Oral Presentations

5' -Splice Site Recognition in Mammalian Cells: Identification of Intronic Silencers Using Functional SELEX

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The detailed mechanisms governing 5' -splice site choice in vertebrate cells are still largely obscure. In particular, it is not understood why “perfect” sites are often ignored and why U1 snRNP binding is not sufficient to promote splicing. To approach these questions, we designed a 5' -splice site competition assay where the proximal site is strong and the distal site is weak. In the base construct, in vitro splicing exclusively uses the proximal consensus site, but distal splicing is activated when the proximal site is disrupted by mutation. We then used a functional SELEX to identify exonic (-18 to -7) or intronic (+11 to +22) sequences that could silence the proximal site. To date we have characterized 38 intronic sequences that down regulate use at the proximal site. Importantly, our experimental design excludes any sequences that could occlude the proximal site via secondary structure formation; indeed all of the selected variants retain the ability to bind purified U1 snRNP at the consensus site. Bioinformatics analysis showed that there are at least four motifs in our SELEX winners and they correlate significantly with “perfect” pseudo 5' -splice sites in human genome. These elements could function by recruiting trans-acting factors that directly down modulate U1 snRNP function or by disrupting communication between the proximal 5' -splice site and 3'-splice site. Biochemical analysis of selected examples will be presented.

Keywords: 5' -Splice Site, Silencer, SELEX

Identification of Proteins Involved in Constitutive Splicing

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The regulation of hepatic glucose-6-phosphate dehydrogenase (G6PD) by diet and polyunsaturated fats involves changes in the rate of splicing of G6PD pre-mRNA. The amount of RNA produced from a G6PD reporter construct containing exons 7 through 13 and surrounding introns is decreased in rat hepatocytes incubated with arachidonic acid. However, RNA from a G6PD reporter constructs lacking exon 12 was not inhibited by polyunsaturated fatty acids. Moreover, exon 12 of G6PD can transfer its regulatory function to the heterologous mRNA, beta-galactosidase, confirming that exon 12 contains a potential exon splicing enhancer sequence. Elucidating the protein(s) responsible for binding to the exon splicing enhancer within exon 12 will help define a new splicing regulatory mechanism.

To identify RNA binding proteins, a G6PD exon 12 RNA probe was used in UV crosslinking assays. Liver nuclear extracts produced bands corresponding to 90, 75, 60, 50, and 37 kDa. The detected bands were protein because they disappeared in the presence of proteinase K. In addition, the pattern of protein binding was similar when the RNA probe was labeled with A, C, G or U. Competition with unlabeled exon 12 probes successfully competed all proteins. Curiously a protein appeared at 25 kDa. The protein at 25 kDa did not appear when a heterologous unlabelled RNA competitor was used; the appearance of this unique protein appeared at the same rate that a larger protein at 37 kDa disappeared. The pattern of detected proteins differed comparing liver versus HeLa cell nuclear extracts. Additional data will be presented as to the identity of the detected proteins bound to exon 12. The overall objective of our research is to understand the regulation of G6PD mRNA splicing thereby providing new information about the regulation of splicing per se and how polyunsaturated fats regulate gene expression through a nuclear posttranscriptional mechanism.

Keywords: Splicing, RNA binding proteins, hnRNPs

Hu proteins regulate the neuron-specific processing of the human calcitonin/CGRP pre-mRNA by inhibiting polyadenylation of the non-neuronal 3' terminal exon

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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. 10:208, Lou H. et al. 1998 Mol Cell Biol. 18:4977, Lou H. et al. 1999 Mol Cell Biol. 19:78,and Zhu, H. et al. 2003 Mol Cell Biol 23:5959) However, regulation of the neuron-specific exon 4 exclusion remains largely unknown.

Our laboratory recently identified Hu proteins, a group of neuron-specific RNA binding proteins, regulate the neuron-specific processing of CGRP. We observed a strong correlation between Hu protein expression and CGRP processing. In cell lines previously used to mimic the CGRP processing including F9, PC12 and CA77, Hu proteins are highly expressed, while in cell lines used to mimic the non-neuronal calcitonin processing including HeLa and CHO, little Hu proteins were detected by western blot analysis. A strong correlation was also observed using the P19 neuron cell model. Dominant negative Hu proteins significantly affected the processing of CGRP in CA77, suggesting that Hu proteins are functionally involved in the neuron-specific regulation of CGRP production.

Hu proteins regulate the CGRP processing by inhibiting the polyadenylation of the alternative terminal exon of calcitonin. We identified a U-rich sequence immediately downstream of the polyA signal of calcitonin terminal exon as Hu binding site. Hu proteins bind to this sequence and inhibit the polyadenylation of this exon by blocking both cleavage and polyadenylation steps. Addition of recombinant Hu proteins decreased the binding of a polyadenylation factor, the cleavage stimulation factor (CstF) 64KD, to the polyA site. PolyA addition assays demonstrated that the ability of Hu proteins to inhibit polyadenylation depended on intact U-rich sequence. We are in the process to identify the underlying mechanism of the polyadenylation-inhibiting function of Hu proteins.

The calcitonin/CGRP pre-mRNA represents the first target of Hu proteins that function as novel RNA processing regulators in the nervous systems.

Keywords: Hu proteins, polyadenylation, calcitonin/CGRP

Pseudouridine Synthases: revisiting and refining a mechanism that seemed settled

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The pseudouridine synthases (psi synthases) catalyze the isomerization of specific uridine residues in RNA to pseudouridine (psi). The psi synthases are grouped into five families that share no global sequence similarity except for a single conserved aspartate residue that is essential for catalysis. Crystal structures have been solved for members of all five families and all five families share a core beta-sheet fold, suggesting that they are homologues and probably operate through the same chemical mechanism [1]. Two mechanisms have been proposed for the psi synthases. In the initial steps of the "Michael mechanism", the Asp is the nucleophile in a Michael addition to the pyrimidine ring. In the initial steps of the "acylal mechanism", the Asp stabilizes an oxocarbenium intermediate by adding to C1' of the ribose ring. In both mechanisms, C5 of the uracilate is positioned near C1' of the ribose ring forming a carbon-carbon bond and ultimately, psi. The Michael mechanism appeared to be established by studying the inhibition of the psi synthase TruA with RNA containing 5-fluorouridine [2]. A hydrated product was observed and was believed to be the product formed through ester hydrolysis of a Michael adduct. To further investigate the mechanism, we studied the interaction of stem-loop RNA containing 5-fluorouridine ([f5U]TSL) and TruB (a psi synthase that is not inhibited by [f5U]TSL [3]). TruB was incubated with stem-loop RNA containing f5U in buffer containing 50% [18O] water, and the enzyme and modified [f5U]TSL were analyzed by mass spectrometry. The hydrated products were also analyzed by 2D NMR. [f5U]TSL is not hydrated through ester hydrolysis when incubated with TruB, showing that the mechanism of the psi synthases is not settled.

References:

1. Mueller, E.G., Chips off the old block. Nat. Struct. Biol., 2002. 9(5): p. 320-322.
2. Gu, X.R., Y.Q. Liu, and D.V. Santi, The mechanism of pseudouridine synthase I as deduced from its interaction with 5-fluorouracil-tRNA. Proc. Natl. Acad. Sci. U. S. A., 1999. 96(25): p. 14270-14275.
3. Spedaliere, C.J. and E.G. Mueller, Not all pseudouridine synthases are potently inhibited by RNA containing 5-fluorouridine. RNA, 2004. 10(2): p. 192-199.

Keywords: Pseudouridine Synthases, 5-fluorouridine, dyskerin

Discriminate RNP Remodeling and Conformational Proofreading by a DEAD-box Protein

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DExH/D proteins, which are involved in virtually all aspects of RNA metabolism, catalyze NTP-driven conformational changes as well as conformational proofreading reactions in large RNA-protein assemblies such as the pre-mRNA splicing machinery. Although their physiological roles require highly selective activities, DExH/D proteins have generally not displayed significant preference towards particular substrates in vitro. It is thus unclear how inherently non-selective RNA and RNP remodeling proteins are turned into highly selective enzymes. Here we identify a molecular mechanism through which inherently non-selective RNA/RNP remodeling capacity of DExH/D proteins can be turned into a highly selective activity. We demonstrate in vitro that the RNA binding protein U1A can confer a high degree of selectivity to the DExH/D protein DED1 without any direct interaction between the two proteins and enable DED1 to carry out a conformational proofreading reaction as well as the selective remodeling of a defined region within a more complex RNP.

Keywords: DEXH/D, RNPase, Selectivity

Primary sequence and RNA secondary structure are important for high affinity CsrA binding

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Csr (carbon storage regulation) is a global regulatory system that controls bacterial gene expression post-transcriptionally. There are at least three components of the Csr system in E. coli: CsrA, CsrB and CsrC. CsrA is an RNA binding protein that plays a central role in repressing several stationary phase processes and activating certain exponential phase functions. CsrB and CsrC are non-coding regulatory RNAs that contain multiple CsrA binding sites. Both CsrB and CsrC are capable of sequestering CsrA and antagonizing its activity. CsrA regulates translation initiation of glgC and cstA mRNAs by blocking ribosome access to their cognate Shine-Dalgarno sequences. All of the known CsrA-regulated mRNAs contain multiple CsrA binding sites, although there is a considerable amount of sequence variation among the CsrA binding sites of different RNA targets. SELEX was carried out to identify high affinity RNA ligands that bind to CsrA. The SELEX-derived consensus was determined as RUACARGGAUGU (where R is any purine). All but one of the RNA ligands that was cloned and sequenced contained GGA in the loop of a short hairpin. Deletion of the conserved GGA severely affected CsrA-RNA interaction. Additional mutant transcripts were examined to investigate the significance of primary sequence and RNA secondary structure on CsrA binding. The above experiments revealed that both primary sequence and RNA secondary structure are important for CsrA-RNA interaction.

Keywords: CsrA, SELEX, RNA binding protein

Transcription pauses in the untranslated leader of Bacillus subtilis trp operon

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The trp RNA-binding attenuation protein (TRAP) down regulates expression of the Bacillus subtilis trpEDCFBA operon in response to tryptophan by transcription attenuation and translation control mechanisms. Tryptophan-activated TRAP has a short time frame to control this regulatory decision. When transcribing the 5'-untranslated leader of the trp operon in vitro, RNA polymerase pauses at two sites. The first site just precedes the critical overlap between the alternative antiterminator and terminator structures (U107). The second pause site is immediately downstream from the trp leader termination points (U144). These pause positions well correspond to their role in the transcription attenuation and translation control, respectively. Pausing at both sites is stimulated by the general bacterial transcription elongation factor NusA. The pause events allow the synchronization of transcription with TRAP binding and/or RNA folding thus providing a correct regulatory response to the intracellular concentration of tryptophan. Substitution of the natural 3' U residues at both pause sites to purines (A and G) compromises pausing more severely than substitution to C. Furthermore, point mutations at position 144 also reduce reduce the footprint of the paused transcription bubble in vivo. Importantly, these mutations result in elevated trp operon expression in vivo, thereby establishing for the first time that RNA polymerase pausing can influence bacterial gene expression.

Keywords: bacterial gene regulation, RNA polymerase pausing, Bacillus subtilis trp operon

Binding and Structural Studies of Bacillus subtilis Antiterminator Bulge/tRNA Complex Formation Using Fluorescence Techniques

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A unique RNA/RNA interaction occurs between uncharged tRNA and the mRNA 5' leader region of many Gram-positive bacterial tRNA synthetase, amino acid biosynthesis, and amino acid transport genes (1). This interaction leads to antitermination of transcription and complete transcription of the gene. Without this interaction (i.e. in the presence of only charged tRNA) transcription termination occurs (2). The current investigations revolve around the structural features involved in the recognition of the tRNA acceptor stem by the antiterminator, containing a unique RNA bulge, in the leader region (3,4). High affinity interactions between the antiterminator and tRNA have been detected using steady-state fluorescence. Using these same fluorescence studies, possible conformational flexibility has been detected in a single-nucleotide, reduced-function variant of the antiterminator. Further fluorescence studies of the interactions of the antiterminator bulge and its variant with the wild-type and variant tRNA as well as aminoglycosides will be presented.

References:
(1) Grundy, F.J.; Moir, T.R.; Haldeman, M.T.; Henkin, T.M. (2002) Nucleic Acids Res. 30, 1646-1655.
(2) Grundy, F.J.; Collins, J.A.; Rollins, S.M.; and Henkin, T.M. (2000) RNA 6, 1131-1141.
(3) Gerdeman, M.S.; Henkin, T.M.; Hines, J.V. (2002) Nucleic Acids Res. 30, 1065-1072.
(4) Gerdeman, M.S.; Henkin, T.M.; Hines, J.V. (2003) J. Mol. Biol. 326, 189-201.

Keywords: mRNA/tRNA interaction, fluorescence, RNA bulge

Application of an Invasive Cleavage Assay to Study the Effect of AU-rich Elements on mRNA Turnover Rates

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Genes encoding proteins such as cytokines, growth factors and proto-oncogenes often produce highly unstable transcripts. The instability of many of these mRNAs is due to the presence of an AU-rich element (ARE) in the 3' UTR. Here we describe the application of a novel technology, the Invader RNA assay, to the study of ARE-mediated mRNA decay. The Invader RNA assay is based on cleavage of a target RNA-specific probe at a unique one-base-overlap structure generated by the simultaneous hybridization of an invasive DNA primer. The product generated by this reaction serves as an invasive primer in a second reaction to generate a fluorescent signal resulting from cleavage between a fluorophore and quencher on a common reporter oligo. The Invader RNA assay is isothermal, is independent of variables introduced by RT-PCR, can be performed on cell lysates, can detect fewer than 10 molecules of RNA per cell and provides absolute quantitation. The basic approach used the tet repressor to control a beta-globin reporter gene in which the 3' UTR and polyadenylation signals were replaced with a multiple cloning site and a strong, synthetic polyadenylation element (SPA). An ARE was inserted into the 3' UTR of some genes to examine the impact of an instability element on mRNA degradation. To apply the Invader RNA assay we first developed and qualified Invader sets for beta-globin mRNA. Plasmids were transiently transfected into LM(tk-) cells that stably express the tetracycline repressor, and mRNA decay was analyzed by assaying changes in steady state over time after addition of tetracycline to the medium. Our results are comparable to those obtained by Northern blot and RPA, indicating the utility of the Invader RNA assay for studying mRNA decay. Supported by NIH grants GM38277 and 55407 to DRS. ELM was supported by Oncology Fellows training grant T32 CA093838 to the OSU Comprehensive Cancer Center.

Keywords: AU-rich element, Invasive cleavage assay, mRNA decay

Tyrosine phosphorylation of PMR1 is required for the targeting and degradation of polyribosome-bound substrate mRNA

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PMR1 is a polysome-associated mRNA endonuclease that catalyzes the degradation of albumin mRNA in Xenopus liver and PTC-containing beta-globin mRNA in murine erythroid cells. PMR1 is part of 2 complexes; a ~680 kDa complex with substrate mRNA (complex I), and a smaller complex (complex II, 1). The C-terminal 100 amino acids contain a complete targeting domain that includes a predicted tyrosine phosphorylation site at position 650. Phosphorylation at this site was confirmed by western blot with phosphotyrosine-specific monoclonal antibody of TAP-tagged protein recovered on IgG-Sepharose. Co-ip experiments confirmed that PMR1 in Xenopus liver was also tyrosine phosphorylated. The phosphorylation site comprises the sequence RDGDRFYY. Mutating each tyrosine to phenylalanine mapped phosphorylation to the second tyrosine, Y650. This mutation was as effective as a targeting domain deletion in blocking polysome targeting of PMR1. A Y650D mutation was also defective in polysome targeting, confirming the requirement for tyrosine phosphorylation at this site. Albumin mRNA was selectively recovered by PMR1-TAP but not PMR1 bearing the Y650F mutation. On glycerol gradients Y650F PMR1 is only present in the lighter complex II that lacks substrate mRNA, confirming that tyrosine phosphorylation is required targeting by PMR1. The impact of tyrosine phosphorylation on PMR1-mediated mRNA decay was examined by co-expressing catalytically active protein together with albumin and luciferase mRNA. While the Y650F mutation had no effect on catalytic activity, it stabilized albumin mRNA. mRNA stabilization also occurred when cells were treated with a general tyrosine kinase inhibitor. These data provide a novel link between endonuclease-mediated mRNA decay and signal transduction, and suggest that an SH2 domain protein brings PMR1 to the translating mRNP.

References:

1. Yang, F and Schoenberg, DR. Mol. Cell. 14: 435-445, 2004

Keywords: mRNA decay, PMR1, tyrosine kinase

Protein-RNA interactions in the central domain of the 30 S ribosomal subunit

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The central domain of Escherichia coli 16 S ribosomal RNA is the binding site for several ribosomal proteins including the primary binding protein, S15. In vitro studies performed in the Williamson lab indicate that only a portion of the central domain- helices 21, 22, and 23 and the three-way junction joining helices 20, 21, and 22, are necessary for binding of this region's proteins. These proteins appear to stabilize the structure and initiate conformational changes for folding the rRNA into the active conformation.

Nucleotides 726-731 (730 loop) form a highly-conserved tetra-loop in helix 23a. Previous studies indicate the 730 loop interacts with S15, but is not essential for S15 binding in vitro. This suggests that the 730 loop helps to stabilize the binding of S15 and the subsequent folding of the entire central domain. To identify nucleotides and structural motifs of the 730 loop essential for the function of the ribosome, nucleotides 726-731 were randomly mutated and functional sequences were selected. Seventy-six unique mutants have been isolated, sequenced, and assayed for function.

Though the 730 loop is highly conserved phylogenetically, multiple clones with high function were obtained. Analysis of the 730 loop clones has revealed a clear preference at every position in the loop. This indicates a certain structural motif is important for the function of the ribosome. Also, significant covariations were found suggesting interactions between several nucleotides in the loop, in particular 726 and 731. To investigate the 726/731 covariation, single and double mutants were constructed. Functional analysis of the 726:731 mutants suggests a base-pair is needed as the closing pair of the 730 loop.

Keywords: 16 S ribosomal RNA, central domain, ribosomal proteins

Structural Origin of the Memory Effect in Single Hairpin Ribozymes

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RNA molecules play a central and active role in numerous biological processes such as translation, splicing, gene expression, and viral replication. In order to become active, RNA molecules fold into native secondary and tertiary structures. A detailed understanding of RNA folding dynamics is therefore needed to understand its biological activity. The hairpin ribozyme provides an excellent model system for studying RNA tertiary structure and dynamics. The natural function of the hairpin ribozyme is the self-catalyzed backbone cleavage and ligation as part of the rolling circle replication of Nepovirus satellite RNA. Previous studies of an engineered minimal two-way junction version of the hairpin ribozyme have revealed heterogeneity in folding and in catalysis. Specifically, the hairpin ribozyme has been shown to exhibit four molecular sub-populations with distinct folding (docking) and cleavage properties. It has been further demonstrated that individual RNA molecules tend to remain within a single sub-population over long periods of time, giving rise to a “memory effect” in docking.
In this study we seek to gain further insight into the structural origin of this “memory effect” observed in the hairpin ribozyme. To this end, we have developed a fluorescence resonance energy transfer (FRET) based native gel mobility assay that enables us to separate it into two major sub-populations. The isolated sub-populations are then further examined using FRET both in bulk solution and under a single-molecule fluorescence microscope. Distinct folding behaviors of the isolated sub-populations are evident when examined under our single-molecule microscope. Furthermore, our measurements reveal that efficient interconversion between sub-populations can be achieved through heating to >60°C. The apparent lack of interconversion at lower temperatures indicates that a large energy barrier exists between the sub-populations and that interconversion may require melting of at least one of the ribozyme’s secondary structure elements.

Keywords: Hairpin Ribozyme, Single Molecule Microscopy, Folding Dynamics

Peripheral sequence elements key to self-cleavage activity of natural hammerhead ribozymes by forming tertiary contacts

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Hammerhead ribozymes (hRz) are small, naturally occurring RNA motifs found mostly in viroids, where they site-specifically cleave RNA during replication. Recent work by Khvorova1, by De la Peña2 and by our lab3 showed that tertiary contacts outside the catalytic core stabilize natural hRz at physiological Mg2+ concentrations. The natural hammerheads cleave well in intracellular assays compared to the minimalist hRzs. The magnesium ion-induced folding occurs in a single step for the natural hRzs, a contrast to the two-step folding seen upon disruption of the loop I and II interaction4. We are exploring the contributions of peripheral sequence elements to catalysis by natural and engineered hRz. Artificial hammerheads containing tertiary stabilizing motifs (TSM) cleave in absence of magnesium with the addition of spermidine or cobalt hexammine and they are more stable at high temperatures. Searches for TSM-containing hammerhead and hammerhead-like sequences in animal database gave several potential hits in Schistosomes, crickets and even rodents and humans. Kinetic studies of the cricket hammerhead show the influence of TSM in the catalytic activity of the molecule under physiological magnesium concentrations (0.5 mM). Mutational studies suggest potential structural models of tertiary contacts in cricket hammerhead.

References:

1) Khvorova A, Lescoute A, Westhof E, Jayasena SD. (2003) Sequence elements outside the hammerhead ribozyme catalytic core enable intracellular activity. 10(9), 708-12.

2) De la Peña M, gago S, Flores R. (2003) Peripheral regions of natural hammerhead ribozymes greatly increase their self-cleavage activity. 22(20) 5561-70.

3) Saksmerprome V, Roychowdhury-Saha M, Jayasena S, Khvorova A, Burke DH. (2004) Artificial tertiary motifs stabilize trans-cleaving hammerhead ribozymes under conditions of sub-millimolar divalent ions and high temperatures.

Keywords: Hammerhead ribozyme, Kinetic study, mutational study

Endogenously expressed multimeric self-cleaving hammerhead ribozymes Ablate mutant collagen *IN CELLULO*

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Hammerhead ribozymes are small catalytic RNA molecules that can be targeted to any RNA molecule containing a putative cleavage site, and can selectively eliminate mutant gene products producing dominant negative effects, such as those found in severe variants of osteogenesis imperfecta. We previously reported the development of a self-cleaving multimeric hammerhead ribozyme (Smicun et al, 2003). A vector was developed (pCOLZ) that used the COL1A1 promoter to drive expression of the multimeric ribozyme (M8Rz547) and its monomeric counterpart (Rz547). The multimeric and monomeric ribozymes in this vector were stably co-expressed in MC3T3-E1 osteoblasts expressing the truncated COL1A1 target transcript pMG155. The multimeric ribozyme exhibited self-cleavage to derivative fragments, including monomers. Increased expression of ribozymes was found in cells expressing pCOLZM8Rz547 compared to the cells expressing pCOLZRz547. A modest reduction of truncated target transcript and protein was seen in cells expressing the ribozyme monomer, while nearly complete ablation of target transcript and protein was produced in cells expressing the ribozyme multimer. Both monomeric and multimeric ribozymes expressed from the pCOLZ vector were found in the nucleus and cytoplasm, and target transcript reduction appeared to be more active in the nucleus than the cytoplasm. A reversion to a more normal collagen phenotype, measured as an increase in fibril diameter and restored fibrillar architecture, and in a decreased rate of collagen turnover, was produced in cells expressing the ribozyme multimer. Self-cleaving multimeric ribozymes expressed from an endogenous promoter are more effective than monomers at eliminating target gene products and restore a normal collagen phenotype *in cellulo*.

Leader Conformation of a Bound Precursor tRNA at the B. subtilis RNase P Holoenzyme Active Site

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Ribonuclease P holoenzyme (RNase P) catalyzes the 5' maturation of precursor tRNAs (pre-tRNAs). The B. subtilis RNase P holoenzyme consists of an RNA component and a small protein component. The RNA component of RNase P alone is catalytically active in vitro, which makes RNase P a ribozyme. The protein cofactor, however, is essential in vivo. Data from crosslinking and kinetic studies have demonstrated that the protein component directly interacts with the 5' leader of the pre-tRNA substrate and that the active site may be localized near the RNA-protein interface. However, there is currently little detailed structural information about the RNase P active site. To further characterize the structure of the RNase P holoenzyme-pre-tRNA complex, we have used time-resolved fluorescence resonance energy transfer (tr-FRET) to measure the distance between two fluorophores in this complex. We have site-specifically labeled several single cysteine mutants of the RNase P protein with tetramethylrhodamine, while labeling pre-tRNA at various positions in the 5'-leader, including the cleavage site, with fluorescein. The RNase P holoenzyme-pre-tRNA ternary complex was formed using RNase P protein and pre-tRNA labeled at various sites and the distance between the fluorophores was measured using tr-FRET. These measured distances allow us to characterize the conformation of the 5' leader and cleavage site of pre-tRNA relative to the RNase P protein in the ternary complex. Based on these data, we have refined a structural model for the RNase P-pre-tRNA complex.

Keywords: RNase P, FRET, ribozyme

The Protein Component of Ribonuclease P from Escherichia coli Enhances Affinity for Both Precursor and Product tRNA

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Ribonuclease P (RNase P), which catalyzes the removal of 5' leader sequences from pre-tRNAs, is a heterodimer composed of an individual protein subunit and a RNA subunit which can bind and catalyze pre-tRNA cleavage and protein subunits. Analyses of the Bacillus subtilis RNase P shows that the protein subunit contacts the 5’ -leader sequence of pre-tRNA, and thus selectively contributes to the affinity of the holoenzyme for pre-tRNA, but have little effects on tRNA product binding. Here, we show that the Escherichia coli RNae P holoenzyme, which has the more common structural feature characteristic of Type A RNA subunit than Type B RNA subunit of B. subtilis holoenzyme, binds tightly to both pre0tRNA and tRNA. Tight binding of product tRNA appears to be a general phenomenom since the holoenzyme also binds tightly to two E. coli tRNAs. Interestingly, a hybrid enzyme containing the E. coli P RNA and the B. subtilis protein subunit also binds product tightly suggesting that enhanced product binding is due to an indirect effects of the protein of RNA-RNA interactions unique to the Type A RNase P RNA. In contrast to tRNA, the product of a model duplex substrate binds weakly to E.coli RNase P, providing evidence that the proein sensitive interactions are within the tertiary structure of tRNA and do not involve the cleavage site. These results provide evidence for novel contributions of the protein subnit to holoenzyme function and suggest that product inhibition may be in important factor in the physiological function of RNase P.

Keywords: RNase P, Holoenzyme, tRNA binding

Mechanism and Control of Allostery in Kin.46, a Self-thiophosphorylating RNA

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Kin.46 was selected for transfer of the thiophosphate from ATP-gamma-S to its own 5' hydroxyl [1,2]. Addition of an exogenous deoxyoligonucleotide complementary to the 3´primer binding sequence (PBS) increases activity 1000-fold. This allosteric activation was an unintentional consequence of including the oligo in the original selection design. The present work examines the structural basis of this allosteric activation using organomercurial PAGE [3] for kinetic analysis. A 5 nt “linker” region connects the activator helix to the substrate-binding internal guide sequence and may stabilize long-range interactions between the linker and nucleotides closer to the catalytic core. Catalytic activity is dramatically reduced by perturbations in this region, either by base pairing through extension of the oligo activator or by changes to the sequence itself. In the absence of effector oligo, attenuation of activity could be partially relieved by shortening the 3' PBS, and Eyring analysis of these truncated constructs revealed that the thermodynamic activation parameters (delta H and delta S of activation) increased with increasing 3' PBS length, suggesting that the effector oligo may increase activity through thermodynamic stabilization of the 3' end of the ribozyme, irrespective of sequence, both in the presence and absence of the appropriate effector oligo. Furthermore, changing the nucleotide composition of the hybrid RNA-DNA activator helix, to all-RNA, all-DNA, or DNA-RNA, only slightly reduces activity, ruling out the possibility of tertiary docking through 2' hydroxyls in the activating helix. A 7 nt RNA substrate can be thiophosphorylated in multiple turnover using a trans configuration of Kin.46 that severs these seven nucleotides from a 20 nt loop (L1) that connects the RNA substrate to the ribozyme in the cis constructs. Deletion analysis of L1 in the trans ribozyme suggests that allostery in the intact Kin.46 may arise from displacement of inhibitory interactions between L1 and the 3' PBS.

References:
1. Lorsch, J. R. and Szostak, J. W. (1994) Nature 371, 31-36
2. Lorsch, J. R. and Szostak, J. W. (1995) Biochemistry 34, 15315-15327
3. Rhee, S. S. and Burke, D. H. (2004) Anal. Biochem. 325, 137-143

Keywords: ribozyme, allostery, phosphorylation

Enzymatic Ligation of Tethered Mononucleotides by DNA Ligase

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DNA ligase seals nicks in dsDNA and is an essential enzyme involved in DNA repair, recombination. DNA Ligases are among the most widely used enzymes in molecular biology and biotechnology today, commonly used for cloning, detection of single nucleotide polymorphisms, and for pathogen identification. Ligase requires a reactive hydroxyl group on the upstream “tag” fragment aligned with a phosphoryl group from the downstream “acceptor” fragment. While this is normally involves Watson-Crick basepairing of DNA bases at the ligation junction, structural contexts other than DNA-DNA duplexes are compatible with enzymatic ligation. RNA fragments can also be joined using T4 DNA ligase when bridged by a complementary oligodeoxynucleotide bridge. We are using ligation-based methods for preparation of substrates and identification of products from selections for ribokinase ribozymes.

We examined enzymatic ligation at a minimal junction in which only a single terminal nucleotide of the donor fragment is capable of annealing adjacent to the acceptor. The junction nucleotide is anchored via a flexible tether attached to a small oligonucleotide that anneals nearby on the bridging oligo. This arrangement mimics the case in which an oligonucleotide is complementary to most of its target, but is significantly mismatched near the ligation junction. We found that this ligation can be driven to near completion under optimal conditions. Using standard ATP concentrations the adenylated intermediate accumulates and is only slowly converted to product. By limiting ATP, much less adenylated intermediate is observed and the ligated product accumulates more rapidly and to a greater extent. We interpret these results in terms of a four component model; i) Gap-sealing of the tethered mononucleotide substrate is slow; ii) Enzyme dissociates from adenylated substrate before sealing gap; iii) Free enzyme is re-adenylated rapidly at high ATP or slowly at low ATP concentrations; iv) Adenylated enzyme binds only poorly to adenylated substrate.

Keywords: ribozymes, DNA ligase, kinase

Directional and Orientational Compensation of Self-Assembling Tecto-RNA

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The design and construction of nano-scale structures using processes that exhibit fundamental physical and chemical control is a major aim of nanoscience and fundamental for nanotechnology. Supramolecular self-assembly offers one of the most general strategies for generating nanostructures and is important in a range of fields: chemistry, physics, biology, materials science, and, increasingly, manufacturing. RNA is fully programmable: Self-assembling RNA molecules can be designed by reverse folding: Design is at the level of 3D structure while synthesis is at the level of sequence. Using this approach, we have developed directional, conformation-specific self-assembly of RNA. We have designed RNA molecules that assemble to form long fibers by equilibrium polymerization. We have designed other RNA molecules that assemble cooperatively to form closed, oligomeric complexes. We can control assembly using the principle of orientational compensation, which we have introduced to understand and predict the behavior of new tecto-RNA molecules. Applying this principle, we can design molecules that form long fibers either with head/tail or with left/right compensation.

Keywords: nanotechnology, RNA folding

Spatial Organization of Nuclear DNA by Nucleolar Gathering of tRNA Genes

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Recent genetic studies suggest that transcriptional silencing of RNA polymerase II near tRNA genes (tgm silencing) is not closely related to other forms of transcriptional silencing in yeast, but rather is somehow linked to spatial organization of genes in the nucleus. Although the nucleolus is normally thought of as a specialized site for ribosome biogenesis, we have also shown this to be the site of early pre-tRNA processing in S. cerevisiae. Recently, we have used in situ hybridization to show that most tRNA genes, while distributed throughout the linear genome map, are found primarily at the nucleolus. This result is consistent with the colocalization and possible coregulation of tRNA and rRNA biosynthetic pathways. The mechanism of this spatial organization is unclear, although genetic and cytological studies show that multiple mutations that compromise ribosomal RNA biosynthesis both disorder the nucleolus and release the nucleolar association of the tRNA genes. This three-dimensional organization of pol III expression is expected to be a central consideration in the spatial organization of information in the nucleus. Currently we are investigating whether other sets of coregulated genes are brought together during active transcription.

Keywords: tRNA gene, nucleolus

Translational control in macrophage inflammation

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Induced synthesis of ceruloplasmin (Cp) by interferon (IFN)-gamma in monocyte/macrophages is halted by a delayed translational silencing mechanism. Previously, we described a 29-nt, IFN-Gamma-Activated Inhibitor of Translation (GAIT) stem-loop structure as the 3'-UTR element required for silencing. Here, we identify the proteins that bind the GAIT element, and investigate their assembly into a functional mRNP. Using genetic and biochemical methods, four GAIT element-binding pro-teins have been identified: ribosomal protein L13a, glutamyl-prolyl-tRNA synthetase (GluProRS), NS1-associated protein-1 (NSAP1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Assembly of the GAIT mRNP occurs in two temporally distinct stages. During the early stage, which is complete within 2 h, GluProRS is phosphorylated and released from its residence in the aminoacyl-tRNA multisynthetase complex. Phosphorylated GluProRS interacts with NSAP1 to form a non-functional, pre-GAIT complex. About 12 h later, L13a is phosphorylated and released from the surface of the 60S ribosomal subunit. Released GluProRS joins GAPDH and the pre-GAIT complex to form the functional GAIT complex that binds the 3'-UTR GAIT element of Cp mRNA and blocks its translation. Thus, L13a and GluProRS have divergent functions in protein synthesis: in their usual residence sites in the 60S ribosomal subunit and the multisynthetase complex, respectively, they support global translation. However, regulated translocation to an inflammation-responsive mRNP results in gene-specific translational silencing. This translational control mechanism may be critical for the resolution of inflammation in IFN-gamma-activated macrophages.

References:

1. Mazumder, B., Sampath, P., Seshadri, V., Maitra, R.K., DiCorleto, P.E., and Fox, P.L. Regulated release of L13a from the 60S ribosomal subunit as a mechanism of transcript-specific translational control. Cell 115: 187-198, 2003.

2. Sampath, P., Mazumder, B., Seshadri, V., Gerber, C.A., Chavatte, L. Kinter, M., Ting, S.M., Dignam, J.D., Kim, S., Driscoll, D.M., and Fox, P.L. Noncanonical function of glutamyl-prolyl-tRNA synthetase: Gene-specific silencing of translation. Cell, in press, 2004.

Keywords: translational control, ceruloplasmin, GAIT

Identification and characterization of new RNA editing substrates in Physarum mitochondria

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Most mitochondrial genes in the slime mold Physarum polycephalum are not encoded by a single open reading frame. Instead, functional mRNAs are created by the co-transcriptional insertion of "extra" nucleotides not found in the genome. These nucleotide insertions, which occur on average every 25 nts in mRNAs and every 40 nts in tRNAs and rRNAs, make it difficult to localize mitochondrial genes using standard programs. Indeed, the current map of the Physarum mitochondrial genome contains large gaps and lacks four genes that are common to virtually all other mitochondria (nad2, and4L, nad6, and atp8). We have developed an algorithm for finding genes whose mRNAs require insertional editing and predicting editing sites. Using this algorithm, we mapped the four "missing" genes and estimated the probability of nucleotide insertion at individual sites. We then designed RT-PCR primers based on these predictions and characterized the mRNAs for these four genes, identifying all editing sites. Interestingly, in addition to the expected nucleotide insertions, we find a specific deletion of three encoded A's at one site within the nad2 mRNA. Primer extension analysis of total RNA pools indicated that these nucleotides are absent from essentially all nad2 mRNAs, making this the first example of deletional RNA editing in Physarum mitochondria.

Keywords: RNA editing, bioinformatics, mitochondria