# RNA – From Mathematical Models to Real Molecules

## 4. Experiments with RNA Molecules

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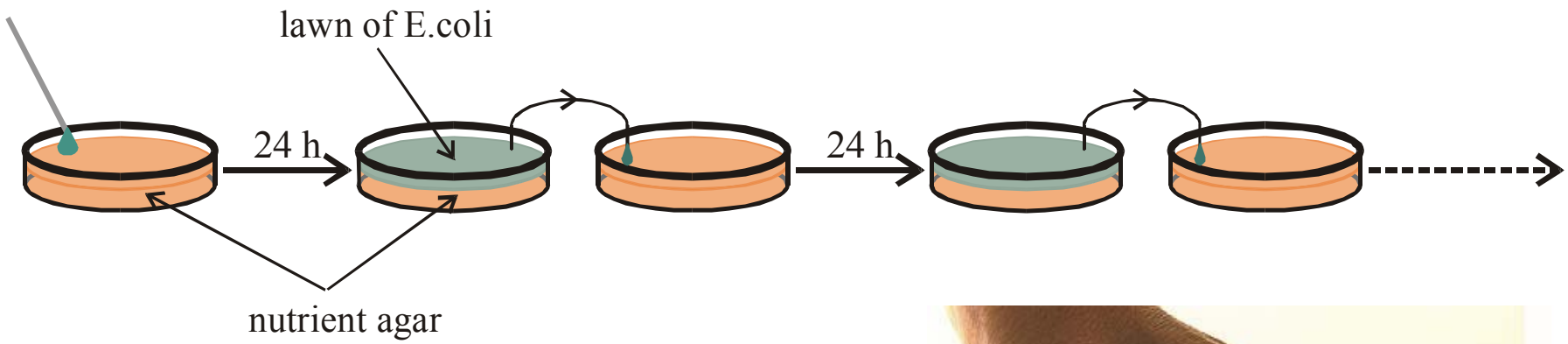
http://www.tbi.univie.ac.at/~pks

## Bacterial Evolution

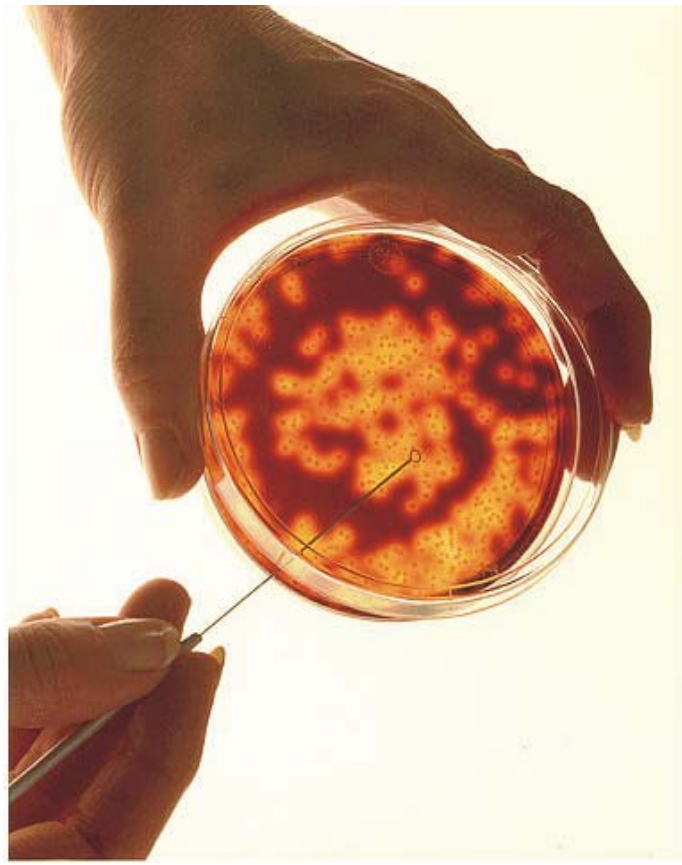
S. F. Elena, V. S. Cooper, R. E. Lenski. ***Punctuated evolution caused by selection of rare beneficial mutants***. Science **272** (1996), 1802-1804

D. Papadopoulos, D. Schneider, J. Meier-Eiss, W. Arber, R. E. Lenski, M. Blot. ***Genomic evolution during a 10,000-generation experiment with bacteria***. Proc.Natl.Acad.Sci.USA **96** (1999), 3807-3812




Serial transfer of Escherichia coli cultures in Petri dishes

1 day Î 6.67 generations
1 month Î 200 generations
1 year Î 2400 generations



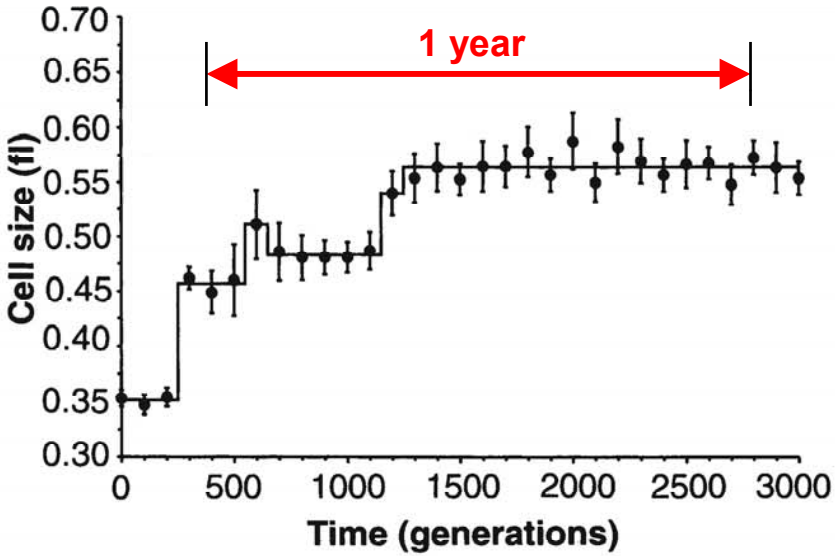



**Fig. 1.** Change in average cell size (1 fl = $10^{-15}$ L) in a population of *E. coli* during 3000 generations of experimental evolution. Each point is the mean of 10 replicate assays (*22*). Error bars indicate 95% confidence intervals. The solid line shows the best fit of a step-function model to these data (Table 1).

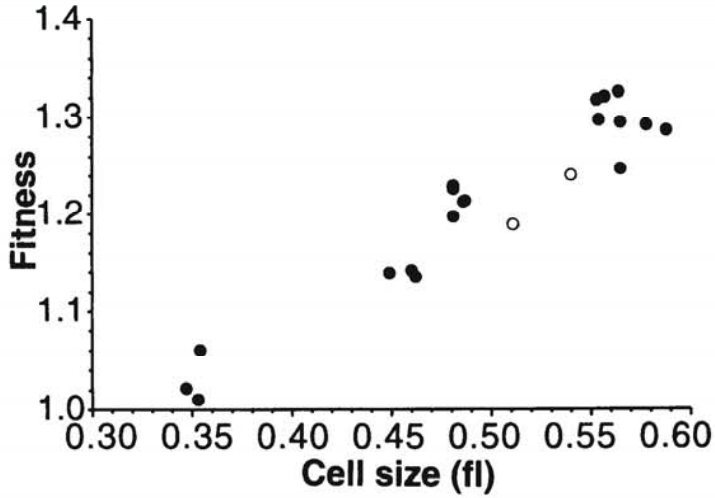
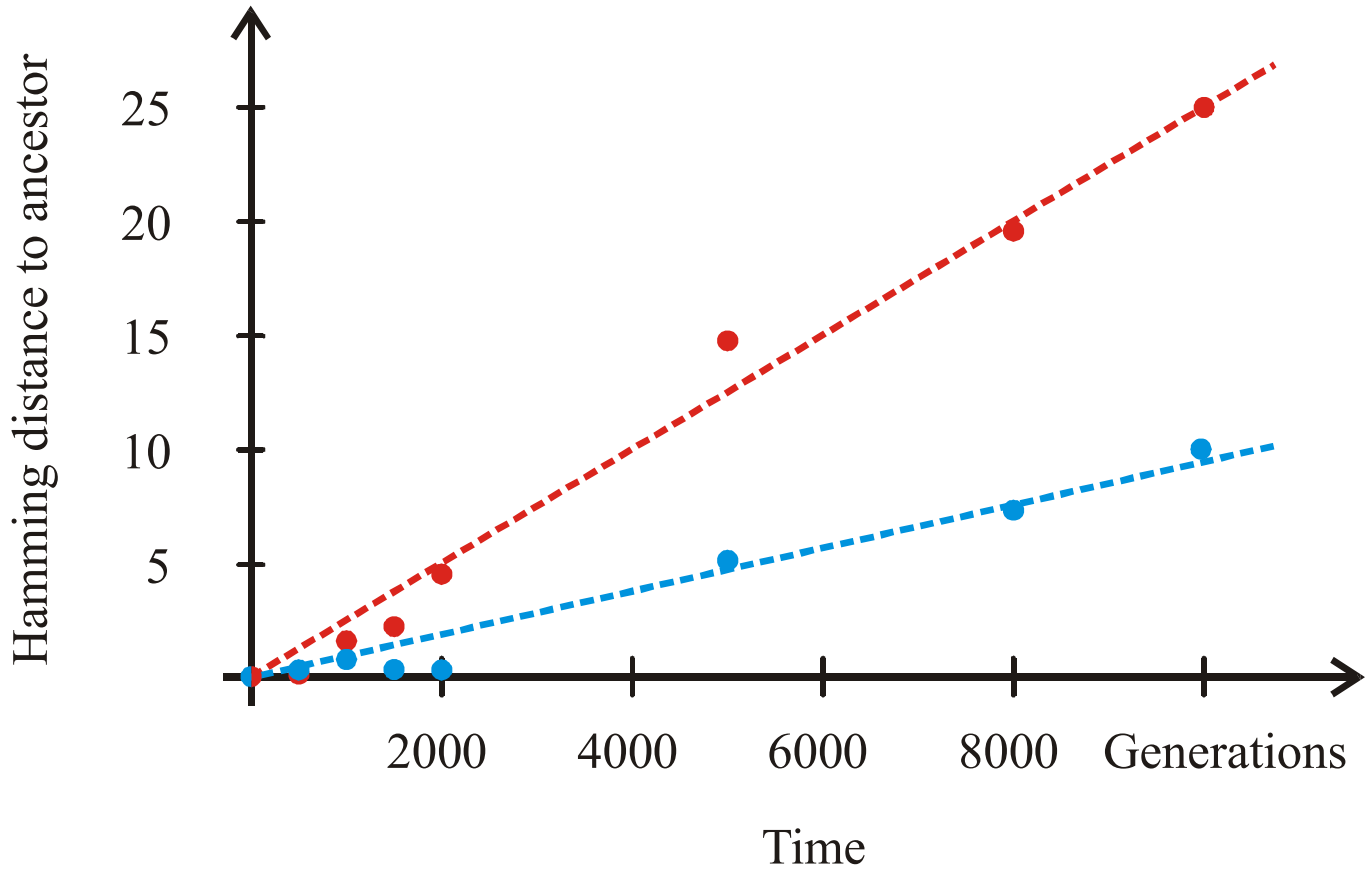



**Fig. 2.** Correlation between average cell size and mean fitness, each measured at 100-generation intervals for 2000 generations. Fitness is expressed relative to the ancestral genotype and was obtained from competition experiments between derived and ancestral cells (*6*, *7*). The open symbols indicate the only two samples assigned to different steps by the cell size and fitness data.

Epochal evolution of bacteria in serial transfer experiments under constant conditions

S. F. Elena, V. S. Cooper, R. E. Lenski. *Punctuated evolution caused by selection of rare beneficial mutants*. Science **272** (1996), 1802-1804




Variation of genotypes in a bacterial serial transfer experiment

D. Papadopoulos, D. Schneider, J. Meier-Eiss, W. Arber, R. E. Lenski, M. Blot. *Genomic evolution during a 10,000-generation experiment with bacteria*. Proc.Natl.Acad.Sci.USA **96** (1999), 3807-3812

# Evolution of RNA molecules based on Qβ phage

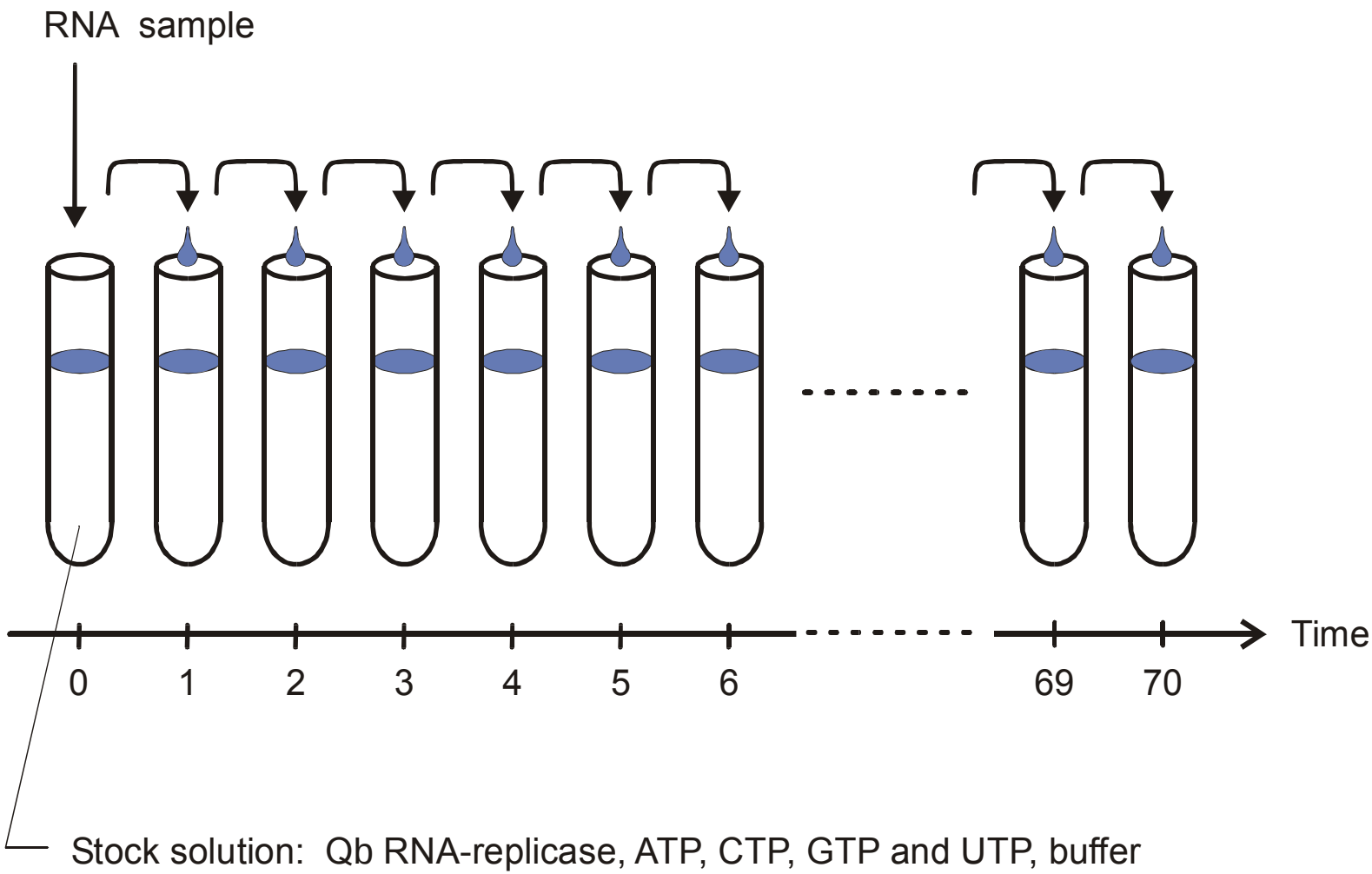
D.R.Mills, R,L,Peterson, S.Spiegelman, ***An extracellular Darwinian experiment with a self-duplicating nucleic acid molecule***. Proc.Natl.Acad.Sci.USA **58** (1967), 217-224

S.Spiegelman, ***An approach to the experimental analysis of precellular evolution***. Quart.Rev.Biophys. **4** (1971), 213-253

C.K.Biebricher, ***Darwinian selection of self-replicating RNA molecules***. Evolutionary Biology **16** (1983), 1-52

C.K.Biebricher, W.C. Gardiner, ***Molecular evolution of RNA*** **in vitro**. Biophysical Chemistry **66** (1997), 179-192

G.Strunk, T. Ederhof, ***Machines for automated evolution experiments*** **in vitro** ***based on the serial transfer concept***. Biophysical Chemistry **66** (1997), 193-202




The serial transfer technique applied to RNA evolution ***in vitro***

Reproduction of the original figure of the serial transfer experiment with Qβ RNA

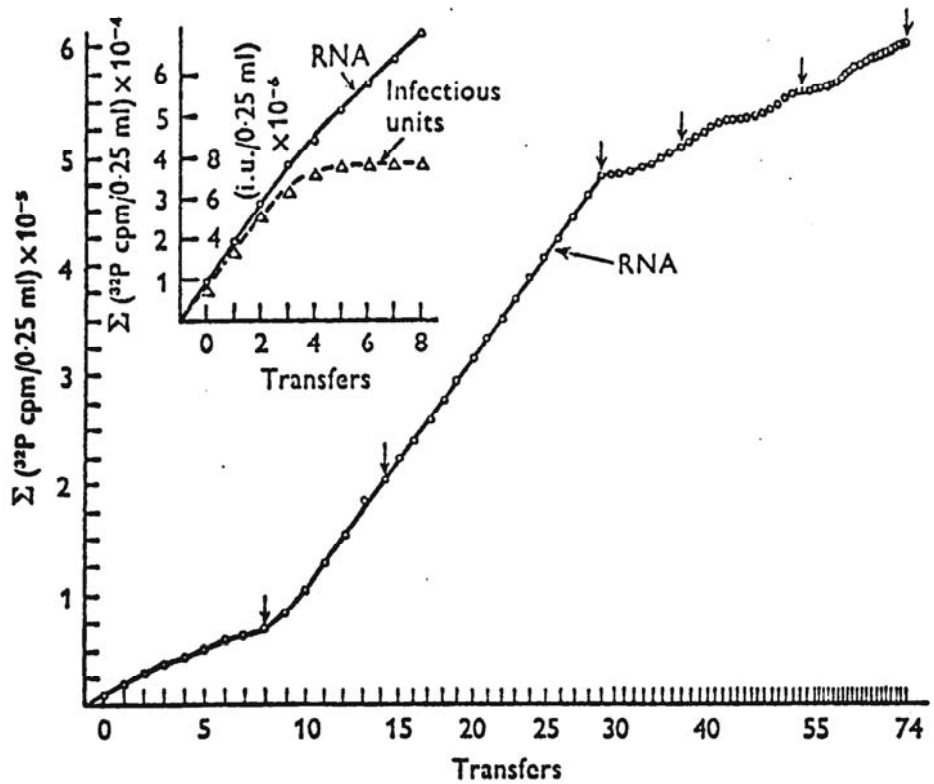
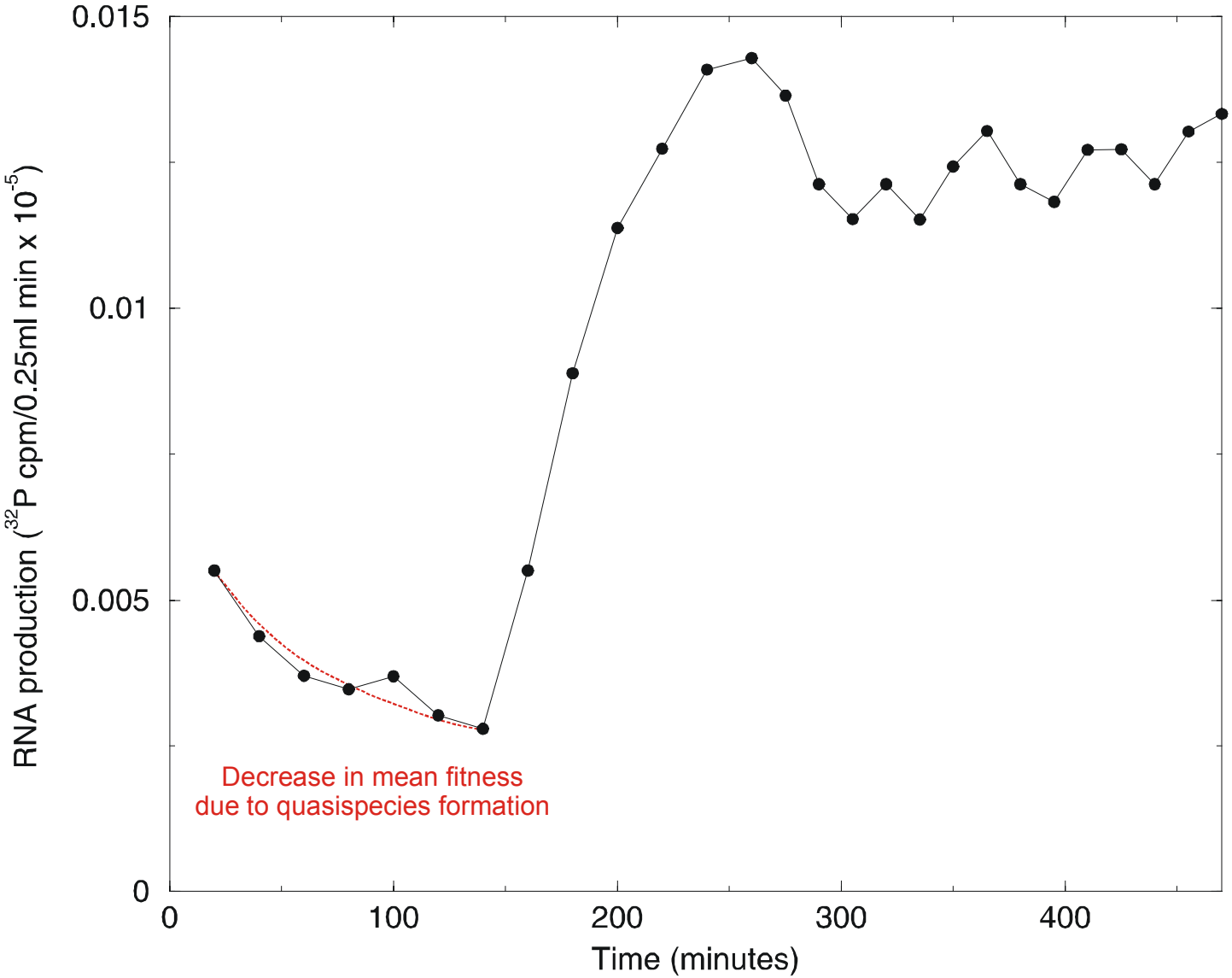



Fig. 9. Serial transfer experiment. Each 0·25 ml standard reaction mixture contained 40 μg of Qβ replicase and ³²P-UTP. The first reaction (0 transfer) was initiated by the addition of 0·2 μg ts-1 (temperature-sensitive RNA) and incubated at 35 °C for 20 min, whereupon 0·02 ml was drawn for counting and 0·02 ml was used to prime the second reaction (first transfer), and so on. After the first 13 reactions, the incubation periods were reduced to 15 min (transfers 14–29). Transfers 30–38 were incubated for 10 min. Transfers 39–52 were incubated for 7 min, and transfers 53–74 were incubated for 5 min. The arrows above certain transfers (0, 8, 14, 29, 37, 53, and 73) indicate where 0·001–0·1 ml of product was removed and used to prime reactions for sedimentation analysis on sucrose. The inset examines both infectious and total RNA. The results show that biologically competent RNA ceases to appear after the 4th transfer (Mills *et al.* 1967).

D.R.Mills, R,L,Peterson, S.Spiegelman, *An extracellular Darwinian experiment with a self-duplicating nucleic acid molecule*. Proc.Natl.Acad.Sci.USA **58** (1967), 217-224




The increase in RNA production rate during a serial transfer experiment

# Evolutionary design of RNA molecules

D.B.Bartel, J.W.Szostak, **In vitro** ***selection of RNA molecules that bind specific ligands***. Nature **346** (1990), 818-822

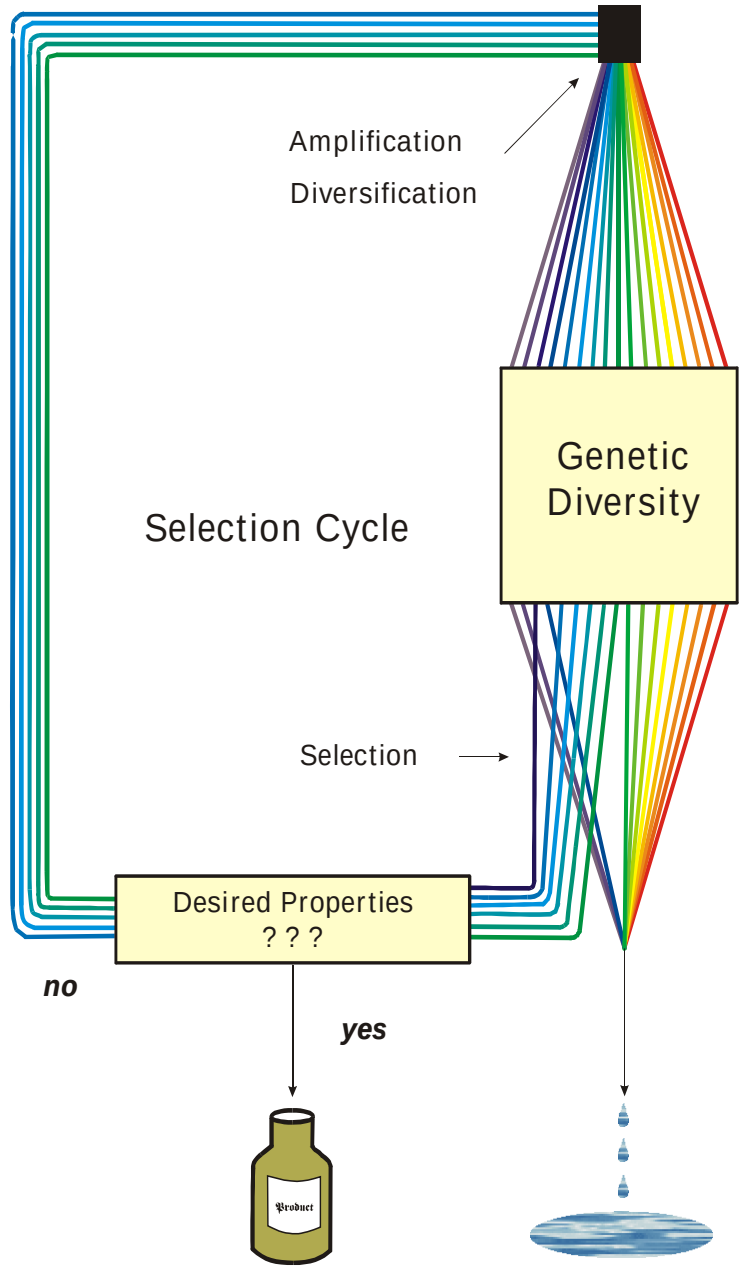
C.Tuerk, L.Gold, **SELEX** - ***Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage*** **T4** ***DNA polymerase***. Science **249** (1990), 505-510

D.P.Bartel, J.W.Szostak, ***Isolation of new ribozymes from a large pool of random sequences***. Science **261** (1993), 1411-1418

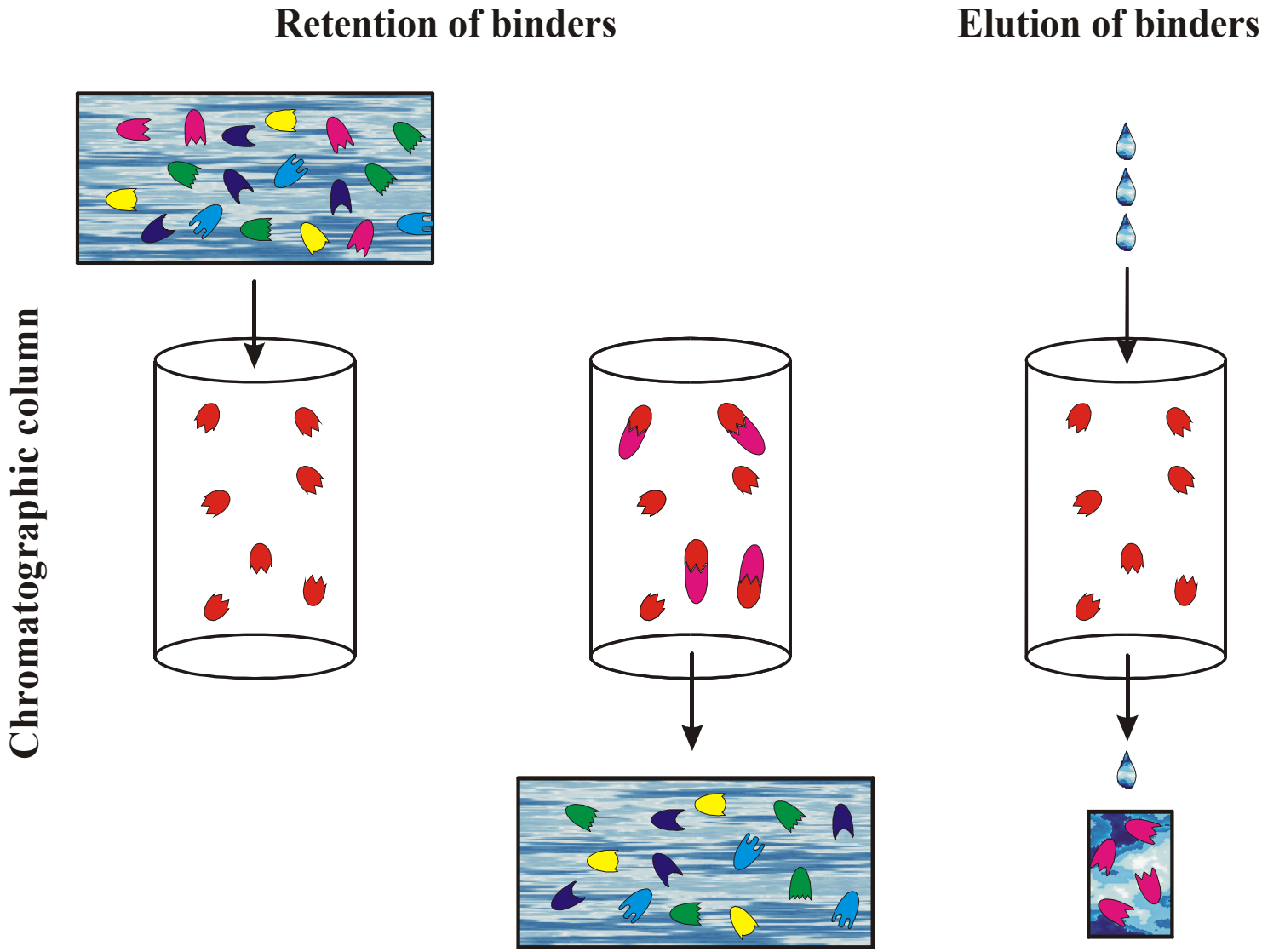
R.D.Jenison, S.C.Gill, A.Pardi, B.Poliski, ***High-resolution molecular discrimination by RNA***. Science **263** (1994), 1425-1429

Y. Wang, R.R.Rando, ***Specific binding of aminoglycoside antibiotics to RNA***. Chemistry & Biology **2** (1995), 281-290

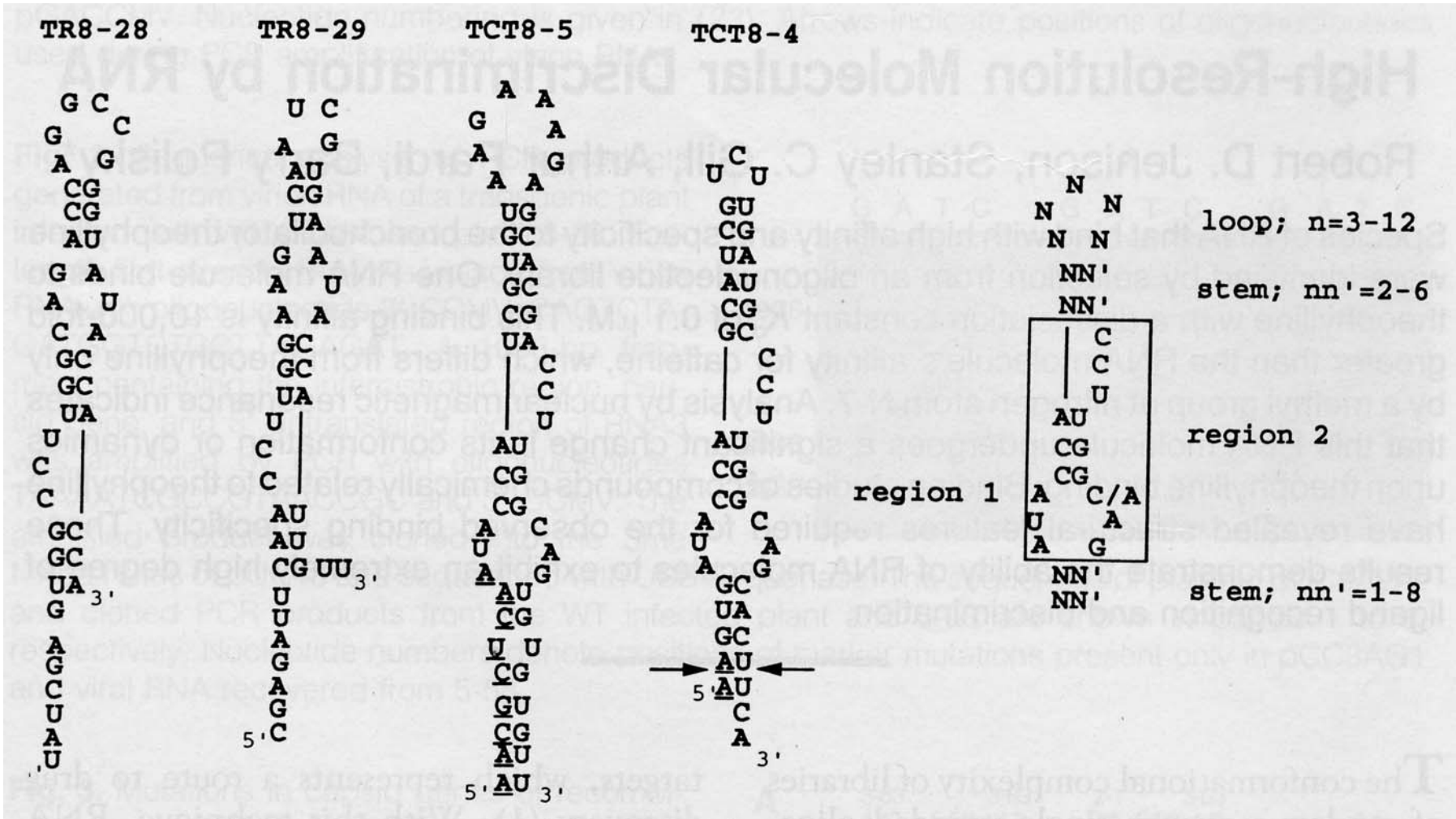
Jiang, A. K. Suri, R. Fiala, D. J. Patel, ***Saccharide-RNA recognition in an aminoglycoside antibiotic-RNA aptamer complex***. Chemistry & Biology **4** (1997), 35-50




Selection cycle used in applied molecular evolution to design molecules with predefined properties

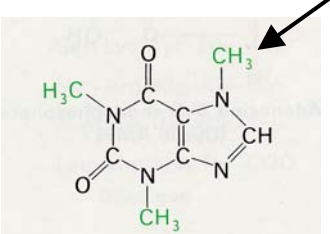



The SELEX technique for the evolutionary design of ***aptamers***

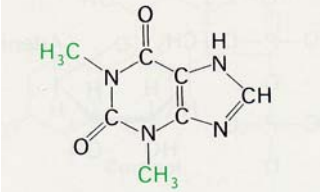



Secondary structures of aptamers binding theophyllin, caffeine, and related compounds

additional methyl group



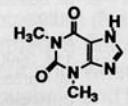
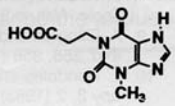
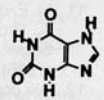
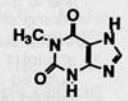
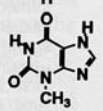
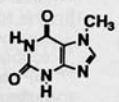
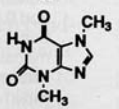
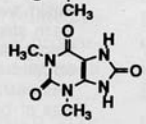
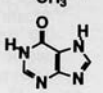
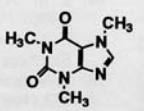
Caffeine
(1,3,7-Trimethylxanthine)



Theophylline
(1,3-Dimethylxanthine)

Dissociation constants and specificity of theophylline, caffeine, and related derivatives of uric acid for binding to a discriminating aptamer TCT8-4

**Table 1.** Competition binding analysis with TCT8-4 RNA. The chemical structures are shown for a series of derivatives used in competitive binding experiments with TCT8-4 RNA (Fig. 2) (*20*). The right column represents the affinity of the competitor relative to theophylline, $K_d(c)/K_d(t)$, where $K_d(c)$ is the individual competitor dissociation constant and $K_d(t)$ is the competitive dissociation constant of theophylline. Certain data (denoted by >) are minimum values that were limited by the solubility of the competitor. Each experiment was carried out in duplicate. The average error is shown.

| Compound | Structure | $K_d(c)$ (μM) | $K_d(c)/K_d(t)$ |
|---|---|---|---|
| Theophylline |  | 0.32 ± 0.13 | 1 |
| CP-theophylline |  | 0.93 ± 0.20 | 2.9 |
| Xanthine |  | 8.5 ± 0.40 | 27 |
| 1-Methylxanthine |  | 9.0 ± 0.30 | 28 |
| 3-Methylxanthine |  | 2.0 ± 0.7 | 6.3 |
| 7-Methylxanthine |  | > 500 | >1500 |
| 3,7-Dimethylxanthine |  | > 500 | > 1500 |
| 1,3-Dimethyluric acid |  | > 1000 | >3100 |
| Hypoxanthine |  | 49 ± 10 | 153 |
| Caffeine |  | 3500 ± 1500 | 10,900 |

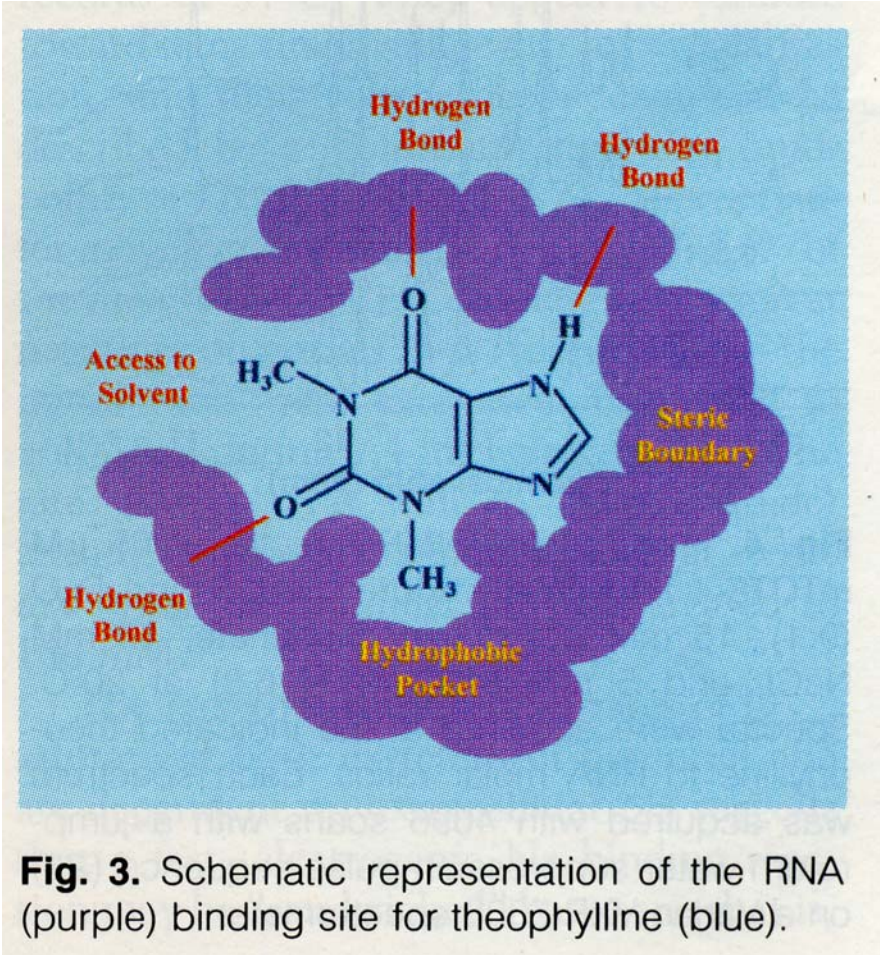



**Fig. 3.** Schematic representation of the RNA (purple) binding site for theophylline (blue).

Schematic drawing of the aptamer binding site for the theophylline molecule

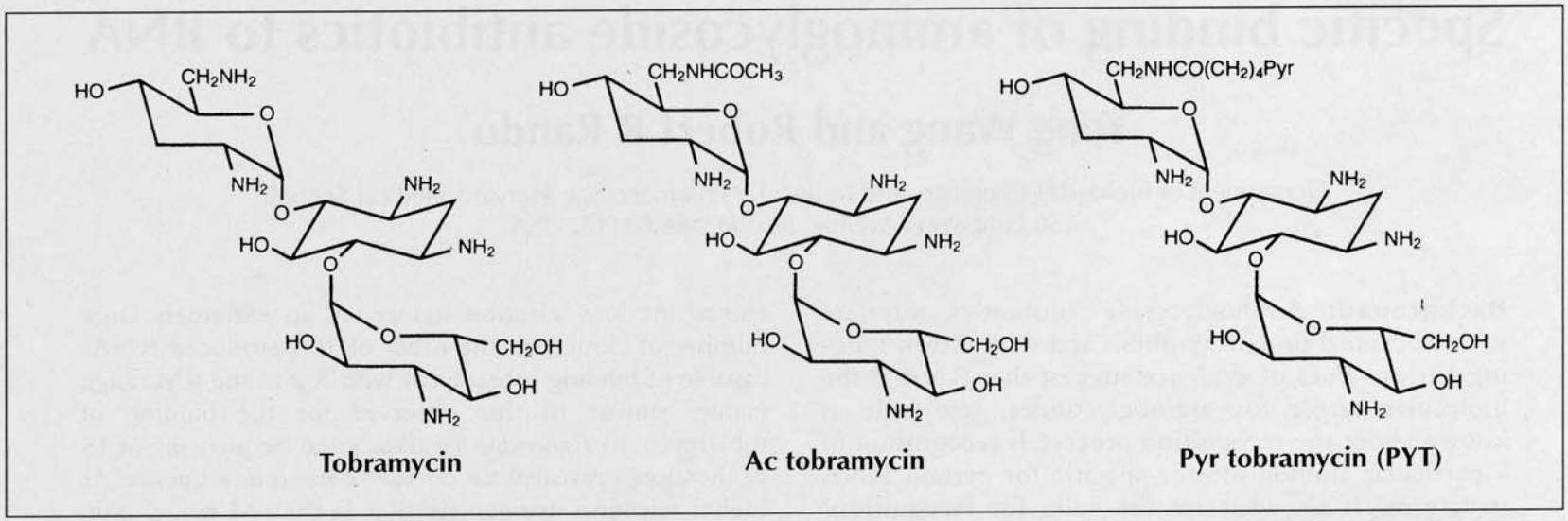
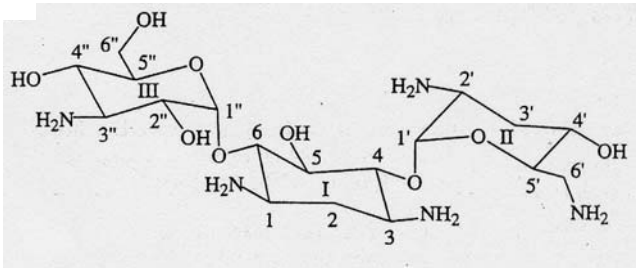


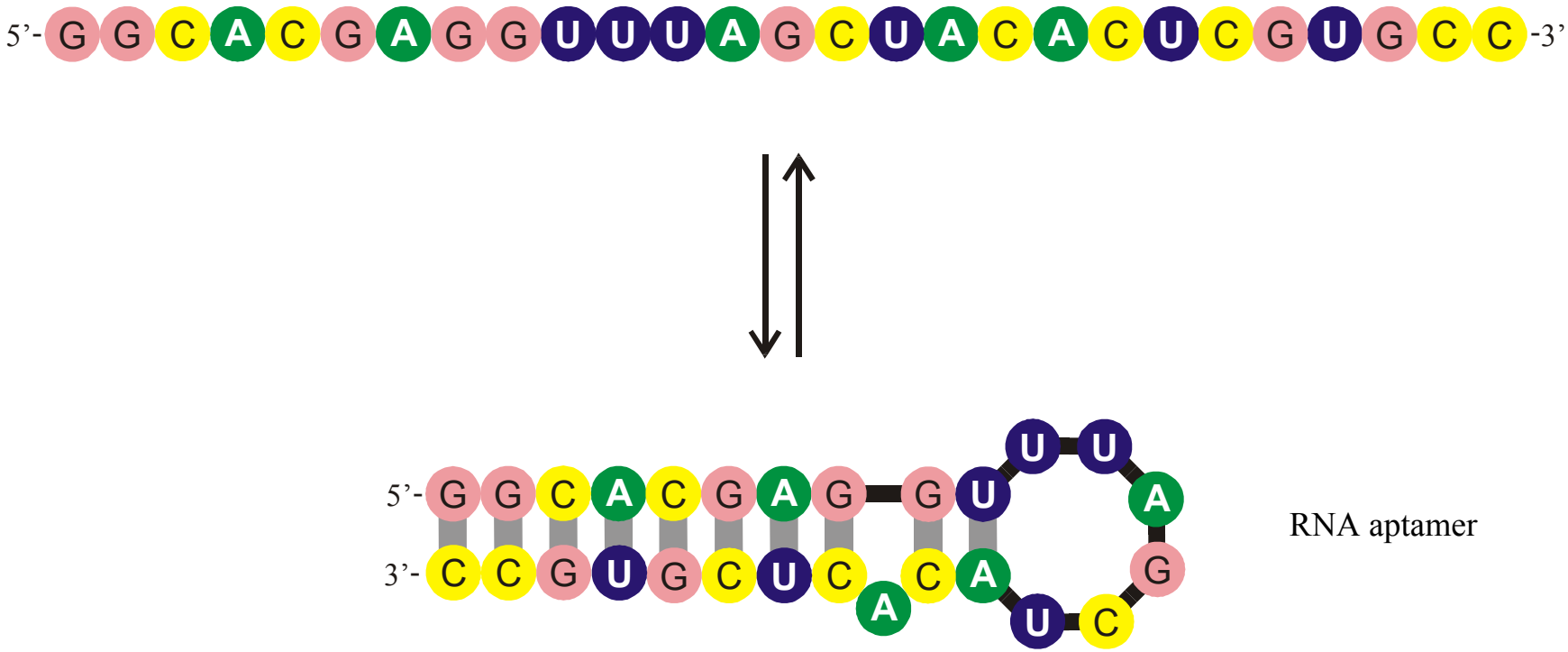

**Fig. 1.** Structures of tobramycin and analogs used in these studies.

Aptamer binding to aminoglycosid antibiotics: Structure of ligands

Y. Wang, R.R.Rando, ***Specific binding of aminoglycoside antibiotics to RNA***. Chemistry & Biology **2** (1995), 281-290



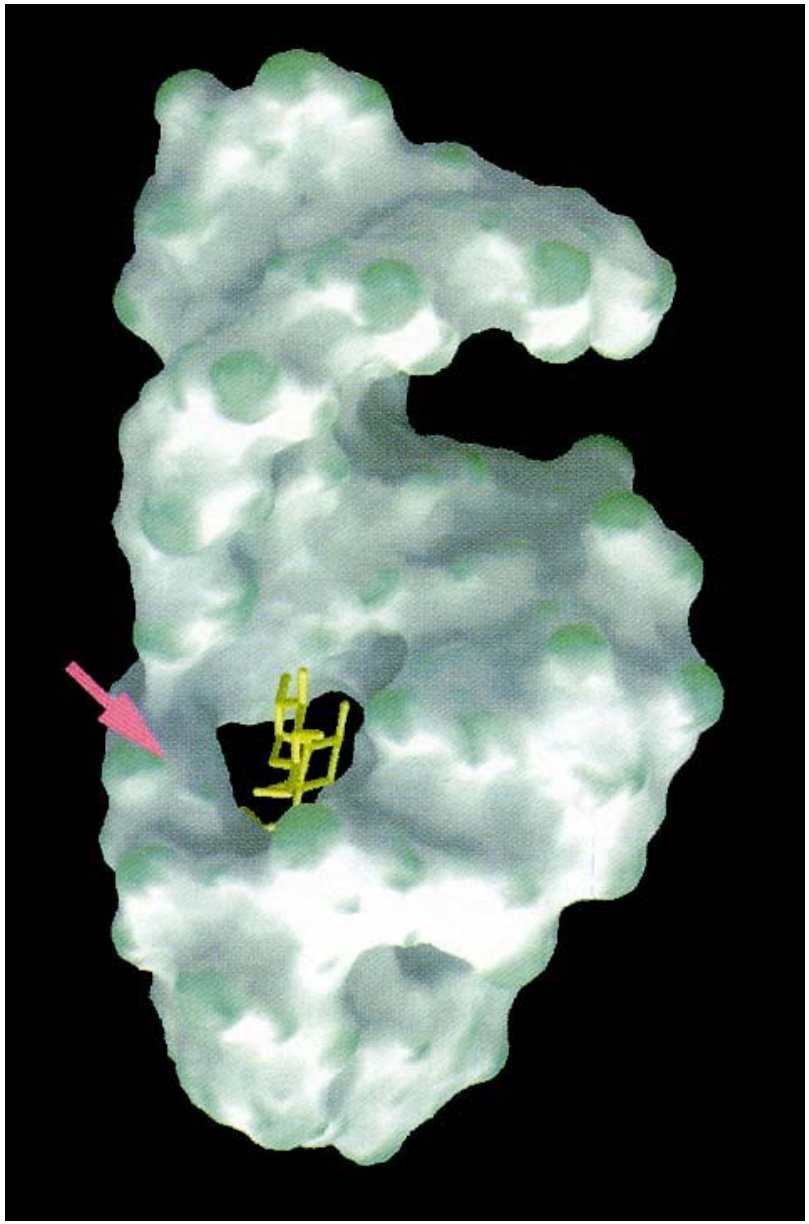
tobramycin




RNA aptamer

Formation of secondary structure of the tobramycin binding RNA aptamer with $K_D$ = 9 nM

L. Jiang, A. K. Suri, R. Fiala, D. J. Patel, ***Saccharide-RNA recognition in an aminoglycoside antibiotic-RNA aptamer complex.*** Chemistry & Biology **4**:35-50 (1997)



The three-dimensional structure of the tobramycin aptamer complex

L. Jiang, A. K. Suri, R. Fiala, D. J. Patel, Chemistry & Biology **4**:35-50 (1997)

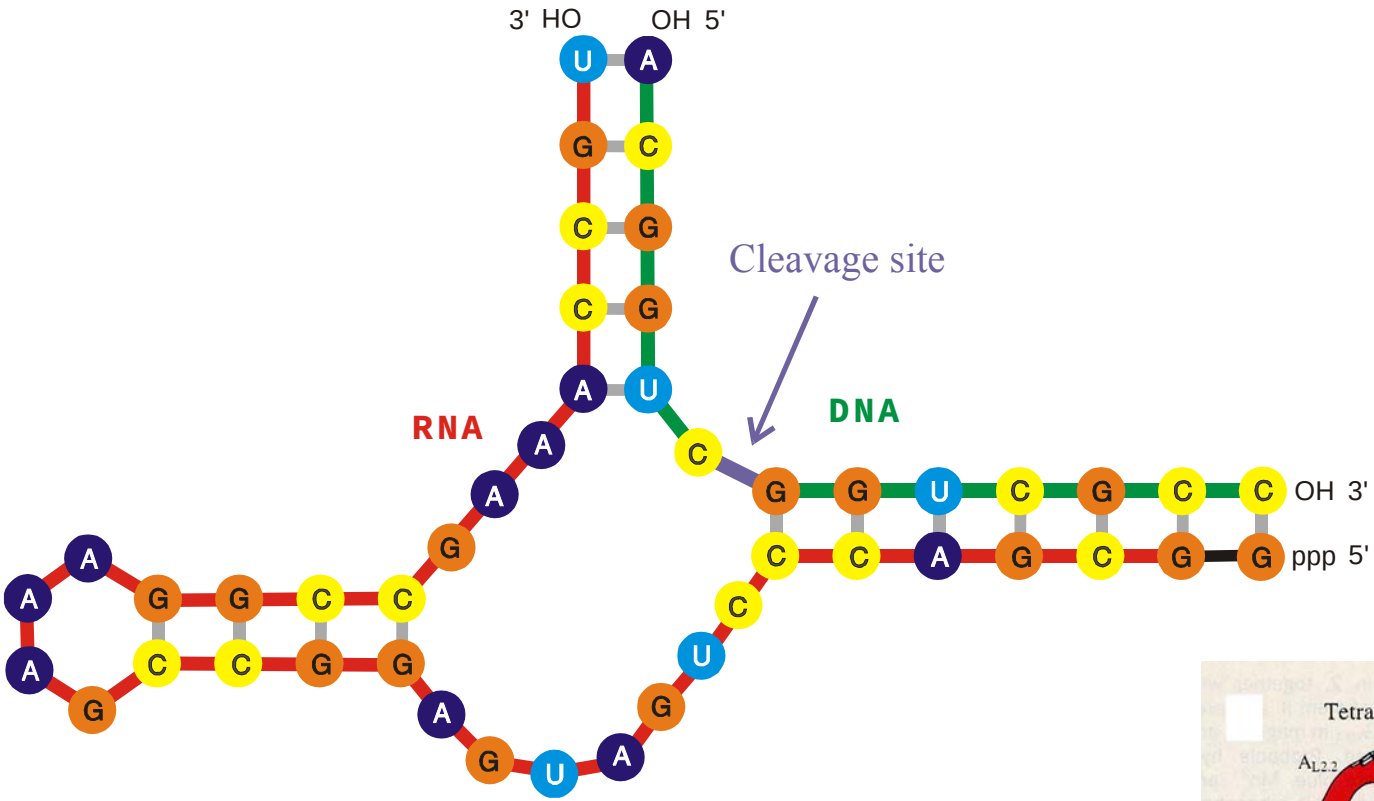
## Hammerhead ribozyme – The smallest RNA based catalyst

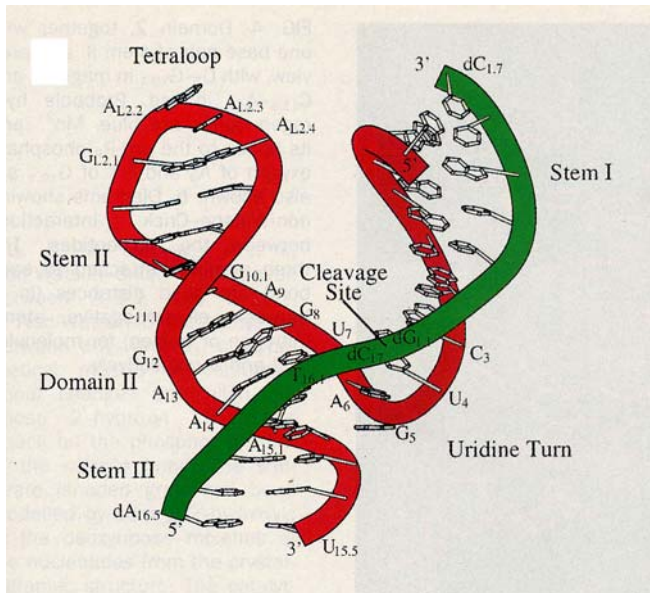
H.W.Pley, K.M.Flaherty, D.B.McKay, ***Three dimensional structure of a hammerhead ribozyme***. Nature **372** (1994), 68-74

W.G.Scott, J.T.Finch, A.Klug, ***The crystal structures of an all-RNA hammerhead ribozyme: A proposed mechanism for RNA catalytic cleavage***. Cell **81** (1995), 991-1002

J.E.Wedekind, D.B.McKay, ***Crystallographic structures of the hammerhead ribozyme: Relationship to ribozyme folding and catalysis***. Annu.Rev.Biophys.Biomol.Struct. 27 (1998), 475-502

G.E.Soukup, R.R.Breaker, ***Design of allosteric hammerhead ribozymes activated by ligand-induced structure stabilization***. Structure **7** (1999), 783-791



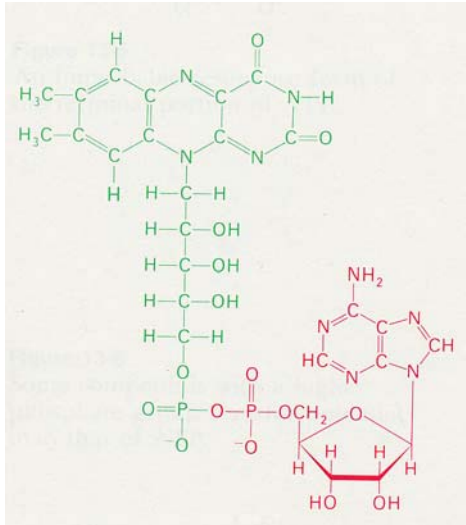




**Hammerhead ribozyme**: The smallest known catalytically active RNA molecule

**Allosteric effectors**:

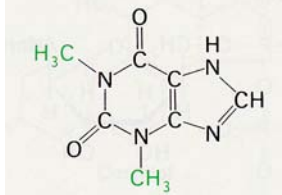
FMN = flavine mononucleotide

H10 – H12



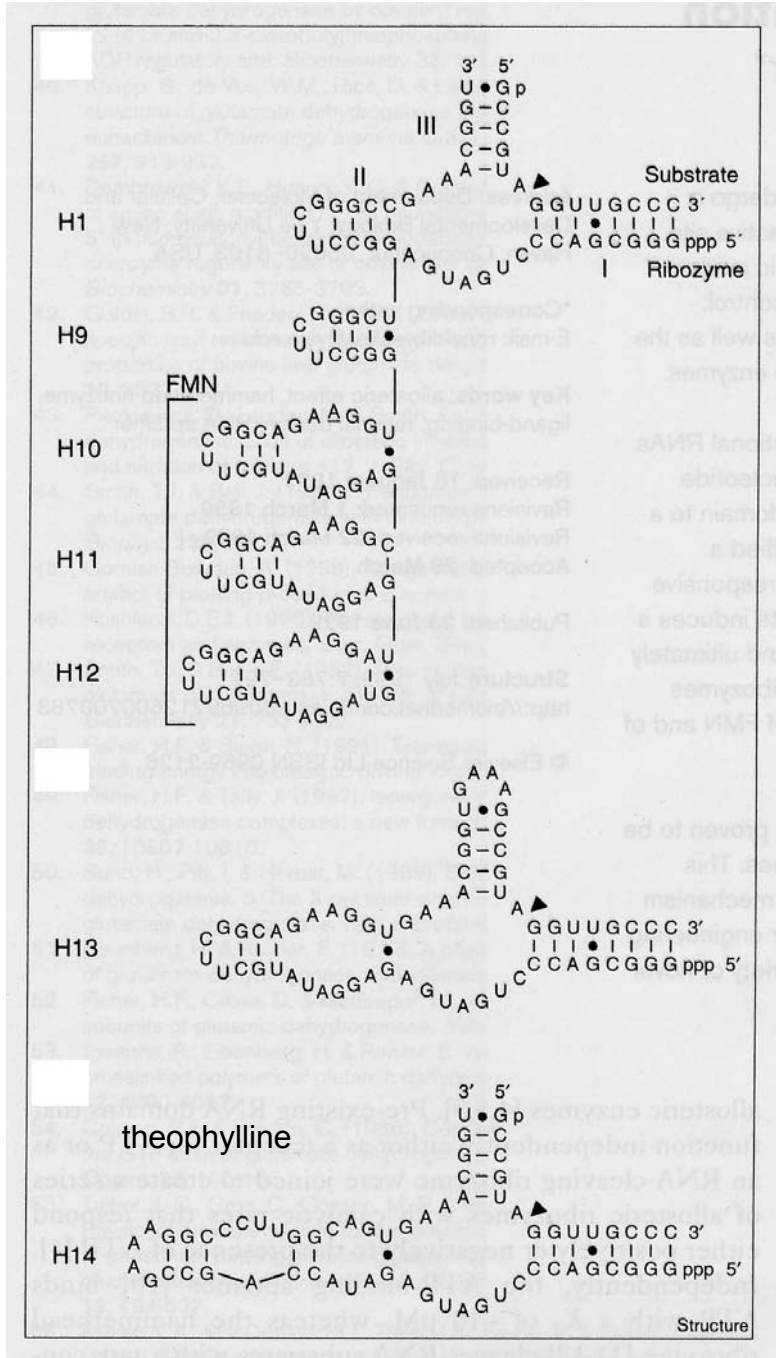
theophylline

H14



Self-splicing allosteric ribozyme

H13




Hammerhead ribozymes with allosteric effectors

*Nature* **402**, 323-325, 1999

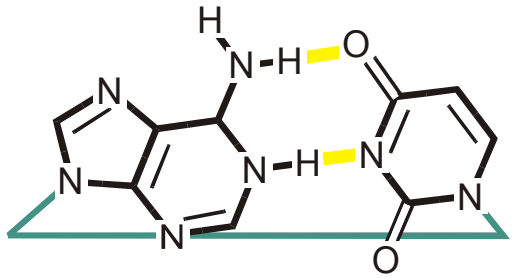
# A ribozyme that lacks cytidine

**Jeff Rogers & Gerald F. Joyce**

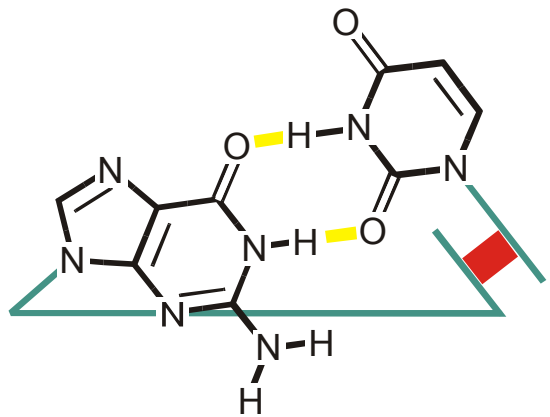
*Departments of Chemistry and Molecular Biology, and the Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037, USA*

**The RNA-world hypothesis proposes that, before the advent of DNA and protein, life was based on RNA, with RNA serving as both the repository of genetic information and the chief agent of catalytic function[1]. An argument against an RNA world is that the components of RNA lack the chemical diversity necessary to sustain life. Unlike proteins, which contain 20 different amino-acid subunits, nucleic acids are composed of only four subunits which have very similar chemical properties. Yet RNA is capable of a broad range of catalytic functions[2–7]. Here we show that even three nucleic-acid subunits are sufficient to provide a substantial increase in the catalytic rate. Starting from a molecule that contained roughly equal proportions of all four nucleosides, we used *in vitro* evolution to obtain an RNA ligase ribozyme that lacks cytidine. This ribozyme folds into a defined structure and has a catalytic rate that is about $10^5$-fold faster than the uncatalysed rate of template-directed RNA ligation.**

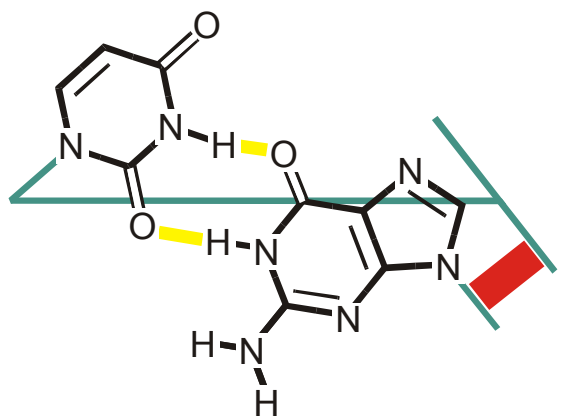
Catalytic activity in the **AUG** alphabet



A=U
(U=A)



G=U



U=G

Base pairs in the **AUG** alphabet

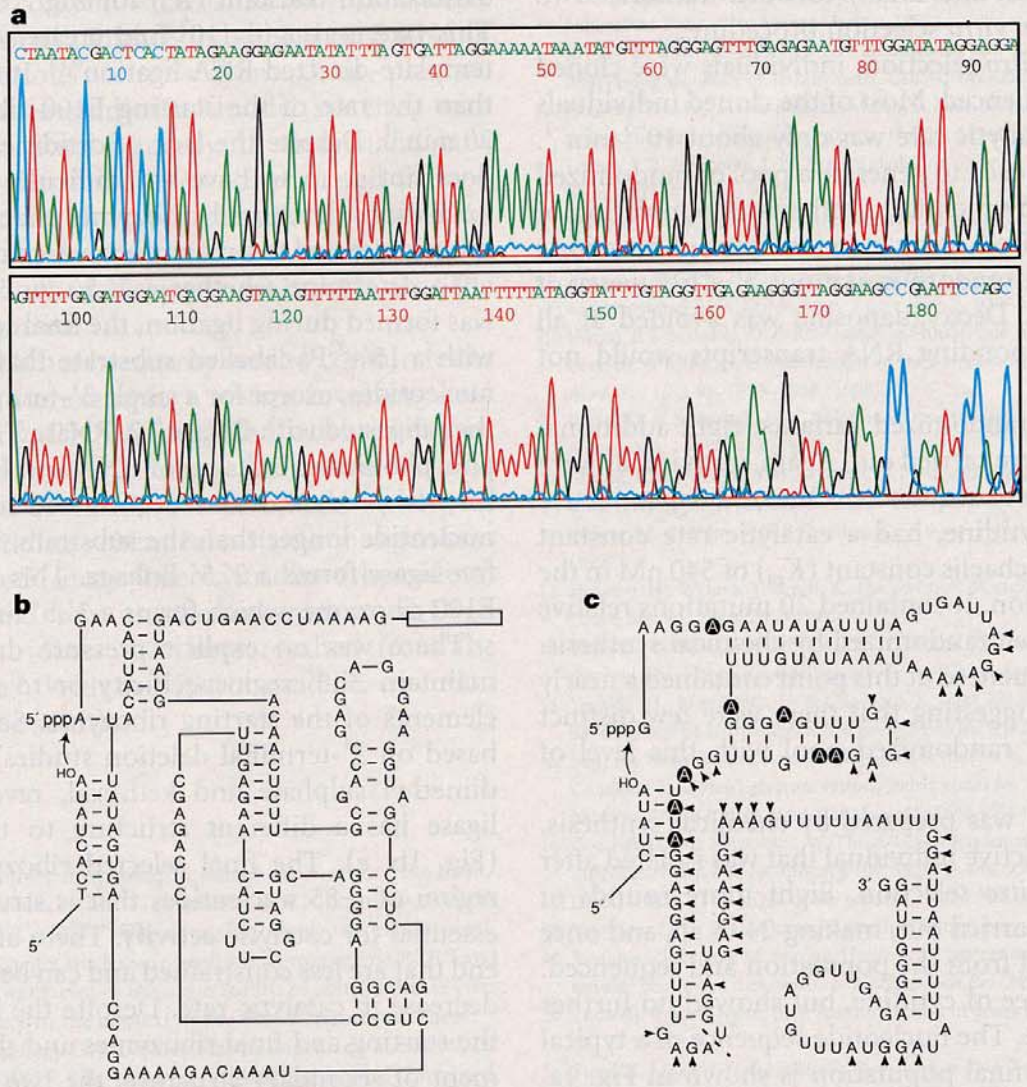



**Figure 1** Composition of the final selected cytidine-free ribozyme. **a**, Sequence trace showing the lack of cytidines at nucleotide positions 19–173. Positions 2–18 correspond to the T7 promoter sequence and positions 174–188 correspond to the downstream vector sequence (pCR 2.1). Automated sequencing was carried out using an ABI model 373 DNA sequencer and was confirmed by manual sequencing of both strands (data not shown). **b**, Secondary structure of the starting ribozyme (E100) based on that of the class I ligase[10]. Box indicates the primer binding site at the 3′ end of the ribozyme. **c**, Secondary structure of the final selected ribozyme based on chemical modification of unpaired adenosine and guanosine residues (carat marks), carried out in the absence of substrate. Highlighted adenosine residues blocked catalytic activity when methylated at N1. Dashed line indicates the site of the largest 3′-terminal deletion that was compatible with catalytic activity.

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# A ribozyme composed of only two different nucleotides

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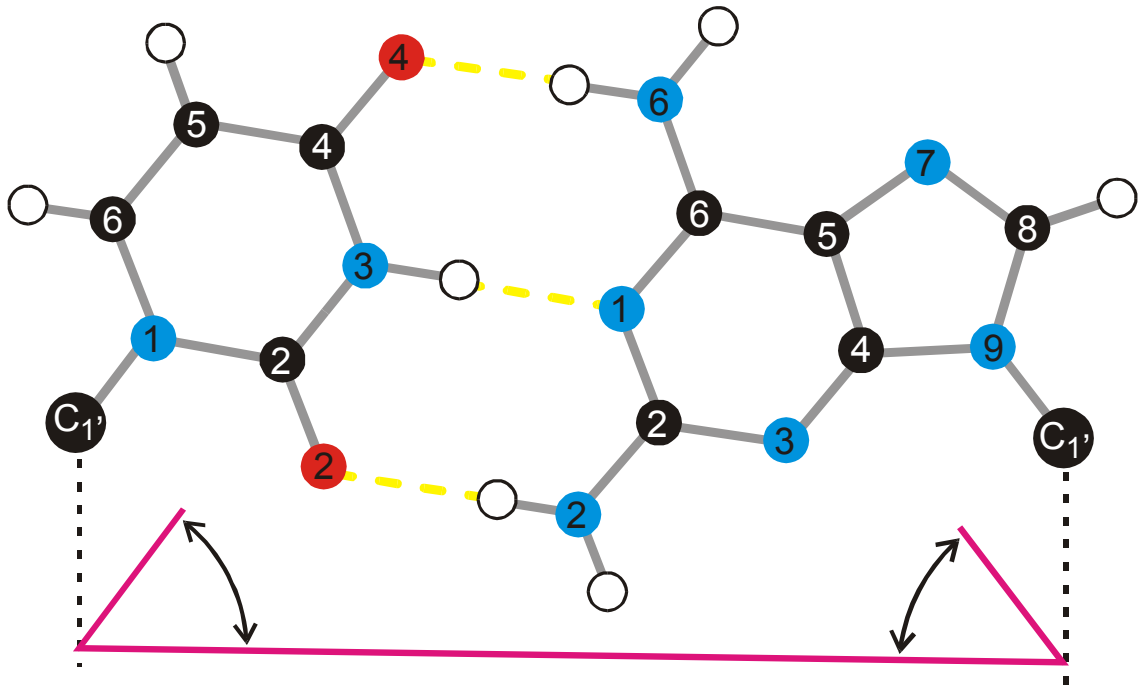
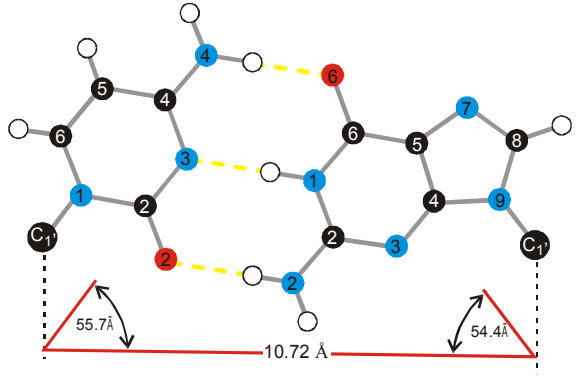
**RNA molecules are thought to have been prominent in the early history of life on Earth because of their ability both to encode genetic information and to exhibit catalytic function[1]. The modern genetic alphabet relies on two sets of complementary base pairs to store genetic information. However, owing to the chemical instability of cytosine, which readily deaminates to uracil[2], a primitive genetic system composed of the bases A, U, G and C may have been difficult to establish. It has been suggested that the first genetic material instead contained only a single base-pairing unit[3–7]. Here we show that binary informational macromolecules, containing only two different nucleotide subunits, can act as catalysts. *In vitro* evolution was used to obtain ligase ribozymes composed of only 2,6-diaminopurine and uracil nucleotides, which catalyse the template-directed joining of two RNA molecules, one bearing a 5′-triphosphate and the other a 3′-hydroxyl. The active conformation of the fastest isolated ribozyme had a catalytic rate that was about 36,000-fold faster than the uncatalysed rate of reaction. This ribozyme is specific for the formation of biologically relevant 3′,5′-phosphodiester linkages.**

Catalytic activity in the **DU** alphabet

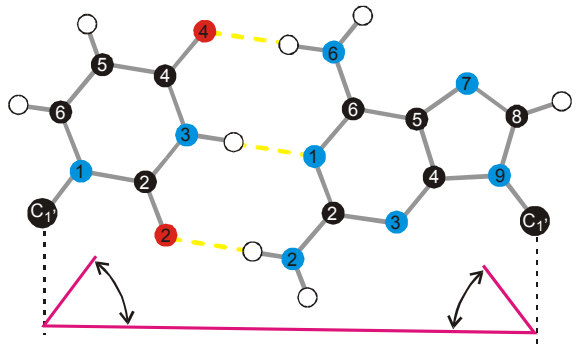



The 2,6-diamino purine – uracil, **DU**, base pair

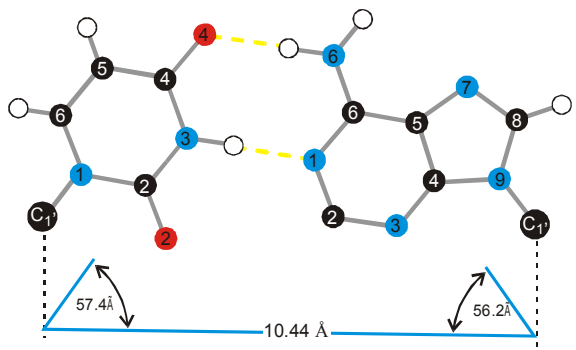
G Í C




D Í U



A = U




Three Watson-Crick type base pairs

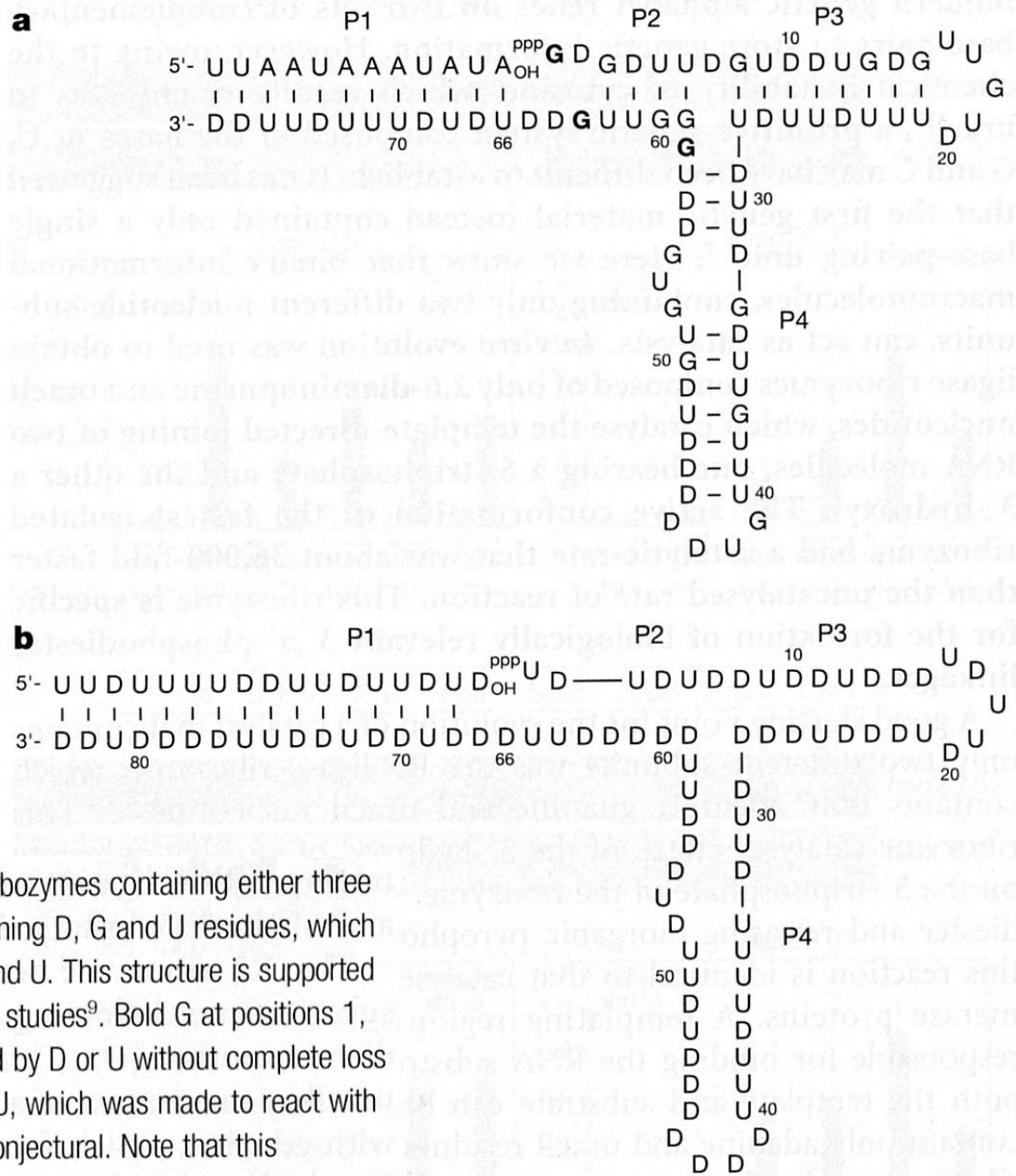



**Figure 1** Sequence and secondary structure of ligase ribozymes containing either three or two different nucleotide subunits. **a**, Ribozyme containing D, G and U residues, which was made to react with a substrate containing only A and U. This structure is supported by chemical modification and site-directed mutagenesis studies[9]. Bold G at positions 1, 58 and 63 indicates residues that could not be replaced by D or U without complete loss of catalytic activity. **b**, Ribozyme containing only D and U, which was made to react with a substrate containing only D and U. This structure is conjectural. Note that this molecule is shortened by one nucleotide at the 5′ end and lengthened by six nucleotides at the 3′ end compared with the ribozyme shown in **a**.

# Evolution of aptamers with a new specificity and new secondary structures from an ATP aptamer

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**ABSTRACT**

**Small changes in target specificity can sometimes be achieved, without changing aptamer structure, through mutation of a few bases. Larger changes in target geometry or chemistry may require more radical changes in an aptamer. In the latter case, it is unknown whether structural and functional solutions can still be found in the region of sequence space close to the original aptamer. To investigate these questions, we designed an in vitro selection experiment aimed at evolving specificity of an ATP aptamer. The ATP aptamer makes contacts with both the nucleobase and the sugar. We used an affinity matrix in which GTP was immobilized through the sugar, thus requiring extensive changes in or loss of sugar contact, as well as changes in recognition of the nucleobase. After just five rounds of selection, the pool was dominated by new aptamers falling into three major classes, each with secondary structures distinct from that of the ATP aptamer. The average sequence identity between the original aptamer and new aptamers is 76%. Most of the mutations appear to play roles either in disrupting the original secondary structure or in forming the new secondary structure or the new recognition loops. Our results show that there are novel structures that recognize a significantly different ligand in the region of sequence space close to the ATP aptamer. These examples of the emergence of novel functions and structures from an RNA molecule with a defined specificity and fold provide a new perspective on the evolutionary flexibility and adaptability of RNA.**

**Keywords: Aptamer; specificity; fold; selection; RNA evolution**

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Evidence for **neutral networks** and **shape space covering**

Evidence for **neutral networks** and **intersection** of apatamer functions

J Mol Evol (2003) 57:299–308
DOI: 10.1007/s00239-003-2481-y

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# Evolutionary Landscapes for the Acquisition of New Ligand Recognition by RNA Aptamers

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**Abstract.** The evolution of ligand specificity underlies many important problems in biology, from the appearance of drug resistant pathogens to the re-engineering of substrate specificity in enzymes. In studying biomolecules, however, the contributions of macromolecular sequence to binding specificity can be obscured by other selection pressures critical to bioactivity. Evolution of ligand specificity *in vitro*—unconstrained by confounding biological factors—is addressed here using variants of three flavin-binding RNA aptamers. Mutagenized pools based on the three aptamers were combined and allowed to compete during *in vitro* selection for GMP-binding activity. The sequences of the resulting selection isolates were diverse, even though most were derived from the same flavin-binding parent. Individual GMP aptamers differed from the parental flavin aptamers by 7 to 26 mutations (20 to 57% overall change). Acquisition of GMP recognition coincided with the loss of FAD (flavin-adenine dinucleotide) recognition in all isolates, despite the absence of a counter-selection to remove FAD-binding RNAs. To examine more precisely the proximity of these two activities within a defined sequence space, the complete set of all intermediate sequences between an FAD-binding aptamer and a GMP-binding aptamer were synthesized and assayed for activity. For this set of sequences, we observe a portion of a neutral network for FAD-binding function separated from GMP-binding function by a distance of three mutations. Furthermore, enzymatic probing of these aptamers revealed gross structural remodeling of the RNA coincident with the switch in ligand recognition. The capacity for neutral drift along an FAD-binding network in such close approach to RNAs with GMP-binding activity illustrates the degree of phenotypic buffering available to a set of closely related RNA sequences—defined as the set's functional tolerance for point mutations—and supports neutral evolutionary theory by demonstrating the facility with which a new phenotype becomes accessible as that buffering threshold is crossed.

**Key words:** Aptamers — RNA structure — Phenotypic buffering — Fitness landscapes — Neutral evolutionary theory — Flavin — GMP

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## Introduction

RNA aptamers targeting small molecules serve as useful model systems for the study of the evolution and biophysics of macromolecular binding interactions. Because of their small sizes, the structures of several such complexes have been determined to atomic resolution by NMR spectrometry or X-ray crystallography (reviewed by Herman and Patel 2000). Moreover, aptamers can be subjected to mutational and evolutionary pressures for which survival is based entirely on ligand binding, without the complicating effects of simultaneous selection pressures for bioactivity, thus allowing the relative contributions of each activity to be evaluated separately.

## A ribozyme switch

E.A.Schultes, D.B.Bartel, Science **289** (2000), 448-452



minus the background levels observed in the HSP in the control (Sar1-GDP–containing) incubation that prevents COPII vesicle formation. In the microsome control, the level of p115-SNARE associations was less than 0.1%.

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50. GST-SNARE proteins were expressed in bacteria and purified on glutathione-Sepharose beads using standard methods. Immobilized GST-SNARE protein (0.5 μM) was incubated with rat liver cytosol (20 mg) or purified recombinant p115 (0.5 μM) in 1 ml of NS buffer containing 1% BSA for 2 hours at 4°C with rotation. Beads were briefly spun (3000 rpm for 10 s) and sequentially washed three times with NS buffer and three times with NS buffer supplemented with 150 mM NaCl. Bound proteins were eluted three times in 50 μl of 50 mM tris-HCl (pH 8.5), 50 mM reduced glutathione, 150 mM NaCl, and 0.1% Triton X-100 for 15 min at 4°C with intermittent mixing, and elutes were pooled. Proteins were precipitated by MeOH/$CH_3Cl$ and separated by SDS–polyacrylamide gel electrophoresis (PAGE) followed by immunoblotting using p115 mAb 13F12.
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# One Sequence, Two Ribozymes: Implications for the Emergence of New Ribozyme Folds

**Erik A. Schultes and David P. Bartel***

**We describe a single RNA sequence that can assume either of two ribozyme folds and catalyze the two respective reactions. The two ribozyme folds share no evolutionary history and are completely different, with no base pairs (and probably no hydrogen bonds) in common. Minor variants of this sequence are highly active for one or the other reaction, and can be accessed from prototype ribozymes through a series of neutral mutations. Thus, in the course of evolution, new RNA folds could arise from preexisting folds, without the need to carry inactive intermediate sequences. This raises the possibility that biological RNAs having no structural or functional similarity might share a common ancestry. Furthermore, functional and structural divergence might, in some cases, precede rather than follow gene duplication.**

Related protein or RNA sequences with the same folded conformation can often perform very different biochemical functions, indicating that new biochemical functions can arise from preexisting folds. But what evolutionary mechanisms give rise to sequences with new macromolecular folds? When considering the origin of new folds, it is useful to picture, among all sequence possibilities, the distribution of sequences with a particular fold and function. This distribution can range very far in sequence space (*1*). For example, only seven nucleotides are strictly conserved among the group I self-splicing introns, yet secondary (and presumably tertiary) structure within the core of the ribozyme is preserved (*2*). Because these disparate isolates have the same fold and function, it is thought that they descended from a common ancestor through a series of mutational variants that were each functional. Hence, sequence heterogeneity among divergent isolates implies the existence of paths through sequence space that have allowed neutral drift from the ancestral sequence to each isolate. The set of all possible neutral paths composes a "neutral network," connecting in sequence space those widely dispersed sequences sharing a particular fold and activity, such that any sequence on the network can potentially access very distant sequences by neutral mutations (*3*–*5*).

Theoretical analyses using algorithms for predicting RNA secondary structure have suggested that different neutral networks are interwoven and can approach each other very closely (*3*, *5*–*8*). Of particular interest is whether ribozyme neutral networks approach each other so closely that they intersect. If so, a single sequence would be capable of folding into two different conformations, would have two different catalytic activities, and could access by neutral drift every sequence on both networks. With intersecting networks, RNAs with novel structures and activities could arise from previously existing ribozymes, without the need to carry nonfunctional sequences as evolutionary intermediates. Here, we explore the proximity of neutral networks experimentally, at the level of RNA function. We describe a close apposition of the neutral networks for the hepatitis delta virus (HDV) self-cleaving ribozyme and the class III self-ligating ribozyme.

In choosing the two ribozymes for this investigation, an important criterion was that they share no evolutionary history that might confound the evolutionary interpretations of our results. Choosing at least one artificial ribozyme ensured independent evolutionary histories. The class III ligase is a synthetic ribozyme isolated previously from a pool of random RNA sequences (*9*). It joins an oligonucleotide substrate to its 5′ terminus. The prototype ligase sequence (Fig. 1A) is a shortened version of the most active class III variant isolated after 10 cycles of in vitro selection and evolution. This minimal construct retains the activity of the full-length isolate (*10*). The HDV ribozyme carries out the site-specific self-cleavage reactions needed during the life cycle of HDV, a satellite virus of hepatitis B with a circular, single-stranded RNA genome (*11*). The prototype HDV construct for our study (Fig. 1B) is a shortened version of the antigenomic HDV ribozyme (*12*), which undergoes self-cleavage at a rate similar to that reported for other antigenomic constructs (*13*, *14*).

The prototype class III and HDV ribozymes have no more than the 25% sequence identity expected by chance and no fortuitous structural similarities that might favor an intersection of their two neutral networks. Nevertheless, sequences can be designed that simultaneously satisfy the base-pairing requirements

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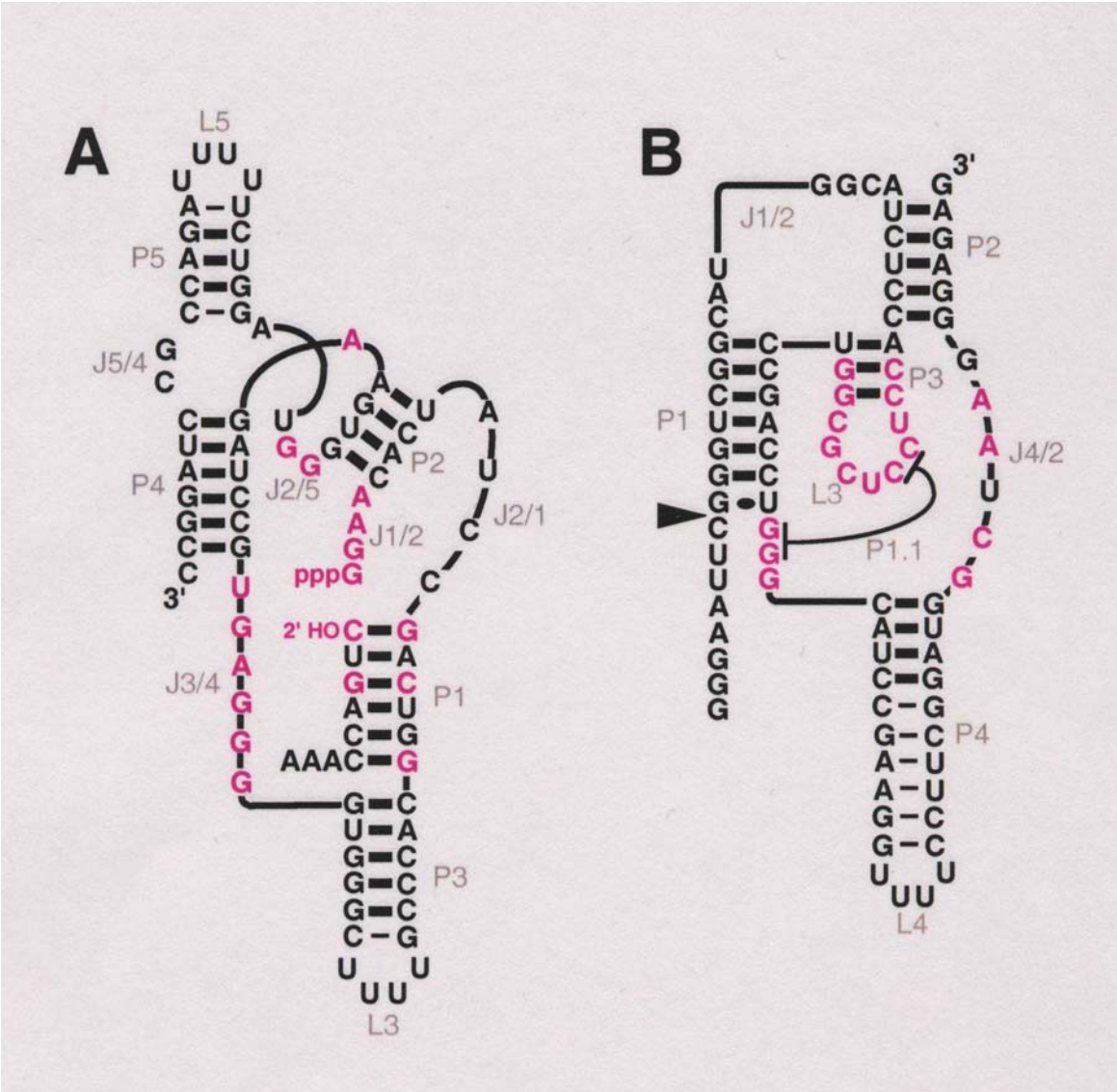
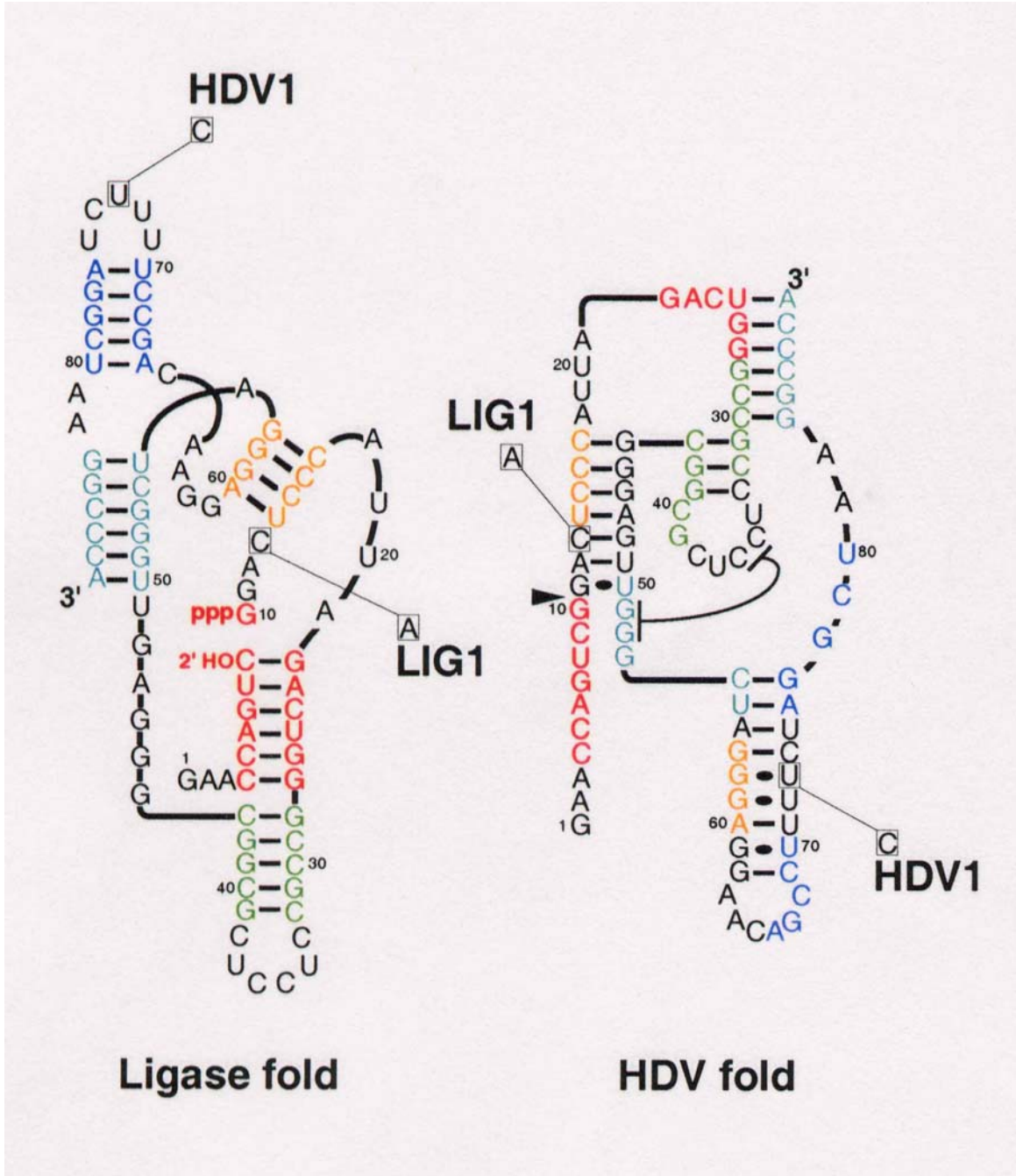
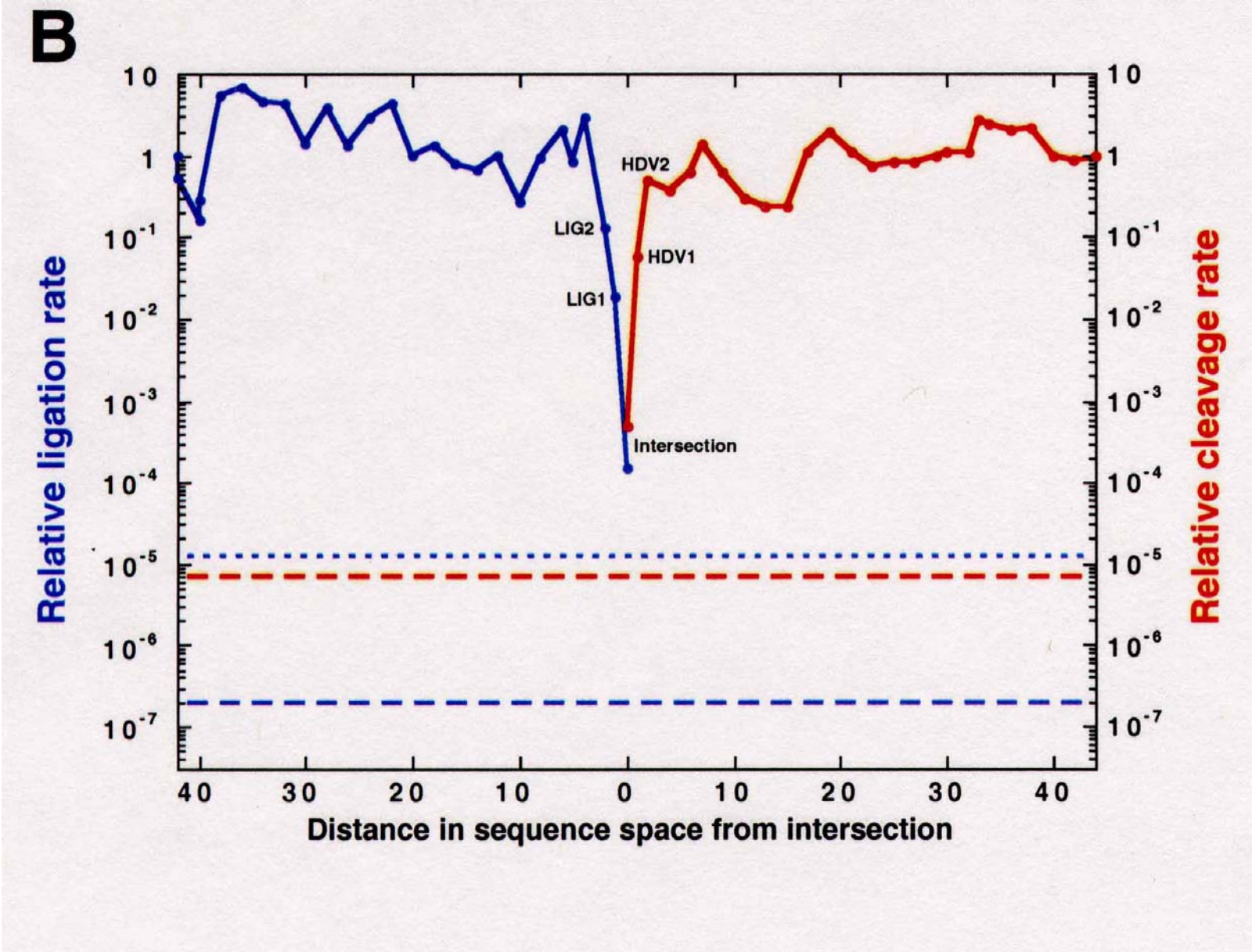



Two ribozymes of chain lengths n = 88 nucleotides: An artificial ligase (**A**) and a natural cleavage ribozyme of hepatitis-d-virus (**B**)

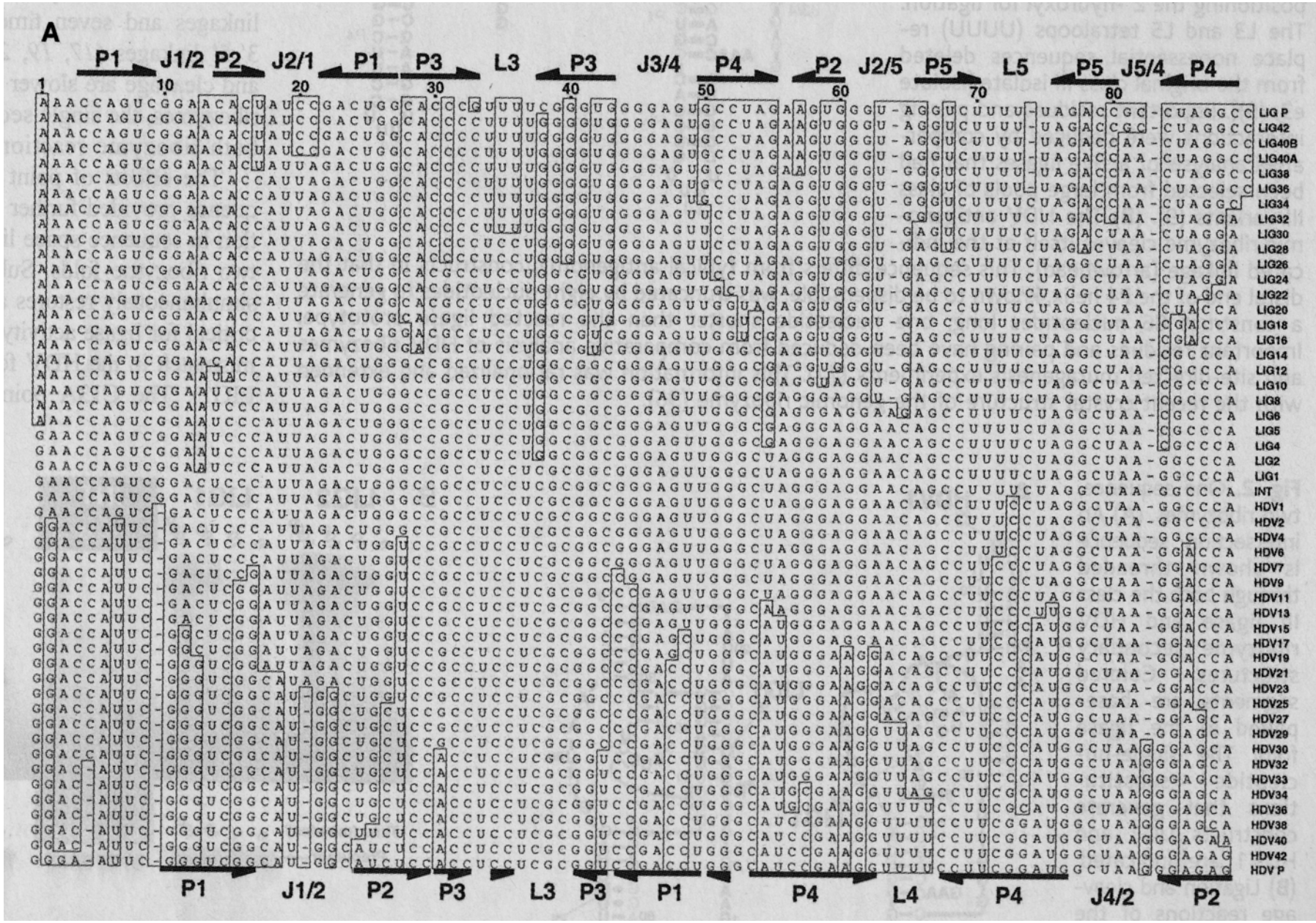
The sequence at the ***intersection***:

An RNA molecules which is 88 nucleotides long and can form both structures






Two neutral walks through sequence space with conservation of structure and catalytic activity




Sequence of mutants from the intersection to both reference ribozymes


Universität Wien

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