# RIBOZYMES: BASIC SCIENCE AND THERAPEUTIC APPLICATIONS

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# Ribozymes: Basic Science and Therapeutic Applications

*Keynote Address*

**A6-001** RECONSTRUCTING ANCIENT FORMS OF LIFE, Steven A. Benner, Department of Chemistry, Swiss Federal Institute of Technology, CH-8092 Zürich, Switzerland

Three decades ago, Pauling and Zuckerkandl pointed out that the biological macromolecules from ancient forms of life might be reconstructed following a rule of "maximum parsimony". Parsimony assumes that descendent biomolecules arose from their ancestral forms via the smallest number of independent evolutionary events. This approach was originally intended to be a theoretical exercise. However, the explosion in the size of the protein and DNA sequence databases allows a sophisticated model of biomolecular evolution to be built, while recent advances in technology allows ancient proteins and nucleic acids from now extinct forms of life to be synthesized and studied in the laboratory. The physical and catalytic behavior of these reconstructed biomolecules can be compared with evolutionary are documented in the fossil record. The result is a powerful research tool for discerning the physiological function of contemporary biological molecules and the origin of biological function, especially in higher organisms. Further, models of ancient forms of life as verified by the behavior of reconstructed biomolecules from these life forms provide inspiration for the synthetic organic chemist, suggesting of new types of molecules that mimic behaviors of natural biomolecules, but that provide tests of chemical principles important for living systems. This talk will proceed backwards in time, starting with the present day ruminants, proceeding back to their Oligocene ancestors as they began the successful conquest of the herbivorous ecological niche in the contemporary world, back further to the time when mammals diverged into the various orders that later were to replace dinosaurs as they succumbed to an unfortunate extinction event, back to the time when aquatic plants invaded terrestrial habitats, and then back to the origin of cellular life as we presently know it. The focus will be on enzymes that interact with nucleic acid and nucleotides, in particular RNA and RNA cofactors.

*Large Ribozymes I*

**A6-002** A TALE OF TWO RIBOZYMES AND TWO TAILS ON A RIBOZYME, Thomas R. Cech[1], Arthur J. Zaug[1], Jennifer A. Davila-Aponte[1,2], Toru Nakamura[1], Yuh-Hwa Wang[3], and Jack D. Griffith[3], [1]Howard Hughes Medical Institute, University of Colorado, Boulder, [2]MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, and [3]Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill.

The *Anabaena* pre-tRNA$^{Leu}$ group I intron was converted to an RNA enzyme that uses a guanosine nucleophile to cleave substrate RNAs with multiple turnover. The small size of this ribozyme (241 nt) and the very short internal guide sequence-substrate duplex (P1 = 3 base pairs) led to the expectation that the activity of this ribozyme might differ from that of the well-studied *Tetrahymena* ribozyme (388 nt, P1 = 6 bp). In fact, the *Anabaena* ribozyme has a second-order rate constant for RNA cleavage [$(k_{cat}/K_m)^S$] that is 250- to 500-fold smaller than that of the *Tetrahymena* ribozyme, and a multiple turnover rate constant at saturating concentration of RNA substrate ($k_{cat}$) that is ~400-fold larger. However, these are apples-and-oranges comparisons, as the two ribozymes have different rate-limiting steps for both kinetic conditions. These kinetic differences are expected from the shorter P1 of the *Anabaena* ribozyme; for example, weaker binding of RNA speeds product release during multiple turnover and thereby overcomes the rate-limiting product release observed for the *Tetrahymena* ribozyme. More detailed mechanistic studies of guanosine binding, stereospecificity, DNA cleavage, and pH-rate profile show that the two ribozymes are fundamentally quite similar. To investigate the three-dimensional structure of group I ribozymes, a helix extension method was developed to determine the relative orientation of hairpin stems in a folded RNA molecule. As a control, the aminoacyl acceptor stem and anticodon stem of yeast tRNA$^{Phe}$ were extended with continuous double-helical segments. Electron microscopic examination showed the expected angle (90°) with a range of ±30°. When the P6 and P8 helices of the *Tetrahymena* ribozyme were each extended by 75 base pairs, the ribozyme retained full RNA-cleavage activity. Thus, its tertiary structure appears to be intact. Electron microscopy revealed that the extended stems were clearly visible as two tails, diverging with an angle of 95 ± 25°. The average angle is very close to that predicted by the Michel-Westhof model of the group I intron core. Thus, helix extension may provide a general method for determining key features of three-dimensional structure in folded RNA molecules.

**A6-003** TERTIARY INTERACTIONS IN GROUP I AND GROUP II INTRONS, François Michel[1], Maria Costa[1], Luc Jaeger[1,2] and Eric Westhof[2], [1]Centre de Génétique Moléculaire du CNRS, 91190 Gif-sur-Yvette, France, [2]Institut de Biologie Moléculaire et Cellulaire du C.N.R.S., 67084 Strasbourg, France.

While catalytic introns from the same group share essentially the same ribozyme core, most group I and group II introns include supplementary RNA domains. Even though these non-core components differ among subgroups, they are subject to strong evolutionary constraints. Genetic and thermodynamic studies of the *sunY* intron of bacteriophage T4 have shown that non-core sections of this intron are necessary to stabilize the correct, active three-dimensional fold of its ribozyme core. The ΔG corresponding to the cooperative transition from secondary structure to overall tertiary folding of the entire intron has been adjusted by natural selection to a slightly negative value under physiological conditions, resulting in optimal coupling between the two splicing steps and maximal specificity of exon ligation. Despite the fact that group I and group II introns have not been optimized for maximal thermodynamic stability, these molecules make frequent use of the same motifs to ensure efficient recognition of separate domains: such instances of convergent evolution betray the fact that the structural repertoire of RNA is a rather limited one. Prominent among frequently used components are terminal loops of the GNRA family. As shown by phylogenetic, kinetic and thermodynamic analyses of group I and group II introns, and also by in vitro selection, interaction between GNRA loops and complementary motifs can be highly specific. As has occasionally happened in Nature, contacts between GNRA loops and their targets can be replaced by classical base pairing between complementary loops, generating so-called 'pseudoknots'. More generally, pseudoknots play a major part in interdomain recognition and novel instances of such interactions, which strongly constrain possible three-dimensional architectures, will be presented for both group I and group II introns.

A6-004 ANTIBIOTIC INTERACTIONS WITH GROUP I INTRON RNA, Renée Schroeder[1], Herbert Wank[1], Katharina Semrad[1], Iris Hoch[1], Uwe von Ahsen[1], Mary G. Wallis[1]and Michael Famulok[2, 1] Institute of Microbiology and Genetics, Vienna Biocenter, A-1030 Vienna, Austria, [2] Institute of Biochemistry of MLU, Munich, Germany.

Small molecules, which contain guanidino groups (arginine, streptomycin, viomycin) can inhibit splicing of group I introns by binding to the guanosine binding-site and by competing with the guanosine cofactor. In addition to these competitive inhibitors, many antibiotics, which are known to be specific inhibitors of prokaryotic protein synthesis (neomycin B, gentamicin, tobramycin) inhibit group I intron splicing with high affinity. Interestingly, only ribosomal A-site specific antibiotics are active on group I introns, raising the question, what do group I intron splicing and ribosomal decoding have in common to make them a common target for the same antibiotics. The similarity of both processes is further underlined by the fact that miscoding-inducing antibiotics also induce mis-splicing. In the T4 phage derived *td* intron, translation and splicing are coupled events, as the intron is inserted into the open reading frame of the mRNA. In order to uncouple splicing from translation we introduced a stop codon in various positions of the upstream exon . Splicing efficiency was greatly reduced, suggesting a role for the ribosome in splicing of group I mRNA introns *in vivo* . For a better understanding of how RNA binds antibiotics we isolated RNA molecules with specific affinities to neomycin B or viomycin via *in vitro* selection. Out of a pool of $10^{15}$ RNA molecules randomized over 74 nucleotides several neomycin B binding motifs and one consensus motif for binding viomycin were selected. Although the initial RNA pool was completely randomized, certain of the selected molecules show sequence homology with naturally occurring RNAs containing antibiotic binding sites.

*Large Ribozymes II*

A6-005 STRUCTURE AND FUNCTION IN RIBONUCLEASE P: CONVERSION OF AN INTERMOLECULAR INTO AN INTRAMOLECULAR REACTION, Norman R. Pace, Daniel N. Frank, Michael E. Harris, James M. Nolan, Bong-Kyeong Oh, and Mary Anne Rubio, Indiana University, Bloomington, IN 47405

RNase P cleaves leader sequences from pre-tRNAs. *In vivo* RNase P is composed of protein and RNA. *In vitro*, at high ionic strength, the bacterial RNase P RNA is active in the absence of the protein moiety. Knowledge of the structure of RNase P RNA is crucial to understanding its action and manipulating its function.

Ongoing phylogenetic-comparative analyses of bacterial RNase P RNA have solved its secondary structure. In the current model of *Escherichia coli* RNase P RNA, for instance, 64% of its 377 nucleotides are engaged in proven helices. Current comparative sequencing efforts seek to define the core of the ribozyme essential for catalysis and to identify tertiary structure contacts. A combination of molecular dynamics computer modeling, and inter- and intramolecular photoaffinity crosslinking is being used to orient RNase P RNA structural elements relative to one another, and thereby to infer the tertiary structure of the RNA. The current version of the RNase P RNA tertiary structure will be reviewed.

The kinetic analysis of RNase P-mediated catalysis has been complicated because product release is normally rate-limiting. Furthermore, the intermolecular nature of the cleavage reaction precludes many applications of *in vitro* selection schemes to the analysis of RNase P. In order to examine and to manipulate RNase P function more effectively, we used the new structural information to design a pair of ribozymes in which circularly permuted RNase P RNAs are covalently linked to a pre-tRNA substrate so that the substrate is positioned at the active site. These "active-site-tethered" pre-tRNA-RNase P RNA conjugates undergo accurate and efficient self-cleavage *in vitro*, with first-order reaction rates equivalent to the rate of the chemical step of the native RNase P reaction.

Unlike most ribozymes, RNase P recognizes its substrate through tertiary RNA-RNA interactions, rather than through extensive Watson-Crick basepairing. However, the development of the active-site-tethered conjugates has led us to create a sequence-specific endonuclease, termed Endo.P. In the Endo.P configuration, the 3' half of the pre-tRNA acceptor stem tethered to RNase P RNA binds exogenous RNA substrates *via* Watson-Crick basepairing; the bound substrate is subsequently cleaved at the predicted site. The demonstration of sequence-specific cleavage by Endo.P expands the potential of RNase P and its derivatives as reagents in gene therapy. (Supported by NIH grant GM34527.)

A6-006 THE CATALYTIC MECHANISMS AND STRUCTURAL FEATURES OF GROUP II INTRON RIBOZYMES, Anna Marie Pyle, William J. Michels, Kevin Chin, Danette Daniels, Justin Green and Dana Abramovitz, Department of Biochemistry and Molecular Biophysics, Columbia University College of Physicians and Surgeons, New York, NY 10032.

Group II introns are essential for the processing of many organellar genes in plants, fungi and other organisms. They have also been widely accepted as models for the catalytic center of the eukaryotic spliceosome. Despite their apparent lack of extended tertiary structure, group II introns contain functionalities for the catalysis of many different chemical transformations. These RNAs appear to rely on networks of noncannonical tertiary interactions to compose their active sites for catalysis. In order to begin detailed studies of structure/function relationships in group II introns, we have further characterized the overall mechanism of group II intron splicing and created multiple-turnover ribozymes from substructures within the intron. We have found that, under all reaction conditions, self-splicing in vitro involves a first step that is partitioned between two competing parallel first-order reactions. Either pathway leads successfully to splicing and has distinct requirements for reaction conditions, including protein cofactors. Both the hydrolytic and the branching pathways can be modeled by ribozymes that have been created to perform these respective reactions intermolecularly. In this way, techniques of molecular biology, chemical synthesis and enzymology have been used to scrutinize the chemical mechanisms and identify functional interactions that contribute to catalysis. The hydrolytic first step path has been studied using a two-part system in which the highly conserved Domain 5 RNA (41 nts) cleaves the 5'-exon/intron boundary in-trans. These studies revealed that D5, despite its apparent lack of base-pairing interactions with the rest of the intron, binds with a $K_d$ of ~ 300 nM (A.M. Pyle & J.B. Green, *Biochemistry* (1994) 33, 2716). The overall reaction rate is limited by the chemical step of hydrolysis rather than conformational changes or off-rate. In order to study this reaction in greater detail, a true ribozyme capable of specifically cleaving small oligonucleotide substrates was derived from the hydrolytic path of the group II intron. The ribozyme contains two subunits: Domain 1 (which base-pairs to the substrate) and Domain 5. The ribozyme specifically cleaves small RNAs with multiple-turnover in a reaction that is also rate-limited by chemistry ($k_{cat}$ = 0.02 $min^{-1}$; $k_{cat}/K_m$ = 3.3 x $10^6$ $M^{-1}min^{-1}$). In addition to revealing the strength of 5'-splice site association with the active site ($K_d$ = 6 nM), this construct has facilitated independent examination of functional groups at the splice site and on D5 that are responsible for catalytic activity and binding. The branch-mediated first step is also being studied using a construct comprised of Domain 5 linked to Domain 6. This RNA is added to 5'-splice site in-trans to catalyze 2'-OH attack by the branch point adenosine. The branch reaction, which is the sum of several competing reactions, is limited by the rate of an internal conformational change. Using these ribozyme constructs, the features important to branching are being differentiated from those that contribute to hydrolysis. Mechanistic data acquired in the course of this study imply that the hydrolytic and branching pathways have distinct biological functions in group II intron processing of RNA.

**A6-007** COMPUTER MODELLING OF RNA STRUCTURE, Eric Westhof, Equipe Modélisation et Simulation des Acides Nucléiques, UPR 9002, Institut de Biologie Moléculaire et Cellulaire du CNRS, 15, rue René Descartes, F-67084 Strasbourg-Cedex, France.

In the field of nucleic acid structure, modelling has a long and famous history, since it extends back to the model of DNA proposed by Watson and Crick in 1953. The amount of hard data which went into the model was not large (axial rise and helical pitch with antiparallel strands because of symmetry). The key discovery was that of isosteric pairings between complementary bases for which the experimental support, Chargaff's rules, was quite indirect. It took, however, another 50 years to unravel the fine details of the DNA helix which govern DNA tertiary structure and recognition (propeller-twist, sequence-induced backbone variations, water and ion binding). For RNA structures, the modelling of the anticodon-codon interaction by Fuller & Hodgson in 1967 and that of a full tRNA by Levitt in 1969 constitute both remarquable achievements. For the anticodon loop, the main assumption was that the number of stacked bases is a maximum. Although the first two residues were wrongly exposed to the solvent, the model provided most of the stereochemical explanations for Crick's wobble hypothesis and for the role of the modified base following the anticodon triplet. Among the dozens of tRNA models put forward, Levitt's model was the only one topologically correct with co-axial stacking of the right stems and some tertiary contacts. The main errors were essentially local (conformation of the T-loop, bases in *syn*). Levitt was the first to use comparative sequence analysis to identify tertiary contacts and molecular mechanics method to refine hand-built structures. In short, the usefulness of RNA modelling resides more in the heuristic power brought about by the suggested topologies or architectures rather than in the fine stereochemical and conformational details. This does not imply that one can be careless about geometry and stereochemistry. On the contrary, the passage from the local structure to the global fold constitutes Achille's heel of RNA modelling. In other words, although there are global constraints that the overall structure must embody (RNA helices are bulky right-handed chiral objects), their links to the fine structure are often too degenerate for uniquely specifying the local environment. Local structure depends on electrostatic forces, stacking interactions, sugar-phosphate conformational energy, base sequence, water and ion binding. The modelling of such an overwhelming amount of mutually coupled interactions is still intractable. This led us to favour interactive modelling techniques coupled with restrained least-squares refinement for building large RNA molecules. This approach has several drawbacks (among others, the heavy reliance on human judgments) which can be partially alleviated by the systematic use of the whole gamut of biochemical and chemical probes available for exploring the atomic accessibilities and by the constant exploitation of comparative sequence analysis. Examples emphasizing the successes and limits of our modelling approach in the field of catalytic RNA (group I introns, M1 RNA of RnaseP, hepatitis delta virus, and hammerhead ribozyme) will be presented. It will be stressed that, rather than tending to absolute predictions, modelling is a tool for producing and visualizing three-dimensional hypotheses which should lead to new experiments and require experimental verification. The size of the structural data base on which most of present RNA modelling rests is, in fact, too limited and the determination of new X-ray (or NMR) structures is becoming an urgent necessity.

## *Therapeutic Applications of Ribozymes*

**A6-008** FACILITATION OF INTRACELLULAR FUNCTIONING OF HAMMERHEAD RIBOZYMES: APPLICATIONS TO TREATMENT OF HIV INFECTION. John Rossi[1], Shawn Westaway[1], Garry Larson[1], Cecile Carbonnelle[1], Don Kohn[2], Chen Zhou[2], Saswati Chatterjee[3], Deepinder Brar[3], K.K. Wong[3], John Zaia[3], Shirley Li[3], and Edouard Bertrand[4]. [1]. Center for Molecular Biology and Gene Therapy, Loma Linda Univ. School of Medicine, Loma Linda, CA, [2]. Childrens Hospital of Los Angeles, USC School of Medicine, Los Angeles, CA., [3]. Division of Pediatrics, City of Hope, Duarte,CA., [4]. Institute Jacob Monod, Paris, France.

In the little over ten years since the discovery that RNA molecules can have catalytic activity, there have been a large number of reports of ribozymes that have been engineered to target and functionally destroy cellular or viral RNAs. There have been mixed levels of success however, in designing ribozymes that function efficiently in cells, and clear indications that *in vitro* kinetic activities of ribozymes often do not always translate into equivalent intracellular activities. A number of problems must be solved before engineered ribozymes can be routinely and successfully used in an intracellular setting. The interactions between ribozymes and their targets need to be maximized; one approach to this problem is to try to ensure that the ribozyme co-localizes with its target RNA inside cells. It is also necessary to understand the effects of interactions of cellular proteins on ribozyme function. Unfortunately, we know very little about how to maximize ribozyme-target interactions inside a cell, the potential effects of cellular and viral RNA binding proteins on ribozyme function, or the signals for directing RNA traffic within the cell. In order to address these problems we have experimented with several different strategies for enhancing the intracellular functioning of ribozymes. One strategy is to co-localize the ribozyme with the viral target by capitalizing on the fact that HIV reverse transcriptase sequesters and localizes cellular $tRNA^{LYS3}$ to the primer binding site of the HIV genome. We have therefore designed and tested a series of chimeric constructs consisting of all or part of $tRNA^{LYS3}$ fused to a hammerhead ribozyme targeted to a cleavage site just upstream of the primer binding site. A series of in vitro binding as well as cell culture viral challenge experiments demonstrate that this is an effective strategy for co-localization of a ribozyme with a retroviral target. We have also developed a series of promoter constructs which are useful for ribozyme expression and directing ribozyme traffic to a particular intracellular compartment. These include viral LTRs, human CMV, U1, U6 and $tRNA^{METi}$. Several of these promoters have been tested in both AAV and retroviral vectors in cell lines, bone marrow $CD34^+$ and peripheral $CD4^+$ cells. Preliminary results with AAV demonstrate that back to back promoter constructs are fully functional, thereby facilitating multi-functional ribozyme expression. Finally, a strategy for co-localizing ribozymes and targets utilizing 3' UTRs is being tested. A model system to test the validity of this approach will be discussed.

**A6-009** TOWARDS THE APPLICATION OF RIBOZYMES TO HUMAN HEALTH, Dan T. Stinchcomb[1], Thale C. Jarvis[1], James D. Thompson[1], Kevin Kisich[1], James A. McSwiggen[1], Dennis Macejak[1], Laverna Alby[1], James K. Belknap[3], Richard A. Majack[3], David F. Ayers[1], Timothy L. McKenzie[1], Pete Stecha[1], Tod Woolf[1], Francine E. Wincott[1], Nassim Usman[1], Elizabeth G. Nabel[2] and J. Joseph Marr[1], [1]Ribozyme Pharmaceuticals, Inc., 2950 Wilderness Pl., Boulder, CO 80301, [2]University of Michigan Medical Center, Ann Arbor, [3]University of Colorado Health Sciences Center, Denver.

Several technological challenges need to be met before ribozymes can be used as therapeutics to treat human disease. We have delivered active ribozymes extracellularly to the appropriate target cells in culture. In these cell culture models, ribozymes significantly inhibit smooth muscle cell proliferation and inhibit the expression of the inflammatory cytokine, TNF-α. Active ribozymes show significantly greater biological activity than catalytically inactive ribozymes or ribozymes with scrambled binding arm sequences. Thus, these ribozymes can bind and cleave their appropriate target RNA in the relevant cellular environment. In some clinical settings, extracellular delivery of ribozymes can be contemplated. For such applications, we have demonstrated that chemically synthesized, stabilized ribozymes can be delivered intact to a number of tissues by intravenous or intraperitoneal injection or by local catheter delivery. In other cases, where long term expression is desired, a gene therapy approach is more relevant. When embedded in RNA transcripts, ribozyme activity is often compromised. We have developed several RNA polymerase III vectors that express high levels of ribozyme-containing transcripts and maintain ribozyme cleavage activity. Further drug development of exogenously delivered ribozymes and endogenously expressed ribozyme-containing genes will permit treatment of diverse diseases, such as restenosis and inflammatory disorders.

*Protein Facilitation of RNA Catalysis*

**A6-010** FACILITATION OF RIBOZYME CATALYSIS AND RNA FOLDING BY RNA CHAPERONES, Daniel Herschlag[1], Mala Khosla[1], Zenta Tsuchihashi[1], Richard L. Karpel[2], Timothy Coetzee[3], and Marlene Belfort[3], [1]Stanford University, Stanford, CA, 94305, [2]University of Maryland, Baltimore, MD,[3]Wadsworth Center, Albany, NY.

When the recognition sequence of a ribozyme is extended beyond a certain length, multiple turnover is slowed and specificity is decreased. We have shown that widely specific RNA binding proteins can act to overcome these fundamental limitations on ribozyme activity. For example, the p7 nucleocapsid (NC) protein of HIV-1 increased multiple turnover of a hammerhead ribozyme by 20 fold and increases specificity up to 100 fold (1). The enhanced turnover has been directly shown to result from catalysis of dissociation of the RNA products from the ribozyme (2). The enhanced specificity appears to arise from catalysis of dissociation of potential RNA substrates, thereby providing the ribozyme a greater ability to choose which substrate to cleave. The hnRNP A1 protein, a peptide polymer derived from its C-terminal consensus domain, and the S12 ribosomal protein also facilitate the hammerhead reaction (2,3).

The action of these RNA binding proteins can be summarized as follows: they exert their effects by accelerating attainment of the thermodynamically most stable species throughout the ribozyme's catalytic cycle. A further example of this is provided by the ability of the NC protein to resolve a misfolded ribozyme/RNA complex that is otherwise long-lived (2). Similarly, the S12 ribosomal protein enhances the splicing of group I introns by facilitating folding prior to the splicing reaction, apparently via nonspecific binding interactions (3).

These and related observations, combined with an analysis of RNA structural features, has suggested that RNA have a fundamental folding problem. The *in vitro* effects of widely specific RNA binding proteins such as NC and hnRNP proteins suggest that these proteins may have a biological role as RNA chaperones that prevent misfolding of RNAs and resolve RNAs that have misfolded, thereby ensuring that RNA is accessible for its biological functions.

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2. Herschlag, D., Khosla, M., Tsuchihashi, Z. and Karpel, R. L. (1994) *EMBO J.* 13, 2913-2924. "An RNA Chaperone Activity of Nonspecific RNA Binding Proteins in Hammerhead Ribozyme Catalysis."
3. Coetzee, T., Herschlag, D., and Belfort, M. (1994) *Genes & Development* **8**, 1575-1588. "*Escherichia Coli* Proteins, Including Ribosomal Protein S12, Facilitate *In Vitro* Splicing of Phage T4 Introns by Acting as RNA Chaperones."

**A6-011** INVOLVEMENT OF THE *NEUROSPORA* MITOCHONDRIAL TYROSYL-tRNA SYNTHETASE IN SPLICING GROUP I INTRONS.

Alan M. Lambowitz, Roland Saldanha, Smita Patel, Christopher Myers, Gerald Wallweber, Yelena Kemel and Mark G. Caprara, Departments of Molecular Genetics, Biochemistry and Medical Biochemistry, The Ohio State University, Columbus, Ohio 43210.

The *Neurospora* CYT-18 protein, the mitochondrial (mt) tyrosyl-tRNA synthetase, functions in splicing group I introns by stabilizing the catalytically-active structure of the intron core. The *Neurospora* mt LSU and *ND1* group I introns are not self-splicing and are completely dependent on the CYT-18 protein for splicing both *in vivo* and *in vitro*. Chemical structure mapping showed that these group I introns can form most of the conserved secondary structure in the absence of the CYT-18 protein, but that CYT-18 is required for formation of the active tertiary structure. Kinetic measurements suggest that the binding of CYT-18 to the mt LSU intron is a two step process, with an initial bimolecular step that is close to diffusion limited ($3.24 \times 10^7$ $M^{-1}$ $s^{-1}$) followed by a slower step (0.54 $s^{-1}$). This biphasic binding might reflect rapid binding of CYT-18 to the unfolded intron, followed by all or part of a slower conformational change leading to the active tertiary structure. After CYT-18 binding, splicing occurs at a rate of 0.0025 $s^{-1}$, comparable to the rate of self-splicing of the *Tetrahymena* LSU intron. The $K_d$ for the complex between the CYT-18 protein and a version of the *Neurospora* mt LSU intron, calculated from $k_{off}/k_{on}$, was < 3 pM. As a result of this tight binding, the CYT-18 protein functions stoichiometrically in *in vitro* splicing reactions due to its extremely slow dissociation from the excised intron RNA. Specific mechanisms may exist for dissociating the protein from the excised intron RNA *in vivo*. The structure of the complexes between the CYT-18 protein and the mt LSU and *ND1* introns were analyzed by a variety of techniques, including chemical structure mapping, modification interference, and SELEX. These experiments showed that (i) CYT-18 can bind with high affinity to a small RNA containing the P4-P5-P6-P6a segment of the intron, but additional sequences in P7-P9 are required for maximal binding, (ii) specific nucleotides at or near the junction of the P4-P6 stacked helix appear to be involved in binding, and (iii) the binding of CYT-18 results in strong protection of the phosphate backbone in P4, P6 and P9. These findings combined with the ability of CYT-18 to suppress structural mutations in different regions the phage T4 *td* intron suggest that CYT-18 binds to each of the two major helices of the group I intron catalytic core and stabilizes them in the correct relative orientiation to form the intron's active site.

**A6-012** HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEINS AS FACILITATORS OF NUCLEAR PRE-mRNA PROCESSING,

Maurice S. Swanson[1], James T. Anderson[1], Scott M. Wilson[1], Annette M. Oberdorf[1], Jill W. Miller[1], Kshama V. Datar[1], and Michael R. Paddy[2], [1]Department of Immunology and Medical Microbiology and the Center for Mammalian Genetics, [2]Department of Anatomy and Cell Biology and the Center for Structural Biology, University of Florida College of Medicine, Gainesville, FL 32610-0266.

RNA polymerase II (pol II) transcripts are extensively processed within the nucleus prior to export of mRNAs into the cytoplasm. These RNA processing events include pre-mRNA capping, splicing, and polyadenylation followed by nucleocytoplasmic export of the product mRNAs. Although heterogeneous nuclear ribonucleoproteins (hnRNPs) are abundant nuclear proteins which directly bind to nascent pol II transcripts, and are believed to play an important role in pre-mRNA processing, their specific nuclear functions have remained obscure. To investigate possible functions of hnRNPs *in vivo*, we have isolated and characterized nuclear polyadenylated RNA-binding (Nab) proteins from *Saccharomyces cerevisiae*[1,2]. The Nab proteins are crosslinked by UV-light to poly(A)+ RNAs *in vivo* , and are localized within the nucleus with a diffuse nucleoplasmic distribution similar to the localization of hnRNPs in human cells. Nab1p is identical to Npl3p/Nop3p previously characterized as a factor required for both protein import into the nucleus and pre-rRNA processing within the nucleolus. Since both the *NAB2* and *NAB3* genes are essential for cell viability, we isolated conditional alleles of these genes to study Nab2p and Nab3p function *in vivo*. The Nab2 protein is required for both the regulation of poly(A) tail length and nucleocytoplasmic export of mRNAs. Loss of wild-type Nab2p by growth of a *GAL::nab2* strain in glucose leads to a dramatic increase in the length of poly(A) tails on mRNAs, and the accumulation of poly(A)+ RNAs within the nucleus. Temperature-sensitive *nab2* strains are also defective in poly(A) tail length and nucleocytoplasmic mRNA export. Long poly(A) tails are added to precursor RNAs using a mutant *nab2* cell extract, but Nab2p is not an essential factor for 3'-end processing *in vitro*. In contrast, loss of Nab3p by glucose repression of a *GAL::nab3* allele does not affect poly(A) tail length, mRNA export, or rRNA processing, but does inhibit pre-mRNA splicing *in vivo*[2]. These results have led to the hypothesis that the Nab proteins are involved in the organization, or packaging, of different regions within nascent pol II transcripts to form discrete RNA-RNP structures which facilitiate subsequent RNA processing reactions.

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2. Wilson, S.M., Datar, K.V., Paddy, M.R., Swedlow, J.R., and Swanson, M.S. 1994. Characterization of nuclear polyadenylated RNA-binding proteins in *Saccharomyces cerevisiae*. *J. Cell Biol.*, in press.

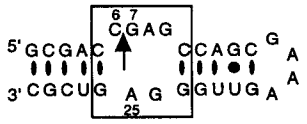
*Structural Biology of Ribozymes: X-Ray Crystallography and NMR*

**A6-013** THREE-DIMENSIONAL STRUCTURE OF A HAMMERHEAD RIBOZYME, Heinz W. Pley, Kevin M. Flaherty, and , David B. McKay, Stanford University School of Medicine, Stanford, CA 94305.

The hammerhead ribozyme is a small catalytic RNA motif made up of three base-paired stems and a core of highly conserved, non-complimentary nucleotides that are essential for catalysis. The x-ray crystallographic structure of a hammerhead RNA-DNA, ribozyme-inhibitor complex has been solved to 2.6 Angstrom resolution. The base-paired stems are A-form helices. The core has two structural domains. The first domain, formed by the sequence 5'-CUGA following stem I, is a sharp turn that is identical in structure to the uridine turn of tRNA. The second domain is a non-Watson Crick duplex of sequence $^{5'\text{-U-G-A-3'}}_{3'\text{-A-A-G-5'}}$. Anomalous scattering measurements with $Mn^{2+}$ and $Cd^{2+}$ identify a divalent ion binding site in the second domain. The phosphodiester backbone of the DNA inhibitor strand is splayed at the phosphate 5' to the cleavage site. The structure suggests that the ribozyme facilitates cleavage by destabilizing both the phosphodiester linkage at the cleavage site and the linkage 5' to it.

**A6-014** STRUCTURAL STUDIES OF A LEAD-DEPENDENT RIBOZYME. Arthur Pardi and Pascale Legault, Department of Chemistry and Biochemistry, University of Colorado at Boulder, Boulder, Colorado, 80309-0215, U.S.A.

Our increasing knowledge of ribozyme function still relies on extremely limited structural information. To address this limitation we are using solution NMR spectroscopy to obtain structural data on ribozymes. State-of-the-art heteronuclear multidimensional NMR is being used to determine the three-dimensional the solution structure of a 30-mer $^{13}C/^{15}N$-labeled lead-dependent ribozyme, known as the leadzyme. The leadzyme is a small RNA motif that undergoes cleavage in the presence of lead at a specific phosphodiester bond within an asymmetric purine-rich internal loop.




This internal loop, which is the site of the catalytic activity, displays a variety of interesting structural characteristics which will be discussed in light of the functional properties of the leadzyme. First, A25, which is in proximity of the cleavage site, has an unusual $pK_a$ of 6.5 which means that this adenine is partially protonated at physiological pH. Thus we have performed structural studies of the leadzyme at pH 5.5 to help understand the effect of protonation. Second, many torsion angles which define the three-dimensional structure of the active site internal loop deviate from their standard A-form conformation. For example, we have identified residues with syn glycosidic angles and/or 2' endo sugar puckers. The internal loop of the leadzyme exhibits significant dynamic behavior on the microsecond to millisecond timescale. Such dynamics appear to be a structural characteristic of many other internal and terminal loops in RNAs. Finally, we have performed NMR studies on several variant leadzyme sequences that also show catalytic activity and NMR structural data on these variants will be compared with the structural data on the "wild type" leadzyme.

**A6-015** STRUCTURAL ELEMENTS IN A HAIRPIN RIBOZYME, Ignacio Tinoco, Jr., Zhuoping Cai, and Jeffrey Kieft, Department of Chemistry, University of California, Berkeley, and Structural Biology Division, Lawrence Berkeley Laboratory, Berkeley, CA 94720.

We are studying the structure of a hairpin ribozyme derived from the minus strand of tobacco ring spot virus satellite RNA [(-)sTRSV], which has been engineered to specifically cleave the HIV-1 RNA in its leader sequence. The catalytically active system contains a 49-nucleotide ribozyme which specifically cleaves and ligates a 14-nucleotide substrate. The proposed secondary structure of the ribozyme-substrate complex consists of four short helices separated by two internal loops (loops A and B). The 63-nucleotide ribozyme-substrate complex is very large for obtaining a detailed three-dimensional structure by NMR. Therefore, we have started by studying the high resolution solution structures of two subunits of the complex: a 28-nucleotide RNA which contains the substrate plus the substrate binding site of the ribozyme, and the remaining 35-nucleotide RNA fragment. We hope that the structures of these subunits will allow the determination of the structure of the complete ribozyme.

The substrate plus substrate-binding site consists of two 14-nucleotide strands that form a symmetric internal loop of eight nucleotides (loop A) with its flanking helices. We selectively $^{13}C$-labeled one strand of the internal loop at C8 of each purine and C6 of each pyrimidine; isotope-edited 2D and 3D experiments were used to facilitate resonance assignments. The cleavage site is at a 5'-CpG-3' step that begins the loop next to a G•C base pair. A cross-strand NOE is seen between the imino proton of guanine in the G•C pair and the H1' proton of the conserved guanosine on the 3' side of the cleavage site. This NOE, plus a pure 2'-endo conformation for the cytidine at the 5' side of the cleavage site, indicate that the cytidine is bulged out. The aromatic protons of the cytosine and the H1' protons of the adjacent G•C base pair are broadened, indicating millisecond dynamics for this part of the loop.

The 35-nucleotide fragment can fold into a hairpin loop of three nucleotides, a stem of three base pairs, and an asymmetric internal loop of 16 nucleotides (loop B) closed by a stem of five base pairs. Photo-crosslinking studies of Butcher and Burke [*Biochemistry* 33, 992 (1994)] suggest that the internal loop can have a structure similar to that of loop E of eukaryotic RNAs [Wimberly et al., *Biochemistry* 32, 1078 (1993)]. The NMR studies are at too early a stage to draw conclusions yet.

*Synthesis of Ribozymes With Improved Therapeutic Potential*

**A6-016** MODIFICATION OF THE HAMMERHEAD RIBOZYME FOR STRUCTURE-FUNCTION STUDIES AND IMPROVED BIOLOGICAL ACTIVITY, Fritz Eckstein, Max-Planck-Institut für experimentelle Medizin, Hermann-Rein-Str. 3, D-37075 Göttingen, Germany.

The spatial orientation of the three helical segments of a hammerhead ribozyme in solution has been derived from fluorescence (FRET) measurements. The molecule has a Y-shape with helices I and II in close proximity. Modification studies show that helix II requires a minimum of 2 base pairs for optimal cleavage efficiency and that the closing loop II can be replaced by non-nucleotidic linkers with a length of approximately 17 atoms. Base modifications in the central core provide no evidence for a G/A double mismatch pair but are consistent with the existence of two structurally distinct G12/A9 and A13/G8 mispairs. Modifications of the 2´-OH groups indicate that those at positions G5 and G8 are essential for activity. Hammerhead ribozymes are well suited for interference with gene expression since they can cleave essentially any RNA containing certain trinucleotide sequences. We have chosen to explore the possibilities of applying synthetic ribozymes by exogenous delivery to cells for this purpose. Synthetic unmodified ribozymes are readily degraded by nucleases present in serum and nuclei. However, replacement of the pyrimidine nucleosides by either their 2´-fluoro- or the 2´-aminonucleoside analogues has a dramatic effect on stability against ribonucleases. Several orders of magnitude in stability can be gained. Additionally, the introduction of phosphorothioates at the termini protects against degradation by exonucleases. Such ribozymes directed against the HIV LTR exhibit some protection against HIV replication. However, a higher efficiency of cellular uptake is desirable. It is expected that attachment of hydrophobic residues such as hexaethylene glycol moieties or cholesterol will help to improve uptake. Attachment of these groups at the 5´-end of the ribozyme does in general not impair catalytic efficiency. The 3D model of the ribozyme should be helpful in the design of new constructs.

**A6-017** MODIFIED OLIGONUCLEOTIDES: HYBRIDIZATION PROPERTIES, PHARMACOKINETIC PROPERTIES AND PHARMACOLOGICAL ACTIVITY, Susan M. Freier, C. Frank Bennett, P. Dan Cook, Stanley T. Crooke, Lendell L. Cummins, Nicholas M. Dean, David J. Ecker, Richard H. Griffey, Charles J. Guinosso, Douglas Kornbrust, Janet Leeds, Elena A. Lesnik, Brett P. Monia, Yogesh S. Sanghvi and Joan E. Zuckerman, ISIS Pharmaceuticals, Carlsbad, California 92008.

Therapeutic utility of unmodified DNA or RNA antisense oligonucleotides is often limited by their susceptibility to endogenous nucleases. We have introduced modifications to the sugar-phosphate backbone of oligonucleotides with the primary goal of improving biostability without reducing the high affinity and specificity associated with Watson-Crick hybridization. Substitution at the 2'-position of deoxyribose can simultaneously increase affinity of oligonucleotides for target RNA and reduce their susceptibility to nucleases. For 2'-fluoro and 2'-alkoxy substitutions, small substituent size correlates with the best affinity and large substituent size correlates with the best nuclease resistance. It is possible, however, to identify 2'-modified oligonucleotides in which both affinity and stability are improved over that of an unmodified deoxy-phosphorothioate. Uniformly 2'- modified oligonucleotides are not effective substrates for RNAse H. They have been used, however, to identify antisense sequences that operate by a non-RNAse H mechanism and allow for RNAse H activity when incorporated into 2'-modified oligonucleotides containing 2'-deoxy gaps. Such molecules are associated with increased potency and prolonged activity in cellular assays for antisense activity. Oligonucleotide charge can be reduced by 2'-amino-alkoxy substitution or replacement of the negatively charged phosphate with a neutral backbone such as the MMI linkage (methyleneoxy-methylimino) or the amide linkage used in PNA (peptide nucleic acid). These modifications can also improve hybridization affinity and increase nuclease resistance. *In vitro* and *in vivo* data on modified oligonucleotides will be presented including hybridization thermodynamics, nuclease susceptibility, pharmacokinetic properties and pharmacological activity.

**A6-018** RNA SYNTHESIS AND RIBOZYMES, Nassim Usman*, Leonid Beigelman, Kenneth Draper, Francine Wincott & James McSwiggen, Departments of Chemistry & Biochemistry, Cell Biology and Enzymology, Ribozyme Pharmaceuticals, Inc., 2950 Wilderness Place, Boulder, CO 80301

A variety of selective and uniform structural modifications have been applied to DNA oligonucleotides to enhance their nuclease resistance.[1] A strategy of uniform modification is not applicable to ribozyme (Rz) modification since some ribose residues in the catalytic core are required for reasonable catalytic activity. Hammerhead (HH) Rzs can tolerate a number of modified residues and still maintain some catalytic activity as well as exhibit better nuclease stability.[2-4] A systematic study of the catalytic activity and nuclease stability of selectively modified 36-mer HH Rzs has resulted in a generic motif containing 5 ribose residues, 29-30 2'-*O*-Me nucleotides, 1-2 other 2'-modified nucleotides at positions U4 and/or U7 and a 3'-3'-linked "cap". Eight different 2'-modified uridine residues were introduced at positions U4 and/or U7 of a specific Rz sequence. From the resulting set of 24 Rzs, several have almost wild-type catalytic activity. More specifically, Rzs containing U4 and U7 $NH_2$ and U4 *C*-allyl substitutions have $k_{cat}/K_M$ = ~6 x $10^7$ $M^{-1}min^{-1}$ and 0.3 x $10^7$ $M^{-1}min^{-1}$ respectively (the parent all-RNA Rz and one containing 2'-*O*-Me binding arms have $k_{cat}/K_M$ = ~4 x $10^7$ $M^{-1}min^{-1}$). The stability of these two Rzs, expressed as a half-life was ~72 h at nM concentrations in a variety of biological fluids including human serum. The overall increase in stability/activity obtained is 14,000-21,000-fold over that of the all-RNA parent Rz (stability half-life of ~0.1 min). A number of improvements have been made to the standard chemical synthetic method for preparing biologically active RNA[5] to synthesize the large numbers and amounts of ribozymes needed to arrive at such stable and active Rzs. Changes in the conditions of coupling and significant changes to the deprotection protocols have resulted in an increase in yield of Rz product and shortened dramatically the overall time required for synthesis and deprotection.

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*Viral and Lipid-Mediated Delivery of Ribozymes*

**A6-019 ADENO-ASSOCIATED VIRUS VECTORS FOR IN VIVO GENE TRANSFER,** Barrie Carter[1], Thomas Reynolds[1], William Guggino[2] and Terry Flotte[2], [1]Targeted Genetics Corporation, Seattle, [2]Johns Hopkins Hospital, Baltimore

Adeno-associated virus (AAV) has several desirable features as a vector for gene therapy. It is a defective human parvovirus, is non-pathogenic, has a linear single strand DNA genome, does not absolutely require dividing cells for gene expression, and may integrate into the host cell genome. AAV vectors contain no viral coding regions, express no viral antigens, can package a maximum of about 4.5 kb of foreign DNA and can be purified and concentrated to high titer. We are developing AAV vectors expressing the CFTR cDNA for gene therapy of cystic fibrosis. AAV vectors give high transduction efficiencies in human airway epthelial cell lines or primary human nasal polyp cells from cystic fibrosis patients. These AAV-CFTR vectors complemented the cystic fibrosis chloride channel defect as measured by functional electrophysiological assays in excised patch-clamp assays. The AAV-CFTR vectors used for the *in vitro* studies were evaluated in an *in vivo* animal model by delivery to the airway surface of New Zealand white rabbits. These studies also utilized an AAV-CFTR vector with an epitope tag expressed at the amino terminus of the recombinant CFTR protein. The presence of this epitope did not alter the normal electrophysiological functioning of the protein to correct the CF defect in the *in vitro* studies and provided a tag to unambiguously identify the recombinant CFTR protein expressed by the vector. In the *in vivo* studies in rabbits,a flexible fiberoptic bronchoscope was used to deliver AAV-CFTR vector particles to the surface of the right lower lobe bronchus. The left upper lobe was used as the control. The presence of AAV-CFTR vector genomes appeared to be limited to the right lower lobe of each of the vector-treated animals as assessed by *in situ* DNA PCR. RNA expression was detectable by PCR in lung homogenates from rabbits sacrificed 3 days, 10 days, 3 months, and 6 months after vector instillation, while none was observed in vehicle-treated controls. CFTR protein expression was detectable by immunohistochemistry and immunoblotting at each of these time points as well. For this immunological detection, an antibody directed to the amino terminal epitope tag was used. Expression was dose dependent. There were no gross morphological or histopathologic changes seen in the lung, liver, kidney, or heart samples examined from any of these animals. These findings demonstrate both biologic activity and safety of AAV-CFTR vector administration to the airway surface, when delivered in a manner analogous to that which would be used for clinical trials of gene therapy in CF patients. For such clinical trials we propose to use an AAV-CFTR vector which does not contain a foreign epitope. This vector is currently in preclinical evaluation using Rhesus monkeys in an in vivo delivery model analogous to the rabbit model. These results will be discussed.

**A6-020 RIBOZYME AND mRNA DELIVERY USING CATIONIC LIPOSOMES,** Robert W. Malone, Gene Therapy Program, Department of Medical Pathology, University of California, Davis 95616.

Cationic lipids can be used to introduce various biologic polymers into cells both in culture and *in vivo*. This discussion will focus on the use of cationic lipids for the delivery of RNA. Three basic topics will be covered: 1) Current models for the mechanism(s) of transfection, 2) Artifacts and pitfalls which are encountered when transfecting mRNA and ribozymes with cationic lipids, and 3) Review and classification of currently available cationic transfection lipids with a focus on which compounds are useful for RNA delivery. Experimental examples will be used to illustrate these three topics. **Current Models for Transfection:** Synthetic cationic transfection lipid preparations spontaneously interact with DNA in solution to form lipid-DNA complexes. When placed onto tissue culture cells, these lipid-DNA complexes interact with the plasma membrane, resulting in lipid fusion with the plasma membrane. Two leading hypotheses for the mechanism of transfection have yet to be differentiated; transfection via direct cytoplasmic delivery and transfection via an endocytic pathway. **Artifacts and Pitfalls:** After formulation as a lipid/polynucleotide complex, the lipids stabilize the polynucleotide. Presumably, this occurs by reducing the accessibility of polynucleotides to nucleases, thereby increasing the chemical half-life of the mRNA or ribozyme in the presence of cells, tissues, or nuclease-containing solutions. This stabilization certainly occurs in solution, probably occurs with complexes which are adherent to cells during transfection, and may extend to endosomes within cells. These stabilized lipid/polynucleotide complexes may serve as a slow release pool of stabilized polynucleotide, or may represent an inactive polynucleotide pool. Furthermore, since the association of lipid/polynucleotide complexes with cells is rapid and efficient, methods which physically examine polynucleotide integrity and subcellular localization by RNA extraction and/or subcellular fractionation may yield misleading results. Cationic lipid treatment can be associated with significant cytotoxicity, and this toxicity may be significantly enhanced by the addition of polynucleotides to the treatment. Therefore, for experiments incolving cationic lipid-mediated transfection, we advocate the analysis of "functional half-life" pioneered by D.R. Gallie, rather than more traditional methods of isolation and physical characterization, with particular attention to the use of controls to assess non-specific effects. **Cationic Transfection Lipids:** A number of cationic lipid transfection compounds have now been developed, with new compounds being developed at a rapid rate. In general, these compounds vary in relative transfection activity both with respect to cell and polynucleotide type. The existing compounds can be classified by both the polynucleotide binding domain and the hydrophobic domain. Polynucleotide binding groups include monoamines (Lipofectin™, DOTAP, DMRIE, DC-Chol) and polyamines (Lipofectamine™, DOGS). Hydrophobic domains are composed of oleic acid side chains (Lipofectin™, DOTAP, Lipofectamine™, PolyGum), shorter chain and/or saturated lipids (DMRIE, DMHME, DLHME, DM-PolyGum, DOGS), and cholesterol (DC-Chol). In general, we find that the monoamine-based, short side-chain lipids are more effective for RNA transfection. **In conclusion**, cationic lipids are useful for many biological applications requiring polynucleotide delivery to cells. Transfected polynucleotides may include DNA plasmids which transcribe ribozyme molecules as well as pre-formed RNA including ribozymes. However, the use of cationic lipid transfection can introduce artifacts which may mislead the unwary researcher.

**A6-021** RIBOZYME-MEDIATED REPAIR OF DEFECTIVE mRNAS BY TARGETED TRANS-SPLICING: RNA BASED MOLECULAR SURGERY, Bruce A. Sullenger, Departments of Experimental Surgery and Genetics, Duke University Medical Center, Durham, North Carolina

The observation that trans-cleaving ribozymes can be targeted to cleave specific RNAs *in vitro* has led to much speculation about their potential usefulness as gene inhibitors. By cleaving targeted mRNAs *in vivo*, such ribozymes can potentially be used to stop the flow of genetic information. Here, I will describe a different application of ribozymes. A group I intron ribozyme can potentially be used to manipulate the flow of genetic information by targeted trans-splicing. Defective cellular transcripts may be repaired, or pathogen-derived transcripts may be altered to encode antagonists to the pathogen using such technology.

To assess the feasibility of this approach, I have tested the ability of the L-21 version of the *Tetrahymena* group I intron ribozyme to repair truncated *lacZ* transcripts with targeted trans-splicing. *In vitro*, the ribozyme can quickly and accurately trans-splice a *lacZ* 3' exon onto a truncated *lacZ* 5' exon to generate an RNA product which encodes the alpha-complement of ß-galactosidase. In *E. coli* , the ribozyme can perform similar reactions which result in the production of translatable *lacZ* transcripts and ß-galactosidase activity.

The ability to alter the sequence of targeted RNAs represents an alternative way of performing gene therapy. Mutant transcript issuing from defective genes can be corrected by targeted trans-splicing. This approach has one major potential advantage over conventional approaches to gene therapy. In the trans-splicing case, one does not have to worry about recapitulating the endogenous expression pattern of a gene after gene transfer. Our progress towards developing such ribozymes to correct mutant ß-globin transcripts from sickle cell anemia patients will be discussed. In addition, such trans-splicing ribozymes offer an alternative approach for inhibiting HIV replication. Viral transcripts can be altered to encode antiviral proteins. With this approach, the ribozyme may have to act upon only a small percentage of HIV RNAs to be effective. Our progress to this end will also be discuused.

*Late Abstracts*

THE CELL NUCLEOLUS: YET ANOTHER RNA MACHINE, Joan A. Steitz[1], Kazimierz T. Tycowski[1], Brenda A. Peculis[1], Mei-Di Shu[1], [1]Dept. of Molecular Biophysics and Biochemistry, Yale University, Howard Hughes Medical Institute, New Haven, CT 06536.

The nucleolus of all eucaryotic cells is inhabited by multiple small nucleolar RNPs (snoRNPs). Some of these are transcribed (by RNA polymerase II or III) from their own independent transcription units, while many more are encoded within the introns of protein-coding genes. U3, U8, U13, and MRP belong to the first class, while U14-U24 belong to the latter. Many, but not all, snoRNAs bind the autoantigen fibrillarin.

Recently, considerable progress has been made in assigning functions to snoRNPs in vertebrate cells. U3, the most abundant snoRNP, acts at an early processing site near the 5′ end of the pre-rRNA transcript and also influences the maturation of 18S rRNA. By exploiting the *Xenopus* oocyte, where a small RNA can be targeted for destruction by injection of a complementary oligonucleotide, we have recently defined roles for U8, U22 and U14. U22 and U14 are required for maturation of 18S rRNA at both ends, whereas U8 is essential for 5.8S and 28S rRNA processing. Thus, direct parallels can be drawn between the mode of rRNA processing in bacteria and eucaryotes.

*In vitro* studies suggest that most intron-encoded snoRNAs arise by exonucleolytic trimming after excision (and debranching) of their host introns. However, many questions regarding this novel class of small RNAs remain. Why are all intron-encoded RNAs destined for the nucleolus? Why have only some snoRNAs been selected to be intron-encoded? What is the mobility mechanism whereby these snoRNAs "move" from intron to intron among highly expressed genes of vertebrate organisms? What is the function of the abundant, polyadenylated, but apparently non-coding, transcript of the host gene for human U22? Does the extensive complementarity exhibited by many intron-encoded snoRNAs to conserved regions of 18S and 28S rRNAs mean that they function as chaperones in ribosome assembly? The state of progress on each of these issues will be discussed.

CRYSTALLOGRAPHIC ANALYSIS OF THE GROUP I INTRON RIBOZYME, Jamie H. Cate[1], Thomas Cech[2], Anne Gooding[2], Craig Kundrot[2], Elaine Podell[2], and Jennifer A. Doudna[1], [1]Dept. of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06522 and [2]University of Colorado, Boulder, CO.

The Group I introns are a class of autocatalytic RNA molecules that catalyze their own excision from precursor transcripts such as ribosomal and transfer RNAs to produce the mature cellular versions of these molecules. There are currently over 200 examples of Group I introns, all of which share a common secondary structure and certain sequence elements. Despite an extensive body of biochemical work, the structures within the RNA that mediate chemistry remain unknown. To understand these ribozymes and general features of RNA architecture in more detail we are investigating the molecular structures and interactions of two structural domains, P4-P6 and P3-P9, within the Group I intron from *Tetrahymena*. The ability to divide the catalytic core into its component domains provides for the first time a means of assaying directly for correct folding and formation of the tertiary contacts required to produce a functional catalytic center. We are currently using x-ray crystallography to determine the high-resolution structure of the 160-nucleotide P4-P6 domain RNA. Crystals of the RNA diffract to 2.4 Å resolution; work is in progress to obtain phasing information by the method of multiple isomorphous replacement. At twice the size of tRNA, the P4-P6 molecule will be the largest RNA molecule for which a crystal structure has been determined. Biochemical work in our laboratory has shown that a functional catalytic center can be reconstituted when the domains are synthesized separately and added together in *trans*. Kinetic and physical characterization of the domain interactions has revealed that the domains bind tightly to each other with an apparent dissociation constant ($K_m$) of 4 nM, indicating the presence of multiple interdomain contacts. Work is in progress to identify the nature and locations of these contacts to facilitate a deeper understanding of the structure and dynamics of the Group I intron ribozyme.

***IN VITRO* EVOLUTION OF RIBOZYMES**, Gerald F. Joyce, Amrita Banerji, Ronald R. Breaker, Richard Bruick, Xiaochang Dai, Alain De Mesmaeker†, Sun-Ai Raillard, and Joyce Tsang; Departments of Chemistry and Molecular Biology, The Scripps Research Institute, La Jolla, CA 92037; † Central Research Laboratories, Ciba-Geigy Ltd., Basel, Switzerland.

The principles of Darwinian evolution can be applied to a large, heterogeneous population of RNA molecules to obtain particular RNAs that have desired biochemical properties, including the ability to catalyze a target chemical reaction. Beginning with the *Tetrahymena* group I ribozyme, we generated a population of $10^{13}$ variants and carried out *in vitro* evolution to develop ribozymes with novel or altered catalytic function. The wild-type *Tetrahymena* ribozyme catalyzes sequence-specific cleavage of an RNA substrate via a $Mg^{2+}$-dependent phosphoester transfer mechanism and has a very limited ability to cleave DNA in a similar manner. In one ongoing lineage, we directed the population of ribozymes through more than 40 successive "generations" of *in vitro* evolution, selecting for DNA-cleavage activity under progressively more stringent selection constraints. By generation 27, catalytic efficiency had improved $10^5$-fold, with $k_{cat} \sim 1\ min^{-1}$. Beginning with generation 28, the lineage was branched, selecting on the one hand for DNA-cleavage activity in the context of a DNA duplex and on the other hand for DNA cleavage in the presence of RNA molecules that act as competitive inhibitors. The 27th-generation DNA-cleaving ribozymes retain the ability to cleave RNA and, fortuitously, have acquired the ability to cleave arabinose-containing oligonucleotide analogues. This general-purpose ability to recognize and cleave a phosphate ester was applied to nucleic acid analogues that contain an amide in place of the phosphate, attempting to translate ground-state interaction with the tetrahedral phosphate to transition-state stabilization of the tetrahedral intermediate of amide hydrolysis. The evolved (but not the wild-type) ribozymes were found to catalyze $Mg^{2+}$-dependent cleavage of an unactivated, alkyl amide, when that linkage is located within an oligodeoxynucleotide or between an oligodeoxynucleotide and a short peptide. The first-order rate constant for RNA-catalyzed amide cleavage, $k_{cat}$, is $0.1 - 1 \times 10^{-5}\ min^{-1}$, corresponding to a rate acceleration of greater than $10^3$ compared to the uncatalyzed reaction. This activity is now being optimized through *in vitro* evolution.

*RNA Active Sites for Fundamental Cellular Reactions: The Spliceosome, Ribosome and Telomerase; Large Ribozymes I*

**A6-100** MUTATIONS AT POSITIONS 13 AND 914 IN *E.COLI* 16S RIBOSOMAL RNA PERTURB VARIOUS FUNCTIONS OF THE RIBOSOME, Léa Brakier-Gingras, Robert Pinard, Marc Côté and Julie Carrière, Département de Biochimie, Université de Montréal, Montréal, Québec, Canada H3C-3J7

To further investigate the role of ribosomal RNA in protein synthesis, site-directed mutagenesis was used to introduce a U→A and an A→U transversion at positions 13 and/or 914, respectively, in *E.coli* 16S rRNA, in the vicinity of the central pseudoknot 17-19/916-918. This structure, which is located at the junction of the three major domains of 16S rRNA, is required for translation initiation. When expressed *in vivo* in a high-copy-number plasmid, under conditions which force cells to depend on plasmid-encoded rRNA for protein synthesis, both mutations impaired cell growth and decreased protein synthesis. Structural studies with mutant 30S subunits revealed that the mutations promote the binding of an oligodeoxyribonucleotide probe to one strand of the pseudoknot, indicating that they destabilize this structure. This could be related to the fact that they decrease the rate of formation of the 30S initiation complex, as showed by toeprinting assays. As we previously showed, both mutations also increase translational fidelity and this could be related to destabilization of another pseudoknot structure, the 530 region pseudoknot, which plays a crucial role in the control of translational accuracy. From studies of the sensitivity to chemical or enzymatic attack of 16S rRNA, naked or within the 30S subunits, we now propose that the destabilization of the central pseudoknot results from a direct effect of the mutations on this structure, whereas the destabilization of the 530 region pseudoknot results from perturbations of protein S12-16S rRNA interactions, S12 being known to convey structural information from the region around the central pseudoknot to the 530 region (supported by the Medical Research Council of Canada).

**A6-101** EDITING OF MUTATED RNA SEQUENCES WITH MODIFIED RNA OLIGONUCLEOTIDES IN *XENOPUS* NUCLEAR EXTRACTS, Jennifer M. Chase[1], Lisa Saccomanno[2], Anthony DiRenzo[1], Chris Shaffer[1], Kurt Levy[1], Christopher Workman[1], Francine E. Wincott[1], Tod M. Woolf[1] & Dan T. Stinchcomb[1], 1. Departments of Cell Biology and Chemistry & Biochemistry, Ribozyme Pharmaceuticals Inc., 2950 Wilderness Place, Boulder, CO 80301. 2. Department of Biochemistry, University of Utah, Salt Lake City

Many genetic diseases are caused by single base substitutions. If RNA editing could be rationally directed to mutated RNA sequences, genetic diseases caused by certain base substitutions could be treated. We have used a synthetic complementary RNA oligonucleotide and modified RNA oligonucletides to direct the correction of a premature stop codon mutation in dystrophin RNA. The complementary RNA oligonucleotide was hybridized to a premature stop codon and the hybrid was treated with nuclear extracts containing the cellular enzyme double-stranded RNA adenosine deaminase (dsRAD). When the treated RNAs were translated *in vitro*, a dramatic increase in expression of a downstream luciferase coding region was observed. The cDNA sequence data are consistent with deamination of the adenosine in the UAG stop codon to inosine by dsRAD. Injection of unmodified oligonucleotide/mRNA hybrids into *Xenopus* embryos also resulted in an increase in luciferase expression. This directed reversion is termed therapeutic nucleic acid editing. In principal, modifications of ribozyme, antisense, or triple-strand technologies could be exploited to edit a variety of mutated RNA or DNA sequences..

**A6-102** EXPLORING TERTIARY INTERACTIONS INVOLVING TETRALOOPS BY IN VITRO SELECTION, Maria Costa, François Michel, Centre de Génétique Moléculaire du C.N.R.S., 91190 Gif-sur-Yvette, France.

An understanding of the mechanisms by which catalytic RNAs fold into their final three-dimensional structure requires, first of all, the identification of long range interactions between secondary structure motifs. Among terminal loops, tetraloops belonging to the GNRA and UNCG families are largely preferred as stems 'caps'. One possible explanation is that these loops take part in tertiary interactions. This hypothesis is supported by phylogenetic analysis of group I introns, which allowed identification of covariations involving GNRA loops. Thus, presence of a particular GNRA loop is often associated with that of a C-G base pair (bp) at position n of a specific helix elsewhere in the molecule, while the third nucleotide of the loop covaries with the bp at position n+1 of the same helix: as a rule, if R=G in the loop, bp n+1 is U-A; if R=A, bp n+1 is C-G. In intron *td* of bacteriophage T4, the interaction implied by the latter covariation seems to exist between loop L2, a GUGA loop, and bp 4 and 5 (C-G and U-A, respectively) of helix P8. We have developed an in vitro selection system to explore tertiary interactions involving tetraloops. The *td* intron was transformed into a substrate-enzyme pair by separating the P1-P2 helices from the rest of the molecule: the P1-P2 substrate is recognized by the rest of the intron and positioned in a suitable way for proper cleavage at the 5' splice jonction. Assuming that the L2-P8 tertiary interaction implied by covariation analysis plays a major role in the recognition and positionning of the P1-P2 piece in the catalytic site of the intron core, it should be possible to select P8 motifs specific for a given L2 loop. Thus, we entirely randomized the P8 hairpin and three different tetraloops were introduced in L2: the wild-type loop, as a positive control, GAAA and UUCG. A population of substrate molecules that had been completely randomized at the four positions of the L2 loop was also tested. Selection was based on the ability of the core to catalyze, by means of its terminal G, a phosphoester transfer reaction after attack of the 5' splice jonction. After four rounds of selection the fraction of reactive core molecules was found to have risen from less than 0.01 to values between 0.075 and 0.22 and each population specifically recognized the P1-P2 substrate it had been presented with during selection. Sequencing, after the third round, of a mixture of 100 individuals from each pool revealed that several sites in P8 were already constrained, in a specific way for each pool.

**A6-103** TANDEM GROUP I RIBOZYMES WITH DIFFERENT FUNCTIONALITY IN A SINGLE, NUCLEAR rRNA INTRON OF *DIDYMIUM IRIDIS*,Wayne A. Decatur, Steinar Johansen*, and Volker M. Vogt, Section of Biochemistry, Molecular and Cellular Biology, Cornell University, Ithaca, NY 14853; *Department of Cell Biology, Institute of Medical Biology, University of Tromsø, 9037 Tromsø, Norway

DiSSU1 is an unusual group I intron in the nuclear small subunit RNA gene of the myxomycete *Didymium iridis*. It is comprised of two catalytic RNA elements (Cell 76: 725-734, 1994): a group I ribozyme (GIR1) in the loop of the paired segment P2 of a second group I ribozyme (GIR2). Using deletion analysis and site-directed mutagenesis of the G-binding site in the individual P7 segments, we have characterized the functions of these two ribozymes. GIR2 is responsible for the self-splicing reaction. GIR1 plays no role in splicing, but catalyzes efficient site-specific hydrolysis at an internal processing site (IPS) positioned where a normal 3' splice site would be with respect to this ribozyme. Presumably this cleavage allows the expression of the ORF for the homing endonuclease I-DirI, which also resides in the P2 loop, succeeding the IPS. GIR2 thus is a unique example of a group I ribozyme whose biological function is hydrolyis and not splicing. The secondary structural pairing P2, although previously shown to be dispensable for self-splicing of group I ribozymes (Biochemistry 28: 6534-6539, 1990), is predicted to be unexpectedly long in the GIR2 of DiSSU1: about 40 base pairs. This extended P2 serves to bring the core of the GIR2 in proximity to the P1 pairing. Currently, we are investigating if GIR2 is capable of splicing *in trans*, relying on the base pairing of this long P2 pairing segment.

A6-104 βAPP mRNA CLEAVAGE BY SYMMETRIC AND ASYMMETRIC HAMMERHEAD RIBOZYMES, Robert Denman, Marilyn Smedman, Niloufar Haque, Ling Kung and Ying Sung, NYS Institute for Basic Research, Staten Island, NY 10314.

Many plant viroid and virusoid RNAs contain a self-cleaving secondary structure motif called the hammerhead composed of three short RNA helices. In a significant number of these structures, the pairing bases in helices I and III which span the cleavage site are asymmetrically distributed. In order to determine whether such a distribution would confer advantageous cleavage properties to *trans*-acting ribozymes designed to cleave and degrade cellular mRNAs, we examined the effect of altering the base-pairing distribution in ribozymes targeted to the amyloid peptide precursor (βAPP) mRNA at position 141. Four ribozymes were formed from two βAPP synthetic mRNA analogs and two ribozyme RNA core sequences. Symmetric ribozymes β133/Rz133 and β125/Rz133 contained 8 bp in helix I and 7 bp in helix III, respectively. Asymmetric ribozyme β125/Rz125 had 13 bp in helix I and 4 bp in helix III, while asymmetric ribozyme β133/Rz125 contained 8 bp in helix I and 4 bp in helix III. The ability of each ribozyme to cleave its substrate RNA was first assessed under single-turnover conditions at 37°C. These studies revealed that only symmetric ribozyme, β133/Rz133, effectively cleaved its substrate. Further studies using a 80°C, 1 min → 37°C, 1 min. temperature cycling paradigm resulted in a 2500-fold enhancement in the $k_{cat}/K_m$ of ribozyme β133/Rz133 and enabled β125/Rz125 and β125/Rz133 cleavage to be detected; however the $k_{cat}/K_m$ values for the latter two ribozymes were 100 and 700 fold less than ribozyme β133/Rz133. Since the distribution and location of pairing bases in helix I and III of β133/Rz133 and β125/Rz133 was the same, the lower activity of the β125-containing ribozymes suggests that higher order structure, similar to that observed with full-length APP mRNA substrates, inhibits ribozyme activity. Nevertheless, despite its relatively low *in vitro* activity, transiently expressed asymmetric Rz125 mRNA specifically and effectively lowered steady-state βAPP levels in Chinese hamster ovary (CHO) cells in culture.

A6-105 NUCLEAR RNASE P: STRUCTURES AND FUNCTIONS, D. Engelke, E. Pagan-Ramos, Joel Chamberlain, Katherine Neville, and A. Tranguch, Department of Biological Chemistry, University of Michigan, Ann Arbor, MI 48109-0606

Ribonuclease P is a ubiquitous ribonucleoprotein endonuclease that recognizes and aligns the cleavage site of its pre-tRNA substrates through tertiary structure contacts, rather than base-pairing. RNase Ps from eubacteria, archaebacteria, and eukaryotic nuclei and mitochondria are ribonucleoprotein holoenzymes, yet the RNA subunit alone from eubacteria is a ribozyme whereas the RNA subunits from other sources are not. To understand the structure and function of the eukaryotic nuclear RNA, we have used phylogenetic structure analysis, structure-sensitive chemical and enzymatic RNA footprinting, and directed mutagenesis. The resulting secondary structure model is strikingly similar to the eubacterial RNA consensus. The most conserved sequences and structural domains are being subjected to saturation mutagenesis to test for their functions in the context of the holoenzyme *in vivo* and *in vitro* An unexpected result of these experiments is that most of the strongly conserved sequence positions are not essential to enzyme function *in vivo*, although mutations at these postions modify various aspects of the RNase P enzymatic activity. One of the less obvious conserved features is an essential internal loop sequence contained within an arm in the center of the RNA. This region does not appear to bind either protein or substrate, yet is strongly conserved in approximately the same place within the structural model in all RNase P RNAs examined. This arm is a tightly folded structural domain that may serve as a coordination site for magnesium at the catalytic center.

A6-106 DERIVATION OF A SMALL EXTERNAL GUIDE SEQUENCE CAPABLE OF INDUCING CLEAVAGE OF TARGET RNA BY HUMAN RNase P. Shaji T. George, Martina Werner, Jeffrey L. Nordstrom and Allan R. Goldberg, Innovir Laboratories Inc., 510 East 73rd Street, New York, N.Y. 10021.

Ribonuclease P (RNase P) is an endogenous ribozyme present in all cells that processes precursor tRNAs to yield their mature 5' termini. The RNase P holoenzyme consists of a protein and an RNA. It has been shown that the RNA component of the enzyme from *E. Coli* is the catalytic moiety. Heterologous RNAs can be targeted for cleavage by RNase P by the use of an external guide sequence (EGS). Hybridization between an RNA and an EGS, resembling three quarters of a tRNA with a single stranded aminoacyl acceptor and D stems complementary to the target RNA, results in the formation of a tRNA-like complex. This complex is recognized by Rnase P as if it were a precursor tRNA, resulting in the cleavage of the target RNA (Yuan et al., 1992). In an attempt to reduce the overall length of the EGS, we tested many truncations of a 63 nt long EGS that induces cleavage of a short Hepatitis B virus RNA sequence. These modifications resulted in an EGS that was only 29 nt long and induced cleavage of the substrate more efficiently that the original one. It was also observed that the anticodon stem and loop were inhibitory for inducing cleavage. Truncations of the T-loop or the aminoacyl acceptor stem were inhibitory for activity but modifications in the variable loop and the D-stem did not alter the activity significantly. Although the requirement of a T-stem was obligatory, the nucleotide sequence and the length of this stem can be altered. These studies have helped to identify guidelines for designing EGSs against target RNA.

Yuan, Hwang & Altman, PNAS, (1992) **89**, 8006

A6-107 TEMPLATE FUNCTION IN THE TELOMERASE RNA OF TETRAHYMENA David Gilley, Margaret S. Lee and Elizabeth H. Blackburn, Department of Micro. and Immun., Box 0414, U. C., San Francisco, San Francisco, CA 94143

The RNP telomerase is an unusual reverse transcriptase responsible for the synthesis of the G-rich strand of telomeric DNA. The telomerase RNA of Tetrahymena contains the template for telomere repeat synthesis. We are studing the functional role of different telomerase RNA domains involved in telomere formation. To achieve this we have introduced telomerase RNA genes with mutations within and proximal to the template domain into the cell and analyzed their *in vivo* and *in vitro* effects. We characterize the telomerase activity in partially purified preparations made from cells transfected with various mutated telomerase RNA genes. In this way, the templating domain was shown to contain seven nucleotides (positions 49 to 43), one nucleotide more than the six nucleotide telomeric repeat. Use of the seven possible templating nucleotides allows primers to anneal and be elongated at alternate positions. We show that base pairing at positions 50 and 51 is directly responsible for positioning the primer for elongation. Another unexpected outcome of these studies was that all alterations within the template reduced long product formation and certain mutations affect enzymatic fidelity. Reduction in the formation of long products *in vitro* cannot be simply explained by lack of or poor annealing capabilities of mutant products. Thus, our *in vitro* studies of mutant telomerase have exposed unexpected enzymatic characteristics of telomerase. This approach is also valuable for determining if a given mutant RNA is stable and assembled *in vivo* into telomerase. Cellular phenotypes of template residue mutations fall into three classes: early lethal, delayed lethal, and those alterations that have no effect on cellular viability. In summary, the templating region of the telomerase RNA can be separated into several functional domains: boundaries of the template directly copied during the synthesis of the G-rich strand, residues involved in annealing functions, and regions involved in exonuclease and termination capacities. This recent work directly supports and extends the current model of telomerase action and has uncovered new facets of the action of this enzyme.

**A6-108** DISSOCIATION OF DUPLEX RNA FORMED BETWEEN HAMMERHEAD RIBOZYMES WITH LONG ANTISENSE FLANKS AND SUBSTRATE RNA, Matthias Homann, Wolfgang Nedbal and Georg Sczakiel, Angewandte Tumorvirologie, Deutsches Krebsforschungszentrum, Heidelberg, Germany

Hammerhead ribozymes with antisense flanks of several hundred nucleotides in length, termed catalytic antisense RNA, have been used successfully to inhibit replication of the human immunodeficiency virus type 1 (HIV-1) in human tissue culture cells. We observed that not only `symmetric` but also `asymmetric` ribozymes, i.e. ribozymes with a long flank forming helix III and a short flank (3 nucleotides) forming helix I were potent inhibitors. The extent of inhibition was decreased when in vitro inactive drivatives or the parental antisense RNAs were tested. This observation was somewhat surprising since catalytic turnover was only expected for ribozymes with short antisense flanks but not for catalytic antisense RNA. One conceivable explanation for the ribozyme-mediated increase of the inhibition of HIV-1 was that dissociation between substrate RNA and the antisense flanks of the ribozyme was underestimated in vivo. Dissociation of duplex RNA could have two consequences: (i) ribozyme-mediated cleavage of the substrate could compete with dissociation and, thereby, increase inhibition and (ii) turnover of catalytic antisense RNA in living cells could be relevant. As a model to investigate RNA duplex dissociation we used an HIV-1-directed hammerhead ribozyme and a complementary target RNA. The design of this tat-directed ribozyme was asymmetric with the catalytic domain at the 5'end of the antisense flank forming helixIII. Association of the ribozyme with different complementary RNAs and dissociation of the formed duplex RNA were measured at pH 7.4, physiological ionic strength and varying temperatures and analyzed by polyacrylamide gelelectrophoresis. The rate constant for association between the tat-directed asymmetric ribozyme and a 645 nucleotides long target RNA was 1.5 x 104 M-1s-1, the cleavage rate constant was 3 x 10-4 s-1, and t1/2 for the dissociation was at least 5 orders of magnitude smaller than the association at the experimental RNA concentrations. However, in the presence of the kationic detergent CTAB, dissociation was enhanced by a factor ranging between 102 and 103. This means that it might be relevant to consider RNA duplex dissociation in living cells.

**A6-109** **SELF-CLEAVING GROUP I RIBOZYMES INVOLVED IN THE EXPRESSION OF PROTEINS ENCODED BY NUCLEOLAR GENOMES**, Steinar Johansen, Anna Vader, Christer Einvik, Wayne A. Decatur* and Volker M. Vogt*, Department of Cell Biology, Institute of Medical Biology, University of Tromsø, 9037 Tromsø, Norway; *Section of Biochemistry, Molecular and Cellular Biology, Cornell University, Ithaca, NY 14853.

The myxomycete protist *Didymium iridis* contains a self-splicing group I intron in its nuclear ribosomal DNA (Cell 76:725-734, 1994). This intron encodes three distinct nucleases: two group I ribozymes (GIR 1 and GIR 2) and one homing-endonuclease (I-*Dir*I). The intron is mobile in genetic crosses, -a process dependent on the intron-encoded I-*Dir*I. GIR 2 is a 'regular' group I ribozyme that catalyses intron excision and exon ligation. GIR 1 is a very small (120 nt) and novel self-cleaving group I ribozyme involved in internal processing of intron RNA, but not intron splicing. Comparative analyses including several nuclear mobile group I introns reveal some conserved features: internal processing sites leading to separate ORF containing RNAs, and a 3'UTR of ORF-RNAs that resemble the snoRNAs. The 3'UTRs contain the highly conserved sequence motifs Box C and/or Box D, known as binding sites of nucleoar proteins in snoRNAs (TIBS 18:131-135, 1993). We propose that the mRNAs of nucleolar homing endonucleases are generated by ribozyme-processing of pre-rRNA, stabilized as RNP-particles by binding to nucleolar proteins and then transported to the cytoplasm for translation. Northern analysis of cellular RNAs sopports this model.

**A6-110** STRUCTURE OF THE FOUR HELIX BUNDLE PROTEIN ROP COMPLEXED WITH TWO COMPLEMENTARY RNA HAIRPINS

Peter S. Klosterman and Thomas A. Steitz, Department of Molecular Biophysics and Biochemistry and the Howard Hughes Medical Institute, Yale University, New Haven, CT 06520

Rop (Repressor of Primer) is a four helix bundle protein, encoded by ColE1 family plasmids, which binds to two complementary plasmid encoded RNA species, RNA I and RNA II. In the absence of RNA I and rop, part of RNA II binds to the plasmid origin of replication, priming DNA replication. When bound to RNA I, the primer portion of RNA II is buried and DNA replication does not occur. The RNA I - RNA II complex is stabilized by rop. Rop also binds to complementary RNA hairpin stem loops which form a small portion of the RNA I and RNA II sequences. This binding is of interest in understanding RNA protein interactions since it appears to be specific to RNA tertiary structure but not sequence. Rop binds equally strongly to stem loop pairs in which the loop sequences have been reversed.

We have identified several conditions giving crystals of rop complexed with two complementary 18 base RNA stem loops. We have complete X-ray diffraction data on native crystals, with good statistics extending to 2.15 Å. The crystal space group is C2, with unit cell dimensions a=77Å, b=44Å, c=154Å, β=99.5°. Self rotation function analysis indicates that there are three complexes in the asymmetric unit. We also have diffraction data from a promising isomorphous heavy atom derivative crystal. Currently we are processing the data and plan to pursue isomorphous replacement and molecular replacement studies to solve the structure of the complex.

**A6-111** CLEAVAGE SITE SELECTION BY M1 RNA, THE CATALYTIC SUBUNIT OF *ESCHERICHIA COLI* RNASE P, IS INFLUENCED BY PH, Joanna Kufel, Leif A. Kirsebom, Department of Microbiology, Uppsala University, Box 581 Biomedical Center, S-751 23 Uppsala, Sweden.

The maturation of the 5'-terminus of a tRNA is the result of cleavage by RNase P. The catalytic subunit of RNaseP is an RNA (designated M1 RNA in *E. coli* ) which under certain *in vitro* conditions cleaves tRNA precursors at the expected position in the absence of the protein subunit. Previous work showed that several features on tRNA precursor such as the identity of the nucleotides at and near the cleavage site, the length and primary structure of the aminoacid acceptor-stem as well as of the T-stem determine the RNase P cleavage site.

To further investigate how M1 RNA localizes its cleavage site we have studied cleavage site selection by M1 RNA, under various reaction conditions using tRNA precursors which are cleaved at two positions. Our results showed that the preference of cleavage site changed with variations in pH or $Mg^{2+}$-concentration. By contrast, no difference in cleavage site selection was observed with increasing pH in the presence of $Ca^{2+}$ as the only divalent metal ion. Depending on the identity of the nucleotide at position "+72", replacement of $Mg^{2+}$ with $Ca^{2+}$ resulted in a change of the main cleavage site irrespective of pH. We conclude that cleavage in the presence of $Ca^{2+}$ compared to cleavage in the presence of $Mg^{2+}$ has different structural requirements at and near the cleavage site. UV cross-linking revealed that close contact points between M1 RNA and its substrate were the same irrespective of pH or the identity of the divalent cation. Our results also showed that the observed pH-effects are due to changes in the catalytic cleavage rates rather than to global, structural rearrangements.

Currently we are using the same precursors to investigate cleavage site selection by mutant M1 RNAs carrying changes in the region known to interact with the two C's in the 3'-terminal CCA sequence of tRNA and suggested to be involved in metal ion(s) coordination .

**A6-112** CHEMOTHERAPY WITH A GROUP I INTRON TARGET, Michael J. Leibowitz and Yong Liu, Department of Molecular Genetics and Microbiology, UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ 08854-5635

Group I self-splicing introns are present in the mitochondrial and nuclear genomes of various pathogenic eukaryotic microorganisms, including the opportunistic fungal pathogen *Pneumocystis carinii* which has nuclear Group I introns. Three "sequevars" of *P. carinii* have been characterized, with sequevar Pc1 harboring such introns in its 16S and 26S rRNA genes, and Pc2 and Pc3 harboring an intron only in their 26S genes. The group I introns (I) catalyze their own excision from primary transcripts in the presence of guanosine substrate (G) with ligation of flanking exons (E1 and E2); the excised linear guanylylated intron also catalyzes its own circularization.

$$\underset{1}{\text{E1-I-E2 + G}} \rightarrow \text{E1 + GI-E2} \rightarrow \underset{2}{\text{E1-E2 + GI}}$$

In reactions *in vitro*, pentamidine and some derivatives as well as tetracycline and ethidium bromide non-competitively inhibit the first reaction, while L-arginine competes with the guanosine substrate. In addition to this inhibitory effect, L-arginine stimulates the second reaction step, with a 17-fold stereospecificity for the L-enantiomer. Oligo-L-arginine peptides are much more potent, with the peptides L-arginine-L-arginine-amide and tri-L-arginine being 10,000 times as potent as inhibitors and 400 times as potent as stimulators. Unlike L-arginine, these peptides also inhibit the cyclization of the linear excised intron, consistent with cyclization occuring by internal attack by the 3'-terminal G residue, which mimicks the G substrate of reaction 1. These *in vitro* effects of small molecules on ribozyme activity suggest that antimicrobial agents might be designed to act on nuclear and mitochondrially-encoded intron ribozymes, which are found in *P. carinii* and other eukaryotic microbial pathogens. *In vivo* experiments to test the physiological consequences of inhibition of splicing are currently in progress.

**A6-113** THE CBP2 PROTEIN PROMOTES THE FOLDING OF A GROUP I INTRON DURING TRANSCRIPTION, Alfred S. Lewin, Lynn C. Shaw and Hymavathi K. Tirupati, Department of Molecular Genetics and Microbiology, University of Florida College of Medicine, Gainesville, FL 32610-0266

The terminal intron of the precursor to cytochrome *b* mRNA (*b*I5) is a group I intron that splices autocatalytically *in vitro* under high salt conditions but requires a protein encoded by the nuclear *CBP2* gene in order to be spliced in yeast mitochondria. This purified protein permits the splicing of *b*I5 under physiological conditions. The protein appears to do this by promoting the correct folding of the RNA under these low-salt conditions. Results of modification/protection experiments indicate that the 5' and 3' splice junctions (P1 and P10), stem P2 and the catalytic core of the intron (P4, P6 and P7) are protected from solvent in the presence of Cbp2 protein in the same manner that they are protected in the high-salt autocatalytic conditions. The "foldase" function of Cbp2 is also reflected in its ability to suppress the effects of mutations in the RNA that block autocatalytic splicing: Point mutations affecting the selection of the 3' splice site (base changes in P9.0 and P10) can be overcome in the presence of Cbp2 but mutations affecting the 5' splice site (P1) are not suppressed by Cbp2. In addition, the protein modifies the folding of the intron during transcription: *b*I5 can be spliced during transcription only in the presence of the protein and mutants of the RNA that are not capable of post-transcriptional splicing can be spliced during transcription. Co-transcriptional splicing reduces hydrolysis at the 3' splice site, suggesting a more efficient coordination of the first and second steps of splicing than in the post-transcriptional reaction. We interpret these results to imply that the tertiary and perhaps the secondary structure of the nascent transcript is modulated by Cbp2.

**A6-114** THE NEWT HAMMERHEAD RIBOZYME: STRUCTURE AND APPLICATIONS, Ettore Luzi, Michela Denti, Simona Casarosa and Giuseppina Barsacchi, Laboratory of Cellular and Developmental Biology University of Pisa, via Carducci 13, Ghezzano (PISA), Italy.

Synthetic transcripts of the "Bgl II" satellite DNA of the European newt *Triturus* undergo self-catalyzed, site specific cleavage *in vitro*, as discovered in the American newt *Notophthalmus*. The reaction requires $Mg^{++}$ and generates products with 5'-hydroxyl and 2',3'-cyclic phosphate groups and is mediated by a "hammerhead catalytic domain". The newt ribozyme is transcribed by the RNA Polymerase II under the control of a Proximal Sequence Element (PSE) and a Distal Sequence Element (DSE); *in vitro* newt monomeric "Bgl II" RNA can *trans* cleave an appropriate substrate RNA, suggesting that *in vivo* it may be involved in RNA processing events as RNP particle. We have identified and characterized the newt ribozyme ribonucleoprotein (newtRzRNP) present in the oocytes of *Triturus*. The newtRzRNP sediments at 12S and specific shifted complexes assemble on monomeric newtRzRNA. UV cross-linking studies identified a polipeptide of about 40 kDa (p40) that specifically crosslinked to the newt ribozyme. In addition, we are studying how to utilize the newt ribozyme as a tool to inactivate specific gene functions. Its presence *in vivo* as a RNP could support its cleaving *in trans*. and the binding of the specific p40 protein could enhance the *in vivo* application of this ribozyme (compared to other modified ribozymes) to inactivate O6-methyl-guanine-DNA-methyl-transferase (MGMT) mRNA in Chinese Hamster Ovary (CHO) cells and of feline immunodeficiency virus (FIV) in mice cells lines.

**A6-115** **EVALUATION OF MODIFIED OLIGORIBONUCLEOTIDE ANALOGUES AS EXTERNAL GUIDE SEQUENCES FOR INDUCING CLEAVAGE OF HBV RNA BY RNase P**, Michael Y.-X. Ma, Shaji T. George, Martina Werner, Umberto Pace, Jeffrey L. Nordstrom and Allan R. Goldberg, Innovir Laboratories, Inc., 510 East 73rd St., New York, NY 10021

Synthetic oligonucleotides and their analogues are currently being investigated as potential human therapeutics. We are developing a new class of oligonucleotide analogues, known as external guide sequences (EGSs), which can hybridize to a RNA target by complementary Watson-Crick base-pairing. The resulting EGS-target RNA complex possesses structural features similar to that of tRNA. This tRNA-like complex is recognized by RNase P, resulting in the cleavage of the target RNA. RNase P is a ubiquitous ribonucleoprotein which cleaves the 5' sequence of precursor tRNA to generate the mature 5' termini. In this communication, we wish to report the effect of chemical modifications of EGSs targeted to the human hepatitis B virus (HBV). These chemical modifications were primarily aimed at enhancing nuclease resistance and cell permeability of EGSs. Starting with a 31 nt unmodified EGS RNA, specific 2' as well as backbone modifications were introduced. The effect of such modifications on the cleavage activity by a purified preparation of RNase P was examined using a short model RNA target sequence derived from the pre-genomic HBV RNA using an *in vitro* cleavage assay. In addition, RNA thermal denaturation experiments and gel shift assays were performed to better evaluate the factors involved in EGS-substrate complex formation and their recognition by RNase P. Preliminary results indicate that modifications at most positions in the EGS are well tolerated by RNase P, whereas others abolish its cleavage activity. Several residues in the T-loop of EGS are particularly critical for inducing target cleavage by RNase P. These results should provide useful insights into the design and development of modified EGSs as potential inhibitors of gene expression.

**A6-116** A PHYLOGENETIC APPROACH TO MODELING RIBONUCLEASE P RNA TERTIARY STRUCTURE. James M. Nolan, Michael E. Harris, Bong-Kyeong Oh, Jiunn-Liang Chen, James W. Brown*, and Norman R. Pace. Department of Biology, Indiana University, Bloomington, IN 47405. *Department of Microbiology, North Carolina State University, Raleigh, NC 27695.

We have mapped the crosslinking sites of RNase P RNAs from three diverse bacterial species to four different nucleotides of its tRNA substrate. By mapping the many sites in RNase P to which tRNA crosslinks, we have obtained data with which to model the tertiary structure of RNase P RNA. We are using a phylogenetic perspective for this analysis. The RNase P RNAs chosen, from *Escherichia coli, Bacillus subtilis*, and *Thermotoga maritima*, differ to some extent in secondary structure. They are, however, homologous and therefore must share a common core tertiary structure. Thus, any valid model of RNase P tertiary structure that satisfies the secondary structure and crosslinking constraints of one species should also be able to accommodate the constraints imposed upon the RNAs from other species.

This assumption is the basis of our strategy for modeling the three-dimensional structure of RNase P RNA, using the YAMMP RNA modeling package, in collaboration with Steve Harvey and Arun Malhotra at the University of Alabama-Birmingham. We have developed a procedure for the extrapolation of coordinates of YAMMP models generated for one species to those for another species. This allows the enforcement of constraints imposed by each species onto a particular YAMMP model. The results of the YAMMP modeling are being used as constraints for all-atom modeling, using MC-SYM, in collaboration with François Major at the University of Montreal, and Thomas Easterwood at the University of Alabama-Birmingham.

**A6-117** PSEUDOURIDINE RESIDUES AT ACTIVE SITES IN RIBOSOMAL RNAS FROM *ESCHERICHIA COLI* TO MAN, James Ofengand and Andrey Bakin, Roche Institute of Molecular Biology, Roche Research Center, Nutley, N. J. 07110

Little is known about the role of pseudouridine (Ψ), the predominant modified nucleotide in both large and small subunit ribosomal RNAs. A prerequisite to understanding the purpose behind Ψ formation in ribosomal RNA is knowledge of their amount and location in the RNA chain. Here we report the localization of Ψ in both cytoplasmic and mitochondrial ribosomal RNAs of a number of species.

Localization of Ψ in RNA was done by a new sequencing method based on primed reverse transcription (Bakin, A. & Ofengand, J., 1993, *Biochemistry*, 32, 9754-9762). Analysis of *E. coli* large subunit (LSU) ribosomal RNA located a total of eight Ψ residues plus one modified Ψ. The 2457, 2504, 2580, and 2605 Ψ residues are in or within 2-3 residues of the peptidyl transferase ring. The other sites, 746, 955 and 1911, 1915, 1917 define two regions known to be also near to the peptidyl transferase center (PTC). A complete survey of *S. cerevisiae* cytoplasmic LSU RNA detected 30 Ψ residues which clustered in the same three regions as in *E. coli* despite the nearly 4-fold difference in total number. In *D. melanogaster*, mouse, and human cytoplasmic LSU RNA, the respective 57, 57, and 54 Ψ residues which were found were also located in the same three regions. The four Ψ detected in *H. halobium* LSU RNA and the five in *B. subtilis* were in two of the three regions. Yeast mitochondrial LSU RNA had only one Ψ residue, corresponding in position to Ψ2580 at the PTC ring of *E. coli* 23S RNA. The single Ψ detected in mouse and human mitochondrial LSU RNA was at the same position as in yeast. These results suggest that (a) clustering of Ψ residues in 2-3 specific regions around the PTC may be universal for LSU RNA and (b) Ψ2580 may be of specific importance at the PTC.

A single Ψ was found in *E. coli* small subunit (SSU) RNA at position 516 in the 516-535 conserved sequence loop. This loop is believed to have a complex, pseudoknotted, tertiary structure and to be critically involved in the recognition between mRNA and tRNA. Ψ formation at the equivalent site in *B. subtilis* has also been found. Analysis of yeast cytoplasmic and mitochondrial SSU RNAs is in progress.

**A6-118** STEREOCHEMICAL SELECTIVITY OF GROUP II INTRON REACTIONS, Mircea Podar, Philip S. Perlman and Richard A. Padgett, Department of Biochemistry, University of Texas Southwestern Medical Center at Dallas, Dallas, TX 75235.

Previously we have shown that group II intron self-splicing proceeds with a phosphorothioate in the Sp configuration at either splice junction but not with a phosphorothioate in the Rp configuration and that both steps of the splicing reaction proceed with inversion of stereochemistry. Group II self-splicing introns can carry out several phosphodiester hydrolysis reactions which are believed to be related to the splicing reactions. The stereochemical specificities of all of the major hydrolysis reactions have been determined using RNAs with stereospecific phosphorothioate substitutions at the cleaved phosphodiester bonds. Hydrolysis of the 5' splice site in an intact intron or a large intron fragment with domain 5 supplied in trans proceeds with an Sp but not an Rp phosphorothioate as does the first step of group II intron self-splicing via RNA branch formation. In contrast, the hydrolysis of the bond immediately following the upstream exon followed by second exon sequences (the reaction called spliced exons reopening, SER), proceeds efficiently with Rp phosphorothioates but not with Sp. A reaction in which the upstream exon is followed by a short intron sequence, that has been used as a model for the first step splicing reaction, also proceeds efficiently with the Rp isomer; however, unlike SER, the Sp isomer shows a slow rate of cleavage, suggesting that the substrate can be used in two different reactions. We conclude that these efficient Rp reactions are not the step one reaction and suggest that they are related to the reversal of the second step of self splicing.These results indicate that, in the absence of significant intron sequences, the upstream exon RNA binds to and is hydrolysed by an active site related to the second splicing step active site.

**A6-119** DETERMINING METAL ION BINDING POCKETS IN THE CATALYTIC CORE OF A GROUP I INTRON, Barbara Streicher[1], Eric Westhof[2], and Renée Schroeder[1]. [1]Institute of Microbiology and Genetics, Vienna Biocenter, A-1030, Vienna, Austria and [2]Institut de Biologie Moléculaire et Cellulaire du CNRS, Université Louis Pasteur, Strasbourg, France

Divalent metal ions promote different hydrolysis reactions in group I introns. While $Mg^{2+}$ induces hydrolysis at the splice sites and promotes cofactor dependent splicing, $Ca^{2+}$, $Sr^{2+}$ and $Pb^{2+}$ catalyse the hydrolysis of a phosphodiesterbond (5' to U940, *id* intron) in the core of the intron. $Mn^{2+}$ has a special status, as it promotes the first step of splicing only, it induces hydrolysis at the 5' splice-site and it also catalyses cleavage of the U940 bond. $Ca^{2+}$ and $Mn^{2+}$ inhibit the second step of splicing competing with $Mg^{2+}$. While the products of cleavage at the splice-sites have 3' OH and 5' phosphate ends indicating attack by an activated water molecule, products of cleavage at the U940 bond have 5' OH and 3' phosphates suggesting attack by its own O2´ hydroxyl group.

Two main metal ion binding pockets (A and B) could be determined by docking metal ions into the 3D structural model. Pocket A accommodates $Mg^{2+}$ and $Mn^{2+}$ for hydrolysis at the 5' splice-site and for splicing. Pocket B accommodates $Ca^{2+}$, $Sr^{2+}$, $Pb^{2+}$ and $Mn^{2+}$ for cleavage at U940. When $Ca^{2+}$ or $Mn^{2+}$ are positioned in pocket B, thereby precluding occupation by $Mg^{2+}$, the second step of splicing ($Mg^{2+}$ dependent) is inhibited.

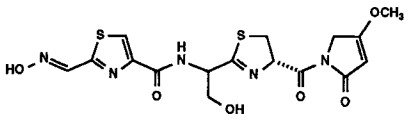
The bulged nucleotide in P7 (C870) is involved in the formation of pocket A. Mutation of this nucleotide to U or G affects hydrolysis at the splice-sites by $Mg^{2+}$, but does not affect hydrolysis at U940. These mutants have elevated $Mg^{2+}$ and guanosine requirements for splicing and their phenotype can partially be rescued by raising the pH.

While the presence of a $Mg^{2+}$ ion in pocket A might be crucial for both steps of splicing, the presence of a $Mg^{2+}$ ion in pocket B is essential for proper folding of the core and/or adequate stabilization of the transition state in the second step only. This points to an asymmetric role in catalysis for the $Mg^{2+}$ ions in pockets A and B. The larger ions ($Ca^{2+}$, $Sr^{2+}$, $Pb^{2+}$) apparently do not function as $Mg^{2+}$ does in pocket A, while being able to compete with $Mg^{2+}$ ions for pocket B. The results suggest that slight changes in coordination due to the binding preferences of each metal ion type are able to shift the cleavage site and alter the mechanism of hydrolysis.

**A6-120** STRUCTURE-FUNCTION STUDIES OF PEPTIDYLTRANSFERASE INHIBITORS

Peter L. Toogood, Li Li, Jessica Hollenbeck and Huong M. Lam, Department of Chemistry, University of Michigan, Ann Arbor, MI 48109.

The naturally occurring antibiotic althiomycin has been shown previously to inhibit *E. coli* peptidyltransferase activity. We are investigating the mechanism of this inhibition, and examining the interaction of althiomycin with *E. coli* ribosomes using affinity labeling and footprinting experiments. Structure-function studies will be described for althiomycin and its derivatives as peptidyltransferase inhibitors, and a model will be proposed for how althiomycin binds to ribosomes. Synthetic analogs of other known peptidyltransferase inhibitors are being prepared as additional probes of the peptidyltransferase active site and as potential antibiotics.



**Althiomycin2**

**A6-121** 2'-HYDROXYL GROUPS IN THE tRNA ANTICODON REGION REQUIRED FOR P-SITE BINDING IN 30S RIBOSOMAL SUBUNITS

Uwe von Ahsen[1,2], Rachel Green[1], Renée Schroeder[2] & Harry F. Noller[1], [1]Sinsheimer Laboratories, University of California at Santa Cruz, Santa Cruz, CA-95064, USA, [2]Institute of Microbiology and Genetics, University of Vienna, Dr. Bohrg. 9, 1030 Vienna, Austria.

The lack of sequence conservation in tRNAs suggests that structural elements confined in functional groups in the backbone of tRNA are required for recognition by the ribosome. A chemically synthesized anticodon stem-loop oligoribonucleotide of $tRNA^{Phe}$ from *E.coli* binds with high affinity to 30S subunits in a mRNA-dependent manner whereas the DNA analogue is not able to bind.

We synthesized pools of anticodon stem-loop oligonucleotides with no variation in sequence but using mixes that contained different 2'-deoxy- to 2'-hydroxyl-phosphoramidite ratios. The different incorporation efficiencies of the two types were determined.

One of the pools which had about half the binding affinity to 30S subunits compared to the pure RNA analogue was used for a selection procedure to isolate those oligonucleotides whose ribose composition allows P-site binding. Subsequently, the selected population of binders was subjected to partial alkaline hydrolysis to identify positions with higher amounts of 2'-hydroxyl nucleotides compared to the hydrolysis pattern of the total pool. Positions were localized with high requirements for 2'-OH by PhosphorImager quantification. To prove these requirements we synthesized oligoribonucleotides containing single 2'-deoxy nucleotides and determined the binding affinity to the ribosomal P site compared to the pure RNA analogue.

To distinguish between results due to the different sugar pucker of the DNA-containing analogue we also synthesized single 2'-methoxy nucleotide-containing oligoribonucleotides. The positions in the anticodon stem-loop region of tRNA bearing crucial 2'-OH groups for ribosome binding will be presented and a possible interaction to the 16S rRNA is suggested.

Finally, a substitution-interference approach using phosphorothioate-containing tRNA was used to identify phosphate groups required for binding to the 30S subunit.

**A6-122** SYNTHESIS OF PRE-tRNA WITH SINGLE Rp- OR Sp-PHOSPHOROTHIOATE MODIFICATIONS AT THE RIBONUCLEASE P CLEAVAGE SITE,

Jens M. Warnecke, Jens Peter Fürste, Roland K. Hartmann, Abteilung Prof. V. A. Erdmann, Institut für Biochemie, Freie Universität Berlin, Thielallee 63, 14195 Berlin, FRG

Specific Rp- or Sp-phosphorothioate modifications of the RNA backbone have been used to analyze the involvement of non-bridging oxygens in the coordination of metal ions in RNA-catalyzed reactions or mRNA splicing [1, 2, 3]. In a previous study, Rp-phosphorothioate modifications were introduced into pre-tRNAs by enzymatic synthesis [4]. Dependent on the individual pre-tRNA analyzed, either severe reductions in cleavage efficiency or aberrant cleavage were observed in processing reactions catalyzed by ribonuclease (RNase) P enzymes [4]. To analyze the role of both, Pro-Rp and Pro-Sp, non-bridging oxygens at the RNase P cleavage site, we have synthesized a *Thermus thermophilus* pre-$tRNA^{Gly}$ with a 7-nt 5'-flank by combining chemical and enzymatic RNA synthesis. Two chemically synthesized RNAs, a 13-mer (carrying the single phosphorothioate modification introduced as described in [1]) and an 11-mer, as well as one enzymatically synthesized RNA, were annealed to so-called bridging DNA oligonucleotides for ligation by T4 DNA ligase [5]. The two ligation sites were located in the D-loop and between acceptor stem and D-stem of pre-$tRNA^{Gly}$. The two diastereoisomeric 13-mers, separated by RP-HPLC, have been identified by digestion with the stereoselective enzymes phosphodiesterase I and nuclease P1. The pre-$tRNA^{Gly}$ variants carrying a single Rp- or Sp-phosphorothioate modification at the RNase P cleavage site are currently analyzed in single turnover kinetics in the presence of $Mg^{2+}$ and $Mn^{2+}$ using *Escherichia coli* RNase P RNA.

1. G. Slim & M. J. Gait (1992) *Nucleic Acids Res.* **19**, 1183-1188.
2. M. J. Moore & P. A. Sharp (1994) *Nature* **365**, 364-368.
3. T. A. Steitz & J. A. Steitz (1993) *Proc. Natl. Acad. Sci. USA* **90**, 6498-6502.
4. D. Kahle, B. Küst & G. Krupp (1993) *Biochimie* **75**, 955-962.
5. M. J. Moore & P. A. Sharp (1992) *Science* **256**, 992-997.

**A6-123** CHARACTERIZATION OF THE EXTERNAL GUIDE SEQUENCE DIRECTED CLEAVAGE REACTION OF TARGET RNA MOLECULES WITH RIBONUCLEASE P

Martina Werner, William J. Iannuccilli, Shaji T. George, Michael Y.-X. Ma & Allan R. Goldberg, Innovir Laboratories, 510 East 73rd Street, New York, NY 10021

External Guide Sequences (EGS) are RNA molecules which are designed to bind to an RNA target forming a structure that will be recognized and cleaved by ribonuclease P (RNase P) (Forster and Altman, 1990*). This technology is a novel approach to oligonucleotide based therapeutics. A number of different EGS constructs directed against the RNA of human hepatitis B virus, both all RNA and chemically modified, were synthesized and tested for activity *in vitro*. The overall cleavage reaction involves several steps which include binding of the EGS to the target, binding of RNase P to the EGS-target complex, cleavage of the target and dissociation of the ternary complex. A 27 nt fragment of the surface antigen coding region of the viral RNA was synthesized and used as target for *in vitro* studies. Equilibrium binding constants of the target molecules and several different EGS constructs were determined using gel-shift assays on non-denaturing polyacrylamide gels. Kinetics of the cleavage reaction were studied under single turnover conditions as well as under multiple turnover conditions to measure the catalytic rate and to determine the rate limiting step. Kinetic experiments were also used to evaluate the properties of different EGS constructs and to select the most active ones. In addition, the EGS constructs were applied to cleave a longer target, an 837 nucleotide long sequence coding for the surface antigen. Because of the secondary structure of the RNA molecule, the long target is less accessible to the binding of the EGS and RNase P. Several strategies are used to induce *in vitro* cleavage of the long target molecule.

*Forster, A.C. & Altman, S. (1990) *Science* 246, 783-786

*Small Ribozymes: Chemistry, Biochemistry and Applications*

A6-200 FISSION YEAST AS A GENETIC SYSTEM FOR THE ANALYSIS OF ARTIFICIAL ANTISENSE AND RIBOZYME GENES. Greg M. Arndt, David Atkins, Margaret Patrikakis, and Jonathan G. Izant. R.W. Johnson Pharmaceutical Research Institute, GPO Box 3331, Sydney NSW 2001, Australia. Tel: +61 2 360 9377, Fax: +61 2 360 9813.

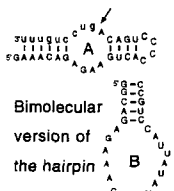
The utility of artificial antisense RNA and ribozyme-mediated gene suppression has been demonstrated in a number of different organisms. Despite the widespread application of these technologies, the design of antisense RNAs and ribozymes remains largely empirical and the critical parameters influencing their function are ill-defined. Unicellular yeasts have traditionally been informative model systems for the study of eukaryotic gene expression but, surprisingly, there have been no convincing demonstrations of antisense RNA or ribozyme activity in *trans* in any yeast species to date. Antisense RNA sequences that affect gene expression in *cis* in *Saccharomyces cerevisiae* fail to interact in *trans*. This suggests that in *S. cerevisiae* some features of RNA metabolism may be intrinsically distinct and raises the possibility that traditional antisense RNA may not function efficiently in these cells. We have chosen to utilize the fission yeast *Schizosaccharomyces pombe* as an experimental host for antisense RNA and ribozyme gene expression. *S. pombe* is evolutionarily diverged from *S. cerevisiae* with many features of gene structure and expression being more similar to those in higher eukaryotes. Our studies have involved the use of antisense RNA in *trans* to suppress the expression of endogenous and heterologous genes capable of conferring screenable cellular phenotypes. We have used a series of antisense RNAs to suppress the expression of a *lacZ* reporter gene in fission yeast. In this system antisense RNA suppression was conditional and reversible, and resulted in greater than 50% reduction in β-galactosidase activity. This study establishes the fission yeast *S. pombe* as an experimental model for the genetic analysis of antisense RNA and ribozyme genes.

A6-201 **RADIATION TARGET ANALYSIS OF RIBOZYMES,** **Steven L. Bernstein[1] and Ellis S. Kempner[2]**
**[1] NEI and [2] NIAMS, NIH, Bethesda, MD 20892**

**Radiation target analysis is a powerful tool for measuring protein molecular mass either in vitro or in vivo. However this technique has not been extended to other macromolecules. Ribozymes are catalytic RNA molecules that can allow direct correlation of RNA size with intrinsic cleavage activity. Hammerhead ribozymes were developed to cleave at one of two specific sites in a murine IRBP mRNA fragment, and were cloned into pBluescript (KS+) plasmid vector. These ribozymes were transcribed from linearized plasmid templates utilizing T3 RNA polymerase to generate RNA molecules with precisely defined sizes. The IRBP mRNA substrate molecule was transcribed as a $^{33}P$ labeled fragment from restriction digested, linearized plasmid vector. Cleavage activity for these two ribozyme species was previously determined (S. Bernstein, submitted). Frozen solutions of these ribozymes were irradiated with high energy electrons at -135°C. After different radiation exposures, the number of remaining ribozyme molecules and the amount of cleavage activity was determined. Densitometric analysis of the remaining ribozyme molecules present as a band on SDS-PAGE. The remaining cleavage activity against the labeled RNA substrate was also determined. The surviving structures and functions were analyzed by target theory, yielding the sizes of the radiation-sensitive units needed for each parameter. Comparison with the known sizes of these molecules (330 and 310 nucleotides respectively) indicates the number of molecules needed for each function, as well as the correlation of radiation sensitivity with the known size of the molecule.**

A6-202 Interdomain Tertiary Interactions in the Hairpin Ribozyme Samuel E. Butcher and John M. Burke, Markey Center for Molecular Genetics, University of Vermont, Burlington, VT 05405

The hairpin ribozyme secondary structure consists of two catalytically essential loop domains (loops A and B) separated by helices. We have used chemical modifying reagents to probe the structure of the ribozyme, and observe magnesium-dependent folding, particularly within the loop domains. To investigate whether the magnesium-dependent folding is the result of local structure (for example, mismatch pairing across loops), or due to higher order tertiary structure (for example, interdomain interactions), we have separated the two loop domains and have investigated the folding patterns of each domain in the presence and absence of magnesium. We find that neither the isolated loop A or loop B domains display magnesium-dependent folding as observed for the intact ribozyme. The isolated loop B domain folds into its native folding patterns in the absence of magnesium, consistent with previous UV cross-linking studies of the independently folding loop B domain. In contrast, the isolated loop A domain does not display the same folding patterns as observed for the intact molecule. Taken together, these data suggest that the observed magnesium-dependent folding of the hairpin ribozyme is due to interdomain (loop-loop) interactions and not simply intradomain mismatch pairings.




To directly demonstrate interdomain tertiary interactions within the hairpin ribozyme, we have been successful in reconstituting catalytic activity with a bimolecular version of the ribozyme consisting of the two isolated loop domains. The resulting reaction occurs only through tertiary interactions and has an observed rate much slower (about 10,000 fold) than the intact ribozyme. The reaction rate can not be rescued with a large molar excess of the loop B domain, and the same observed rate is obtained with a range of loop B concentrations. These data indicate that the slow reaction rate is not because of weak binding affinity. The data are consistent with a large fraction of the loop A molecules being misfolded and in slow exchange with a cleavable loop A conformer.

A6-203 THE DETERMINATION OF STRUCTURAL CONSTRAINTS IN THE LEADZYME DOMAIN P.Chartrand, N.Usman[1] and R.Cedergren.
Département de biochimie, Université de Montréal, Montréal, QC, CANADA, [1]Ribozyme Pharmaceuticals Inc., Boulder, Co 80301.

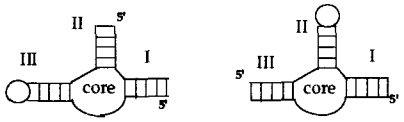
The leadzyme boasts one of the smallest catalytic domains presently known, consisting of a purine-rich asymmetrical internal loop of six nucleotides within an RNA helix: 5'-helix-$C_1GAG_4$-helix-loop-helix-$G_7A_8$-helix-3'. Thus, this small molecule is not only a good candidate for chemical modification, but also for computer modeling using the MC-SYM program developed in our laboratory. RNA chemical synthesis has provided analogs of the leadzyme containing modified nucleotides in the asymmetric loop which allow the determination of constraints for 3-D modeling.

We have used inosine, 2-amino purine and 7-deaza-guanosine to probe the amino group, the ketone group and the N7 position of the guanosine base, respectively. For adenosines, we used purine riboside and 7-deaza-adenosine analogs to probe the amino group and the N7 position. Based on the oligomers, $G_2$ of the sequence CGAG does not seem to be related directly to catalysis since its replacement by G analogs does not affect leadzyme activity. On the other hand, the N7 position of $A_3$, the amino group and the N7 of $G_4$ are important. In the dinucleotide GA, the amino group and the N7 of $G_7$ are also important.

Further analysis of the structure is in progress using chemical modification agents. (Supported by MRC of Canada)

**A6-204** SEARCHING FOR TERTIARY INTERACTIONS THAT *STABILIZE* THE HAMMERHEAD GROUND STATE. Beatrice Clouet-d'Orval and Olke C. Uhlenbeck, Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215.

The hammerhead can be divided into two regions, the conserved core and the helices. In order to investigate the importance of potential tertiary interactions within the core, mutations in an appropriate hammerhead known to greatly reduce the rate of cleavage, were tested for their effects on the free energy of the ground state using competitive inhibition and direct binding experiments. The results indicate that core mutations do not affect the stability of the E·S complex of the hammerhead in configuration 1. A similar result was found with a hammerhead with a deoxynucleotide at the cleavage site. This suggests that the tertiary interactions required to form the core are either weak or are not established in the ground state of configuration 1. In order to investigate whether a hammerhead in configuration 2 might show stabilizing tertiary interactions in the core, a new hammerhead ribozyme was designed. The kinetic properties of this new hammerhead must satisfy several kinetic characteristics before these experiments can be done.




configuration 1 configuration 2

**A6-205** INFLUENCE OF RNA STRUCTURE ON RIBOZYME ACTIVITY, Carolyn Gonzalez, Varykina G. Thackray, and James A. McSwiggen, Ribozyme Pharmaceuticals, Inc., Boulder, Colorado 80301.

Most ribozymes recognize their targets through Watson-Crick base-pairing interactions. As a consequence, self-structure within either the ribozymes or the target sequences can block binding of the ribozyme to its target sequence and thereby reduce cleavage activity. We are employing both computational and experimental approaches in a study of how RNA structure affects ribozyme activity.

RNA folding algorithms predict the lowest free energy structures based on a set of thermodynamic parameters obtained from measurements on short RNA duplexes. While these rules give reasonable predictions of secondary structure for a small set of highly structured RNAs, they remain largely untested for predicting the structure of RNAs in general. In addition, ribozyme-mediated RNA cleavage is a kinetic process (as with all enzymes) and kinetic behavior is difficult to model using (equilibrium) thermodynamic considerations alone. We have begun to address these issues by examining the ability of hammerhead ribozymes to cleave a series of short oligonucleotide substrates containing varying degrees of predicted self-structure.

**A6-206** SELF-PROCESSING RIBOZYME CASSETTES AS TOOLS FOR THE STUDY OF RIBOZYME EXPRESSION AND STABILITY, John F. Gustofson, Pamela A. Pavco, Bharat M. Chowrira, and James A. McSwiggen, Ribozyme Pharmaceuticals, Inc., Boulder, Colorado 80301

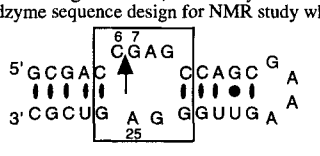
Ribozyme expression cassettes were constructed which generate trimmed, trans-acting ribozymes from longer transcripts through the action of a downstream cis-acting ribozyme. This self-processing system produces small, well-defined trans-acting ribozymes with minimal, nonproductive, intramolecular structure. These cassettes also permit direct comparison of different ribozyme expression vectors without the need to compensate for different transcription initiation and termination sequences. In earlier work (Chowrira, et al (1994) J. Biol. Chem. in press), expression cassettes were created that contained a T7 promoter and that encoded a single trans-acting ribozyme followed by either a hammerhead, hairpin, or hepatitis delta virus cis-acting ribozyme. All three ribozyme motifs functioned efficiently when transcribed *in vitro*, although slight differences were observed in the efficiency of self-processing for the different motifs. When transiently expressed in cultured mouse cells, the same specific cleavage products were observed. In addition, the relative efficiencies of *in vitro* self-processing between the three ribozyme constructs was maintained *in vivo*. Thus, the cellular milieu did not differentially alter the activity of the three ribozyme motifs.

In this second study, we have made a set of hammerhead self-processing cassettes that are expressed from either a T7 promoter or a human U6 promoter. Using this set we ask whether the same trans-ribozyme sequences are more stable in the cytoplasm or the nucleus and whether additional 3' and 5' hairpin sequences can stabilize the trans-ribozyme in these two compartments.

**A6-207** ADVANTAGES OF HETERONUCLEAR NMR SPECTROSCOPY FOR STRUCTURE-FUNCTION INVESTIGATION OF RIBOZYMES. Pascale Legault and Arthur Pardi, Department of Chemistry and Biochemistry, University of Colorado at Boulder, Boulder, Colorado, 80309-0215, U.S.A.

Because standard homonuclear NMR techniques are limited to small RNA molecules ( ≤ 15-mer), no ribozyme three-dimensional structure has been solved to date by NMR spectroscopy. However, this size limitation can now be overcome due to the recent application of heteronuclear NMR spectroscopy to $^{13}C/^{15}N$-labeled RNAs. We will describe the multiple advantages of this NMR technology by presenting its application to a 30-mer lead-dependent ribozyme, termed the leadzyme.

The leadzyme is a small cleavage motif which is specifically cleaved in the presence of lead. Cleavage occurs at the C6pG7 phosphodiester bond within an asymmetric purine-rich internal loop closed by two stable helices. In order to understand the structural and functional determinants of the cleavage reaction , we have synthesized NMR samples of LZ2, the leadzyme sequence design for NMR study which is presented below.




Heteronuclear NMR methods applied to the $^{13}C/^{15}N$-labeled LZ2 have proven critical for the resonance assignment of the active site by allowing the assignments of protons within the internal loop which could not be assigned by standard homonuclear NMR methods. In addition the heteronuclear NMR methods have allowed the identification of additional constraints which define the structure around the cleavage site and which could not be obtained from homonuclear NMR methods alone. In brief, heteronuclear NMR methods allow a three-dimensional structure of the leadzyme to emerge. $^{13}C$ NMR techniques have also been used to measure adenine $pK_a$'s in the leadzyme in order identify potential general acid-catalysts involved in the cleavage reaction. It was found that A25 has a $pK_a$ of 6.5, 2.6 units closer to physiological pH then the $pK_a$ of the corresponding mononucleotide 5'AMP. Its potential mechanistic role will be discussed in light of the structural information.

**A6-208** 3-D Modeling of the Leadzyme Domain

François Major, Marielle Foucrault and Robert Cedergren*

Département d'informatique et département de biochimie*, Université de Montréal, C.P. 6128, Succ. Centre-Ville, Montréal, Québec, Canada H3C 3J7

The leadzyme is an example of a class of enzymes composed of RNA. In the presence of Pb2+, this ribozyme catalyzes the cleavage of a specific phosphodiester bond. The knowledge of the three-dimensional folding of this domain is one of the essential prerequisites to understand its reaction mechanism. However, the absence of precise structural information makes the modeling of its structure problematic. In addition, previous experiments using sequence variants have not allowed the convergence to a clear structural hypothesis. Using the secondary structure and considering thermodynamic aspects, we have derived seven different structural hypotheses which were evaluated using the MC-SYM program. Thousands of models were produced and classified based on structural properties of each nucleotide in the sequence. This classification has allowed the identification of less than 25 structural clusters from a original set of more than 20,000 models. A representative example of each class was refined by energy minimization using molecular mechanics potentials. The five best models were examined and further studied by interactive computer graphics. These models of the catalytic domain feature two non-Watson-Crick base pairs which serve to promote a high degree of base stacking in the internal loop. One model has two stacked base triples. Furthermore, this and another model are supported by the previous data obtained with sequence variants.

(Supported by the Medical Research Council of Canada)

**A6-209** ROLES OF ALTERNATIVE CONFORMATIONS OF A HAMMERHEAD RIBOZYME IN ITS NATURAL SETTING: A REPLICATING SATELLITE RNA, W. Allen Miller[1,2,3], Lada Rasochova[1,2], Michelle Aulik[1,3] and Stan Silver[4]. [1]Plant Pathology Dept., [2]MCDB and [3]Genetics Progams, Iowa State University, Ames, IA 50011. [4]Currently: Plant Pathology Dept., University of Georgia, Athens, GA

The sequence comprising the self-cleavage structure in the satellite RNA (satBYDV RNA) of barley yellow dwarf virus exists in equilibrium between a self-cleaving hammerhead and a favored pseudoknot-like conformation which contains three stacked helices. The sequence self-cleaves very slowly, presumably only when the pseudoknot basepairing "breathes", allowing transient formation of a hammerhead. Site-directed mutations that disrupt the pseudoknot and favor the hammerhead increase the self-cleavage rate hundreds-fold, and compensating mutations reduce the self-cleavage rate in proportion to the predicted stability of the restored pseudoknot (Miller and Silver (1991) NAR 19, 5313). These results led us to predict that this sequence has two functions in satBYDV RNA replication: (i) self-cleavage via the hammerhead, and (ii) some other function via the pseudoknot. To test this model, we constructed a dimeric clone from which infectious satBYDV RNA could be transcribed in vitro (Silver et al. (1994) Virology 198, 331). The mutations that favored either the hammerhead or the pseudoknot or disrupted both structures were introduced into this clone. Trancripts from these clones, along with helper virus, were co-transfected into protoplasts. The fast-cleaving mutants replicated very poorly, supporting our hypotheses that the pseudoknot conformation serves a role in satBYDV RNA replication, and that rapid cleavage is not essential. Mutants with both conformations disrupted were also nonviable, but, surprisingly, a mutant lacking the unpaired base at the 5' side of the cleavage site replicated well, even though it self-cleaved extremely slowly. This work enhances our understanding of RNA replication and ribozyme structure and function, leading toward our goal of designing gene-targeted ribozymes that are replicated by the virus whose RNA they are designed to cleave.

**A6-210** HAIRPIN RIBOZYMES DIVIDED AT THE HINGE REGION IN THE ACTIVE DOMAIN, Eiko Ohtsuka, Yasuo Komatsu and Ikuyo Kanzaki , Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, 060 Japan

A Hairpin ribozyme with 50 nucleotides (E50) was divided at the hinge region (A14-A15). The large fragment was connected with the 5'-end of a substrate RNA (10 mer) using a linker ACCCCC or ACCC to yield 59 mer and 57 mer. These substrate (S59 and S57) cleaved in the presence of 15 mer or 19 mer RNA that contained the original 14 mer plus one or five nucleotides at the 3'-end. However, when the 3'-nucleotide pairs with the linker, cleavage rates were decreased significantly. Extent of the inhibition was greater in the case of S57 with a shorter linker. This type of hairpin ribozyme provides a new model for bent structures of nucleic acids.

**A6-211** INFLUENCE OF DEGREE OF COMPLEMENTARITY BETWEEN RIBOZYME AND TARGET ON CLEAVAGE OF FBN1 mRNA BY ANTISENSE HAMMERHEAD RIBOZYMES, LA Phylactou (1,2), P Tsipouras (1), M Godfrey (3) and MW Kilpatrick (1), Department of Pediatrics, UCONN Health Centre, Farmington CT (1) Department of Clinical Genetics, University of Birmingham UK (2) and UNMC, Omaha NE (3).

Hammerhead ribozymes can act in *trans*, with ribozyme and substrate being two different oligoribonucleotides with regions of complementarity. The degree of complementarity between ribozyme and substrate may influence the efficiency of cleavage. This might offer an approach towards the therapy of dominant genetic diseases, if ribozymes can be designed to selectively cleave mutant alleles. Our disease-model is Marfan syndrome which is caused by mutations in the fibrillin (FBN1) gene, many of which act in a dominant-negative fashion. To investigate the potential of the approach, antisense hammerhead ribozymes specific for the 5'-end (FBN1-RZ1) and the middle (FBN1-RZ2) of the FBN1 mRNA were synthesized by *in vitro* transcription. Both FBN1-RZ1 and FBN1-RZ2 can efficiently cleave their target *in vitro*, at 37°C and 50°C and 1:1 and 10:1 ribozyme to substrate ratios, to a similar extent. Substrate turnover was shown in the case of FBN1-RZ2. Two additional ribozymes containing mismatches against their target (U-C in the 3'-arm and C-A in the 5'- end arm) showed different reduced cleavage efficiencies. To determine the effect of the different ribozymes on fibrillin production, ribozymes were bound to transferrin-polylysine conjugate and delivered by receptor-mediated endocytosis into cultured fibroblasts. Total RNA was extracted from the cells 24 and 48 hours post transfection and the presence of ribozyme verified by RNase protection. Immunohistochemistry showed a ribozyme-specific reduction in the amount of fibrillin secreted by the cells. The influence of the mismatches on ribozyme specific fibrillin reduction, and therefore the potential of hammerhead ribozymes to discriminate between closely related target sequences, is being determined.

A6-212 AN IMPROVED VERSION OF THE HAIRPIN RIBOZYME CAN FUNCTION AS A RIBONUCLEOPROTEIN.

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We have introduced the R17 bacteriophage coat protein binding site into the haipin ribozyme. This construct provides a model system for studying the effects of specific protein binding of an RNA enzyme *in vitro*. Furthermore, engineered ribonucleoproteins may be used to localize a ribozyme *in vivo*. Here we show that extending helix 4 of the hairpin ribozyme to include an R17 bacteriophage coat protein binding site improves by two fold the rate of the cleavage catalysis and by 20 fold the rate of the ligation reaction. A UV cross-linking assay indicates that the 2-fold enhancement correlates directly with a stabilization of the tertiary structure of the ribozyme's large internal loop (loop B). We have shown that coat protein binding does not alter the kinetic parameters of the reaction, and that the protein remains bound during catalysis. These studies indicate that the hairpin ribozyme can be engineered to efficiently function as a ribonucleoprotein *in vitro*.

A6-213 DIVALENT CADMIUM RESTORES CATALYSIS OF A THIOPHOSPHATE-SUBSTITUTED HAMMERHEAD RIBOZYME. Edmund C. Scott and Olke C. Uhlenbeck, Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO 80309.

The hammerhead ribozyme requires millimolar concentrations of $Mg^{++}$ to catalyze the cleavage of its substrate at 0.2 $min^{-1}$ at pH 6.5. The number of the essential metal ions, their role in the mechanism of the reaction, and the ligands to which they are coordinated are being clarified by the use of phosphorothioate substitution. Replacement of the pro-Rp oxygens 5' to the 10 adenosine residues of the hammerhead ribozyme leads to a reduction in the rate of cleavage by five hundred fold, while apparent substrate binding is unchanged. Micromolar concentrations of $Cd^{++}$ in the presence of 10mM $Mg^{++}$ can be used to restore the rate of cleavage to within ten fold of the rate catalyzed by the unsubstituted sequence in the same solvent. Replacement of the single pro-Rp oxygen at the cleavage site with sulfur also reduces the rate of cleavage by about five thousand fold, and this hammerhead can also be restored by micromolar concentrations of $Cd^{++}$. Site specific thiophosphate substitutions are being made in order to determine the location of the specific sites of $Cd^{++}$ binding, and whether or not the metals at these sites play a structural or catalytic role. Restoration of catalytic activity by other divalent metal ions, such as $Mn^{++}$ and $Co^{++}$, which coordinate to sulfur more readily than does $Mg^{++}$, but less well than $Cd^{++}$, is also being investigated.

A6-214 A THREE-NUCLEOTIDE HELIX I IS SUFFICIENT FOR FULL ACTIVITY OF A HAMMERHEAD RIBOZYME : ADVANTAGES OF AN ASYMMETRIC DESIGN, Martin Tabler, Matthias Homann*, Sergia Tzortzakaki and Georg Sczakiel*, Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology Hellas, P.O. Box 1527, GR-71110 Heraklion/Crete, Greece and *Forschungsschwerpunkt Angewandte Tumorvirologie, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 242, D-69120 Heidelberg, Germany

*Trans*-cleaving hammerhead ribozymes with long target-specific antisense sequences flanking the catalytic domain share some features with conventional antisense RNA and are therefore termed "catalytic antisense RNAs". Sequences 5' to the catalytic domain form helix I and sequences 3' to it form helix III when complexed with the target RNA. A catalytic antisense RNA of more than 400 nucleotides, and specific for the human immunodeficiency virus type 1 (HIV-1), was systematically truncated within the arm that constituted originally a helix I of 128 base pairs. The resulting ribozymes formed helices I of 13, 8, 5, 3, 2, 1 and 0 nucleotides, respectively, and a helix III of about 280 nucleotides. When their *in vitro* cleavage activity was compared with the original catalytic antisense RNA, it was found that a helix I of as little as three nucleotides was sufficient for full endonucleolytic activity. The catalytically active constructs inhibited HIV-1 replication about fourfold more effectively than the inactive ones when tested in human cells. A conventional hammerhead ribozyme having helices of just 8 nucleotides on either side failed to cleave the target RNA *in vitro* when tested under the conditions for catalytic antisense RNA. Cleavage activity could only be detected after heat-treatment of the ribozyme substrate mixture which indicates that hammerhead ribozymes with short arms do not associate as efficiently to the target RNA as catalytic antisense RNA. The requirement of just a three-nucleotide helix I allows simple PCR-based generation strategies for asymmetric hammerhead ribozymes. Advantages of an asymmetric design will be presented.

A6-215 CHEMICAL SYNTHESIS OF URIDYL (3′-5′) THYMIDINE PHOSPHATE ANALOGUES AS MODELS FOR RIBOZYME SUBSTRATES, James B. Thomson, Bhisma K. Patel and Fritz Eckstein, Max-Planck-Institut für Experimentelle Medizin, D-37075 Göttingen Germany.

Hammerhead ribozymes direct cleavage of an RNA substrate, at the 3′- side of a variety of nucleotide triplets, generating a 2′,3′-cyclic phosphate and a 5′-hydroxyl group. Incorporation of 5′- modified nucleotides into the ribozyme substrate, immediately 3′- to the cleavage triplet, introduces a new leaving group. Its effect upon the rate of cleavage can then be established. Such compounds could also be utilised in a comparitive study between ribozyme- and RNaseA-catalysed cleavage. We have synthesised 5′-thioadenosine- and 5′-thiouridine 5′-triphosphate, by two independent chemical methods, as well as 5′-aminoadenosine 5′-triphosphate. None of these triphosphates could be incorporated into RNA under standard *in vitro* transcription conditions. Therefore, we synthesised 5′-Up-*S*T-3′ and 5′-Up-*NH*T-3′ dimers which can be subsequently ligated into a ribozyme substrate. They can also be utilised directly to study the stability of such internucleotide linkages.

**A6-216** A THREE-DIMENSIONAL MODEL FOR THE HAMMERHEAD RIBOZYME FROM FLUORESCENCE RESONANCE ENERGY TRANSFER MEASUREMENTS, T. Tuschl[§], C. Gohlke[≠], T. M. Jovin[‡], E. Westhof[*] and F. Eckstein[§], [§]Max-Planck-Institut für experimentelle Medizin, D-37075 Göttingen, Germany; [≠]Department of Molecular Biology, Institute for Molecular Biotechnology e. V., D-07745 Jena, Germany; [‡]Max-Planck-Institut für biophysikalische Chemie, D-37077 Göttingen, Germany; [*]Institut de Biologie Moléculaire et Cellulaire du CNRS, F-67084 Strasbourg, France

For the understanding of the catalytic function of the hammerhead ribozyme, a three-dimensional model is essential. Synthetic constructs were labeled with fluorescence donor fluorescein and acceptor tetramethylrhodamine located at the ends of one of the two strands constituting the ribozyme molecule. Distance relationships were established between different pairs of helices of various length. The FRET efficiencies were determined and correlated with measurements of a reference set of labeled RNA duplexes. Based on vector algebra analysis, FRET efficiencies were predicted as a function of the relative helical orientations in the various ribozyme constructs and compared with the experimental values. The data were most consistent with a Y-shaped arrangement of the ribozyme with helices I and II in close proximity and helix III pointing away. Using these orientational constraints and information from biochemical studies, molecular modeling was carried out and led to a three-dimensional structure of the complete ribozyme.

**A6-217** PHOSPHOROTHIOATE CONTAINING RIBOZYMES; SYNTHESIS AND PURIFICATION Francine Wincott, Anthony DiRenzo, Kurt Levy, Peter Haeberli, Susan Grimm & Nassim Usman,* Department of Chemistry & Biochemistry, Ribozyme Pharmaceuticals Inc., 2950 Wilderness Place, Boulder, CO 80301

A major advantage of chemically synthesized ribozymes is that site-specific modifications may be introduced at any position in the molecule. This approach provides flexibility in designing active ribozymes that are also more stable. The perceived drawback of exogenous ribozyme delivery, instability *in vivo* resulting from degradation by cellular nucleases, may be directly addressed using this site-specific chemical modification strategy. A systematic study of the catalytic activity and nuclease stability of selectively modified hammerhead ribozymes has resulted in the identification of a generic motif containing 5 ribose residues and 31 2′-modified sugars. This substructure has been further elaborated to include phosphorothioate linkages. Although oligodeoxyribonucleotides containing phosphorothioate linkages have been studied extensively, similarly substituted RNAs or ribozymes have not been explored rigorously. The synthesis and purification of these ribozymes will be discussed.

**A6-218** MODIFICATIONS REQUIRED FOR RNA OLIGONUCLEOTIDES TO EFFECTIVELY BIND mRNA TARGET SEQUENCES IN CELLS, Tod M. Woolf, Suzy Brown, Jennifer M. Chase, Antony DiRenzo, Kurt Levy, Chris Shaffer, Danuta Tracz, Francine E. Wincott, Nassim Usman, & Dan T. Stinchcomb, Departments of Cell Biology and Chemistry & Biochemistry, Ribozyme Pharmaceuticals Inc., 2950 Wilderness Place, Boulder, CO 80301

Few studies have dissected the requirements for an exogenously delivered synthetic RNA to bind a target mRNA sequence in cells. We investigated the effect of RNA nucleotide content on the ability of phosphorothioate chimeric oligonucleotides to bind and direct the RNaseH-mediated cleavage of a *ras* target sequence. For ease of detection, the *ras* target sequence was fused in frame to the coding sequence of luciferase. Monia, *et al.* [1] have previously shown that an oligonucleotide bearing 2′-*O*-methyl nucleotides and phosphorothioate linkages at the ends effectively inhibits a similar *ras*/luciferase fusion target. Here we show that an oligonucleotide bearing 2′-*O*-methyl nucleotides without phosphorothioate linkages at the ends has virtually no inhibitory activity. Insertion of RNA nucleotides in the middle of the phosphorothioate DNA sequence significantly reduced, but did not abolish, its inhibitory activity. At these internal positions, 2′-*O*-methyl substitutions were sufficient to completely restore inhibition. Finally, disrupting duplex formation with a 2′-*O*-methyl-substituted RNA loop had little effect on inhibitory activity. These data suggest that RNA molecules containing phosphorothioate linkages at the 5′- and 3′-ends and internal 2′-substitutions should bind to targeted sequences as effectively as phosphorothioate DNA. Such modifications may be required for exogenously delivered RNA to optimally inhibit cellular functions through ribozyme, antisense, or decoy mechanisms.

1. Monia,B.P.; Johnston,J.F.; Ecker,D.J.; Zounes,M.A.; Lima,W.F.; Freier,S.M. *J. Biol. Chem.* **1992**, , 19954-19962.

**A6-219** THE ROLES OF FOUR NUCLEOTIDES FOR THE AUTOLYTIC REACTION OF RIBOZYMES DERIVED FROM HEPATITIS DELTA VIRUS (HDV) RNAS, Wu, Huey-Nan, Institute of Molecular Biology, Academia Sinica, Nankang, Taipei, TAIWAN

The autolytic domains of both senses of hepatitis delta virus (HDV) RNA are highly homologous in sequence and they fold into similar secondary structures. Two domains differ at four locations in helix 3, loop 3 and $J4/2$, and the roles of these genomic or antigenomic specific sequences in self-cleavage reaction were investigated in this report. To accomplish this, we conducted site-specific mutation to replace the genomic specific residue to that of the antigenomic one or *vice versa* and investigated the effect. However, to simplify data interpretation, both HDV autolytic domains have been modified by internal deletion-substitution beforehand. The results illustrate that an A:U pair at the first basepair of helix 3 may stabilize the ribozyme molecule and elevate autolytic activity. A loop 3 of genomic or antigenomic sequence that differs from each other by one residue may affect the preferred sequence(s) of the third residue of $J4/2$. Nevertheless, the combinations of genomic loop 3-$J4/2$ as well as antigenomic loop 3-$J4/2$ of all natural HDV isolates may result in high autolytic activity.

*Therapeutic Applications of Ribozymes*

**A6-300 POL III PROMOTER-BASED EXPRESSION OF EXTERNAL GUIDE SEQUENCES DIRECTED AGAINST HEPATITIS B VIRUS RNA.** Maria Lorena Abate, Shrin Siony, Shaji T.George and Allan R.Goldberg, Innovir Laboratories, 510 E 73rd St. New York, NY 10021.

Ribonuclease P (RNase P) normally cleaves precursor tRNAs to form the mature 5' termini. Heterologous RNAs can be targeted for cleavage by RNase P if an appropriate external guide sequence (EGS) is present. An EGS possesses structural and sequence similarities to tRNA and contains sequences that are complementary to the chosen target RNA. The complex between the EGS and target RNA partially mimics the tertiary structure of the precursor tRNA thus enabling the enzyme to cleave the target RNA. We have used a vector based expression system to investigate EGS mediated cleavage of target RNA in cells. EGSs designed against different sites on HBV (Hepatitis B Virus) RNA have been previously tested in-vitro in our laboratory. Here we report the use of an Epstein-Barr virus-based vector to express EGSs under the control of a human U6 snRNA Pol III promoter. The EGSs were directed to the sequences in the overlapping HBV RNAs that code for surface antigen (HBsAg) and polymerase. Vectors encoding EGSs were transfected by liposomes into hepatoma cell lines expressing HBV. EGS RNA and HBV RNA over a period of several days were monitored by RNase protection assay. HBV DNA levels were determined by Southern blot analysis. HBsAg and the viral DNA in the culture medium were detected by ELISA and slot blot hybridization respectively. The effectiveness of the various EGSs directed against different HBV RNA sites will be discussed.

**A6-301** INHIBITION OF PROSTATE TUMOR CELL GROWTH USING RIBOZYMES DIRECTED AGAINST INTERLEUKIN-6, Stephen J. Freedland, Zhanna Zadourian, Jian-Hua Li, Michael Bennett, Holger M Borchers, Tim Larson, Paul H Gumerlock, Robert Malone, and Kent Erickson, University of California, School of Medicine, Davis, CA 95616.

Interleukin-6 (IL-6) has been shown to act as an autocrine growth factor for the human prostate tumor cell line, DU145. The purpose of these experiments was to suppress proliferation of prostate tumor cells by blocking the production of IL-6 protein. Antisense oligonucleotides against IL-6 mRNA specifically decreased the production of IL-6 as detected by ELISA in the human prostate tumor cell line relative to sense oligonucleotides. That decreased IL-6 production correlated positively with decreased tumor cell growth rate as measured by a mitochondrial enzyme, MTT. This suggests that DU145 inhibition of IL-6 specifically suppressed proliferation. We have designed three linear hammerhead ribozymes with 14 base target recognition domains directed against various sites of the IL-6 mRNA. Novel cationic liposome preparations, such as Polygum, and methods for efficient polynucleotide transfection in the absence or presence of 2% serum were developed. The aim was to achieve the optimal IL-6 suppression with minimal cellular toxicity. Several of these cationic lipids were not toxic to DU-145 by themselves nor were the ribozymes. However, the combined lipid and small nonsense RNA together induced moderate levels of toxicity which was dose dependent. In current studies we are assessing the effect of these ribozymes with select lipids on prostate growth rate as compared to antisense oligonucleotides. (Supported by NCI grants CA47050 and CA57813 and Deutsche Forschungsgemeinschaft Bo1223/1-1).

**A6-302** RIBOZYME-MEDIATED INHIBITION OF SENDAI VIRUS P GENE EXPRESSION, Denise K. Gavin and Kailash C.Gupta, Department of Immunology and Microbiology, Rush University, Chicago, IL 60612

The Sendai virus polycistronic P/C mRNA encodes the P and C proteins in alternate reading frames. To study the role(s) the P gene encoded proteins during Sendai virus infection a "hammerhead" ribozyme (Rz1) was designed to cleave the 5'UTR of the P/C mRNA. Activity of Rz1 was examined both *in vitro* and *in vivo*. Rz1 specifically cleaved 80% of the P/C RNA *in vitro* at a target:ribozyme ratio of 1:1 under near physiological conditions. Coexpression of Rz1 and P/C in the cytoplasm of cells using the T7-vaccinia virus expression system showed 80-90% inhibition of P and C protein synthesis. However, it was observed that ribozyme activity was inhibited by nonhybridizing extraneous bases (NEB) cotranscribed from the vector. A number of ribozyme constructs were then designed to study the effect of NEB as well as the influence of hybridization arm length on ribozyme activity. Reducing the number of NEB to a minimum substantially enhanced ribozyme activity *in vitro* $T_{1/2}$ dropped nearly 7-fold, and overall extent of cleavage increased 30%. However, reducing the length of the hybridization arms from 13- to 9-mers only marginally enhanced ribozyme activity. These findings suggest that the limiting step of the cleavage reaction is the initial interaction between ribozyme and substrate and that NEB can interfere with this interaction.

(This work was supported by a research grant from NIH (AI30517).

**A6-303** RIBOZYME-BASED ANTIVIRALS. John R. Gebhard[1]*, Zheng Xing[2], and J. Lindsay Whitton[1], [1]The Scripps Research Institute, La Jolla CA and [2]Cornell University, Ithaca, NY

We have previously shown that stable intracellular expression of ribozymes specific for the genome of an RNA virus can effect a 100-fold reduction in virus yield. This reduction is virus-specific, in that a closely-related virus whose sequence differs at the ribozyme cleavage site is unaffected. Many obstacles, however, remain in developing these promising agents for therapeutic purposes. For example, viruses with a DNA genome may be more problematic targets, as the genome itself is refractory to ribozyme assault. Furthermore, viruses often replicate in very specific cellular locations, and thus, to maximize the antiviral effects of ribozymes, it will be important to target the ribozyme (a) to the correct intracellular compartment, (b) in sufficient concentration, and (c) at an appropriate time with respect to the virus infection. We have fulfilled the above three requirements by expressing a "conditional-lethal" antiviral ribozyme from the genome of the target virus.

Using vaccinia virus (a poxvirus, with a DNA genome) as a model, we have targeted the mRNA of the host-range gene K1L. This gene is not required for growth in simian (Vero) cells, but is required for growth in human (HeLa) or rabbit (RK) cells. The ribozyme-containing virus can therefore be propagated in Vero cells, titrated, and its ability to infect and replicate in HeLa and RK cells determined. On each cell type we have compared the growth of ribozyme-containing virus with that of a mutant in which the K1L gene is deleted. We find that the ribozyme diminishes growth in all three cell types, and in RK cells the ribozyme effects a 3-log (1000 fold) decrease in production of infectious virus. The effect of the ribozyme, however, is less marked in HeLa cells, where only a 1-log (90%) reduction is seen.

Thus we demonstrate, first, that a ribozyme targeted to the mRNA of an essential gene-product of a DNA virus, when delivered in quantity, to the correct cellular location and at the correct time, can greatly diminish production of infectious virus. Second, the antiviral affect of the ribozyme may be in part cell-type dependent. If this latter phenomenon is general, it holds profound implications for the potential therapeutic value of these agents and perhaps for their 'classical' antisense counterparts.

**A6-304** ENHANCEMENT OF RIBOZYME-MEDIATED RNA CLEAVAGE BY THE NUCLEAR ENVIRONMENT

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In order to examine the influence of the nuclear environment on the hammerhead ribozyme catalysis, ribozymes targeted against HTLV-I *tax* RNA were incubated with nuclei isolated from a Tax-transformed mouse fibroblast cell line. Ribozyme-mediated cleavage of the nuclear RNA was monitored by the RNase protection assay. Comparison of the cleavage efficiencies for the isolated RNA without proteins with that of the RNA present in the nuclei revealed a 30-fold enhancement of the ribozyme-mediated cleavage by nuclear proteins. Examination of the cleavage kinetics suggest a facilitation of the formation of the active ribozyme-target RNA complex. However, the ribozyme activity in nuclear suspensions was strongly dependent on the stability of the particular ribozyme against endogenous nucleases, as an unmodified ribozyme hardly yielded any cleavage products. A ribozyme containing exclusively 2'-deoxynucleotides in its stems cleaved the target RNA by its catalytic activity in the absence of protein, but caused degradation in their presence by induction of nuclear RNase H. These results demonstrate not only the beneficial influence of some chemical modifications on the intracellular ribozyme stability but also on the mechanism of ribozyme-dependent RNA degradation.

**A6-305** REVERSION OF MULTIDRUG RESISTANCE IN A PANKREATIC CANCER CELL LINE BY A SPECIFIC HAMMERHEAD RIBOZYME

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ABSTRACT (Poster Session)

A major problem in cytostatic treatment of many tumors is the development of multidrug resistance (MDR). This is most often accompanied by the overexpression of a membrane transport protein, P-glycoprotein and its encoding mRNA. To reverse this phenotype in cell-cultures, we constructed a specific hammerhead ribozyme possessing catalytic activity to cleave the 880 triplet-sequence GUC in exon 21 of the mdr1 mRNA. We could demonstrate that the constructed ribozyme is able to cleave a reduced substrate mdr1 mRNA (240 b) at the GUC position under physiological conditions in a cell-free system. A DNA sequence encoding the ribozyme gene was then incorporated into a mammalian expression vector (pHßAPr-1-neo) and transfected into the human pancreatic carcinoma cell line EPP85-181RDB, which was resistant against daunorubicin and expressed the MDR phenotyp. The expressed ribozyme decreased the level of mdr1 mRNA expression, inhibited the formation of P-Glycoprotein, and reduced the cell's resistance against daunorubicin dramatically. That means the resistant cells were 1600-fold more resistant than the parental cell line EPP85-181P, whereas those cell clones which showed ribozyme expression were only about 5-fold more resistant than the parental cell line.

**A6-306** A RIBOZYME GENE TO RESTORE FERTILITY IN ARTIFICIALLY MALE STERILE PLANTS, Eric Huttner, Andreas Betzner, Wyatt Paul(*), Rod Scott(*), Denise Garcia(#) and Pascual Perez(#), Groupe Limagrain Pacific, RSBS, GPO Box 475 Canberra ACT 2601 Australia, (*) Department of Botany, University of Leicester, Leicester, LE1 7RH, United Kingdom, and (#) Biocem SA, 24 Avenue des Landais, 63170 Aubière, France.

Ribozymes are new useful tools to modulate gene activity. Gene therapy or delivery of synthetically made ribozymes are two possibilities of using them in therapeutic applications. In plants however the use of ribozymes will mostly involve the construction and transfer to the plant of a gene expressing the ribozyme adequately. As part of our global effort to use genetic engineering for crop improvement, we are investigating the use of customised hammerhead ribozymes (1) to modify various plant characteristics.

One gene we are targeting is an artificial male sterility (AMS) gene. To take advantage of heterosis, many crop plants are hybrids, but hybrid seed production is often a costly process involving emasculation of the female parent. Therefore male sterile plants are useful to facilitate hybrid seed production, and different AMS genes have been described. We use a secreted tobacco ß-1,3-glucanase which, when expressed in the tapetum of the developing anther, prematurely dissolves the callose wall surrounding the developing microspores. At the time of microspore release, the microspores burst, and no functional pollen is formed (2). When the harvested product is a seed or a fruit, it is often desirable to restore fertility in the hybrid plant. We are trying to restore fertility in AMS tobacco and *Arabidopsis thaliana* plants using a ribozyme gene directed against the ß-1,3-glucanase mRNA.

We have designed hammerhead ribozymes against the AMS gene and will show *in vitro* activity data. Ribozyme genes using various expression strategies have been built and transformed into AMS plants. The sterility phenotype of the plants is currently analysed and will be described. It is hoped that analysis of the plants phenotype will also allow us to compare the efficiency of different expression mechanisms.

(1) Haseloff and Gerlach, 1988, Nature, *334*, 585-591
(2) Worrall *et al.*, 1992, The Plant Cell, *4*, 759-771

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**A6-307** THE EFFECTS OF HAMMERHEAD RIBOZYMES TARGETED AGAINST BCR-ABL mRNA.

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In chronic myelogenous leukaemia (CML) the reciprocal translocation of the long arms of chromosomes 9 and 22 results in the formation of the Philadelphia chromosome and a unique BCR-ABL fusion gene. The resulting BCR-ABL protein has a deregulated tyrosine kinase activity and is believed to play a crucial role in the pathogenesis of CML. Two BCR-ABL mRNAs, b2a2 and b3a2, can be generated depending on the exact translocation point.

We have designed hammerhead ribozymes against each type of BCR-ABL mRNA to cleave at sites close to the fusion. Shortened substrates derived from the full length cDNAs were used for the *in vitro* ribozyme reactions. Ribozymes designed to cleave the mRNA 9 nucleotides 3' of the fusion have been shown to be non-specific for their BCR-ABL substrate *in vitro*. The addition of denaturant (urea) or an increase in reaction temperature or ratio of ribozyme to substrate lowers the ribozymes' specificity. However neither ribozyme cleaves an unrelated RNA. The effect of substrate size had little effect on the reaction.

Experiments are currently underway with a ribozyme designed to cleave 3 nucleotides 5' of the b3a2 fusion in the hope that this will prove to be more specific.

A6-308 INHIBITION OF VASCULAR SMOOTH MUSCLE CELL PROLIFERATION BY HAMMERHEAD RIBOZYMES TARGETING C-MYB, Thale C. Jarvis, Laverna J. Alby, Ira R. von Carlowitz, Fran Wincott, Pamela A. Pavco, Amber Beaudry, John Gustofson, James A. McSwiggen, Leo Beigelmann, Chris Shaffer, Anthony DiRenzo, Kurt Levy, Nassim Usman and Dan T. Stinchcomb, Ribozyme Pharmaceuticals, Inc., Boulder, CO 80301

The hyper-proliferative response of vascular smooth muscle cells following coronary angioplasty is widely thought to be the primary mechanism responsible for the neo-initimal thickening associated with restenosis. C-*myb* is a proto-oncogene that is involved in cell-cycle regulation and is expressed in smooth muscle cells in a tissue specific manner. Using a smooth muscle cell proliferation assay, we have demonstrated reproducible inhibition of proliferation by exogenously delivered hammerhead ribozymes designed to cleave c-*myb* mRNA. Ribozymes targeting several different hammerhead sites in the c-*myb* coding region have been tested and show varying degrees of efficacy. In all cases, inactive versions of the same ribozymes (in which the catalytic cores have been mutated) show little or no inhibition. Therefore the effect appears to be mediated by catalytic cleavage of the message, rather than merely an anti-sense effect of the binding arms of the ribozyme. The most efficacious ribozymes show comparable levels of inhibition on rat aortic smooth muscle cells, porcine aortic smooth muscle cells and human aortic smooth muscle cells. Inhibition is dose-dependent with an $IC_{50}$ of approximately 50-100 nM. Data on a variety of different modified ribozyme chemistries as well as several different cationic lipid delivery vehicles will be presented.

A6-309 HAMMERHEAD RIBOZYMES AGAINST HUMAN PAPILLOMA VIRUS (HPV) TYPE-18: IN VITRO STUDIES

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Hammerhead ribozymes constructed in order to cleave the E6/E7 oncogene transcripts of HPV-18 were shown to be highly efficient against the synthetic target oligonucleotides. In the present study these hammerhead ribozymes ( targeted at sites 123, 309 and 669 of HPV type-18 transcript) proved effective against HPV transcripts from HeLa cells.

To compare the efficiencies of ribozymes, hybridization of these ribozymes with the *in vitro* transcribed HPV E6/E7 genes as function of time and concentration of ribozyme were evaluated. When increasing amounts of ribozymes were used, the hybridizing efficiency of Rz 309 was about 1.5 fold and 2-6 fold higher than Rz 123 and Rz 669 repectively. When ribozymes were incubated with HPV transcripts of E6 and E7 oncogenes for varying time intervals, hybridizing activity continued significantly upto 1-2 hours and then reached a plateau. The ribozymes are now being tested against E6/E7 RNA expressed in a bacterial system and are compared with an antisense RNA.

A6-310 **SUPPRESSED EXPRESSION OF *LacZ* AND HBV X GENES BY HAMMERHEAD RIBOZYMES AND DETECTION OF A CLEAVAGE PRODUCT IN *E. COLI*,** **Changwon Kang and Eunsung Junn, Department of Life Science, Korea Advanced Institute of Science and Technology, Taejon 305-701, Republic of Korea**

**Several hammerhead ribozymes were designed to target and cleave the *lacZ* mNRA produced from the plasmid M13mp18 and hepatitis B viral X gene mRNA fused to *lacZ* mRNA in intra- and intermolecular configurations. A hammerhead ribozyme was designed to target the GUC sequence of the *Sal*I site in the multicloning region of the M13mp18. It successfully cleaved the *lacZ* mNRA *in vitro* and in *E. coli* and thus suppressed the *lacZ* expression in *E. coli*. The *in vitro* cleavage activity of the ribozyme was confirmed by detecting cleavage products. Suppression of the *lacZ* expression in *E. coli* was shown by difference in the color of plaques, when recombinant M13mp18 derivatives carrying either the ribozyme gene plus target gene or the target gene alone were transfected into *E. coli* cells. The *in vivo* cleavage product was also detected from the total RNA isolated from white plaques. Analysis of the *in vitro* and *in vivo* cleavage products revealed the exact cleavage sites. Three different ribozymes were also designed to target the GUC sequence in 3 positions of HBV X gene. Only one of them successfully cleaved the HBV X mRNA in both intra- and intermolecular configurations *in vitro*. Although it suppressed the expression of the *lacZ* gene translationally fused to the HBV X gene in intramolecular fashion, it failed to do so in intermolecular fashion in *E. coli*, according to β-galactosidase assay of cell extracts and phenotypic observations.**

A6-311 INHIBITION OF TNF-α SECRETION BY MURINE MACROPHAGES FOLLOWING *IN VIVO* AND *IN VITRO* RIBOZYME TREATMENT, Kevin O. Kisich, Peter F. Stecha, Holly A. Harter, and Dan T. Stinchcomb, Ribozyme Pharmaceuticals, Inc., 2950 Wilderness Place, Boulder, CO.

Tumor necrosis factor-α (TNF-α) is a pleiotropic cytokine produced primarily by cells of the granulocyte-macrophage lineage, and CD4+ T-cells. The overproduction of TNF-α is associated with autoimmune diseases such as rheumatoid arthritis, psoriasis, and chronic inflammatory bowel disease. We have evaluated the potential of ribozymes directed against TNF-α mRNA to selectively inhibit TNF-α production by murine macrophages in tissue culture and *in vivo*. Following cationic lipid-mediated delivery of ribozymes to primary macrophages in culture, anti-TNF-α ribozymes were more effective inhibitors of TNF-α secretion than catalytically inactive ribozyme controls. Inhibition of TNF-a secretion was proportional to the dose of ribozyme administered, with an $ED_{50}$ of 130 nM. Cationic lipid/ribozyme complexes were also delivered to murine peritoneal macrophages *in vivo*. Following intraperitoneal injection the macrophages in the peritoneum accumulated approximately 25% of the injected dose in 3 hours. Peritoneal macrophages were harvested 3 hours after injection by peritoneal lavage and then were stimulated with LPS in tissue culture. Administration of the catalytically active ribozyme inhibited LPS stimulated TNF-α secretion by approximately 50% relative to an inactive ribozyme control. IL-1α secretion and cell viability were not affected by anti-TNF-α ribozyme treatments in tissue culture or *in vivo*. Northern analysis of TNF-α mRNA extracted from macrophages following *in vivo* ribozyme treatment revealed the accumulation of ribozyme specific TNF-α mRNA degradation products. These data demonstrate that catalytic ribozymes are potent inhibitors of specific gene products *in vivo*.

A6-312 **COMPARISON OF THE EFFECTS ON K 562 CELLS OF RIBOZYMES TARGETED AGAINST BCR/ABL AND BCL-2 mRNAs.** *W. Lange, M. Daskalakis, S. Scheid, G. Dölken and J. Finke. Abt. Hämatologie und Onkologie, Med. Univ.-Klinik Freiburg, Hugstetter Str. 55, D-79106 Freiburg, Germany.*

Chronic myelogenous leukemia (CML) is characterized in most cases by the Philadelphia chromosome, which is caused by a reciprocal translocation of the c-ABL gene from chromosome 9 and the BCR gene from chromosome 22. The resultant BCR/ABL fusion gene and its subsequent biological sequelae play a crucial role in the development of CML. In order to interrupt the production of the CML associated $p210^{BCR/ABL}$ protein at the molecular level, highly specific ribozymes were designed which exclusively cleave the BCR/ABL B3A2-type mRNA as it is present in the CML cell line K562. In addition a ribozyme targeted against BCL-2 mRNA was developed; expression of the BCL-2 gene prevents programmed cell death, apoptosis. Depending on the structure of several ribozymes tested we were able to inhibit the proliferation rate of K562 cells with different efficiencies. Due to decreased inhibition after 48 hours with single lipofections of phosphodiester ribozymes repeated lipofections at 48 hour intervals were necessary. Modified nuclease resistant ribozymes, however, were almost as effective as their unmodified counterparts. When K562 cells were incubated only in the presence of a ribozyme targeted against BCL-2, we observed the same degree of reduction on the proliferation rate as with a ribozyme against BCR/ABL. Both ribozymes together did not show any additional effects. It might be possible, that BCR/ABL acts via BCL-2. Future applications of the above ribozymes might be the development of molecular purging methods in the context of new treatment modalities for CML and other leukemias.

A6-313 STUDIES OF THE EFFICACY OF RIBOZYMES TARGETING α-LACTALBUMIN mRNA IN STABLE CELL LINES AND TRANSGENIC MICE.

Phil L'Huillier+, Solange Soulier, Marie-George Stinnakre & Jean-luc Vilotte. Laboratoire de Genetique Biochimique et cytogenetique, INRA-CRJ, 78352, Jouy-en-Josas, Cedex, France. +On leave from: AgResearch, Ruakura Agricultural Centre, Private Bag, Hamilton, New Zealand.

Hammerhead ribozymes (Haseloff & Gerlach, 1988) with 12 nucleotide antisense flanks, and targeting five sites along the bovine α-lactalbumin (α-lac) mRNA have previously been expressed in the cytoplasm of cells using the T7-vaccinia virus. Ribozymes targeting one site adjacent to the 3' poly A signal and two 'apparent' stem-loop regions reduced the level of α-lac mRNA by 80-90%. However to achieve this ribozyme-mediated reduction in target RNA levels, it was necessary to deliver a 1000 fold excess of ribozyme over target (L'Huillier, Davis & Bellamy 1992).

In this report we will describe current studies in which the efficacy of the above ribozymes is being evaluated in transgenic mice or stably transformed cells in culture. In transgenic mice, ribozyme sequences have been inserted into transgene constructs designed to direct expression to the mammary gland. Mice transgenic for ribozymes targeting both mouse and bovine α-lac mRNA have been created and are currently being evaluated.

Stable cell lines transcribing α-lac and ribozyme sequences have been created by a two step process whereby initially, sequences encoding for α-lac were intergrated into the genome of 3T3 cells and subsequently, episomally-replicating plasmids delivering ribozyme sequences were stably transfected. At a ratio of ribozyme:target of 10-100:1, the level of α-lac mRNA was reduced by 50-60% for the comparable ribozymes described above. However a catalytically-inactive ribozyme demonstrated near-equal efficacy as the active molecule targeting the same site.

Haseloff, J & Gerlach, W L (1988) Nature 334: 585-591.
L'Huillier, P J, Davis, SR & Bellamy, AR (1992) EMBO J 11: 4411-4418.

A6-314 RETROVIRAL GENE TRANSFER OF ANTI-RAS RIBOZYME INTO TUMOR CELLS WITH ACTIVATED RAS ONCOGENE, Mingxia Li, Herinder Lonial, Ron Citarella, Robert Kramer, Department of Tumor Biology, Medical Research Division, American Cyanamid Company, Pearl River, NY 10965

The hammerhead ribozymes targeting the activated H-Ras (12 val) oncogene were cloned into Moloney murine leukemia virus based double copy retroviral vectors under the control of a human tRNA promoter. The efficiency and specificity of these ribozymes in mediating the cleavage of the H-Ras transcript was first analyzed in a cell-free system. The retroviral vectors were constructed to carry selectable markers of Neo or PAC gene to enrich the transduced target cells. The ribozymes were expressed in human EJ, murine 3T3 and rat intestinal cells with or without the activated H-Ras oncogene. Up to 60% reduction in ras protein was observed in these cells using a H-ras specific antibody. The malignant potential of these cells was determined in nude mice, where we observed up to a 90% reduction in tumor growth of cells transduced with functional ribozyme. Our results indicate the usefulness of retroviral vector mediated anti-H Ras ribozyme expression in vivo.

A6-315 GENERATION OF A MAMMALIAN CELL LINE DEFICIENT IN GRP INDUCTION THROUGH EXPRESSION OF A grp94 mRNA-TARGETED RIBOZYME, Edward Little and Amy S. Lee, Department of Biochemistry and Molecular Biology, University of Southern California, Los Angeles, CA 90033

In response to depletion of intracellular $Ca^{2+}$ stores or a blockage of N-linked protein glycosylation, such as that caused by the calcium ionophore A23187 or tunicamycin, the glucose regulated proteins (GRPs) are specifically induced in mammalian cells. The GRPs are a set of endoplasmic reticulum (ER) localized proteins related to the heat shock protein (HSP) family. GRP94 is an abundant glycoprotein, and exhibits $Ca^{2+}$ binding and protein chaperoning properties. Using a hammerhead ribozyme targeted against grp94 mRNA and driven by a stress-inducible promoter, we have generated a cell line deficient in its ability to induce GRP94. The effect of this ribozyme as determined by the level of intact grp94 mRNA is mediated by the cleavage of the grp94 message just downstream of the initiation codon. Surprisingly, this cell line also exhibits impaired GRP78 inducibility. Thus, suppression of GRP94 induction causes a simultaneous suppression of GRP78 induction, suggesting that GRP94 induction may be part of a signalling pathway from the ER to the nucleus. The ribozyme expressing cells also show increased sensitivity to ER $Ca^{2+}$ depletion caused by A23187 or thapsigargin, an ER-$Ca^{2+}$ ATPase inhibitor, but not to tunicamycin. This increased sensitivity to ER $Ca^{2+}$ depletion indicates that GRP94 has an important protective role in cells under $Ca^{2+}$ stress conditions, but may not be sufficient to protect cells against glycosylation block stress.

A6-316 DOUBLE RIBOZYME MEDIATED CLEAVAGE OF INFLUENZA A NP-vRNA, Annette Menke and Gerd Hobom, Institute for Microbiology and Molecular Biology, Justus-Liebig-University, Giessen, Germany, D 35392
Various hammerhead-type ribozymes directed against the influenza NP-vRNA near-terminal 5' sequence have been constructed and their catalytic activities have been analysed *in vitro* and *in vivo*. For cleavage at two neighbouring GUC-sites present in this nearly invariant region of influenza segment 5 sequence we designed several constructs containing one or two cleavage centers, in tandem covalent linkage. Double-headed ribozymes were able to cut at both cleavage sites in single-step reactions in considerably increased rates and in true catalytic turnover, surpassing any of the related single-headed constructs in substrate association and facilitated product dissociation kinetics *in vitro*.
*In vivo* analysis of ribozyme activity was performed in transient cDNA transfection into COS cells followed by challenge with influenza virus infection. For ribozyme delivery various strategies of expression by RNA polymerase II were used, in comparison with expression by RNA polymerases I or III. In any of these conditions double-centered ribozymes again rated distinctly superior over their single-centered analogs, resulting in up to 99% as opposed to 80% of viral inhibition rates, in particular modes of ribozyme RNA expression.

A6-317 **EXTERNAL GUIDE SEQUENCES THAT DIRECT HUMAN RIBONUCLEASE P TO CLEAVE THE FUSION JUNCTION OF THE PML/RARα RNA OF ACUTE PROMYELOCYTIC LEUKEMIA**, Jeffrey L. Nordstrom, Shaji T. George and Allan R. Goldberg, Innovir Laboratories, Inc., 510 East 73rd St., New York, NY 10021
Acute promyelocytic leukemia (APL) is associated with a chromosomal translocation that fuses genes for a putative transcription factor (PML) and the retinoic acid receptor α (RARα). PML/RARa has been hypothesized to function as a dominant negative oncogene whose product blocks myeloid differentiation. External guide sequences (EGSs) are oligoribonucleotides that form complexes with target RNAs in such a way that the target RNA may be cleaved by RNase P. We are exploring the possibility of using EGSs to specify the cleavage of PML/RARα mRNA in APL cells. Since EGS design criteria have not been fully formulated, EGSs with various structures were tested for their ability to target the cleavage of a 51 nt fragment of PML/RARα mRNA by a purified preparation of human RNase P *in vitro*. EGSs based on the structure of a human $tRNA^{GLY}$ were more effective than ones based on a E. coli $tRNA^{TYR}$; the most effective ones lacked the entire anticodon arm. The EGS recognition sequence was altered so as to target cleavage to different sites near the fusion junction. Cleavage was obtained at sites that were 4 and 5 nts, but not 6 and 9 nts, downstream from the fusion junction. These data confirm that the base paired region between the EGS and its RNA target must conform to some sequence requirement to be effectively cleaved by RNase P.

A6-318 RIBOZYME AND ANTISENSE CONSTRUCTS CONFER RESISTANCE TO HIV-1 INFECTION IN HEMOPOIETIC CELLS, Lun-Quan Sun, Jason Smythe, Jagdeesh Pyati, Wayne Gerlach and Geoff Symonds, The R.W. Johnson Pharmaceutical Research Institute, GPO Box 3331, Sydney, NSW 2001, Australia
A hammerhead ribozyme targeted to the HIV-1 tat gene and an antisense sequence complementary to sections of R, U5 and the 5' region of gag gene were designed and cloned into MoMLV-based retroviral vectors. To test the anti-viral efficacy of these constructs, amphotropic therapeutic retroviruses were used to transduce both hemopoietic cell lines and primary CD4+ enriched peripheral blood lymphocytes (PBLs). The ribozyme and antisense sequences were constitutively expressed in both T cell lines (for over 6 months) and PBLs, and no apparent cellular cytotoxicity was observed in the transduced cells. HIV-1 replication was markedly inhibited in both pooled T cells and PBLs challenged with either laboratory HIV-1 strains such as IIIB, or primary clinical HIV-1 isolates. These results indicate the potential applications of ribozyme and antisense technology in HIV-1 gene therapy.

A6-319 DEVELOPMENT OF HAIRPIN RIBOZYMES FOR TGF-β1, TGF-β2, AND TYPE II TGF-β RECEPTOR WITH DEFINED 3' TERMINI BY AUTOCATALYTIC PROCESSING, Roy Tarnuzzer, Robert Stiff and Gregory Schultz, Department of Ob/Gyn, University of Florida, Gainesville, FL 32610
Excessive formation of scar tissue is a major complication following several types of surgeries including trabeculectomy for glaucoma. The transforming growth factor beta proteins (TGF-β1 and TGF-β2) appear to be key regulators of scar formation due to their ability to increase synthesis of extracellular matrix proteins and TIMPs while decreasing synthesis of matrix metalloproteinases. TGF-βs bind to three distinct plasma membrane proteins on target cells. The type II TGF-β receptor is a serine/threonine kinase that is required for generation of TGF-β response. We constructed three hairpin ribozymes for TGF-β1, TGF-β2 and type II TGF-β receptor mRNAs using an autocatalytic plasmid which contains a hairpin ribozyme that generates processed catalytic hairpin RNAs with defined 3' termini (Gene 122:85-90). cDNA sequences for TGF-β1, TGF-β2 and type II TGF-β receptor were searched for hairpin ribozyme cleavage sites defined by the consensus sequence NNRYNG(A,T,C)Y(G,T,C)NNNNN. Potential cleavage sites were screened for sequences most similar to that of the wild type hairpin ribozyme and within the coding region of each message. Oligonucleotides were synthesized as two overlapping oligonucleotides of 39 and 37-nt. The DNA sequences with specificity for type II TGF-β receptor, TGF-β2 and TGF-β1 were: CCG GGG CCC **ACA CAC TGA GAA GTG AAC CAG AGA AAC ACA**; CCG GGG CCC **TGA AGT AGG AGA AGT AGA ACC AGA GAA ACA CAC**; and CCG GGG CCC **TAG CCA CAG CAG AAG TAG** ACC CAG AGA AAC ACA, respectively. Sequences in bold are complementary to and specific for the target mRNAs. Each of these 39-nt specific oligonucleotides were annealed to a universal 37-nt oligomer (GCC ACG CGT ACC AGG TAA TAT ACC ACA ACG TGT GTT T) composing the 3' structural domain of the hairpin ribozyme and the overhangs filled using Taq polymerase. Products were digested with ApaI and MluI and ligated into pHC plasmid similarly digested. Positive clones were screened and are being assessed for autocatalytic processing and the ability to cleave target mRNAs. Supported in part by NIH grant EY05587 and US Army contract DAMD-17-91-C-1095.

**A6-320** OPTIMISING HAMMERHEAD RIBOZYMES FOR USE AGAINST HIV *IN VIVO*, Stephanie A. Thompson, Philip Griffin and William S. James, Sir William Dunn School of Pathology University of Oxford, OX1 3RE, UK.

Trans-acting ribozymes can be used to ablate the expression of chosen genes in a sequence specific manner. This provides a potential mechanism for anti-viral gene therapy. Investigations into ribozyme potency *in vivo* have shown that most are non-inhibitory, even when they exhibit catalytic activities *in vitro*. We have undertaken a systematic approach to identify factors which are important for the activity of ribozymes *in vivo*. For these studies we used a family of ribozymes which share a common cleavage site within the HIV tat gene but have different lengths of complimentary flanking regions (FCR). These vary in length between 9 and 562 nucleotides.
Short ribozymes with FCRs of 9 nucleotides were found to be the only members of the family to show some degree of catalysis *in vitro* at 50°C. Ribozymes with FCRs of 33 nucleotides had high initial reaction rates but were not catalytic. At 37°C the short ribozymes had little activity, however, ribozymes with 33 nucleotide FCRs still had high initial reaction rates although they remained non-catalytic.
Ribozymes were transferred to an *in vivo* expression system using retroviral vectors and challenged with HIV. The ribozymes with FCRs of between 30 and 40 nucleotides inhibited HIV replication by more than 99.9%. Short ribozymes were not inhibitory.
By analysing asymmetric ribozymes *in vitro* we found that a long 3' FCR alone produced a ribozyme with a high initial reaction rate. We examined the duplex potential of the target RNA, which involves calculating the tendency of each nucleotide to be involved in an RNA secondary structure. From this analysis we predict that the region of the target RNA complimentary to the 3' FCR of the ribozyme will have an extended region of single-strandedness at 37°C . We believe that it is this region of single stranded RNA which is available for inter-RNA base pairing that is responsible for the *in vivo* potency of the longer ribozymes.
The conclusions drawn from these studies are that long ribozymes, and those which have a high initial hybridisation rate with the target RNA, are most likely to be inhibitory. The catalytic potential, i.e. turnover, of a ribozyme appears to be unnecessary for effective inhibition.

**A6-321** BIOLOGICAL FUNCTION STUDIES OF GROWTH HORMONE RECEPTOR USING A RIBOZYME APPROACH, Xinzhong Wang and John J. Kopchick, Department of Biological Sciences, Molecular and Cellular Program and Edison Biotechnology Institute, Ohio University, Athens, OH 45701
Growth hormone (GH) is an important agent involved in vertebrate growth and metabolism. It stimulates somatic growth by a variety of effects on target tissues. It has been clearly shown that GH is involved in regulating metabolic processes which include lipid, nitrogen, carbohydrate, and mineral metabolism.
Like most polypeptide hormones and growth factors, GH acts by binding to a receptor(s) on target cells. The GH receptor (GHR) has been shown to be a plasma membrane associated glycoprotein. Investigation of GH structure and GHR interactions combined with the subsequent intracellular signal transduction pathways have lead to an initial understanding of the mechanism(s) of GH action. However, a problem in the GH/GHR research is the lack of a natural GH responsive system which lacks endogenous GHR. In order to establish such a system for studying the GH and GH/GHR interaction relative to cell differentiation and growth, we selectively generated an ribozyme construct (mGHR-RZ) which should "attack" the mouse GHR mRNA. Expression of mGHR-RZ was directed via the mouse metallothionien-I transcriptional regulatory sequences. The mGHR-RZ was successfully expressed in mouse L cells which contain low but detectable levels of mGHR. RT-PCR analysis revealed that the endogenous mGHR mRNA in mGHR-RZ containing cells was suppressed to non-detectable levels. An GH-specific response assay (GH stimulated tyrosine phosphorylation of ~95,000 dalton proteins) indicated that the mGHR was not functional when mGHR-RZ was expressed in these cells. With this approach, a natural GH responsive cell line lacking endogenous GHR has been established and provides a system for the study of the biological function of GHR and its analogs when re-introduced into these cells (This work was partially supported by grant from NRICGP/USDA 91-37206-6738 and NIH grant DK42137-01A2, as well as a grant from Innovations in Drug Development, Inc.).

*In vitro Selection of Ribozymes With New Activities; Viral and Lipid-Mediated Delivery of Ribozymes*

**A6-400** A HIGH FREQUENCY OF FUNCTIONAL RIBOZYMES DERIVED FROM A 'BASAL' HAMMERHEAD RIBOZYME VECTOR.
Lars Ährlund-Richter, Sten Larsson, Michael Andäng, Department of Biosciences at Novum, Karolinska Institute, 141 57 Huddinge, Sweden.

Using a 'basal' hammerhead ribozyme construct (Larsson *et al*, N.A.R. 22:2242-8, 1994), we have targeted mRNA from three different genes (β2M, WT1 and H19) in cell lines or transgenic mice. Target mRNA sequences ranging between 12 - 19 nucleotides were selected for the ribozyme-substrate interactions.

Effects on target mRNA levels were observed for five out of six tested ribozymes (β2M: 4/4, and H19: 1/2) after stable transfections into cell lines. Three out of three ribozymes (β2M: 1/1 and WT1: 2/2) were efficient when tested in transgenic mouse models.

This paper will discuss the unexpected high frequency of functional ribozymes, the design of our basal ribozyme vector, and the consequences from altering 5' flanking sequences of the ribozyme.

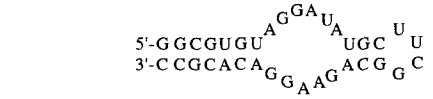
A6-401 IN VITRO SELECTION OF RNA LIGANDS TO p53 BASED ON AN ELECTROPHORETIC MOBILITY SHIFT ASSAY, Matthias Dobbelstein[1] and Thomas Shenk, Howard Hughes Medical Institute, Department of Molecular Biology, Princeton University, Princeton, NJ 08544

The tumor suppressor gene product p53 has previously been described as an RNA binding protein (Oberosler et al., EMBO J. 12, 2389-2396, 1993). However, no sequence specificity has been reported so far. We therefore selected RNA ligands to p53 from a randomized pool of RNA sequences. The iterative selection procedure comprised reverse transcription of the RNA molecules, PCR amplification of the corresponding DNA, in vitro transcription by T7 RNA polymerase, and an electrophoretic mobility shift assay as the selection step: after incubation of the RNA pool with purified p53, the RNA-Protein complexes were separated from the free RNA molecules by polyacrylamide gel electrophoresis. The band containing the RNA-p53 complex was isolated and the RNA was purified from the gel. These RNA molecules were then subjected to the next amplification step. After ten rounds of selection and amplification, the final RNA pool was enriched for sequences binding to p53 with high affinity even under stringent conditions. Requirements on RNA sequence and structure as well as implications on the functions of both p53 and cellular RNA molecules will be discussed.

1) Supported by the Stipendium fuer Infektionsbiologie of the German Cancer Research Center

A6-402 STRUCTURAL CHARACTERIZATION OF A FMN BINDING RNA BY NMR, Pei Fan and Dinshaw Patel, Cellular Biochemistry and Biophysics Program, Memorial Sloan-Kettering Cancer Center, 1275 York Ave., New York, NY 10021

There is currently great interest in defining the higher order folding of RNA when bound to ligands ranging from cofactors to antibiotics, peptides and proteins. This approach has been facilitated by *in vitro* selection and evolution methodologies that have identified RNA molecules that specifically interact with immobilized ligands. Recently, a 35mer RNA has been identified that binds specifically to the cofactor flavin mononucleotide (FMN) (Burgstaller & Famulok, 1994). The sequence and predicted secondary structure of this FMN binding RNA is shown below:



We have used *in vitro* transcription with T7 polymerase (Millligan & Uhlenbeck, 1989) to generate milligram amounts of the FMN binding 35mer RNA. Proton NMR detects a conformational transition in the 35mer RNA on complex formation with FMN. The structural basis of this transition is currently being elucidated by a combined NMR-computational study on $^{13}C$ and $^{15}N$ labeled RNA with the eventual goal of defining the intermolecular interactions stabilizing complex formation.

Burgstaller, P. & Famulok, M. (1994) *Angew. Chem.* **33**, 1084-1087.
Milligan, J. F. & Uhlenbeck, O. C. (1989) *Methods in Enzymology* **180**, 51-62.

A6-403 **RIBOZYME MEDIATED INHIBITION OF EXPRESSION OF PORCINE LEUKOCYTE 12-LIPOXYGENASE.** Jia-Li Gu, Dange Veerapanane, Rama Natarajan, Lisa Thomas, Wei Bai, John Rossi, Jerry Nadler. Department of Diabetes, Endocrinology and Metabolism, City of Hope National Medical Center, Duarte, CA 91010

Recent studies suggest that activation of a 12-LO pathway of arachidonic acid is involved in angiotensin II (AII) induced steroidogenic and vascular actions. The leukocyte type 12-LO is expressed in human adrenal glomerulosa and vascular smooth muscle and that this form of 12-LO is upregulated by AII in both of these tissues. There are currently no specific pharmacologic inhibitors for the leukocyte type 12-LO. We have designed and synthesized a RNA/DNA chimeric hammerhead 12-LO ribozyme with two terminal phosphorothiorate internucleotide linkages. The ribozyme was tested at varying temperatures and amounts using a 210 nucleotide 12-LO RNA substrate. This substrate was specifically digested into two fragments by concentrations of ribozyme as low as 10ng but not by the antisense oligonucleotide which is located in the same region. The ribozyme was active at 37°C and highly stable in cell culture conditions when added with a Tranfectam reagent. To determine the effect of the ribozyme in cultured cells, the ribozyme was introduced into porcine VSMC by Transfectam reagent. We used reverse transcriptase polymerase chain reaction analysis to evaluate the level of porcine leukocyte 12-LO mRNA in these treated cells. The results derived from several repeat experiments consistently demonstrated that the ribozyme at concentration of 1 μM inhibited 12-LO mRNA level by ~70%. Therefore, this 12-LO ribozyme should prove to be a highly valuable tool to evaluate the role of the leukocyte 12-LO pathway in mediating AII-induced adrenal and vascular actions.

A6-404 COMPARATIVE STABILITY, IN VITRO ACTIVITY AND CELLULAR UPTAKE CHARACTERISTICS OF HAMMERHEAD AND HAIRPIN RIBOZYMES TARGETED AGAINST c-myc PROTOONCOGENE mRNA SUBSTRATE
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Sequence-selective cleavage of target mRNAs may have potential therapeutic applications. In this laboratory we have been investigating the potential use of ribozymes in cleaving c-myc for potential anticancer therapy.
In this study we have compared both hammerhead and hairpin ribozymes with respect to their stability in serum and cell culture medium, in-vitro cleavage activity against a c-myc mRNA substrate, and their relative abilities to traverse cellular membranes.
Ribozymes and c-myc RNA substrates were synthesized on a Cruachem DNA/RNA synthesiser using fpmp-phosphoramidite chemistry.
Ribozymes were initially HPLC purified and then deprotected according to the manufacturers protocol (Cruachem). Ribozymes were [$^{32}P$]- 5'-end labelled using gamma-[$^{32}P$]-ATP and polynucleotide T4 kinase (Gibco). and subsequently purified by polyacrylamide gel electrophoresis. In-vitro activities of the 32 mer hammerhead ribozyme sequence and a 50 mer hairpin ribozyme directed against the same GUC cleavage site within a 27 mer c-myc RNA substrate were compared as a function of the metal ion concentration in 50mM TRIS pH7.5.
A minimum threshold of 10mM $MgCl_2$ was required for cleavage by the hammerhead sequence.Significant activity was only observed when hammerhead ribozyme concentrations were similar to or greater than the substrate concentrations. Activity was independent of temperature within the narrow range of 20-40°C. Preliminary stability studies suggest that unmodified all RNA ribozymes are extremely unstable for exogenous delivery to target cells. Uptake studies in U87 cells suggest that modified RNA structures, e.g. DNA-RNA and 2'-O-Methyl RNA-RNA chimeric modified hamerhead and hairpin ribozymes, enter living cells by active endocytosis and that this mechanism may be similar to that observed for antisense DNA oligonucleotides.

A6-405 MOLECULAR BASIS OF *in vitro* SELECTION OF THE ATP BINDING RNA, Feng Jiang, Radovan Fiala, David Live, Dinshaw Patel, Cellular Biochemistry and Biophysics Program, Memorial Sloan-Kettering Cancer Center, 1275 York Ave., New York, NY 10021

RNA molecules which display ligand binding and catalytic properties comparable to those of proteins and enzymes are rapidly being discovered by the methods of *in vitro* selection and evolution. This has led to the discovery of a 40mer RNA that binds ATP with great affinity and specificity (Sassanfar & Szostak, 1993).The ATP binding 40mer RNA was synthesized by *in vitro* transcription with T7 RNA polymerase (Milligan, et al. 1989), and its structure was studied by NMR spectroscopy. Sequence specific imino proton assignments of the 40mer RNA identified two base-paired stems, indicating a secondary structure consistent with that predicted by the sequence analysis of a panel of ATP binding RNAs (Sassanfar & Szostak, 1993). Upon the addition of ATP, seven additional imino proton resonances from loop residues were observed suggesting that the ATP binds the 40mer RNA at the loop and stabilizes the loop structure. Heteronuclear NMR experiments of the $^{13}C$ and $^{15}N$ labelled RNA with/without ATP are being carried out to identify the interaction between the ATP and the 40mer RNA.

Milligan, J. F. and Uhlenbeck, O. C. (1989) Synthesis of small RNAs Using T7 RNA Polymerase *Methods in Enzymology* **180**, 51-62.
Sassanfar, M. & Szostak, J. W. (1993) An RNA motif that binds ATP *Nature* **364**, 550-553.

A6-406 RECEPTOR-MEDIATED DELIVERY OF ANTISENSE HAMMERHEAD RIBOZYME REDUCES THE AMOUNT OF FIBRILLIN SECRETED FROM CULTURED FIBROBLASTS, MW Kilpatrick (1), LA Phylactou (1,2), M Godfrey (3), G Wu (4) and P Tsipouras (1), Departments of Pediatrics (1) and Internal Medicine (4), University of Connecticut Health Center, Farmington CT 06030, University of Nebraska Medical Center, Omaha NE (3) and Department of Clinical Genetics, University of Birmingham UK (2).

Mutations in the fibrillin gene on chromosome 15 (FBN1) are responsible for Marfan syndrome (MFS), a systemic genetic disorder of the connective tissue. Many such FBN1 mutations appear to act in a dominant-negative fashion, raising the possibility that reduction of the amount of product from the mutant FBN1 allele might be a valid therapeutic approach for MFS. As the first step in the development of such an approach, we have investigated the ability of antisense hammerhead ribozymes to down-regulate the FBN1 gene product. A DNA fragment coding for an antisense hammerhead ribozyme (FBN1-RZ1) specific for the 5'-end of the FBN1 mRNA has been cloned into the vector pBSIISK+ to allow large scale *in vitro* synthesis of the ribozyme. The FBN1-RZ1 ribozyme can efficiently cleave its target *in vitro*, in a magnesium dependent manner, at both 50°C and 37°C. To determine the effect of FBN1-RZ1 on cultured cells, the ribozyme was bound to a transferrin-polylysine conjugate and delivered by receptor-mediated endocytosis into cultured dermal fibroblasts. RNase protection was used to demonstrate the presence of the FBN1-RZ1 ribozyme in total RNA isolated from fibroblasts harvested 24 and 48 hours following transfection. To demonstrate the effect of FBN1-RZ1 on fibrillin production, cultured fibroblasts were stained with an anti-fibrillin antibody following transfection with FBN1-RZ1 transferrin-polylysine complex. Visualisation of the antibody by fluorescence microscopy demonstrated that transfection with FBN1-RZ1 dramatically reduces the amount of fibrillin produced by cultured fibroblasts.

A6-407 *IN VITRO* SELECTION OF APTAMERS THAT CAN SPECIFICALLY BIND THE HDV ANTIGEN, P.K.R. Kumar[1,3], Michael Lai[2] and Andrew D. Ellington[1], [1]Department of Chemistry, Indiana University, Bloomington, IN. 47405, USA; [2]Howard Hughes Medical Institute, University of Southern California School of Medicine, Los Angels, CA 90033-1054, USA; [3]National Institute of Bioscience and Human Technology, MITI, 1-1 Higashi, Tsukuba Science City 305, Japan.

While studies have previously suggested that the hepatitis delta virus antigen can specifically bind to hepatitis delta virus RNA, no binding sites for the delta antigen have been determined. Since multiple delta antigens can bind at many locales along the viral genome, *in vitro* selection was used to determine what sequence or structural commonality might exist between RNAs that could interact specifically with the delta antigen. Starting from a RNA pool that had a 120 base random sequence core, aptamers that could specifically bind delta antigen were selected. The aptamers could bind to the delta antigen from two- to -five-fold better than a wild-type substrate, and could recognize both large and short forms of the protein. Sequence analysis revealed that most of the high-affinity aptamers contained two base bulge sequences embedded in extended helical regions. Minimal RNA ligands that were derived from the aptamer sequences and contained two base bulge structures could bind specifically to the delta antigen. These results should allow high affinity sites on the viral genome to be mapped, and may provide insights into the regulation of hepatitis delta virus replication.

A6-408 REDUCED β2-MICROGLOBULIN mRNA LEVELS IN TRANSGENIC MICE EXPRESSING A DESIGNED HAMMERHEAD RIBOZYME.

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We have generated three artificial hammerhead ribozymes, denoted 'Rz-b', 'Rz-c' and 'Rz-d', with different specificities for the mouse beta-2-microglobulin (β2M) mRNA. Stable transfections of the ribozyme into a mouse cell-line (NIH/3T3) revealed up to 80% reduction of β2M mRNA substrate in individual transfected clones.

Rz-c was selected for a study in mice and three Rz-c expressing transgenic families were established and analysed. Expression of the ribozyme transgene was tested for and detected in lung, kidney and spleen. Ribozyme expression was accompanied with reduction of the β2M mRNA levels in heterozygous (Rz+/-) animals. The effect was most pronounced in lung with more than 90% β2M mRNA reduction in individual mice (Larsson *et al* N.A.R. 22:2242-8, 1994).

Preliminary results also indicate reduction of β2M protein levels in the heterozygous (Rz+/-) animals. At present time homozygous (Rz+/+) animals are being analysed for β2M mRNA and protein expression. Results from these analysis will be discussed.

A6-409 IDENTIFICATION OF METAL CLEAVAGE SITES IN HUMAN BRAIN TRANSCRIPTS, Daniela Marazziti, Rafaele Matteoni+, Elisabetta Golini, Angela Gallo and Glauco P. Tocchini-Valentini, Istituto Biologia Cellulare CNR, Roma, Italy, +EniChem SpA, Monterotondo, Roma.

Divalent metal ions are involved in ribozyme function, both in promoting proper folding of the RNA and directly participating in catalysis. We have investigated the presence of specific divalent metal cleavage sites in pools of human brain cDNA transcripts. cDNA obtained from a collection of human brain mRNA was cloned into a transcription vector. Pools of cDNA clones of length 250-280 bp were transcribed in vitro and the RNA assayed for the presence of lead-specific cleavage sites. We identified one transcript specifically cleaved in the presence of ≤1 mM $Pb^{++}$. The cleavage site was mapped by primer extension and direct sequencing of the cleavage product. Secondary structure prediction by computer analysis placed the $Pb^{++}$ cleavage site in the single stranded region of an asymmetric internal loop within an helical hairpin. Similar structural motifs have been reported for in-vitro selected, $Pb^{++}$-dependent ribozymes. Database analysis with search keys combining sequence and secondary structural elements revealed the presence of homologous motifs in gene transcripts of various metal-binding proteins. We prepared oligonucleotide templates corresponding to the "conserved" putative cleavage motif. The templates were transcribed in vitro and tested for cleavage by metal ions. Specific cleavage at the original site was observed in the presence of $Pb^{++}$. The effect of other divalent metals was also studied.

A6-410 BIODEGRADABLE POLYMER DEVICES FOR THE SUSTAINED EXOGENOUS DELIVERY OF RIBOZYMES

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Ribozymes have been shown to inhibit gene-expresion in a sequence-specific manner by causing cleavage of target mRNA. Studies to date suggest that RNA subtsrates are relatively unstable for exogeous delivery and that for sustained efficacy repeated admistration is likely but clinically undesirable. For these reasons, exogenous delivery systems which can protect ribozymes form ribonuclease digestion and simultaneously provide sustained delivery over extended time periods may be useful for the biopharmaceutical application of RNA ribozyme nucleic acids. Biodegradable polymer matrices offer this potential and have been evaluated for the potentail exogenous delivery of ribozymes in our laboratory. In this preliminary study we have evaluated the potential use of solvent cast films ( 100μm) of poly L-lactic acid (PLA) polymer of molecular weight 690,000. A 32 mer hammerhead ribozyme antisense to the human *c-myc* oncogene exon 2, was used to demonstrate sustained release of the ribozyme from the PLA film *in vitro* and to confirm that the polymer protects the entrapped ribozyme from degradation prior to release. The ribozyme (Cruachem) was deprotected and 5' end labelled with $^{32}P\gamma ATP$ and bacteriophage T4 polynucleotide kinase. The radiolabelled ribozyme was incorporated into a 2 % polymer-chloroform solution, before casting of the film. Release of the oligonucleotide was monitored using liquid scintillation counting and the stability of the free and polymer entrapped ribozyme in foetal calf serum was monitored using denaturing polyacrylamide gel electrophoresis. The released ribozyme from the polymer matrix was reacted with its substrate to ensure it was still capable of cleaving the active site on *c-myc* sequence. The *in vitro* release profiles suggest that the entrapped ribozyme was released biphasically from the polymer films, characterised by an initial rapid burst of release followed by a more sustained release which extended for several weeks. The polymer entrapped ribozymes were resistant to degradation from serum nucleases over a 14 day period, compared to free ribozyme which was rapidly degraded. The *in vitro* cleavage activity of the polymer released hammerhead was similar to free ribozyme suggesting that the polymer device fabrication procedure did not affect the biological properties of the RNA.

A6-411 Analysis of functional structure of HDV ribozyme by modification interference and in vitro selection strategies

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HDV ribozyme derived from human hepatitis delta virus can catalyze the self-cleavage reaction in the presence of $Mg^{2+}$ ions with the same fashion of hammerhead and hairpin ribozymes. But there are no structural homologies between these ribozymes. In order to elucidate the functional structure of HDV genomic ribozyme we have studied by in vitro mutagenic analyses and chemical probing method. These results supported pseudoknot secondary structure model and identified important bases for cleavage reaction. These bases are all located in single stranded regions (SSrA, B and C) in this structure and furthermore they are all conserved in HDV antigenomic ribozyme.

Recent several studies on ribozymes have suggested that ribozyme is also one of metal enzymes. To elucidate the binding site of $Mg^{2+}$ ions for catalytic reactions in HDV ribozyme, we generated modification interference analysis with partially thio-substituted HDV ribozyme and $Pb^{2+}$ cleavage analysis in the presence of $Mg^{2+}$ ions. We could identify important phosphates, that is -1, 0 which are located in cleavage site, 21 in SSrC region and 75 in SSrB region. These phosphates seem to be close together for folding of active conformation and agreed with recently proposed tertiary structure model.

HDV ribozyme may have good advantage for therapeutic application because of its origin. *Trans*-acting HDV genomic ribozyme based on pseudoknot secondary structure can cleave 13-nts substrate. Although this *trans*-acting HDV genomic ribozyme was very less active compared to antigenomic one, activity increased 100 fold by extending the stem II base pairings from 5 to 8. Based on this molecule we are now trying to select higher active HDV ribozyme by in vitro selection procedure.

A6-412 EFFECTIVE RIBOZYME DELIVERY IN PLANT CELLS, Rhonda Perriman[1,3], W.J. Peacock[3], E.S Dennis[3] and George Bruening[1,2] ; Centre for Engineering Plants resistant against pathogens (CEPRAP)[1], Dept. of Plant Pathology, University of California, Davis[2], and CSIRO Division of Plant Industry, Canberra Australia[3]

This project investigates the utility of highly expressed linear or tRNA-embedded ribozymes (Rz) as a means of inactivating specific target RNAs in plant cells. Hammerhead Rz and antisense (As) sequences have been incorporated into a tyrosine tRNA and compared with the analogous linear molecules. To further optimise the levels of Rz and As transcripts *in vivo*, we have used an autonomously replicating vector. *In vitro*, the linear Rz was able to induce significantly greater levels of cleavage of the designated target RNA, chloramphenicol acetyl transferase (CAT), than the tRNA-embedded Rz. In contrast to this, *in vivo* CAT activities show that the tRNA-embedded Rz is able to reduce CAT activity to 15% of control levels. This is considerably more effective than the linear Rz and control As sequences. A mutated, non-cleavable CAT target sequence did not exhibit the increased reduction in CAT activity in the presence of the tRNA-embedded Rz. The tRNA Rz construction was initially made with functional RNA polymerase II and III promoter sequences. Mutagenesis of these promoters has revealed the most active Rz transcript is derived from the RNA polymerase III promoter. Finally analysis of CAT mRNA accumulation in the presence of the tRNA Rz indicates that the reduction in CAT activity is consistent with *in vivo* cleavage of the CAT mRNA.

**A6-413** ALPHAVIRUS MEDIATED DELIVERY OF RIBOZYME THERAPEUTICS, John M. Polo, David A. Driver, and Thomas W. Dubensky, Dept. of Viral Therapeutics, Viagene, Inc., 11055 Roselle St., San Diego, CA 92121

The use of catalytic "ribozyme" RNA molecules to inhibit gene expression has shown increasing potential for therapeutic applications. However, the efficacy of this approach is highly dependent on efficient delivery systems which result in abundant intracellular expression of the ribozyme. To address these requirements, we have developed an RNA virus-based vector system using the prototype alphavirus, Sindbis virus. Replicon expression cassettes which function as either RNA or cDNA molecules are delivered into a wide range of target cells as packaged vector particles or naked vector nucleic acid. Following delivery, production of high levels of the desired therapeutic occurs via an internal Sindbis-specific promoter. The application of this Sindbis expression system for various therapeutic agents, including ribozymes, will be discussed.

**A6-414** LACK OF ISOTOPE EFFECTS IN HAMMERHEAD RIBOZYME REACTIONS, Kazunari Taira,[1,3] Shinya Sawata,[1,4] Satoshi Nishikawa,[1] Makoto Komiyama,[2] [1]National Institute of Bioscience and Human Technology, MITI, 1-1 Higashi, Tsukuba 305; [2]Department of Chemistry and Biotechnology, Faculty of Engineering, University of Tokyo, Hongo, Tokyo 113; [3]Institute of Applied Biochemistry and [4]Institute of Materials Science, University of Tsukuba; Tsukuba Science City 305, Japan.

Since ribozymes are being recognized as metalloenzymes, much attention has been focused on their detailed reaction mechanisms involving $Mg^{2+}$ ions. Base-catalysis mediated by $Mg^{2+}$-hydroxide was proposed by Uhlenbeck's group based on pH-rate profiles of various metal ion-catalyzed reactions for the hammerhead ribozyme. In order to examine whether a proton transfer process occurs in the transition state of hammerhead ribozyme reactions, *e.g.* by $Mg^{2+}$-hydroxide-mediated catalysis, we have measured isotope effects for the 32-mer ribozyme (R32) at pH 6.0. In this system, the chemical cleavage step has unambiguously been proven to be the sole rate-limiting step ($k_{cleav}$). The cleavage rate constant in $H_2O$ ($k_{cleav(H)}$) was 4.4 times larger than the corresponding value in $D_2O$ ($k_{cleav(D)}$). The apparent isotope effect of 4.4 ($k_{cleav(H)}/k_{cleav(D)}$) might be taken as the evidence that supports an involvement of a proton transfer in the transition state. However, since the concentration of $Mg^{2+}$-OD in $D_2O$ is a few times lower than that of $Mg^{2+}$-OH in $H_2O$, the reduction of the active species, $Mg^{2+}$-OD, in $D_2O$ can be the sole cause of the slower ribozyme reactions in $D_2O$. Indeed, the estimated value ($K_a^{H_2O}/K_a^{D_2O}$) for a water molecule coordinated to $Mg^{2+}$ ion with its $pK_a^{H_2O}$ of 11.4 turned out to be 4.5. The observation of no isotope effects is pertinent to the idea that (i) it is the second step of the reaction which involves the cleavage of $P\text{-}O_{(5')}$ bond that is the overall rate-limiting step and (ii) a $Mg^{2+}$ ion acts as the Lewis acid to stabilize the second step of the reaction by directly coordinating to the leaving 5'-oxygen. It is thus possible that hammerhead ribozymes use the general double metal ion catalysis as proposed by Steitz and Steitz.

**A6-415** DESIGN OF POL III-DRIVEN RIBOZYME EXPRESSION VECTORS, James D. Thompson, Bharat M. Chowrira, James A. McSwiggen, Timothy L. McKenzie, Terra A. Malmstrom, Louis Ganousis, David F. Ayers, Ribozyme Pharmaceuticals, Inc. Boulder CO 80301

Key to the development of ribozyme expression vectors is the design of transcription units that both accumulate in target tissues and exhibit good ribozyme activity. We have determined general rules for obtaining maximum ribozyme activity in the context of complex transcription units. In addition, we have identified an RNA structural motif that, when incorporated into a tRNA-based expression system, significantly enhances accumulation of pol III transcripts. Retroviral vectors containing the modified tRNA expression system were used to express anti-HIV ribozymes in human peripheral blood T cells and T cell lines. Ribozyme expression in cultured peripheral blood lymphocytes was demonstrated by Northern blot analysis. Ribozyme cleavage activity was readily detectable in total RNA from transduced human T cell lines, and expression was stable for at least 3 months in the absence of selection.

**A6-416** A NEW CLASS OF MOLECULES FOR EXPLORING THE CATALYTIC POTENTIAL OF RNA

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The limited variety of known ribozyme-catalyzed reactions in nature raises the possibility that RNA structure is unsuitable for a broad range of catalysis. However, *in vitro* selection and *in vitro* evolution techniques have successfully generated artificial ribozymes with new catalytic activities (1,2). To investigate the role of RNA structure in catalysis of novel chemical reactions, we have designed a new class of RNA molecules, similar in many respects to antibodies. These molecules have a constant scaffold region of ~260 nucleotides, derived from the *Tetrahymena* group I intron, that folds in a stable, unique structure (3). Two variable regions of 30 and 50 nucleotides are constrained by the scaffold to be localized close in space (4,5), with the possibility of forming a catalytic site. Binding sites at either end of the molecule allow alignment of appropriately modified oligonucleotides for *in vitro* selection schemes. A DNA pool with a diversity of approximately $10^{16}$ has been constructed, and this diversity can be increased at least 10-fold by shuffling between the two variable regions after PCR amplification. A fraction of the pool has been transcribed *in vitro* and is being used for selection of ribozymes that catalyze RNA ligation and amide bond forming reactions.

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*Late Abstracts*

CATALYTIC COMPETENCE OF HAMMERHEAD MINIZYMES WITH REDUCED LOOP STRUCTURE, Serguei V. Amontov[1,2] and Kazunari Taira[1,3], [1]National Institute of Bioscience and Human Technology, [2]National Institute for Advanced Interdisciplinary Research Agency of Industrial Science & Technology, MITI; [3]Institute of Applied Biochemistry, University of Tsukuba; Tsukuba Science City 305, Japan.

For a long time hammerhead ribozymes with deleted stem II were considered as degenerated cripple structures and attracted less interest due to extremely low activity compared to full size ribozyme. In this work a number of hammerhead mini-ribozymes with reduced loop II have been constructed and their activities were determined. It was found that the catalytic activity of these structures varied from completely inactive to surprisingly highly active minizymes(about 15% from full size ribozyme). Although, it was found that the bases $G_{10.1}$ and $C_{11.1}$ are very important for the catalysis, but base pairing between them is not imperative prerequisite to the catalytic competence as it was postulated before. Minizyme-like structures could be considered as most simple and convenient model for investigation of catalytic mechanism.

CLEAVAGE OF AN AMIDE BOND BY A RIBOZYME

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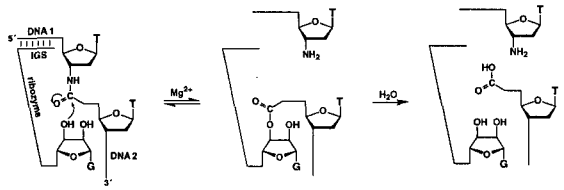
Since the discovery of RNA enzymes in nature, there has been considerable interest in exploring the catalytic potential of RNA in the laboratory. A broad functional capacity seems plausible not only on chemical grounds, but also in light of the presumed role of RNA during the early history of life on earth. A variant form of a group I ribozyme, optimized by *in vitro* evolution for its ability to catalyze $Mg^{2+}$-dependent phosphoester transfer reactions involving DNA substrates, also catalyzes the cleavage of an unactivated, alkyl amide when that linkage is presented in the context of an oligodeoxynucleotide analogue. Substrates containing an amide bond that joins either two DNA oligos, or a DNA oligo and a short peptide, are cleaved in a $Mg^{2+}$-dependent fashion to generate the expected products. The 5′ cleavage product was identified as $GGCCCTCT_{NH_2}$ based on comparison with authentic material – both compounds have identical mobility in denaturing polyacrylamide gels and are comparably derivatizable at the primary amine with sulfosuccinimidyl-6-(biotinamido) hexanoate. The first-order rate constant for RNA-catalyzed amide cleavage, $k_{cat}$, is $0.1 - 1 \times 10^{-5}$ $min^{-1}$ for the DNA-flanked substrates, corresponding to a rate acceleration of greater than $10^3$ compared to the uncatalyzed reaction.




RIBOZYME-MEDIATED HIGH RESISTANCE AGAINST POTATO SPINDLE TUBER VIROID IN TRANSGENIC POTATOES, Xi-Cai Yang, Yin Yie, Feng Zhu, Yu-Le Liu, Yi-Tang Yan and Po Tien. Institute of Microbiology, Chinese Academy of Sciences, Beijing 100080, China.

So far, ribozymes have mainly been used in attempts to inhibit RNA viruses which have an exclusively cytoplasmic replication cycle. Although it is assumed that ribozymes move from the nucleus into the cytoplasms to cleave the RNAs, we postulated that ribozymes could be more effective to cleave the RNA of viruses and viroids which undergo a nuclear phase during their replication, for example, potato spindle tuber viroid (PSTVd). Two hammerhead ribozymes were designed to cleave the PSTVd RNA of 359 bases. The first ribozyme R(-) was aimed at a putative binding site of DNA dependent RNA polymerase II within the T1 domain. The second one R(+) was targeted towards the C domain containing a postulated binding site of DNA-dependent RNA polymerase III and a region involved in the procesing of viroid replicative intermediates.

In order to study the in vivo inhibition of PSTVd replication by the ribozymes, the R(-) or R(+) dimers were cloned into a plant expression vector, pRok2. Transgenic potato plants were obtained by Agrobacterium tumefaciens mediated transformation of the tuber slices. A total of 34 transgenic plants were generated with the pRok2-R(-) D. Analysis of RNA from the transgenic plants by a return polyacrylamide gel electrophoresis showed that 23 lines were completely free of the PSTVd RNA accumulation after the PSTVd infection. These results were confirmed by northern hybridization analysis of total RNA from the transgenic lines using a PSTVd cDNAs as a probe. In some lines the ribozyme cleavage products were observed, suggesting the ribozyme functioned in vivo. The complete absence of PSTVd RNAs in most of the transgenic lines after infection suggests that the R(-) ribozyme confer the transgenic potatoes a high resistance to PSTVd infection.