemical functionality found in amino acid side chains relative to RNA nucleotides. However, there may be an upper limit as to the size of the RNA that can be determined using the U1A approach. We have attempted using this approach with group II introns (400–650 nucleotides) and were unable to generate crystal hits.

We have also found construct variation to be essential for group II intron structure determination. For the eukaryotic group II intron lariat (622-nucleotides), over 200 constructs were screened against the 48-condition Natrix Screen (Hampton Research) to obtain well-diffracting crystals. We have tested a wide variety of screens and have found Natrix to be the most successful for large RNAs. It should be also noted that the Natrix screen is derived from Scott et al. (1995).

One of the major concerns when varying the sequence of an RNA target for crystallization is the requirement that the mutations not affect the intrinsic biological activity. In the case of group II introns, this is accomplished by analyzing the sequence conservation in the consensus secondary structure and modifying only regions that lack conservation among all the members of the structural class. Figure 1 shows the secondary structure of the group IIB intron from the brown algae *Pylaiella littoralis* (*P.li.LSUI2* intron). The solvent-exposed and non-conserved regions of the secondary structure are capped with either stable UNCG or GNRA tetraloops. This promotes crystallization by: 1) reducing the flexibility of these extraneous sequences that would inhibit crystallization; and 2) in the case of GNRA tetraloops, adding a potential intermolecular binding site for crystal contact formation. An engineered GNRA tetraloop can be seen forming an essential lattice contact in the crystal structure of the eukaryotic group II intron lariat (Figure 2). The position of this GNRA tetraloop is varied in different constructs by placing it in domains IIa, III, or IV. All sequence variants of the GNRA sequence are tested to increase the chances of the tetraloop docking into a neighboring molecule in the lattice. This results in a many possible permutations of constructs. We were able to generate many crystal forms with different space groups and unit cell dimensions (Figure 3). However, only one of these crystal forms diffracted to 3.7 Å, which resulted in the crystal structure of the *P.li.LSUI2* intron.

2.3. *In vitro* transcription

RNAs are synthesized through *in vitro* run-off transcription using T7 RNA polymerase, using linearized plasmid DNA as templates. A T7 promoter directly precedes the group II intron sequence with a restriction enzyme site located at the end for linearization. This construct is cloned into a pUC57 plasmid, which is then transformed into DH5α cells. Plasmid DNA is isolated and purified using maxipreps (Sigma-Aldrich), followed by restriction enzyme digestion to linearize the template. One to eight mL of *in vitro* transcription reaction is prepared by mixing 10× transcription buffer, nucleotide triphosphates (NTPs), template DNA, T7 RNA polymerase, inorganic pyrophosphatase (ThermoFisher), and Triton X-100. *In vitro* transcription reactions are incubated at 37°C for 3 hours to overnight. Some group II introns require the addition of supplemental magnesium, after the transcription reaction is complete, to initiate splicing.

2.4. Non-denaturing purification

In our experience, large RNAs that are purified by denaturing methods and refolded afterwards do not crystallize as readily as RNAs purified using non-denaturing methods. This is likely due to the fact that refolding protocols can result in some fraction of the RNA being misfolded. In addition, the requirement for the testing of many constructs requires high-throughput purification, which is not possible with the standard procedure using denaturing polyacrylamide gels to isolate RNA. We instead utilize a non-denaturing purification procedure that was first developed and used for the structure determination of the bacterial group IIC intron [2]. This involves the sequential digestion of the completed transcription reactions with Turbo DNase (Ambion) and Proteinase K (ThermoFisher) to remove DNA and proteins, respectively. Inorganic pyrophosphate precipitate is removed by centrifugation and the supernatant (containing RNA) is passed through a 0.2 μ m syringe filter. The RNA is then buffer exchanged through a 100 kDa cut-off Amicon Ultra centrifugal filter to remove NTPs and proteinase K. On the same centrifugal filter, the buffer is exchanged six times to RNA storage buffer (5 mM sodium cacodylate, pH 6.5 and 10 mM magnesium chloride) and the RNA is concentrated. Most RNAs are stable and soluble at >10 mg/ml concentration. However, we have found that some RNAs form irreversible hydrogels or white precipitates at high concentrations. Although this aggregation can sometimes be ameliorated by changing the ionic conditions of the storage buffer, it usually requires testing other constructs or introns exhibiting better solubility. We store purified group II intron RNA at room temperature and find that it is stable for at least six months as evidenced by the formation of crystals and gel analysis to verify the lack of degradation. Folded RNAs should not be frozen as it leads to denaturation and misfolding of RNA.

2.5. Crystallization setup

We have found that Natrix (Hampton Research) is the most successful crystallization screen for large RNAs. The lower concentration of MPD and PEGs utilized in Natrix prevent the excessive precipitation we have observed in other screens. We have also tested Natrix 2, Crystal Screen 1 and 2, and Index Screen (all from Hampton Research) and have found excessive precipitation with large RNAs. Natrix 2 is a nucleic acid-specific screen, however it is biased towards the crystallization of smaller RNAs, as evidenced by the higher concentration of precipitants relative to the original Natrix formulation.

Crystal trays are set by mixing 0.4 μ l of each condition with 0.4 μ l of 0.5 mM spermine and 0.4 μ l of 10 mg/ml RNA in a 96-well sitting-drop crystallization plate (Hampton Research) with 100 μ l of each condition in the well. The trays are incubated at constant temperature for at least a week before checking for crystal hits using a light stereomicroscope. If a given construct does not yield any hits in the initial screening, we do not set additional crystallization drops for this sequence. Instead, we further vary the construct and screen again using Natrix.

It is important to screen at both 22°C and 30°C. Screening at these two temperatures results in hits in different conditions and can generate new crystal forms. In general, hits are obtained much more quickly at 30°C compared to 22°C. This is a pattern that was seen for the majority of the crystallization constructs. Repeated attempts at screening at 4°C yielded

unsatisfactory results with far fewer hits and greatly extended growth times on the order of months. We have found that screening at temperatures below 22°C provides no advantages for the crystallization of large RNAs.

2.6. X-ray data collection and screening

Crystals are stabilized and cryoprotected by gradually increasing the precipitant concentration in the drop before looping and freezing in liquid nitrogen. We have found that 30% ethylene glycol is an effective and gentle cryoprotectant for all PEG conditions found in Natrrix. For conditions containing lithium sulfate and ammonium sulfate, we use saturated lithium sulfate as a cryoprotectant. All alcohol-containing conditions can be supplemented with 30% MPD for cryoprotection.

Crystals of large RNAs must first be screened for diffraction before data collection. High intensity x-rays are needed at the screening stage as it enables visualization of weak, high-resolution diffraction spots from very small crystal hits. As a result, undulator beamlines at synchrotrons are preferable as they provide the high intensity and small beam sizes that are ideal for small, weakly diffracting RNA crystals. As an example, the first crystallization hits obtained for the *P.li.LSUI2* intron were a mass of microcrystals and did not exhibit any visible high resolution spots using 20% beam intensity at the undulator beamline at NE-CAT 24-IDC (Argonne National Laboratory). However, increasing the intensity to 100% revealed faint and diffuse diffraction spots to 4.5 Å. As a result, this crystal form was targeted for optimization and resulted in the 3.7 Å structure of the group II intron lariat.

The next stage after diffraction optimization is to obtain phasing data to calculate experimental electron density to allow building of an initial model. This is done through heavy atom soaks and collection of anomalous datasets at multiple wavelengths (peak, inflection, and remote wavelengths). Ytterbium is especially effective for the phasing of group II intron structures as it has an especially strong anomalous signal and usually requires only the peak wavelength to obtain experimental density of high quality for large RNAs [1, 2]. For both the group IIB and IIC intron crystal structures, Yb³⁺ was found to bind with high occupancy only to the active site, which contains highly coordinated Mg²⁺ ions for catalysis [1, 2]. Yb³⁺ has similar octahedral coordination geometry as Mg²⁺, however it only seems to displace Mg²⁺ ions from binding sites where the majority of coordination sites are occupied by RNA ligands. Yb³⁺ also specifically binds the active site of the evolutionarily unrelated group I intron, which also employs a two-metal-ion mechanism of catalysis [17, 18]. Therefore, Yb³⁺ can not only assist with phasing, but is also likely to highlight Mg²⁺ ions in RNA that have significant biochemical and/or structural roles.

Minimization of radiation damage was also essential during anomalous data collection for the structure determination of the eukaryotic group II intron lariat. Anomalous datasets collected from a single position on the crystal yielded experimental maps that were difficult to interpret even though the diffraction did not appear to exhibit radiation damage based on the spot profiles. This hurdle was overcome through the use of a helical data collection strategy in which the beam is moved horizontally along the crystal to spread the radiation dose along the length of the rod-shaped crystal [19]. This data collection technique allowed

the collection of an Yb³⁺ anomalous dataset that resulted in an experimental density map suitable for model building of the *P.li.LSUI2* intron.

2.7. Modeling and structure refinement

The modeling was first initiated in the A-form helical regions with the most easily interpretable regions of the experimental density map. The phylogenetically predicted secondary structure was essential in determining the lengths of A-form helices that were fit into the density using rigid body refinement within COOT[20, 21]. As the density improved with further refinement, it became possible to model regions containing non-canonical base pairs and junctions. Modeling of RNA nucleotides in these regions was done using COOT with the RCrane plugin [22, 23]. Specifically, the rotamer library built into RCrane greatly accelerated the model building process. RCrane allows the user to select from a variety of rotamers for a specific nucleotide position. The displayed rotamers are consistent with the user-defined positions of the nucleobase and phosphate, which are based on a visual inspection of the density. Typically, the base and phosphate are quite prominent features of RNA density maps and can be easily identified.

The initial experimental map only allowed for the placement of ~60% of the *P.li.LSUI2* intron sequence with high confidence. Density for the rest of the molecule remained difficult to decipher. We used a process of iterative molecular replacement-single-wavelength anomalous dispersion (MR-SAD) using the model based on the initial experimental map derived from the Yb peak anomalous dataset. After many iterations of manual modeling followed by MR-SAD, this resulted in a drastic improvement of density for the remainder of the molecule and allowed structure refinement to be initiated.

Most crystal structures of large RNAs are at a moderate resolution of ~3–4 Å. This presents a challenge for modeling individual nucleotides at this resolution. We have found that careful analysis of F_o-F_c difference maps is especially important in this case. Modeling of the *P.li.LSUI2* intron was greatly aided by the fact that it was possible to differentiate pyrimidines from purines at 3.7 Å through inspection of the F_o-F_c difference map. For example, a purine modeled as a pyrimidine resulted in a positive peak emanating from the nucleobase. In contrast, this difference was difficult to determine from the 2F_o-F_c map alone. As a result, this allowed modeling of the catalytic core of the intron with high confidence and also eliminated all of the significant positive and negative peaks in the F_o-F_c difference map. It should be added that it is not possible to determine the difference between pyrimidines and purines using the experimental density map at this resolution. One must also keep in mind that the F_o-F_c difference map can contain some bias. Nonetheless, we still found that it served as an excellent guide in the model building and refinement process, in terms of the identification of sequence register.

Initial rounds of refinement resulted in relatively high R_{free} values that were difficult to improve using standard structure refinement techniques. Deformable elastic network (DEN) refinement [24] was found to be essential in significantly improving the R_{work}/R_{free} values. DEN refinement was designed to significantly improve refinement of low resolution structures; given the improvement observed with the *P.li.LSUI2* intron, it is also very effective at moderate resolution as well. The final R_{free} for this structure was 27.4%.

3. Conclusions

This review discusses some of the major challenges in the structure determination of the 622-nucleotide group II intron from *Pylaiella littoralis*. This workflow for this project is shown in Figure 4. The most time-consuming component of this methodology is generating a crystal form that diffracts to high resolution. It is essential to start with a structured RNA target that lacks large single-stranded regions as this flexibility prevents crystallization and causes solubility problems. It has been our experience that flexible RNAs are much more likely to form hydrogels during the concentration step of the protocol.

This review focused on structure determination of large RNAs using x-ray crystallography; however cryo-electron microscopy (cryo-EM) has recently undergone a revolution with the advent of direct electron detectors. For example, cryo-EM has been used to solve the atomic models of the ribosome [25, 26] and the spliceosome [4, 5, 27–31]. It is likely that the techniques regarding sample preparation and solubility screening described in this review could be applied to the structure determination of large RNAs using cryo-EM as well.

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Highlights

- sub-millisecond imaging of random motion of fluorophores in live cells
- using diffusion coefficients as imaging parameters
- mapping protein-protein interactions in live cells

Figure 1. Secondary structure of the *P.li.LSUI2* group II intron from the brown algae *Pylaiella littoralis*. Highlighted in red are nonconserved loops that were truncated and capped with tetraloops for crystallization.

Figure 2.

A portion of the crystal lattice from the structure of the eukaryotic group II intron lariat (PDB: 4R0D). Circled in black and highlighted in red are engineered tetraloops in domain II forming crystal contacts with neighboring molecules.

Figure 3.

Images of crystals of the *P.li.LSUI2* group II intron obtained from the construct variation procedure. The crystals depicted in this figure either diffracted to low resolution or did not exhibit any diffraction. Therefore, determining the specific space group of these crystals was not possible due to an insufficient number of diffraction spots. Other crystal forms diffracting to higher resolution could be indexed in different space groups. The crystals range in size from 50 to 500 μm .

Figure 4.
Overall workflow for the crystallographic structure determination of a group II intron.