Bio-Physical Studies of Modified Ribosomal RNAs

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Our group is interested in the intersubunit bridge region of the ribosome, which involves interactions between the 16S and 23S rRNAs. The 16S rRNA makes contacts with 23S rRNA through the A site, which is also an important site for tRNA binding and a known target site for aminoglycoside antibiotics. The 23S rRNA contacts 16S rRNA through helix 69 (H69), which also interacts with the A- and the P-site tRNAs. H69 is a dynamic region of the ribosome and contains conserved pseudouridine residues at positions 1911, 1915, and 1917. In order to better understand the role of the modified nucleosides, pseudouridine and its analogues, 3-methylpseudouridine and 3-methyluridine, were synthesized and incorporated into RNA at specific locations. The stabilities and structures of the RNAs were examined by using thermal melting, circular dichroism, and NMR spectroscopy. We are also working on the pH effects on the structure of loop region and contribution of pseudouridine towards the pH-dependent stability.

Keywords: Pseudouridine, Melt curves, Circular Dichroism

Identification of novel protein-protein interactions with Arabidopsis FIP homologue using phage display

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Polyadenylation of messenger RNAs is a complex and well-coordinated process involving a number of protein factors. Identification of protein-protein interactions involved in this process is key to understanding of this major event in 3'end formation of mRNAs. FIP (Factor Interacting with Poly(A) polymerase) is one such factor serving as an important bridge between many cleavage and polyadenylation factors including poly(A) polymerase.

The Arabidopsis thaliana homologue of FIP seems to interact with both At homologues of cleavage and polyadenylation specificity factor/s (CPSF30, etc.) and cleavage stimulation factor (Cstf77) in yeast two-hybrid assays. As an alternative approach to understanding of the interactions of this protein with other factors, we resorted to screening of randomized combinatorial seven amino acid peptide-library through phage display. For this purpose we made a GST-fusion protein containing 483 amino acids of N terminal end and subsequently cleaved the tag with thrombin. The tag free protein was used as a bait protein for phage display. This experiment yielded high affinity binding peptides presumably present in interacting protein partners.

One such binding peptide sequence obtained is found in At Cstf77 homologue, which had previously been shown to interact with FIP NTD in two hybrid assays. Subsequently, site directed mutagenesis of this sequence eliminated this interaction. One of the high scoring peptide was found in constans-like protein, and two other binding peptide sequences were seen at different positions of CHP Zn finger protein containing DC1 domain. Yeast two hybrid assays with these genes have shown positive interaction with FIP NTD. In vitro pull-down assays have confirmed the yeast two-hybrid data with Cstf 77 (both wt and mutant), constans- like protein and CHP Zn finger. Currently mutagenesis of probable binding peptide sequences in the latter two proteins is in progress. These results suggest that FIP might be interacting with other proteins involved in various other nuclear processes such as transcription.

Keywords: Polyadenylation, Phage display, FIP

Determination of Structural Interaction Between T Box Antiterminator-tRNA Complex: Chemo-enzymatic probing

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The Bacillus subtilis tyrS gene is a member of a family of Gram-positive bacterial genes, which are controlled by tRNA-mediated transcriptional antitermination in response to starvation for a particular amino acid. This family of Gram-positive genes comprises a large set of aminoacyl-tRNA synthetase, amino acid biosynthesis and amino acid transport genes. The T box genes have a highly structured 5'-untranslated leader region of 200-300 nucleotides, which includes a transcription terminator signal and a competing antiterminator structure. As a result of the starvation of the cognate amino acid, the resulting uncharged tRNA interacts with the leader region in two places to stabilize the antiterminator. An interaction between the 'specifier codon' and the anticodon of the tRNA is a classical codon:anticodon base-pairing and is the first of these interactions. The second interaction occurs between the acceptor end of uncharged tRNA with four residues in the bulge region in the antiterminator.. This second interaction is proposed to stabilize the antiterminator, preventing the formation of a competing terminator helix and allowing RNA polymerase to continue transcription of the structural gene. The stabilization of the antiterminator would preclude terminator formation, thus, allowing complete transcription of the mRNA. Since the secondary structure of the leader mRNA and that of the tRNA are critical to the proposed mechanism of regulation, it is of interest to determine the structure of both RNAs in complex. For these studies, chemical and enzymatic probing have been employed in the identification of the bases involved in the stabilization of the antiterminator and the conformational changes that take place in tRNA during formation of the complex with the antiterminator. T1, V1, DMS and in-line probing methods have been used. The use of these probing techniques on the complex between tRNA and AM1A have shown differences in the enzymatic cleavage and chemical modification patterns in comparison to tRNA alone. These results indicate that binding of the AM1A to the tRNA introduces structural changes in the tRNA some of which extend beyond the known interaction of base pairing with the four nucleotides at the 3' end of the tRNA acceptor stem.

Keywords: Chemo-enzymatic probing, T Box Antiterminator-tRNA complex

Zinc dependant cleavage of the RNase P RNA backbone in the presence of protein substrate

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Metal ion mapping in RNA has primarily been done through the use of lead and magnesium cleavage. However, these techniques have mainly identified metal binding sites in areas not involved in catalysis. We have now begun using the combination of zinc (II) and cobalt hexamine to allow the formation of both holoenzyme and holoenzymeopre-tRNA complexes but does not support catalysis without the addition of divalent ions such as magnesium (I) or zinc(II). We have chosen to use zinc (II) to map metal binding sites since this metal activates the cleavage activity of RNase P even thought the pKa of zinc-water is higher than that of lanthanides such as terbium. Under this new set of conditions we are able to monitor both the kinetics of the RNase P reaction and metal binding. We have mapped zinc-dependent cleavage of RNase P RNA in varying pre-tRNA concentrations, pre-tRNA leader length and time. In the RNase P holoenzyme, metal ion affinity is dependent on the length of the 5' leader, with affinity increasing as the leader length increases from one to five nucleotides in length. Zinc-dependent cleavage of RNase P RNA is observed in areas that are important for catalysis, such as the P4 helix; however, the extent of cleavage decreases upon addition of pre-tRNA. Furthermore, cleavage is inhibited to a greater extent as the length of the pre-tRNA increases up to five nucleotides, contrary to the behavior of the affinity of the catalytic metal ion. These experiments demonstrate that the addition of pre-tRNA decreases the interaction of zinc(II) ions with the 2' hydroxyl groups in P4 that lead to cleavage of pre-tRNA. These results suggest that the catalytic metal ion does not form an inner-sphere complex with (and/or deprotonate) any of the 2' hydroxyl groups in the active site of RNase P RNA. Furthermore, substrate binding protects RNase P from non-specific metal cleavage.

Keywords: RNase P, Metal binding, Catalytic RNA

Chemo-Enzymatic Probing of Antiterminator Models of Bacillus subtilis tyrS: Insights into structure-function relationship of T box transcription antitermination

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Bacillus subtilis tyrS is an example of an aminoacyl tRNA synthetase gene that is regulated by the transcription antitermination mechanism found in many Gram-positive bacterial genes related to amino acid biosynthesis, amino acid transport and tRNA synthetase. The 5' untranslated region of the mRNA can form two mutually exclusive structures, terminator and antiterminator. The terminator, a stem loop structure, is the thermodynamically more favorable structure and causes the termination of the transcription whereas the antiterminator allows the complete transcription of the gene. The antiterminator, which has a seven nucleotide bulge in between two helices (A1 and A2) is thermodynamically less stable, but is stabilized in the presence of cognate uncharged tRNA via base pairing. The tRNA anticodon interacts with the “specifier sequence” in the 5' untranslated region and the acceptor end interacts with the first four nucleotides in the bulge of the antiterminator, thus presumably adding to the thermodynamic stability of the antiterminator. Chemo-enzymatic probing of in vitro antiterminator models of Bacillus subtilis tyrS, AM1A and AM1A(C11U), was performed to understand the structure-function relationship of the interaction of the tRNA acceptor end with the antiterminator. The probing studies have revealed structural differences between antiterminator model RNAs that correlate with functional differences. Most notably, G13 and G15 in the A2 helix of AM1A and AM1A(C11U) are differentially reactive to the T1 enzyme. This difference correlates with known structural changes observed via NMR due to the C to U substitution at position 11. The same substitution resulted in a large decrease in the antitermination efficiency in vivo as well as tRNA binding affinity in vitro. The chemo-enzymatic probing of the complex hints at identification of the complex, but is consistent with fluorescence experimental data that indicate the complex is in a dynamic equilibrium. This dynamic equilibrium makes it challenging to observe the complex via standard chemo-enzymatic probing methods.

Keywords: Chemo-enzmatic probing, Anitermination mechanism

Discrimination of cognate and noncognate substrates at the active site of class II aminoacyl-tRNA synthetases

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Aminoacyl-tRNA synthetases (aaRS) are responsible for promoting the correct attachment of amino acids to their cognate tRNAs. Within the two structurally unrelated classes of aaRS, lysyl-tRNA synthetase (LysRS) is the only example known to exist in both classes. In order to better understand the role of the amino acids responsible for L-lysine binding in the active site of the class II LysRS (LysRS2), we studied the lysS encoded Escherichia coli protein. Based on the structure of L-lysine bound in the active site of E.coli LysRS2 (lysU, heat-inducible gene), several variants were generated by site-direct mutagenesis. The steady-state kinetic parameters for L-lysine and ATP in the aminoacylation reaction were determined for these LysRS2 variants. These data allowed the assignment of the specific role of the key residues in the active site of LysRS2 upon binding of L-lysyne and ATP. We previously demonstrated that LysRS2 is more susceptible to inhibition by certain noncognate amino acids than LysRS1. To further investigate the function of each residue in the active site towards the specific discrimination against noncognate amino acids and L-lysine, the KI values for S-(2-aminoethyl)-L-cysteine and 5'-O-[N-(L-lysyl)-sulfamoyl] adenosine were determined for each variant. Taken together, these data provide a clear picture of how LysRS2 binds L-lysine and discriminates against non-cognate amino acids, and also provides insight into the origin and evolution of the two forms of LysRS.

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Keywords: L-lysine, synthetase, tRNA

Towards a Comprehensive Model of the Role of the Closing Base Pair in the Stability of Nucleic Acid Hairpins

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The hairpin is the most common secondary structure adopted by RNA and is involved in folding, protein interactions, and tertiary structures. Tetraloops are the most common hairpins, and our lab has used temperature-gradient gel electrophoresis (TGGE) to identify and characterize families of stable RNA tetraloops, including the YNMG family where Y = C or U and M = C or A.(1) The YNMG studies along with others have shown that hairpins are thermodynamically coupled to their loop closing base pairs (LCBs).(1,2,3) The goal of the present study is to determine the physical and molecular origins of this coupling. Based on the functionalities presented by the coupled bases, we hypothesize that the coupling is electrostatic in origin. Preliminary nonlinear Poisson-Boltzman (NLPB) calculations and UV melting experiments performed on UUCG LCB variants support this hypothesis. Free energies from the melting experiments correlate with NLPB calculations, as loops with complementary electrostatics are more stable than their non-complementary counterparts. Preliminary UV melts on loops containing carbon-linker insertions also suggest that the coupling between the loop and LCB is electrostatic in nature. We will present our latest findings on a range of RNA hairpins that have the potential for stable interactions between the loop and LCB.

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Keywords: Hairpin, YNMG, Loop Closing Base Pair

Utilization of 4S-U to identify ribosome sites in close association with the translation initiation codon of leaderless mRNA

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Formation of translation initiation complexes on leaderless mRNA occurs in the absence of signals upstream to the 5'-terminal start codon. The specific interactions between the translation initiation region of leaderless mRNA and the prokaryotic ribosome that occur in the early stages of translation initiation have not been fully characterized. Primer extension inhibition assays (toeprints) have shown that 70S ribosomes bind leaderless mRNA more strongly than 30S subunits in vitro, suggesting a novel mechanism for the formation of translation initiation complexes on leaderless mRNAs. In the current study, bacteriophage lambda leaderless cI mRNA containing 4-thiouridine (4S-U) is used in site-specific cross-linking assays to identify leaderless mRNA-ribosome interactions during formation of the initial ternary complex with Escherichia coli ribosomes. 4S-U is a uridine derivative that has high photoreactivity with both amino acids and nucleotides and can be photoactivated by wavelengths above 300 nm. Gel shift assays have shown that a RNA 20-mer of the leaderless cI coding sequence, with 4S-U at the +2 position of the AUG start codon, binds 70S ribosomes in a tRNA-dependent manner. Photoactivation of 4S-U produced cross-links to rRNA and ribosomal and/or ribosome-associated proteins from 70S ribosomes and 30S subunits. The formation of crosslinks to proteins from 70S ribosomes was not reduced in the presence of a pre-mixed competitor RNA, suggesting that the cI-4S-U 20-mer RNA identified proteins that specifically interact with the initiation codon. An enrichment scheme has been devised to isolate crosslinked proteins from a ternary complex. Efforts are underway to identify these proteins by MALDI-TOF mass spectrometry and to identify crosslinked sites to rRNA. Identification of ribosomal proteins and rRNA positions in close association with leaderless mRNA will help characterize the interactions that contribute to translation initiation.

Keywords: Leaderless mRNA, Ribosome, Translation

Trans-cleaving hammerhead ribozymes with artificial tertiary stabilizing motifs

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Tertiary stabilizing motifs (TSMs) between terminal loops or internal bulges facilitate folding of natural hammerhead ribozymes (hRz) at physiological concentrations of divalent magnesium. However, both substrate and enzyme strands contribute nucleotides to the TSMs of trans-cleaving hRz, complicating the design of hRz that exploit TSMs to target specific mRNA. We employed two strategies to overcome this limitation. FIRST, dividing stem I of the sTRSV hRz into two segments yielded a trans-cleaving ribozyme that efficiently cleaved several small RNA substrates at MgCl2 concentrations as low as 0.2mM. SECOND, we used SELEX to identify new, artificial TSMs that are less sensitive to sequence context. Nucleotides in loop II or in a bulge within the ribozyme strand of stem I were randomized while the interaction partner was held constant. All nucleotides of the substrate pair with the ribozyme, minimizing their possible recruitment into the TSM, as such recruitment could constrain choice of candidate target sequences. Six cycles of selection identified cis-acting ribozymes that were active in 0.1 _M MgCl2. The selected motifs partially recapitulate TSMs found in natural hRz, suggesting that the natural motifs are close to optimal for their respective contexts. Ribozyme "RzB" showed enhanced thermal stability by retaining trans-cleavage activity at 80 degrees in 10 mM MgCl2 and at 70 degrees in 2 mM MgCl2. A variant of ribozyme "RzB" with a continuously paired stem 1 rapidly lost activity as temperature was increased. The selected motifs are modular, in that they permit trans-cleavage of several substrates in submillimolar MgCl2, including two substrates derived from the U5 genomic region of HIV-1. The new, artificial tertiary stabilized hRz are thus nearly independent of sequence context and enable for the first time the use of highly active hRz targeting almost any mRNA at physiologically relevant magnesium concentrations.

Keywords: hammerhead ribozyme, metal ions in RNA folding, SELEX

Preparation and fluorescence binding assays of tRNA minihelix analogs with T box antiterminator model RNA

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The T box transcriptional regulatory system found in Gram positive bacteria regulates expression of many genes that encode for aminoacyl-tRNA synthetase, as well as, amino acid biosynthesis and amino acid transport gene products. Genes regulated by this mechanism have highly conserved structural and sequence elements within the 5' untranslated region of their mRNA. The key to the regulation is the sequence specific and structurally specific interaction of uncharged cognate tRNA with the leader region of the mRNA. In the absence of tRNA, a terminator (stem-loop structure) forms in the mRNA and terminates transcription. A mutually exclusive antiterminator (bulge structure) is only formed in the presence of uncharged cognate tRNA and is stabilized by base pairing with the acceptor end of tRNA. The purpose of this study was to investigate whether minihelix analogs of tRNA could bind the antiterminator RNA with the same specificity and affinity as the full tRNA. Four minihelices were prepared to mimic the T stem loop and acceptor stem of tRNA either with or without a bulged base between the stem sequences. The resulting model tRNAs (minih-UCCA, minih-UCCA_U8, minih-ACCA and minih-ACCA_U8) were prepared by in vitro transcription and fluorescence binding assays were used to determine the binding constants to the antiterminator model RNA AM1A. The fluorescence binding assays were carried out using the prepared minihelices, 5'-fluorescein labeled AM1A and 5'-fluorescein labeled AM1A (C11U). The results indicate that the minihelix tRNA analogs bind as well as the microhelix tRNA analogs and that the bulged nucleotide between the two stems is important for maintaining functionally relevant binding specificity.

Keywords: Fluorescence, tRNA-mRNA Interaction, Minihelix

Characterization of model substrates of E. Coli Rnase P holoenzyme

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The enzyme RNase P is responsible for processing all of the tRNA precursors in the cell. RNase P is a ribonucleoprotein enzyme and thus provides an important model system for understanding how RNA and protein act together to achieve biological specificity. Although many of the interactions necessary for tRNA recognition are known, a complete understanding of how the enzyme distinguishes between cognate and non-cognate substrates is poorly understood. We and others have investigated this issue by characterizing the recognition of simplified model substrates. Previous studies have focused on analysis of model substrate recognition by the catalytic RNA subunit, however, the substrate recognition properties of the holoenzyme appear to be significantly different. Accordingly, we have examined cleavage specificity and binding thermodynamics of the E. coli RNase P holoenzyme for a series of simple helical substrates. We find that model substrates that contain the acceptor stem and T-stem portions of tRNA are bound with high affinity by the holoenzyme. However, a substrate with a 11bp helical structure is cleaved with complex biphasic kinetics and also show significant miscleavage. Interestingly, simply increasing the length of the duplex substrate to 22bp results in monophasic kinetics and cleavage only at the correct cleavage site. The rate of cleavage of the model substrate under single turnover conditions is pH sensitive, similar to native substrates, suggesting that reactions involving both classes of substrates have the same rate limiting step. Importantly, the product of the helical substrate binds much more weakly than tRNA products suggesting that interactions with tRNA structure contributes to binding affinity. Such model substrates are very useful for physical studies of the enzyme, thus characterization of their properties is likely to continue to shed light on the mechanisms by which RNase P accomplishes substrate recognition and catalysis.

Keywords: RNase P, holoenzyme, substrate

Conformational Change of Self-thiophosphorylating Ribozymes with FRET

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The Kin. 46 ribozyme can transfer the thiophosphate from ATP-gamma-S to its own 5' hydroxyl end in the presence of oligonucleotide effector. This oligomer is complementary to the 3' primer binding sequence (PBS) used in the amplification steps during the original selection for activity. The activator helix formed by the PBS and the oligo effector is connected by a 5nt "linker" region to the substrate-binding internal guide sequence and stabilizes a long-range base-paring interaction between the 5 nuclotides of the linker and those closer to the catalytic core. According to our results, the activator helix stabilizes the active conformation of the ribozyme by stabilizing the interaction between the linker and complementary nucleotides within the active site. To know the nature of the activator helix stabilization, the fluorescence resonance energy transfer (FRET) is exploited. Ribozymes derived from the Kin. 46 by internal deletions, have 3 or 4 strands and we have built Cy3- and Cy5-labeled ribozymes (single and double-labels). Some of permutations of the ribozymes are less active than without the dyes, and one is nearly dead but by reversing the locations of the dyes near wt activity comes back. They appear from native gels to fold into a single conformation. Results will be presented on the assembly and initial spectroscopic analysis of these ribozymes.

Keywords: Self-thiophosphorylating ribozyme, Cy3 and Cy5, FRET

Perturbation of cleavage site divalent metal ion affinity by helix P4 mutations in bacterial RNase Phelix P4 mutations in bacterial RNase P: Perturbation of cleavage site divalent metal ion affinity by helix P4 mutations in bacterial RNase P

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The bacterial tRNA processing enzyme ribonuclease P (RNase P) is a ribonucleoprotein composed of an RNA subunit that contains the enzyme's active site, and a protein subunit involved in substrate binding. The RNA subunit contacts the tRNA aminoacyl stem and loop while the protein subunit interacts with the 5' leader sequence. However few contacts have been defined between RNase P and functional groups at or immediately flanking the scissile phosphate, leaving an important gap in our understanding of substrate recognition and elements that compromise the enzyme's active site. Three conserved regions of the ribozyme are thought to lie in close proximity to the scissile phosphate, J5/15, J18/2, and helix P4. Cross-linking and mutagenesis studies have established an interaction between the N(-1) base of the pre-tRNA substrate and J5/15, while cross-linking and chemical protection studies have demonstrated proximity of the pre-tRNA leader sequence to conserved nucleotides in J18/2. In contrast, while helix P4 has been shown to bind divalent metal ions critical to catalytic function, there is no physical evidence demonstrating the proximity of helix P4 to the scissile phosphate. We have examined characteristics of divalent metal ion binding to the scissile phosphate in the context mutations in J5/15, J18/2 and helix P4. We find that in contrast to modest affects on cleavage site divalent metal ion affinity due to changes in J5/15 and J18/2, large effects on cleavage site divalent metal ion affinity result from mutations within helix P4. Consistent with these observations, the same mutations within helix P4 alter the apparent affinity, cooperativity and specificity of divalent metal ions required for catalysis. Taken together, these findings provide evidence for both the proximity of helix P4 to the scissile phosphate and a potential role of this universally conserved region of the ribozyme in catalytic function.

Keywords: Ribozyme, Bacterial RNase P, Catalysis

Comparative Analysis of Human and Mouse tRNA Genes

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tRNA biogenesis has been thoroughly studied in bacteria and yeast, including transcription, and amino-acylation. Very limited information is available regarding tRNA gene organization and the tRNA pathway in most eukaryotes. Preliminary information has been reported that tRNA genes in Xenopus laevis as well as Salmo salar(Atlantic salmon) and Salmo trutta (brown trout) are found in tandem repeats. Further studies have shown that Xenopus tRNA genes are clustered and developmentally regulated. In contrast, very little is known about the nature of tRNAs in mammals.

A combination of in silico developments facilitates the identification and characterization of mammalian tRNA genes and gene products. The genomes of both Homo sapiens and Mus musculus are functionally complete. Also, two programs, tRNAscan-SE and Aragorn, that employ unique algorithms are available for scanning DNA sequences for tRNA genes. tRNAscan-SE searches for base pairing of clover leaf stems, poly-T RNA polymerase III (polIII) termination sequence, and A and B boxes which are polIII transcription factor recognition sites. Aragorn scans genomes and analyzes predicted secondary structure for tRNA homologies.

Eddy and Lowe (1997) published results of tRNAscan-SE tRNA genes from the human genome. Since Aragorn (2004 Canback and Laslett) uses a different algorithm, we are using it to predict human tRNA genes and compare the results. Further, we are scanning the mouse genome with both algorithms for comparison with the human distribution. It is hoped that the prediction of gene families will facilitate experimental characterization of the tRNA pathways in mammals.

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Keywords: tRNA, Development

RNA Binding by the 30 kDa subunit of Cleavage and Polyadenylation Stimulation Factor in Arabidopsis thaliana

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The Arabidopsis thaliana homolog of mammalian Cleavage and Polyadenylation Specificity Factor, 30 kDa (CPSF 30) is an active part of the Arabidopsis polyadenylation complex. It is understood in plants that every mRNA contains 3 signals at the 3' end necessary for polyadenylation to occur. These three are: 1) the cleavage sites which is the point where precursor RNA is cleaved and adenosine residues are added, 2) the NUE (near upstream element, 6-10 base pairs long, situated 10-40 bases 5' of the cleavage site) which is a signaling element that is A rich and thought to possibly function similarly to the highly conserved AAUAAA sequence in mammals, and 3) the FUE (far upstream element, that can be as large at 100 nucleotides and lie anywhere from 13 to 100 nucleotides 5' of the NUE) which is a U-rich region with multiple UG motifs. CPSF 30 showed binding of relatively equal strength to wild type RNA (containing all polyadenylation signals) and RNA in which the NUE signal has been altered and is no longer effective; however, CPSF 30 displays decreased binding to RNA containing no active signals. This suggests CPSF 30 preferentially binds the FUE region of mRNA. RNA binding was also examined in the presence of polynucleotide competitors. CPSF 30 showed little to no binding to wild type RNA in the presence of Poly G and Poly U and reduced binding in the presence of Poly A, indicating the protein has an affinity for these poly neucleotides over any of the polyadenylation signals. The preference for Poly G and Poly U further supports the hypothesis that CPSF 30 binds at the FUE region of mRNA.

Keywords: CPSF 30, Polyadenylation, Arabidopsis

Mass Spectrometric Characterization of Pseudouridines in Ribosomal RNA

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Mass spectrometry can be used for the characterization of the post-transcriptionally modified nucleoside, pseudouridine, in RNA. Since pseudouridine is mass-silent, it has been necessary to develop appropriate experimental techniques for the direct analysis of this modified nucleoside. Our approach uses 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide (CMC) to derivatize pseudouridine residues prior to mass spectrometric analysis. In the course of these studies, we found that several groups were able to raise antibodies against CMC-derivatized oligonucleotides. We are currently developing an antibody-based affinity purification method using the existing CMC-based approach to selectively recognize and isolate pseudouridine residues in ribosomal RNA. Here, we present results from the development of this methodology and describe how such a methodology can be incorporated into our general strategy for RNA characterization by mass spectrometry.

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Keywords: mass spectrometry, pseudouridine, affinity purification

A possible role of two proteins, Imp3p and Imp4p, in the recruitment of the small subunit processome to the pre-rRNA

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Eukaryotic ribosome biogenesis is a dynamic and multistep process involving the assembly and disassembly of RNA-protein and RNA-RNA complexes. Considering the temporal complexity of this process and its importance to cell growth, co-factors are expected to regulate the formation and dissociation of key transient RNA-protein and RNA-RNA interactions. Even though RNA duplexes can form spontaneously, cells often use proteins to stimulate hybridization for various reasons: the site of hybridization is buried, the hybridization is too slow or the duplex is unstable. In this study, we focus on the formation of short duplexes between the U3 small nucleolar RNA (U3 snoRNA) and the precursor-rRNA (pre-rRNA). These hybrids are prerequisites for three endonucleolytic cleavages that initiate small ribosomal subunit biogenesis by releasing the 18S rRNA precursor from the larger pre-rRNA. The most likely role of these RNA hybrids is to guide the U3 snoRNA and its associated proteins, designated the small subunit processome [1] (SSUP), to the target cleavage sites on the pre-rRNA. We discovered using mutagenesis studies as well as binding and annealing assays that two essential proteins of the SSUP, Imp3p and Imp4p from S. cerevisiae, have the apparently unique ability to mediate duplex formation in vitro at one of the two essential U3 snoRNA-pre-rRNA base-pairing sites by stabilizing an otherwise unstable hybrid. Only one of the two proteins, Imp4p, facilitates duplex formation at the other essential base-pairing site by unfolding a conserved stem[2]. We hypothesize that the role of Imp3p and Imp4p is to help recruit the SSUP to its target, the pre-rRNA. Imp3p and Imp4p are the first proteins demonstrated to promote duplex formation for RNA-RNA interactions that are essential for ribosome biogenesis and hence for cell growth.

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Keywords: ribosome biogenesis, RNA annealing, RNA-protein complexes

Construction of an imitating nano-motor driven by six ATP-binding RNAs of bacterial virus phi29---Applications in therapy and diagnosis of cancers

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A switchable imitating DNA-packaging motor was constructed in vitro[1, 2]. The motor is driven by six synthetic ATP-binding pRNA(packaging RNA) molecules[3, 4] that bind to the connector[5] and function in a manner similar to the driving of a bolt with a hex nut[6, 7]. Conformational change and sequential action of the RNA with five-fold (viral capsid)/six-fold (pRNA hexamer) mismatch ensure continuous rotation of the motor, with ATP as energy[7].

A 5-μm DNA was packaged using this synthetic imitating motor[8, 9]. One ATP was used to translocate two bases of DNA. The DNA-filled capsids were subsequently converted into as much as 10^9 pfu/ml of infectious virus[10]. Direct observation revealed that the motor can tow a bead more than 500 nm in diameter.

The 3D structures of pRNA monomer, dimer and hexamer have been probed by photo affinity crosslinking[11-14], chemical modification interference[11,15,16], nuclease probing [14,17-19] cryo-atomic force microscopy [11, 15, 20] and computer modeling[20]. pRNA's size and shape can be controlled and manipulated at will to form stable dimers, trimers [21, 22] and arrays[22] for nanotechnological applications[22]. The motor can be turned off and turned on again[1].

The formation of ordered structural arrays of the motor complex and its components and the ease of RNA dimer, trimer, and hexamer manipulation with desired shape and size make this motor system a promising tool for use as building block for nanodevice and/or for gene delivery. Efficient inhibition of the growth of cancer or cancer cell was demonstrated in cell cultures or animal models through the use of motor nanoparticle to deliver therapeutic siRNAs and/or ribozymes to breast cancer cells, human oropharyngeal epidermoid carcinoma KB cells, leukemia model T cells, lung cancer cells, or hepatitis B virus-infected cells.

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The Role of the Nearly Invariant Histidine in the Pseudouridine Synthase TruB

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Pseudouridine synthases (Psi synthases) have been grouped into five families based upon sequence alignments. The five families share little sequence similarity to each other, but they all contain five short conserved motifs. Only one residue in motif II (Asp-48 in Escherichia coli TruB) is completely conserved among all Psi synthases. Nearly all members of the TruB family of Psi synthases have a histidine residue prior to motif II, His-43 in E. coli TruB. To examine the role of this residue, site-directed mutagenesis was used to generate H43Q, H43N, H43A, H43G, and H43F TruB. Most of the substitutions seriously impaired the enzyme, but all of the altered TruB retained significant activity. Interestingly, substitution with phenylalanine had only a minor effect. To understand the role of H43 more fully, pH dependences of wild-type, H43A and H43F TruB were determined. The wild-type enzyme displays a typical bell-shaped profile. Substitution of His-43 perturbs the pH profile, but it remains bell-shaped. The ascending limb of the pH profile is assigned to Asp-48, and the descending limb is tentatively ascribed to an active site tyrosine residue, the bound substrate uridine, or the bound product pseudouridine.

Keywords: Pseudouridine, Pseudouridine Sythnase, RNA Modification

Hu proteins disrupt polyadenylation of alternative 3' terminal exons in neurons

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Alternative RNA processing of calcitonin/calcitonin gene-related peptide (CGRP) occurs via regulation of inclusion or exclusion of an alternative 3'-terminal exon, exon 4, which is included in thyroid C cells and excluded in neuronal cells. We have learned a great deal about the non-neuronal pathway. (Lou H. et al. 1996 Genes Dev. 10(2):208, Lou H. et al. 1998 Mol Cell Biol. 18(9):4977, Lou H. et al. 1999 Mol Cell Biol. 19(1):78,and Zhu, H. et al. 2003 Mol Cell Biol 23(17) 5959.) However, regulation of the neuron-specific exon 4 exclusion remains largely unknown.

Our laboratory recently identified a group of novel RNA processing regulators in the nervous system. Hu proteins regulate the neuron-specific processing of CGRP by inhibiting the polyadenylation of the alternative terminal exon of calcitonin. These proteins bind to a U-rich sequence immediately downstream of the polyA signal of the calcitonin terminal exon and inhibit both the cleavage and polyadenylation of this exon. To investigate the underlying mechanism, the interaction between Hu proteins and polyadenylation factors were analyzed by both co-immunoprecipitation and GST/His pull down assays. Hu protein was co-immunoprecipitated with CstF 64kD in the mouse brain nuclear extract. Additionally, GST/His pull down assays were carried out using 35S-labeled in vitro translated polyadenylation factors. These experiments demonstrated a direct interaction between Hu proteins and two polyadenylation factors, CstF 64kD and CPSF 160kD, and no interaction was observed with the 68kD subunit of the cleavage factor CF Im. These data strongly suggest that Hu proteins inhibit polyadenylation of the calcitonin exon by disrupting the formation of the polyadenylation complex.

In summary, our studies have uncovered a new paradigm of regulation of alternative RNA processing in neurons.

Keywords: Hu proteins, polyadenylation, calcitonin

Synthesis and Structure of Modified and 15N-Enriched Pseudouridine RNAs

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Pseudouridine is the most abundant modified nucleoside found in nature. This C-glycoside and its variants are found in many types of ribonucleic acids including ribosomal RNAs (rRNAs). The synthesis of pseudouridine and its derivatives with 15N at specific sites will allow for NMR studies to be carried out. Such studies are important in order to better understand the structure and dynamics of modified rRNAs, such as helix 69 in the large subunit rRNA. We have completed the synthesis of [3-15N]pseudouridine using a selective deprotection strategy, and carried out the synthesis of [1,3-15N]pseudouridine by coupling 5-iodo-2,4-[1,3-5N]dimethoxypyrimidine to a protected gamma lactone. Reduction, ring-closing, and deprotection reactions lead to the final 15N-enriched pseudouridine product. Several of the intermediates in the synthesis can be used to generate 1-methylpseudouridine and 1-methyl-3-(3-amino-3-carboxypropyl)pseudouridine. Using phosphoramidite chemistry, the modified pseudouridines are then incorporated site-specifically into a variety of RNAs and used for biophysical studies.

Keywords: pseudouridine,RNA, ribosome, modified nucleosides, helix 69, dynamics

Identifying Highly Specific DNA Ligands to Escherichia coli Helix 69

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Escherichia coli (E. coli) 23S ribosomal RNA (rRNA) contains a hairpin region (helix 69 or 1920 loop) of two naturally abundant modified nucleosides, pseudouridine at positions 1911 and 1917, and a 3-methylated pseudouridine derivative of unknown function at position 1915. Interestingly, pseudouridines appear to be conserved in the ribosomes of a broad range of organisms and are proposed to have functional importance because of their presence in peptidyl transferase centers (PTC). We propose that inhibition of key biomolecular interactions at this site could lead to the development of potential drugs that may disrupt protein synthesis and/or ribosomal assembly within pathogenic bacteria. The goals of this project are to generate and characterize DNA molecules that exhibit both specificity and tight binding to helix 69. These highly specific DNA ligands, or aptamers, will be obtained by using Systematic Evolution of Ligands by Exponential Enrichment (SELEX). In this study, we report amplification of an 84-nt DNA library with a 40-nt random nucleotide region against synthesized and biotinylated hairpins (helix 69) from 23S rRNA immobilized to streptavidin-coated 96-well plate. SELEX results generated a DNA library diversity of 1E12 to 1E9 molecules. After further reduction of the diversity of the library, aptamers from selected rounds will be cloned, sequenced, and binding assays against helix 69 will be determined.

Keywords: SELEX, PCR, aptamers, modified nucleosides, helix 69, DNA and RNA, ribosome

In vitro analysis of the AGC triad of U6

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Both the spliceosome and group II introns contain a highly conserved AGC nucleotide motif. The AGC triad in group II introns has been implicated experimentally in catalysis. While AGC mutations in U6 snRNA have a devastating effect on pre-mRNA splicing, it is unclear what role the triad plays. Our previous genetic experiments show that the AGC triad base-pairs with U4 and U2 to form U4/U6 stem I and U2/U6 helix Ib, a candidate component of the catalytic core. However, some mutations in the AGC triad are refractory to suppression, suggesting the triad plays an additional, unknown role in splicing. Most notably, pyrimidine substitutions at the central nucleotide of the triad (U6-G60) are refractory to suppression. Interestingly, as the guanosine in the catalytic AGC triad of group II introns also requires a purine, the purine-dependence of U6-G60 may similarly reflect a function in catalysis.

To investigate the possible role of the AGC triad in catalysis and to stage the interactions of the triad with U2 and U4, we are analyzing the defects of AGC mutants in vitro. We found that U6-G60A, which blocks 5' splice site cleavage in wild-type extract, is suppressed when reconstituted in extract that restores U2/U6 helix Ib and U4/U6 stem I. This result indicates that formation of one or both of these structures is critical for splicing in vitro and supports our previous in vivo results. We are currently exploiting this system to stage the requirement for U2/U6 helix Ib and U4/U6 stem I formation. We are performing a similar analysis with U6-A59 mutants to understand the role of U6 in the second chemical step of splicing. Our analysis will provide insight into the requirements of the functions of the triad and address whether these functions are consistent with a catalytic role for the invariant AGC triad.

Keywords: spliceosome, snRNA, U6

Structure-Function Studies Of The T Box Regulatory System Found In Gram-Positive Bacteria

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Many Gram-positive bacterial genes are regulated by the T box transcription antitermination mechanism. The antitermination mechanism involves an RNA-RNA interaction between uncharged tRNA and the mRNA leader region of the gene transcript. The mRNA leader region of genes controlled via this mechanism contains highly conserved structural as well as sequence elements. The tRNA is recognized by at least two regions of the leader: the specifier sequence which binds the anticodon of the cognate tRNA and the antiterminator that binds the acceptor end of the uncharged tRNA. The base pairing of the tRNA acceptor end and the antiterminator (containing a seven nucleotide bulge) stabilizes the folding of the antiterminator; thus, inhibiting the formation of the more stable, mutually exclusive, terminator (a stem loop). Previous studies of this system have shown that the secondary structure of the leader region and tertiary structure of the antiterminator as well as the complex with the uncharged tRNA play a fundamental role in antitermination in vivo and tRNA binding in vitro. Interest in the stability and versatility of RNA structure formation revealed the importance of cations in its folding process. Divalent cations and, more recently, monovalent cations have proven crucial in the structural integrity and biological function of RNA. The hypothesis of this research is that the presence of divalent cations, such as magnesium, will affect the structure of the antiterminator and play a critical role in antitermination by affecting the tRNA/mRNA binding process and function. These studies are important in order to understand the structure-function relationship of this novel regulatory system. Results from a range of biophysical techniques (NMR, UV, CD,) will be reported.

Keywords: RNA, metal cations

Role of the WHEP-TRS linker domain in binding of glutamyl-prolyl-tRNA synthetase to the ceruloplasmin 3'-UTR GAIT element

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Glutamyl-prolyl-tRNA synthetase (GluProRS) is a bifunctional aminoacyl-tRNA synthetase (ARS) that catalyzes the ligation of Glu and Pro to cognate tRNAs. GluProRS consists of the two catalytic domains separated by a central linker of three tandem, 50-amino acid WHEP-TRS domains. Single WHEP-TRS domains are present in four other chordate ARSs but not in other proteins. WHEP-TRS domains bind weakly and non-specifically to RNA but their specific function is not known. They do not contribute significantly to aminoacylation suggesting a role in non-canonical ARS functions. We have shown that GluProRS has a non-canonical activity as a gene-specific inhibitor of translation. Following activation of U937 monocytic cells by interferon (IFN)-gamma, GluProRS is phosphorylated and released from the multisynthetase complex. Released GluProRS joins with 3 other proteins to form the GAIT (interferon-Gamma-Activated-Inhibitor of Translation) mRNP which binds to the 29-nt GAIT element in the 3'-UTR of ceruloplasmin (Cp) mRNA and silences its translation. GluProRS directly contacts the GAIT element and we here investigate the domain responsible for binding. UV-crosslinking and RNA EMSA showed that recombinant GluProRS linker containing only the three WHEP-TRS domains bound the GAIT element. Affinity and specificity were determined by surface plasmon resonance (BIAcore system) using immobilized GAIT element. Linker and full-length protein bound the GAIT element with similar high affinity (Kd ~5 nM), but they did not bind an inactive, mutated GAIT element or tRNA(lys) indicating high specificity. Excess recombinant linker suppressed in vitro translational silencing activity of lysates from IFN-activated cells, confirming that linker binding is critical for function. Thus, the GluProRS linker plays a key role in inflammation-responsive translational silencing of Cp. Our results are the first to show a specific function of any WHEP-TRS domain and suggest that the domain may be involved in non-canonical functions of other ARSs.

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Keywords: WHEP-TRS domain, GluProRS, GAIT element

Characterization of the catalytic effects of the MT-A70 subunit of mRNA (N6-adenosine) methyltransferase

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The goal of this project is to better characterize enzymatically the two different isoforms, produced by alternative splicing of the mRNA-(N6-adenosine)-methyltransferase; an enzyme that catalyzes the methylation of internal adenine residues within mRNA using S-adenosyl-L-methionine (AdoMet) as the methyl group donor. mRNA (N6-adenosine)-methyltransferase is a complex structure, composed of two large protein components; MT-A (200kDa) and MT-B (875kDa). The MT-A component consists of two smaller protein subunits, one of which is the 70kDa MT-A70 protein. MT-A70 has been shown to be the subunit which binds the AdoMet substrate (and is therefore suspected to contain the active site of the enzyme).
In an attempt to better characterize enzymatically the different isoforms of MT-A70, the proteins were expressed in prokaryotic and eukaryotic cells. cDNA molecules for the genes (cloned and archived in Genbank) were amplified using PCR and after digestion, ligated into plasmid vectors that were used to express the protein.
For expression in a prokaryotic system, the genes were ligated into the plasmid pQE60, which was subsequently transformed into E.coli. The positive clones were then cultured in the presence of IPTG, to induce expression of the protein. After purification of the MT-A70 subunit, the efficiency of binding of the isoforms to the AdoMet was examined by UV-cross-linking the isoforms with 35S labeled 8-azidoadenosyl-L-methionine. Cross-linking was observed, however binding of the ligand was found to be non-specific.
Over-expression of the protein in a eukaryotic system was accomplished by transfecting the cDNAs for the MT-A70 isoforms into mouse sarcoma cells using a pCMV-Sport6 vector. The resulting clones from the transfections, were screened for over-expression of the desired gene using ribonouclease protection assays (RPAs) and the clones that showed the greatest over-expression of the gene encoding the MT-A70 protein isoforms were cultured and nuclear extracts were prepared and analyzed for methyltransferase activity. A comparison of the methyltransferase activity was then made between the clones over-expressing either of the two isoforms of MT-A70 and controls. Results have shown that the identity of the MT-A70 isoform in the assembled methyltransferase complex had little effect on the specific activity of the enzyme or the kinetic values of the two substrates (RNA or AdoMet) in the reaction.

Keywords: methyltransferase (MT), MT isoforms, enzymatic activity

Magnesium Ion-Induced Effects on Structure and Dynamics in Helix 27 of 16S rRNA from Escherichia coli

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Helix 27 of Escherichia coli 16S ribosomal (r)RNA has been the focus of a long-standing debate concerning the existence of a three-base-pair shift in its base pairing pattern, from the 912-885 to the 912-888 form, during prokaryotic translation [1,2,3,4]. To explore the likelihood that the alternative 912-888 structure is indeed a stable conformation, and perhaps was a functional structure at some time during evolution [5], we have characterized the modes of magnesium ion binding in isolated helices representing both helix 27 conformers by solution NMR spectroscopy, time-resolved FRET, and Tb3+ footprinting. We find that metal binding sites exhibited in the two molecules are diverse in both location and affinity, and have very different effects on global structure of the two constructs. Specific locales of Mg2+ sequestering have provided insight into dynamic and structural features of the unknown 912-888 structure, and imply that two modes of binding exist for Mg2+ in both of the helix 27 conformations. We also discover that metal binding affinity is directly related to the dynamics of the local RNA architecture of our helix 27 constructs, and we believe that this may perhaps be a general characteristic of RNA-metal interactions.

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Keywords: Magnesium ion-binding, NMR spectroscopy, RNA structure and dynamics

Analysis of modified nucleosides of 970 loop in Escherichia coli 16S ribosomal RNA

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E. coli 16S ribosomal RNA contains a total of 11 modified nucleotides, most of which are located at or near regions of the rRNA known to be involved in the decoding process. The 970 loop of 16S rRNA is located near the ribosomal P site and contains two modified nucleotides, m2G 966 and m5C 967. Biochemical and structure studies suggest that the 970 loop plays an important role in the decoding process, as well as in tetracycline binding. To examine the possible role played by these modified nucleotides in protein synthesis, single mutations at each site were constructed and cloned into a genetic system developed in the Cunningham lab. This genetic system allows for the functional assay of a single species of ribosomes in vivo without interference from host ribosomes and without interfering with the normal protein synthesis activities of the cell. Interestingly, single mutations at positions 966 and 967 have a higher level of fluorescence than wild type.

The effect of these mutations on nucleotide modifications at other sites in the 16S rRNA were analyzed by reverse-phase high-performance liquid chromatography. For this analysis, high salt washed 30S subunits were isolated, the 16S rRNA, was enzymatically hydrolyzed, and the 11 different nucleosides were separated by HPLC. To quantify the effects of mutations in the 970 loop on methylation at positions 966 and 977, the peak areas of each modified mucleoside from both wild-type and mutant ribosomes are being measured. The complete analysis of effect of these modified nucleotides in the 16S rRNA between wild type and mutant is in progress.

Keywords: modification, 970 loop, ribosomal RNA

Post-transfer editing in vitro and in vivo by the beta-subunit of phenylalanyl-tRNA Synthetase

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Translation of the genetic code requires attachment of tRNAs to their cognate amino acids. Errors during amino acid activation and tRNA esterification are corrected by aminoacyl-tRNA synthetase-catalyzed editing reactions, as extensively described for aliphatic amino acids. The contribution of editing to aromatic amino acid discrimination is less well understood. We show that phenylalanyl-tRNA synthetase mis-activates tyrosine and that it subsequently corrects such errors through hydrolysis of tyrosyladenylate and Tyr-tRNAPhe. Structural modeling combined with an in vivo genetic screen identified the editing site in the B3/B4 domain of the beta-subunit, 40 Å from the active site in the beta-subunit. Replacements of residues within the editing site had no effect on Phe-tRNAPhe synthesis but abolished hydrolysis of Tyr-tRNAPhe in vitro. Expression of the corresponding mutants in Escherichia coli significantly slowed growth, and changed the activity of a recoded beta-galactosidase variant by misincorporating tyrosine in place of phenylalanine. This loss in aromatic amino acid discrimination in vivo revealed that editing by phenylalanyl-tRNA synthetase is essential for faithful translation of the genetic code.

References:
EMBO J. in press

Keywords: Editing, Genetic code, Phenylalanine

Compilation of mRNA Polyadenylation Signals in Arabidopsis Revealed a New Signal Element and Potential Secondary Structures

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Using a novel program SignalSleuth and a database containing authenticated poly(A) sites, we analyzed the composition of mRNA polyadenylation signals in Arabidopsis, and reevaluated previously described cis-elements within the 3'-UTR regions, including 'Near Upstream Elements' (NUE) and 'Far Upstream Elements' (FUE). As predicted, there are absences of high-consensus signal patterns. The AAUAAA pattern topped the NUE patterns and was found within the predicted location to only ~10-12% of 3'-UTRs. In addition, a new set of polyadenylation signals flanking both sides of the cleavage site, named here as 'Cleavage Elements' (CE), was identified. These cis-elements were not previously revealed by conventional mutagenesis, and are contemplated as a cluster of signals for cleavage site recognition. Moreover, a single-nucleotide profile scan on the 3'-UTR regions unveiled a distinct arrangement of alternate domains of U and A-rich nucleotides, which led to a prediction of the formation of secondary structures. Using an RNA secondary structure prediction program, mFold, we identified three main types of secondary structures on the sequences analyzed. Surprisingly, these observed secondary structures that were disrupted in previously constructed mutations in these regions have demonstrated interruption of usage of the poly(A) sites. These results enable us to revise the current model of plant polyadenylation signals and to develop probabilistic prediction tools to find the most probable cleavage sites at the 3'-end of plant genes.

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Keywords: Polyadenylation signals, mRNA 3, mRNA secondary structures

The Stability of Group I and Group II Single Nucleotide Bulges

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The thermodynamic parameters of oligonucleotide sequences containing single nucleotide bulge loops were determined through optical melting in 1M NaCl. Group I bulge loops, bulges which are not identical to either of the neighboring nucleotides, and Group II bulge loops, where the identity of the single nucleotide bulge is ambiguous because it has the same identity as a neighboring nucleotide, were analyzed. Previously, a model for determining the stability of purine and pyrimidine Group I and Group II bulge loops was determined (1). Twenty Group I sequences with purine bulges and eighteen with pyrimidine bulges were previously studied and the $\Delta G^{\circ}_{37,\,bulge}$ was determined to be between 2.7 kcal/mol and 5.4 kcal/mol and between 2.9 kcal/mol and 5.0 kcal/mol respectively. Further studies also analyzed the stability of Group I bulge loops closed by a G-U, or wobble base pairing. The $\Delta G^{\circ}_{37,\,bulge}$ of thirty one RNA duplexes with Group I single nucleotide bulges closed on one or both sides by a G-U wobble pair was determined to be between 1.2 kcal/mol and 7.1 kcal/mol. In addition to Group I bulge loops, six Group II purines and nine Group II pyrimidines were also previously studied. The $\Delta G^{\circ}_{37,\,bulge}$ ranged from 3.2 kcal/mol and 4.9 kcal/mol and 2.2 kcal/mol and 4.3 kcal/mol for Group II purines and pyrimidines respectively. Previously, it was determined that Group II sequences with G-U nearest-neighbors were equal in destabilization to Group II sequences with Watson-Crick paired nearest neighbors. But, only three sequences of this nature have been studied with $\Delta G^{\circ}_{37,\,bulge}$ between 2.4 and 3.6 kcal/mol. The thermodynamic stability of Group II bulges and Group I and II bulges enclosed by G-U base pairing is being studied to revise a model to determine stability.

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Keywords: bulge, thermodynamics

How Loop-Loop Interactions in a Trans-Acting Hammerhead Ribozyme Enhance Function

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Hammerhead ribozymes are found in a number of plant pathogenic viroids and virusoids, where they are involved in genome replication of the pathogen. The hammerhead was one of the first ribozymes to be characterized, and it has been the focus of much study in both basic research and in clinical trials targeting viral diseases and cancer. The exact mechanism of the cleavage reaction, however, is still elusive. The recent discovery of loop-loop interactions between stems I and II of the hammerhead ribozyme(1,2) has raised many questions about the role that these interactions play in the folding and catalysis of this well-studied ribozyme.

Initial work on the loop-loop interactions focused on cis-acting constructs, with the interacting loops capping stems I and II.(1,2) Here, we use a trans-acting ribozyme, with internal loops in stems I and II, that has been modified from the natural avocado sunblotch viroid sequence. We show that in this construct, as in the cis-acting one, the interaction between stems I and II allows this hammerhead to cleave faster, at lower magnesium concentrations, than the standard, minimal hammerhead ribozyme. Additionally, the internal loops facilitate labeling of stems I and II with donor and acceptor fluorophores, allowing us to study structure and dynamics using time-resolved fluorescence resonance energy transfer (tr-FRET), without disrupting the loop-loop interactions. We anticipate that comparing our results with those from our earlier work on the loop-free hammerhead ribozyme(3) will provide important insight into the effect(s) of the loop-loop interaction on activity, folding, and dynamics as a function of ionic strength, pH, and temperature.

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Keywords: hammerhead, ribozyme, FRET

Cloning and Characterization of the Arabidopsis thaliana Poly(A) Polymerase Gene Family

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Mature, functional messenger RNAs (mRNAs) arise from nascent RNAs that have undergone extensive processing. One of these processes is the addition of a 3' poly (A) tail. This step involves an endonucleolytic cleavage at a cleavage signal then subsequent addition of a string of adenosine residues 3' of the cleavage site. Our lab has focused on the 3' end processing factors involved in plant poly (A) tail formation. To better understand how the poly (A) tail is added to nascent mRNAs, I have been characterizing the poly (A) polymerase (PAP) genes found in Arabidopsis thaliana. PAP is the enzyme responsible for the addition of the poly (A) tail during mature mRNA formation. Arabidopsis has four PAP genes located on chromosomes I, II, III, and IV. Alignment of the PAP proteins show that enzymes arising from chromosomes I, II, and IV appear to be closely related, but the chromosome III gene, when compared to the other three PAPs, is truncated and is missing the nuclear localization signals found in the other PAPs. Also, the three similar genes have alternative splicing around intron 6, which is not seen in PAP III. The structure, expression, and alternative splicing of these four PAP genes will be addressed as well as the localization of the PAP proteins.

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Keywords: RNA processing

Trypanosoma brucei Mitochondrial PPR Proteins in RNA Processing

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A new class of proteins, characterized by Pentatricopeptide Repeat (PPR) motifs, have been recently identified in plants. These proteins contain multiple 35-amino acid consensus sequence repeats that are proposed to form a superhelix capable of binding a strand of RNA [1]. They have been identified in all eukaryotes, though they vary in number. While over 400 putative PPR proteins have been identified in plants, they are less abundant in other Eukaryotes ranging from 2 in Drosophila melanogaster to 19 in Trypanosoma brucei [2]. All PPR proteins characterized to date appear to be involved in RNA processing pathways in the organelles. One of the best characterized PPR proteins, maize Crp1, has been found to be required for translation of petA and petD mRNAs as well as the processing of the petD mRNA from a polycistronic precursor in the chloroplast. Our lab is interested in mitochondrial RNA processing in T. brucei and found it interesting that next to plants, T. brucei has the highest number of predicted PPR proteins at 19. Further database research indicates that most of these 19 proteins are predicted to be mitochondrially targeted. Therefore we have begun to characterize 2 of the putative mitochondrial PPR proteins, PPR1 and PPR2, in T. brucei. These proteins contain 11 and 10 PPR motifs respectively and were found to be homologous to a wheat PPR protein, p63, a putative transcription factor in wheat mitochondria. We begin our studies with RNA interference (RNAi) experiments to study the effect down regulation of these messages has on growth, mitochondrial mRNA levels, and mitochondrial DNA (mtDNA) copy number. Initial results indicate that PPR1 mRNA knockdowns result in a slow growth phenotype. We are currently characterizing mitochondrial RNA levels to study the effect on mitochondrial biogenesis through Northern blot and Real Time PCR analysis. Through these studies we hope to identify relevant RNA processing factors of mitochondrial messages.

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Keywords: PPR protein, Trypanosoma brucei, mitochondrial RNA processing

Engineering of bacteriophage phi29 pRNA for bottom-up assembly of RNA nanostructures and arrays for potential applications in nanotechnology

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The DNA packaging motor of bacteriophage phi29 comprises an essential RNA molecule called pRNA, which functions by forming a hexameric ring around the connector. Ring formation involves hand-in-hand interaction of left and right RNA loops, and the assembly pathway is from dimer to hexamer. We designed modified pRNAs with engineered self-assembly properties to explore the use of pRNA as a building block for nanotechnology by bottom-up nanostructure fabrication. RNA with its structural and functional complexity, easy production and manipulation, and straightforward engineering of supramolecular structures by basepairing has an excellent but underexplored potential to complement protein and DNA nanobiotechnology. pRNA with its unusual structural and functional features is an ideal starting material for RNA nanotechnology. The hand-in-hand interaction which dictates the self-assembly properties of pRNA is based on basepairing of four nucleotides from left and right pRNA loops. By making complementary mutations in these loops, we engineered pRNA molecules that could form stable dimers and trimers in a protein-free environment with very high efficiency. The dimers and trimers were isolated by ultracentrifugation or by purification from native polyacrylamide gels. The 5'/3' paired ends of pRNA form a double helix which is essential for phi29 DNA packaging, but not involved in pRNA oligomerization. Modifications of this domain including extensions, deletions and circular permutations allowed the formation of self-assembled nanostructures with various sizes and shapes. End-to-end fusion of pRNA gave rise to building blocks which were able to self-assemble to micron-size RNA superstructures and arrays. A critical factor in end-to-end fusion was the number of half-turns in the central double-helical region. Our results with pRNA demonstrate that RNA has the potential to serve as versatile building block in nanobiotechnology.

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Keywords: bacteriophage phi29, nanobiotechnology, supramolecular assembly

A test of the model to predict stability of six-nucleotide RNA hairpin loops closed with a GU base pair

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In this study, the thermodynamic parameters of six-nucleotide RNA hairpin loops closed with a GU wobble base pair were studied using optical melting in 1 M NaCl. The loops had the sequence GACGXUAAUYUGUC with XY being the locus for ten possible mismatch combinations (underlined nucleotides are those included in the hairpin loop). A nearest neighbor analysis showed that the free energy values associated with the hairpin loop ranged from 4.3 to 4.8 kcal/mol. The model used to predict the free energy values of the hairpin loops was $\Delta G^{o}_{37L(n)} = \Delta G^{o}_{37i(n)} + \Delta G^{o}_{37MM}$ - 0.8 (if first mismatch is UU unless the loop is closed with a wobble base pair or GA) - 0.8 (if first mismatch is GG and the loop is closed on the 5' side by a purine)[1,2]. The term $\Delta G^{o}_{37L(n)}$ represents the total free energy associated with loops of n-nucleotides (n ¡Ý 4). The ΔGº37i(n) term is the initiation free energy value for the loop and the ΔG^{o}_{37MM} term is the free energy value associated with the interaction between the closing base pair and the first mismatch of the loop[1]. The results of this study indicate the model can accurately predict the thermodynamic parameters of six-nucleotide RNA hairpins closed with a GU base pair.

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Keywords: hairpin loop, wobble base pair, RNA secondary structure

RNA Helix Defects Modulate the Position and Number of Binding Registers of the Protein Kinase PKR

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Many RNAs containing helix defects can regulate PKR. To understand how helix defects influence PKR binding, studies were performed on the dsRNA binding domain of PKR (p20), and TAR RNA from HIV–1. Previous studies showed that 16 bp is the minimal length of perfect dsRNA required for binding p20, and that 22–24 bp is required for binding two p20s. We show that binding p20 to TAR leads to a 1:1 complex but does not afford efficient formation of a 2:1 complex as verified by mixing experiments involving p20, an electrophoretically distinct fusion protein of p20, TAR, and TAR with 30 3'-adenosines. In contrast, binding to a 24 bp perfect dsTAR efficiently leads to a 2:1 complex. Both dsRBMs are required for high–affinity binding to 16–24 bp perfect dsRNAs, TAR and dsTAR. Gel-mobility shift assays reveal straightening of certain TAR bulge mutants, and directed hydroxyl radical mapping of TAR affords two sets of cleavages. These results support modulation of p20 binding registers by RNA–bulges. We present a model for how RNA bulges affect PKR binding

Keywords: PKR, dsRBD, TAR RNA

Endonuclease-mediated decay of normal and nonsense-containing beta-globin mRNA generates degradation intermediates with a 5' cap

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Beta-thalassemia is an inherited disorder of hemoglobin production that results from the presence of a premature termination codon (PTC) in the body of beta-globin mRNA. We previously showed that both normal and nonsense-containing beta-globin mRNA is degraded by an endonuclease-mediated pathway in erythroid cells [1], and the enzyme responsible for this is functionally similar to Xenopus PMR1 [2]. The latter study showed that cycloheximide dramatically stabilized PTC-containing beta-globin mRNA, resulting in a product whose size increased rapidly, then decreased over time. Analysis of poly(A) tail length on these mRNAs showed that cycloheximide caused the rapid lengthening of the poly(A) tail of PTC-containing mRNA that shortened over time. This was confirmed by S1 nuclease protection and primer extension analysis of the 5' ends of these mRNAs. Based on immunoprecipitation with a monoclonal antibody to the trimethyl cap Maquat and coworkers previously reported that the endonuclease-generated decay intermediates contained a 5' cap [3]. The possibility remained that the modification observed in that earlier study was not a true cap, so we re-examined this using eIF4E, since the structure of this protein permits binding only of a cap and not other modified nucleotides. RNA from MEL cells expressing both normal and PTC-containing beta-globin mRNA was bound to Gst-eIF4E, eluted with M7-GDP and analyzed by S1 nuclease protection. In agreement with earlier data, Gst-eIF4E selectively recovered both full-length mRNA and the identified endonuclease-generated decay intermediates. These data indicate that endonuclease cleavage of this mRNA must be followed by cytoplasmic recapping, a phenomenon that is thought to be restricted to newly transcribed mRNA. Supported by PHS grant R21 DK67035.

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Reverse Transcriptase Pausing at 2' Adducts

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Reverse Transcriptases (RTs) pause to varying extents when encountering initiation sites, secondary structures, homopolymers and chemical modifications. Our current work examines pausing at an internal 2' phoshorylated guanosine generated by self-thiophosphorylating ribozymes. We have compared the readthrough efficiencies of reverse transcriptases from several retroviruses: Moloney Murine Leukemia Virus (MMLV), Avian Myeloblastosis Virus (AMV), and homo- and heterodimer forms of the Type 1 Human Immunodeficiency Virus (HIV). Reactions are monitored by performing a time course reverse transcription assay using templates that contain a 2' hydroxyl (control), 2' thiophosphate, or 2' thiophosphate+biotin. AMV and MMLV RTs pause significantly at the large 2' adducts. Homodimer and heterodimer forms of HIV RT both pause at the homoadenosine tract immediately upstream from the target guanosine even when the guanosine does not contain a 2' thiophosphate modification. Pausing at homopolymer sequences has been previously demonstrated and can explain our results for the unmodified RNA. For HIV RTs, more pausing at the modified guanosine is observed with the heterodimer (p51/p66, H+) than with the homodimer (p51/p51, H-). Furthermore, HIV homodimer does not appear to exhibit significant pausing when it encounters a 2' modification. Addition of streptavidin to the biotinylated RNA greatly increases the size of the adduct yet only slightly increases pausing for all RTs. Our results suggest that RNase H plays a role in HIV RT pausing at 2' adducts, perhaps by stabilizing the RT-primer/template complex such that readthrough cannot occur efficiently. In contrast, the more distributive homodimer is able to dissociate from the primer/template more readily than the heterodimer and thus reinitiates to polymerize through the 2' adduct.

Keywords: Reverse Transcription, Self-thiophosphorylating Ribozyme, RNase H

An Unanticipated Relationship Between PMR1 and the Cytoskeleton-Associated Proteins Mena/VASP

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PMR1 is an endonuclease that targets and degrades specific polysome-bound substate mRNAs, To identify its associated proteins stable lines of HeLa (Tet-off) cells were prepared expressing a catalytically-inactive form of PMR1 with a C-terminal TAP tag. In the current study PMR1-TAP containing complexes present in Hela S100 were isolated by tandem affinity chromatography, and the recovered proteins were identified by MALDI and LC-MS/MS. The proteins identified in this screen included alpha-actinin, alpha-spectrin, actin, GTPase-activating protein, Mena (the mammalian ortholog of *enabled*, and VASP (vasodilator stimulated phosphoprotein). Mena and VASP are structurally-related proteins involved in signal transduction through the actin cytoskeleton. Each protein contains a central proline-rich domain flanked by two ena-vasp homology (EVH) domains that interact with vinculin/zyxin and actin, respectively. The association of these proteins and actin with PMR1 was confirmed by Western blot analysis of TAP-recovered protein. Confocal microscopy with phalloidin and antibody to the myc epitope tag on PMR1 identified co-localized sites of actin and PMR1. The specificity of the interaction of PMR1 with Mena/VASP was demonstrated by TAP recovery of proteins following depolymerization of the actin cytoskeleton with Latrunculin A. Actin was no longer recovered with PMR1, but this treatment had no impact on the recovery of Mena or VASP. Neither Mena nor VASP were recovered with GFP-TAP or the TAP tag alone, again indicating a specific association of these proteins with PMR1. The association of Mena with PMR1 was maintained in both sucrose gradients used to separate polysome-bound complexes and glycerol gradients of PMR1-containing complexes released from polysomes by EDTA or puromycin treatment. Targeting of PMR1 to polysomes requires phosphorylation of a C-terminal tyrosine, and the polyproline region of Mena and VASP is a nexus for a number of signal transduction pathways, several of which involve tyrosine kinases. We propose that Mena and VASP bring PMR1 together with a tyrosine kinase whose activity 'licenses' this enzyme to target its polysome-bound substrate mRNA**.**

Keywords: mRNA degradation, Polysomal ribonuclease 1, Mena

Characterizing the determinants of restrictocin specificity

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The sarcin homologue restrictocin efficiently kills many tumor and virus infected cells by crossing cell membranes and catalyzing the cleavage of a single phosphodiester bond located in the sarcin/ricin loop (SRL) of rRNA. Cleavage by this ribotoxin disrupts ribosomal binding of elongation factors, inhibits protein synthesis, and ultimately triggers apoptotic cell death. Our studies investigate how sarcin-like ribotoxins are able to recognize and cleave only one of the thousands of phosphodiester bonds found in ribosomes. An understanding of an enzymatic reaction requires not just snapshots of structural intermediates along the reaction pathway, but also knowledge of when contacts form and, importantly, the significance of each one. Previous crystallographic studies of enzyme-inhibitor complexes have identified protein contacts to the RNA substrate. To determine which of these contacts underlie specificity, we made and kinetically characterized ten mutant ribotoxins designed to selectively disrupt each contact. A picture is emerging from our studies that formation of the initial Michaelis complex is driven by nonspecific electrostatic RNA-protein interactions. In contrast, target site-selection involves only a small subset of interface contacts that occur during a subsequent event either preceding or during the rate-limiting step.

Keywords: endoribonuclease, protein-RNA recognition, translation

Investigation of the solvent accessible sites of the ribosome by limited proteolysis and mass spectrometry

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Limited proteolysis is widely used to investigate the structure of proteins [1]. It also has been used to investigate the surface topography of ribosomal subunit [2]. Two dimensional (2D) gel electrophoresis has been the method of choice for the analysis of digested peptides from ribosome. However, data interpretation is difficult and sometimes impossible using 2D gel electrophorises due to the complexity of the sample and low resolution.

Mass spectrometry is a powerful tool for the analysis of biological molecules such as proteins and nucleic acids. We used the power of mass spectrometry to investigate the limited proteolytic digestion products and intact proteins from E.coli ribosome. Even though the crystal structure of the ribosome is available for a few species, this approach can be used to investigate the topography and solvent accessible sites of ribosomes from other species for which there is no crystal structure available.

Using MALDI-MS, a direct read-out of all digested products and intact proteins from this macromolecular complex is available in a single experiment. On the other hand, liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) and tandem mass spectrometry (LC-ESI-MS/MS) give us more information about the digested products including their sequence. It is assumed that intact proteins are either not solvent accessible or are protected by interactions with rRNAs. Together, this approach provides structural information relating to solvent accessibility and protein stability through interaction between proteins and RNAs within the ribosome.

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Keywords: limited proteolysis, ribosome, mass spectrometry

tRNA genes' effecct on nearby recombination frequency

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It is becoming increasingly clear that spatial organization of genetic information in nuclei strongly affects the behavior of genes. Transfer RNA (tRNA) genes, which are transcribed by RNA polymerase III, are distributed throughout eukaryotic genomes and are frequently found as multicopy families. Recently we have found that the widely scattered tRNA genes in the yeast, Saccharomyces cerevisiae, are clustered near the nucleolus. This spatial organization initiates an ordered biosynthetic pathway for the tRNA genes, but the localization also appears to affect the expression of neighboring genes.

One other possible effect of tRNA genes is that when they are being actively transcribed they will have an effect on the rate of recombination in the surrounding DNA. Two observations suggested that we explore this possibility. First, recombination among the repetitive tRNA genes would be detrimental to survival, and so suppression might have a selective advantage. Second, the repetitive retrotransposon elements insert preferentially next to tRNA genes, suggesting that this position in the genome might have a selective advantage. We have been looking at two different scenarios. The first tests recombination rates between two identical tRNA genes, and the second examines the effects of neighboring tRNA genes on recombination between LTRs of Ty retrotransposons.

Keywords: tRNA, recombination

A random combinatorial approach to identifying proteins that interact with Arabidopsis polyadenylation factor subunits

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During the formation of the 3' ends of mRNA, the cleavage and polyadenylation specificity factor (CPSF) is required for 3' cleavage of the transcript as well as for subsequent polyadenylation (Elliott, et al.,2003).CPSF purified from calf thymus or HeLa cells is a large protein complex containing subunits of 160, 100, 70, and 30 kDa, referred to as CPSF-160, CPSF-100, CPSF-70, and CPSF-30, respectively (Jing Zhao, et al.,1997). Antibodies raised against CPSF-100 coimmunoprecipitate all four subunits of CPSF, confirming their association as a complex (Jenny, et al., 1994). Interestingly, in Arabidopsis CPSF100 was found to interact with a portion of a nuclear poly(A) polymerase. To understand the identification of new protein-protein interactions between Arabidopsis CPSF 100 and other polyadenylate factors a 7-mer M13 phage display expression library was screened against Arabidopsis CPSF 100 purified on CBD affinity column. Sequencing of the interacting Phage after 3rd panning revealed several interacting peptide sequences. Blast search against Arabidopsis protein database with these peptide sequences has revealed that some of these peptides have homology to other poly adenylate factors like CPSF 160 and CstF 50 These and other possible interactions are being confirmed using two-hybrid and directed mutagenesis.

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Studying mRNA/gRNA interaction through ITC

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Trypanosomatid RNA editing consists of insertion and deletion of uridine residues in several mitochondrial mRNAs. This process is specified by base pairing with small transcripts termed guide RNAs. gRNAs are complementary to cognate mRNAs and their interaction is fundamental for the editing proteins to perform a series of reactions yielding mature translatable mRNAs. However, the temporal association of gRNAs with mRNAs and protein recognition is still obscure. It is of considerable importance to elucidate the kinetics and thermodynamics of mRNA/gRNA complexes to better understand RNA editing. The editing domains have different primary sequence and hence different structural forms. Therefore, each mRNA/gRNA interaction may have a unique binding behaviour. Characterizing the affinity and specificity of these interactions will provide important information on mechanisms and regulation of RNA editing. Our laboratory studies two contrasting mRNAs. The mRNA for the cytochrome subunit b (CYb) undergoes limited editing only in the insect form of the parasite. The ATPase subunit 6 (A6) mRNA is extensively and constitutively edited throughout the trypanosome life cycle. From gel band-shift assays, we previously found an apparent KD of 5-10 nM for A6/gA6, which is one order of magnitude smaller than for the CYb pair. Surface plasmon resonance (SPR) experiments also confirm the binding affinity of the A6 pair being higher than for the CYb pair. The aim of the present study was to use isothermal titration calorimetry (ITC) to investigate the thermodynamics of the interaction between gA6 and its cognate mRNA. We were expecting to get nearly tight binding upon injection of gA6 into a solution of A6, as observed for the gel band-shift and SPR assays. However, our preliminary ITC results are contradictory.

Keywords: kinetoplastid, RNA editing, isothermal titration calorimetry

Functional differences among the seven 16S rRNA genes from Escherichia coli

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The Escherichia coli DH5 genome contains seven ribosomal operons. The 16S rRNA sequences from operons rrnA, rrnB, and rrnE are identical. The 16S rRNA from each of the four other ribosomal operons (rrnC, rrnD, rrnG, and rrnH) contain unique sequence heterogeneities. Thus, there are five different 16S rRNA sequences in E. coli. To determine if these sequence heterogeneities alter the function of 16S rRNA, each of the five different sequences was cloned into a genetic system developed in the Cunningham lab. This genetic system allows for the functional analysis of a single species of rRNA without affecting host protein synthesis.

Clones expressing the five different 16S rRNA's were assayed for ribosome function, in vivo, at different temperatures, pHs, and ionic strength. The 16S rRNA from operons rrnC and rrnD are functionally identical to rrnB 16S rRNA under all conditions tested. The 16S rRNA from operon rrnG, however, is approximately 20% more active than the rrnB 16S rRNA and the rrnH 16S rRNA is approximately 20% less active under standard growth conditions (37^0C, aerobic growth in LB medium). When tested under different ionic conditions and pH, however, the 16S rRNA of rrnG decreased in function with increasing ionic strength or pH while the 16S rRNA of rrsH increased in function. These data show that the sequence heterogeneities present in E. coli rRNA results in different levels of ribosome function in response to changing environmental conditions. It is possible that this diversity in rRNA sequence provides a selective advantage for E. coli in it's natural environment.

Keywords: 16S ribosomal RNA, operon

A trans-acting ribozyme that phosphorylates exogenous RNA at precise internal 2' hydroxyls

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Selection and evolution of self-phosphorylating ribozymes[1] and deoxyribozymes[2] has been reported, including a ribozyme that catalyzes multiple-turnover thiophosphorylation of a small RNA substrate[1]. We have launched selections for ribozymes that phosphorylate small molecule targets. Random sequence RNA was ligated to DNA oligonucleotides tethered to the small-molecule phosphorylation target, and these were incubated with (gamma)-thio-ATP. Active species were partitioned in a polyacrylamide gel on a layer of [(N-acryloylamino)phenyl] mercury (APM)[3]. By itself, this protocol does not discriminate between thiophosphorylation of the intended target vs. internal 2'-thiophosphorylation, so we employed a novel method to eliminate most internal products. However, even this method does not prevent recovery of RNAs that phosphorylate 2' OH near the 5' end of the RNA, and all of the recovered species exhibited internal modification. (We have since shifted to a photocleavage method that rigorously excludes internal phosphorylators.)

Activity assays for several truncated RNAs identified the essential secondary structure of the dominant RNA motif. Upon separation into ribozyme and substrate strands, the ribozyme strand can internally phosphorylate any exogenous RNA carrying the sequence 5' GGAAAA(U) 3'. Most of the riboses in the substrate strand can be replaced with deoxyribose. Partial alkaline hydrolysis with hybrid RNA/DNA substrates identified the precise modification site. The ribozyme also catalyzes removal of the phosphate either through a reverse reaction (in the presence of ADP) or through hydrolysis by solvent. The forward reaction shows a significant preference for gamma-thio-ATP over ATP, and is independent of pH over a wide range (approx. 5-9), consistent with a dissociative reaction mechanism that is rate-limited by formation of a metaphosphate transition state. Divalent metal ions are required, with slight preference of $Mn^{2+} > Mg^{2+} > Ca^{2+}$. Lack of reactivity in $[Co(NH_3)_6]^{3+}$ indicates a requirement for inner sphere contact with the metal ion, either for structural stabilization or for catalysis.

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Inhibition of HIV Reverse Transcriptase by RNA Aptamers

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In vitro selection techniques have identified RNA aptamers to HIV-1 group M subtype B (M:B) reverse transcriptase (RT) [1, 2]. These aptamers bind with high affinity (low nanomolar K_ds) to the template-binding region of the enzyme [3] and disrupt RT activity by what is believed to be competitive inhibition. We are interested in the effectiveness of RNA aptamers to inhibit RT function across different viral clades. It is currently unknown how well aptamers selected against HIV-1 M:B RT will inhibit less-closely related HIV and SIV RTs. Expression of aptamers to inhibit viral replication in cell culture suggests that the natural sequence diversity of HIV-1 subtypes may be sufficient to confer resistance to a subset of currently available aptamers [4]. We surveyed a set of aptamers for their ability to inhibit DNA-dependent DNA polymerization by HIV-1 M:B RT, and identified a group of sequences having IC_{50}s of less than 10nM. These aptamers are characterized by simple pseudoknot structures having loops, stems, and bulges of different sizes which may afford a variety of contacts to the RT surface. The aptamers will be assayed for inhibition of HIV and SIV RTs *in vitro* in order to determine their cross-clade utility. We are utilizing conditionally replicating viruses to insert different RT genes into a common HIV-1 M:B background allowing aptamer effectiveness to be monitored in cultured cells. We expect to learn how well RNA aptamers inhibit RT function among distantly related strains, and to begin to understand whether specific positions within the RT are able to confer aptamer resistance.

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Efforts Towards Highly Shifted pKa Values in Nucleic Acids

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The catalytic activity of RNA would be greatly enhanced by cationic bases, which could confer histidine- and lysine-like behavior. Thus, we are investigating the feasibility of large pKa shifts in nucleic acids. We recently described a technique to determine pKa values in nucleic acids.(1) In this method, a specific phosphorothioate is incorporated near a cationic AC mismatch, and the change in 31P chemical shift upon nucleobase protonation is followed as a function of pH. This is a convenient method since the substitution moves the chemical shift downfield by ca. 50 ppm to an isolated region of the spectrum. One of the driving forces for pKa shifting appears to be RNA and DNA folding made possible upon protonation.(2) Currently, we are trying to maximize pKa shifting by maximizing the extent of structure formation upon protonation. Since RNA and DNA folding is exothermic, lowering the temperature of reaction should drive folding. Consistent with this idea, preliminary studies show that lowering the temperature from 37 to 10 C raises the pKa from 6.9 to 7.5. Likewise, model systems are being developed to incorporate cooperativity of base pairing. This is being done by placing mismatches penultimate to the terminus of a hairpin. Initial studies on double mutant cycles reveal the importance of context in that maximal cooperativity is observed for penultimate mismatches as compared to internal mismatches. Overall, it appears that highly shifted pKa's may be attainable in simple secondary structures by adjusting solution conditions and sequence.

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Keywords: pKa, mismatch, Protonation

Effects of the Bulge Loops on Thermodynamic and Structural Properties of the Duplex

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Thermodynamic parameters and structural features of two double-stranded bulge-loop oligonucleotide were explored via optical melting and NMR spectroscopy. The core duplex 5'CGACGCAG3'/5'CUGCGUCG3' was identical for both bulges. Bulge loops were introduced in the middle of the first strand between CG. Earlier studies reported that bulges with at least one identical nearest-neighbor are more stable giving rise to the differentiation of Group I and Group II bulges based on the identity of their nearest neighbors (1). The two studied bulges were as follows: one bulge (A) with no identical nearest neighbors (Group I) and one bulge (G) with at least one identical nearest neighbor (Group II). A greater stability of Group II bulges was attributed to the conformational freedom of the bulge, which can change position with its identical nearest neighbor increasing the entropy of the system (2). Comparison of NMR spectra for the core duplex (3) revealed a downfield shift of two imino-protons after the introduction of the Group I bulge. This is probably due to the decreased stacking of the CG leading to the decreased shielding of the imino-protons by the ring currents of the adjacent aromatic bases (4). Assignment of the imino-protons via temperature-varied NMR experiments and Nuclear Overhauser Effect experiments are still in progress.

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Keywords: Bulge Loops, Group I / Group II, Thermodynamics and NMR

Developing Isostericity Matrices: A Tool for RNA Structural Alignment

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RNA molecules play dual roles, transmitting genetic information and catalyzing chemical reactions. They fold into compact 3D structures, stabilized by tertiary interactions mediated by *motifs -- ordered arrays of non-Watson-Crick base pairs* -- that change much more slowly during evolution than their sequences. Structural analysis and classification of RNA motifs are key for 3D homology modeling and accurate sequence alignment, which in turn is crucial for accurate phylogenetic analysis. We have constructed Isostericity Matrices (IM) to organize the base pairs in each of the 12 geometric families into isosteric subsets. The IM identify base substitutions that can occur in motifs while preserving 3D structure. IM were applied to analyze hairpin loops observed in representative x-ray crystal structures (3.05 Angstrom resolution or better) from the Nucleic Acids DataBank (NDB) up to December 2003. For each hairpin loop we catalogued these structural features: the type and position of non-Watson-Crick base pairs, base-to-backbone H-bonds, locations of changes in strand orientation, key stacking interactions, and positions of extruded bases. Our analysis indicates that the definitions of previously described hairpin families should be expanded and identifies new families of hairpin motifs, closed by a wide range of non-Watson-Crick base pairs. Of the hairpin loops analyzed, results of the GNRA hairpin loop will be discussed since it has been examined more extensively. It was found that GNRA loops in the ribosome largely interact in the minor grooves of helices as expected. We also identified loops with single inserted bases that otherwise do no distort the GNRA motif. New sequence alignments were proposed based on the understanding of the location of insertions and where they have been observed in homologous rRNA sequences.

Functional Expression of Mycobacterium tuberculosis in Escherichia coli

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Tuberculosis (TB) kills two million people per year and is a leading cause of death from infectious disease worldwide. The World Health Organization estimates that one third of the global population is infected with the etiologic agent of TB, Mycobacterium tuberculosis. Currently there are nine recommended drugs for the treatment of M. tuberculosis that affect a wide range of targets, however resistance to each drug has been identified. In M. tuberculosis antibiotic resistance appears to be exclusively due to chromosomal mutations that alter the drug target. For target mutations to lead to resistance they must prevent drug binding yet maintain the normal biological function of the drug target. Thus, if all of the biologically active mutations in a M. tuberculosis drug target in could be easily identified this information could be used to develop new antimicrobials that are not susceptible to the development of resistance.

NMR and X-ray crystallography have shown that ribosomal RNA (rRNA) is composed of structurally independent subdomains accessible to drugs even when fully folded, making bacterial rRNA an ideal drug target. A genetic system developed in the Cunningham lab has already been used to identify all of the viable mutants in several functionally important regions of E. coli 16S rRNA. Here we report the cloning and expression of M. tuberculosis rRNA in this genetic system. Functional analyses of E. coli/M. tuberculosis rRNA hybrids produced ribosomes that are <5% to 132% as active as wild-type E. coli ribosomes in vivo.

Keywords: ribosomal RNAs, translation, M. tuberculosis

Regulation of Glucose-6-Phosphate Dehydrogenase (G6PD) pre-mRNA Splicing-Localization of the Regulatory Element

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Hepatic G6PD expression is regulated at a nuclear post-transcriptional level by various hormonal as well as dietary factors. The amount of G6PD mRNA in primary rat hepatocytes increases 3-4 fold in the presence of insulin; however addition of arachidonic acid to the medium containing insulin inhibits insulin induction about 50%. The insulin stimulation of G6PD mRNA uses the PI3 kinase pathway. In this regard the PI3 kinase inhibitor LY294002 inhibits, the insulin-mediated induction of G6PD, whereas a MAP kinase inhibitor abolishes the inhibitory effect of fatty acid. From these results we have hypothesized that arachidonic acid might be interfering with insulin signal transduction by activating the MAP kinase signal cascade. We have found that the inhibition of expression of G6PD in mice fed a high fat diet involves the slow spicing of intron 11, which can target G6PD pre-mRNA for degradation. To determine the cis-acting element in G6PD pre-mRNA involved in this regulation we transfected fragments of the mouse G6PD genomic DNA into primary rat hepatocytes. Transfection of the constructs with 5' deletion of exons and introns demonstrated that the RNA reporter was regulated by arachidonic acid when the constructs contained exon 12 through the 3' end of gene. The inhibitory effect of arachidonic acid for these constructs requires the presence of both exons and introns in the RNA reporters, consistent with regulation due to splicing of the pre-mRNA. Ligation of exon 12 through the 3' end of gene to the beta-galactosidase gene or the G6PD cDNA resulted in inhibition of the chimeric reporter RNA by arachidonic acid. In contrast, chimeras containing only intron 12 and exon 13 of the G6PD produced RNA that was not inhibited by incubation with arachidonic acid. To define the cis-acting element, constructs with block mutations across exon 12 were transfected into rat hepatocytes, mutations between 43 and 72 nt of exon 12 abolished the response. Current experiments are aimed at further defining the sequence of this element and the proteins that bind to it.

This work is supported by a grant from the NIH.

Keywords: RNA splicing, arachidonic acid, PI3 kinase

Functional reconstitution of the RNase P holoenzyme from Pyroccocus furiosus, a thermophilic archaebacterium

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Ribonuclease P (RNase P) is a ribonucleoprotein complex essential for tRNA maturation in all three domains of life. In bacteria, RNase P is composed of one catalytic RNA subunit and one protein cofactor. Studies on archaeal RNase P have shown that its activity is associated with one RNA subunit and four protein subunits. In eukarya, nuclear RNase P is associated with one RNA subunit and at least nine protein subunits. Why do archaeal and eukaryal RNase P require mutiple protein subunits, respectively, while only one protein cofactor suffices for the function of bacterial RNase P? What is the role of each protein subunit in archaeal and eukaryal RNase P? These queries can be addressed at least in part if we can reconstitute fully functional archaeal or eukaryal RNase P holoenzymes in vitro. Although successful partial reconstitution has been reported for archaeal and human RNase P, there is a dire need for further improvement since the reconstituted RNase P holoenzymes in these studies displayed very poor activity (compared to the native versions) and no multiple turnover. Towards the dual objectives of optimizing in vitro reconstitution and establishing structure-function relationships, we have used recombinant techniques to obtain the RNA and protein subunits of RNase P from Pyroccocus furiosus (Pfu), an archaeon, and succeeded in reconstituting a functional RNase P holoenzyme that exhibits robust multiple turnover in vitro. Moreover, the incremental activity of reconstituted RNase P observed upon sequential addition of the various protein subunits demonstrate that the RNA subunit in the holoenzyme could adopt a catalytically active conformation with a partial suite of protein subunits and that it becomes progressively more active with addition of other protein subunits. We are currently investigating various biochemical properties of the native and in vitro reconstituted RNase P holoenzymes. Results from these studies will be discussed.

Keywords: RNase P, RNP complex, precursor tRNA processing

Analysis of the secondary structure of the RNA subunit of human RNase MRP

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RNase MRP is a eukaryotic ribonucleoprotein that cleaves pre-rRNA. The holoenzyme contains a single, putatively catalytic, RNA and several identified protein components. The related RNase P processes pre-tRNA and shows large structural and functional similarities to RNase MRP. RNase MRP is believed to be a eukaryotic paralog of RNase P; due to both common structural domains in their RNA components and the large overlap of protein content common to both RNPs.

We have conducted a biochemical probing study into the secondary structure of the isolated H. sapiens MRP RNA. RNase probes specific for both single strand and double stranded regions of RNA structure have been employed in addition to the lead (II) ion probe.

Here we present the results of our secondary structure probing analysis of the H. sapiens MRP RNA. Our results confirm the secondary structure predicted by a previous phylogenetic analysis (1).

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Keywords: RNase MRP, structure probing

Regulation of 5' splice sites

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Alternative pre-mRNA splicing events are controlled by specific cis-acting elements within the transcript. These regulatory sequences are generally present in the 3' exon and modulate utilization of the upstream 3' splice site. In many cases serine/arginine-rich splicing factors (SR proteins) have been shown to interact with positive-acting elements (exonic splicing enhancers, ESE) and are required for splicing activation. We have established a system in which to study the mechanism of action of ESEs that regulate 5' splice sites by employing the MS2-RS fusion protein system. Interestingly, in contrast to numerous models, we do not see recruitment of the U1 snRNP to regulated 5' splice sites. The action of ESE-bound SR proteins upstream of a regulated 5' splice site requires their phosphorylation and occurs at a point in spliceosome assembly beyond formation of the initial U1 + U2 snRNP-containing complex. In addition, the presence of ESE-bound SR proteins does not alter the stability of these early complexes. Our results suggest that the U4/6•5 tri-snRNP may be the moiety recruited to 5' splice sites by ESE-bound SR proteins. Recently, we have begun employing a system to directly test this hypothesis. We have generated FLAG-tagged versions of the three U4/6•5 tri-snRNP specific proteins that contain RS domains. These proteins will be expressed in HEK 293T cells, with these transfected cells used to generate splicing-competent whole cell extracts. We will perform in vitro splicing reactions in these extracts and determine whether the recruitment of the U4/6•5 tri-snRNP is dependent upon the presence of an activating MS2-RS fusion protein. Intriguingly, our results to date point to a novel mechanism of pre-mRNA splicing regulation.

Keywords: RNA, Splicing, Regulation

NMR studies of archaeal RNase P

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Ribonuclease P (RNase P) is an essential ribozyme that cleaves the 5' leader sequence of precursor-tRNA to yield mature tRNA. Archaeal RNase P consists of an RNA subunit (P RNA) – which is catalytically active in vitro – and several associated proteins that are necessary for activity in vivo. Four proteins have been identified in RNase P from Pyrococcus furiosus (Pfu) that interact with the catalytic RNA. These four proteins are homologous to four proteins found to be associated with human RNase P: Rpp21, Rpp29, Rpp30 and Pop5.

The long-term goal of this project is to elucidate the structural and functional role of each protein or RNA subunit in the ribonucleoprotein complex and the nature of the interactions between them. Our work has focused on Pfu Pop5, a ~14 kDa protein which we have shown to enhance the activity of an in vitro reconstituted Pfu RNase P system. We aim to solve the structure of Pfu Pop5 by NMR spectroscopy. Amide (backbone) and carbon (backbone & side chain) resonance assignments have been completed and proton assignments are in progress. The structure will be solved by obtaining distance restraints using NOE spectroscopy and using them in structure calculations. Interestingly, analysis of chemical shift data collected from Pfu Pop5 suggests a pattern of secondary structure elements which is strikingly similar to those predicted for human hPop5 and Rpp14 (two proteins which are part of the human RNase P complex).

Keywords: RNase P, ribozyme, NMR

Direct Evidence for Intraprotein and Protein–RNA Disulfide Adducts during 4-thiouridine Biosynthesis

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The biosynthetic pathways of nucleosides and cofactors containing sulfur remain largely unelucidated. ThiI is an enzyme common to the biosynthesis of thiamin and 4-thiouridine (s4U) in tRNA, and ThiI's role in s4U generation is becoming one of the best elucidated cases of sulfur transfer. We have proposed two mechanisms by which ThiI generates s4U, and they differ primarily in which sulfur nucleophile attacks tRNA (1,2). Previous work from our lab provided strong evidence that Cys-456 cycles between thiol(–SH), persulfide(–SSH), and disulfide (with Cys-344) forms (3). We now present experiments using MALDI-MS that provide direct evidence for disulfide and ThiI–tRNA adduct formation during s4U biosynthesis.

Disulfide formation was postulated based on the requirement of a reductant for turnover of ThiI, and single turnover assays that resulted in production of essentially one s4U for each ThiI. Single turnover conditions were used to generate the disulfide bond between Cys-456 and Cys-344 in preparation for MALDI-MS. After a single turnover, trypsin is used to digest ThiI, which should leave the peptide fragments linked by a disulfide bond. We have observed the predicted disulfide-linked species, and it collapses to the individual peptides containing Cys-344 and Cys-456 upon reduction.

To distinguish between the alternative mechanisms, we sought the ThiI–tRNA adduct predicted by the mechanism with a nucleophilic persulfide group. We used ThiI that lacks Cys-344, which should leave the enzyme "stuck" as the adduct in the absence of reductants. A ThiI–tRNA adduct was visible by gel shift, and this complex was digested using either RNase A or RNase T1, followed by trypsinolysis prior to MALDI-MS analysis. The expected disulfide-linked peptide-RNA fragments are detected, and the addition of reductant eliminates the adduct. These results provide direct evidence for the ThiI–tRNA intermediate and strongly suggest that ThiI follows a mechanism in which the persulfide form of Cys-456 attacks uridine-8.

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Keywords: sulfer transfer, tRNA modification, MALDI-MS

Characterization of Eukaryotic-Specific Conserved Elements of RNase P RNA

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RNase P is a ubiquitous endoribonuclease responsible for the cleavage of 5-prime leader of precursor tRNAs (pre-tRNAs). Although the protein composition of RNase P holoenzymes varies significantly among Bacteria, Archaea and Eukarya, the holoenzymes have essential RNA subunits with several sequences and structural features that are common to all three kingdoms of life. Additional structural elements of the RNA subunits have been found that are conserved in eukaryotes, but not in bacteria, and might have functions specifically required by the more complex eukaryotic holoenzymes. In this study, we have mutated four eukaryotic-specific conserved regions in the yeast nuclear RNase P RNA, and characterized the effects of the mutations on cell growth, enzyme function and biogenesis of RNase P. Mutations in three different regions cause defects in holoenzyme assembly, localization and pre-tRNA processing in vivo and in vitro. These data suggest the importance of eukaryotic-specific conserved regions of RNase P RNA in maturation and function of the holoenzyme.

Keywords: tRNA processing

NMR and biochemical studies of archaeal RNase P protein subunits: Pfu P29 and P30

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Ribonuclease P (RNase P) is an essential ribozyme that cleaves 5' leader sequence of precursor-tRNA to yield mature tRNA. Archaeal RNase P consists an RNA subunit (P RNA) – which is catalytically active in vitro – and several associated proteins that are necessary for activity in vivo. Four proteins have been identified in RNase P from Pyrococcus furiosus (Pfu) that interact with the catalytic RNA. These four proteins are homologous to four proteins found to be associated with human RNase P: Rpp21, Rpp29, Rpp30 and Pop5.

The long-term goal of this project is to elucidate the structural and functional role of the protein and RNA subunits in the ribonucleoprotein complex and the nature of the interactions between them. Our work is focused on protein-protein interactions and protein-RNA interactions. Since Rpp29 is the best-studied subunit in archaeal RNase P, we use Pfu P29 as the reference in NMR study of interactions. Amide (backbone) and carbon (backbone & side chain) resonance assignments have been completed and proton assignments are in progress. Crosslinking experiments show that P21 interacts with P29, and Pop5 interacts with P30, in agreement with published yeast two-hybrid data. Pfu P30 (24.5 kDa) is the largest protein subunit in Pfu RNase P and has been shown to be critical for activity in a Pfu RNase P in reconstitution assay. Despite the challenge of protein's size, we collected a promising HSQC spectrum.

Keywords: RNase P, archaebacteria, ribozyme

Sequence Specificity in the Interaction Between pre-tRNA and the Protein Component of Bacterial Ribonuclease P

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The ribonucleoprotein enzyme ribonuclease P (RNase P) removes the 5' leader sequences from all cellular precursor tRNA molecules, a role which requires a uniquely broad substrate specificity. In Bacteria, this specificity arises from the combined contributions of a single, catalytic RNA subunit and a single protein subunit (P protein). Previous studies indicate that a central cleft in the structure of P protein interacts with the 5' leaders of pre-tRNA substrates, increasing substrate affinity and decreasing the requirements for monovalent and divalent metal cations in a leader length dependent manner (For review see 1 and 2). To better understand how P protein contributes to RNase P substrate specificity, we examined the possibility of a leader sequence preference in interactions with pre-tRNA substrates. We find that systematic variation of nucleotides -4 through -10 can alter substrate binding to the B. subtilis RNase P holoenzyme by up to 6-fold. Notably, these variations in binding affinity are not observed with the E. coli holoenzyme, suggesting that any sequence preference may be species-specific. Intriguingly, this observation is also consistent with genomic analysis of leader sequences which indicates an A/U preference at N(-4) for B. subtilis, and little sequence preference for nucleotides N(-3) through N(-5) for E. coli RNase P. Also consistent with genomic analysis, pre-tRNA with a four nucleotide leader containing an A at N(-4) has 20-fold higher affinity for B. subtilis RNase P than a similar substrate with a G at N(-4). This sequence preference suggests a direct contact between the protein and N(-4). Further experiments are underway to determine the nucleotide base functional groups and protein amino acids involved in the discrimination between A and G at N(-4) by B. subtilis RNase P.

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Keywords: RNase P, Protein, Substrate Binding