

Evolution *in vitro*

Theorie und Praxis der Herstellung maßgeschneiderter Moleküle

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Vortragsreihe zum Jahr der Chemie

TU Ilmenau, 22.11.2003

Web-Page for further information:

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

	Generation time	10 000 generations	10 ⁶ generations	10 ⁷ generations
RNA molecules	10 sec	27.8 h = 1.16 d	115.7 d	3.17 a
	1 min	6.94 d	1.90 a	19.01 a
Bacteria	20 min	138.9 d	38.03 a	380 a
	10 h	11.40 a	1 140 a	11 408 a
Higher multicellular organisms	10 d	274 a	27 380 a	273 800 a
	20 a	20 000 a	2 × 10 ⁷ a	2 × 10 ⁸ a

Time scales of evolutionary change

- 1. Controlled evolution experiments with bacteria and RNA**
- 2. Optimization in the RNA model**
- 3. Sequence-structure maps, neutral networks, and intersections**
- 4. Selection experiments and design of RNA molecules**

- 1. Controlled evolution experiments with bacteria and RNA**

2. Optimization in the RNA model

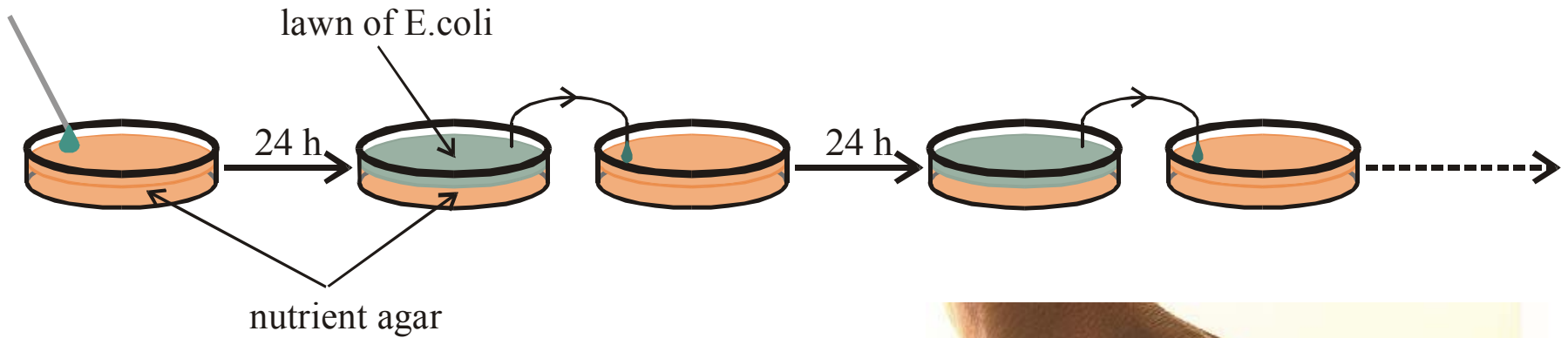
3. Sequence-structure maps, neutral networks, and intersections

4. Selection experiments and design of RNA molecules

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

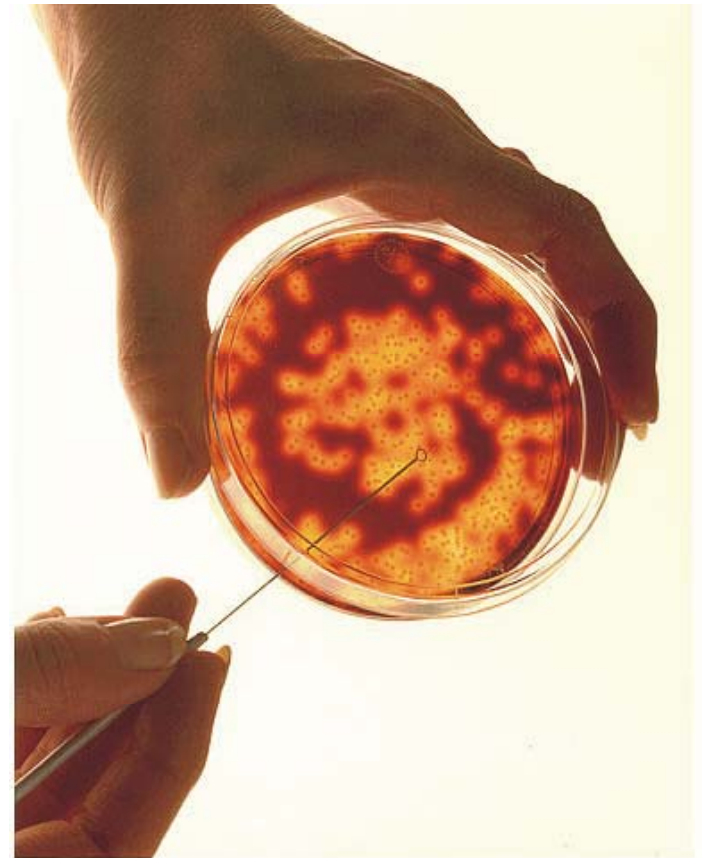


Serial transfer of *Escherichia coli* cultures in Petri dishes

1 day ^a 6.67 generations

1 month ^a 200 generations

1 year ^a 2400 generations



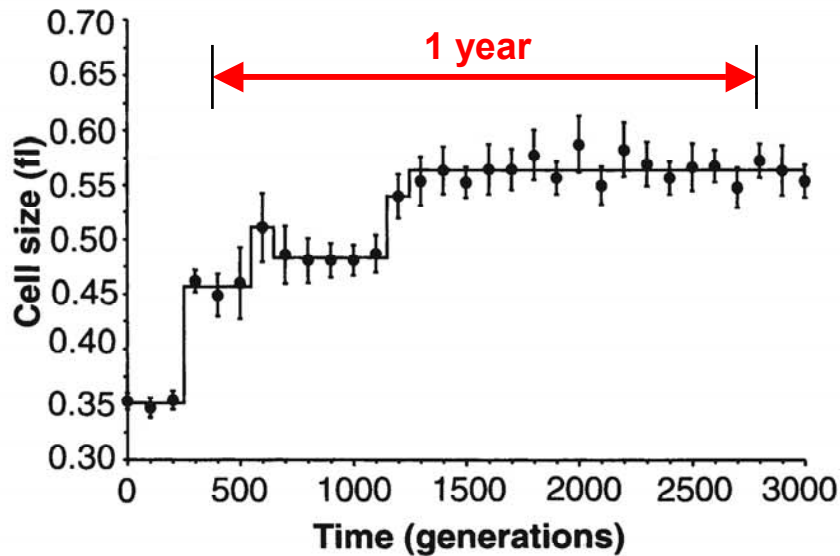


Fig. 1. Change in average cell size (1 fl = 10^{-15} L) in a population of *E. coli* during 3000 generations of experimental evolution. Each point is the mean of 10 replicate assays (22). Error bars indicate 95% confidence intervals. The solid line shows the best fit of a step-function model to these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

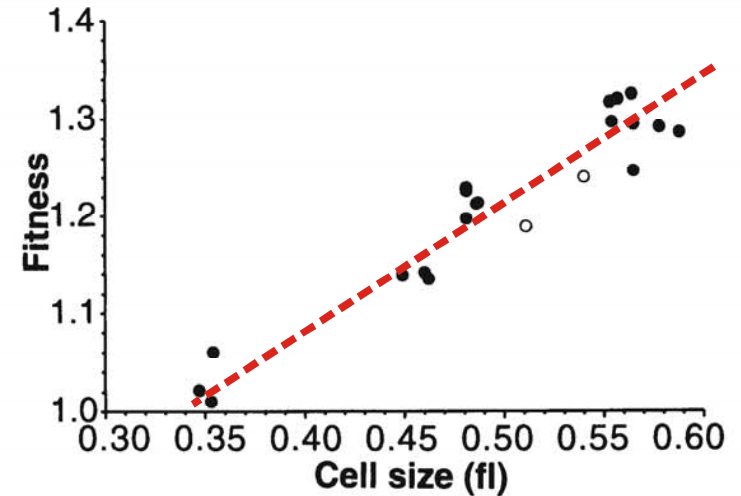
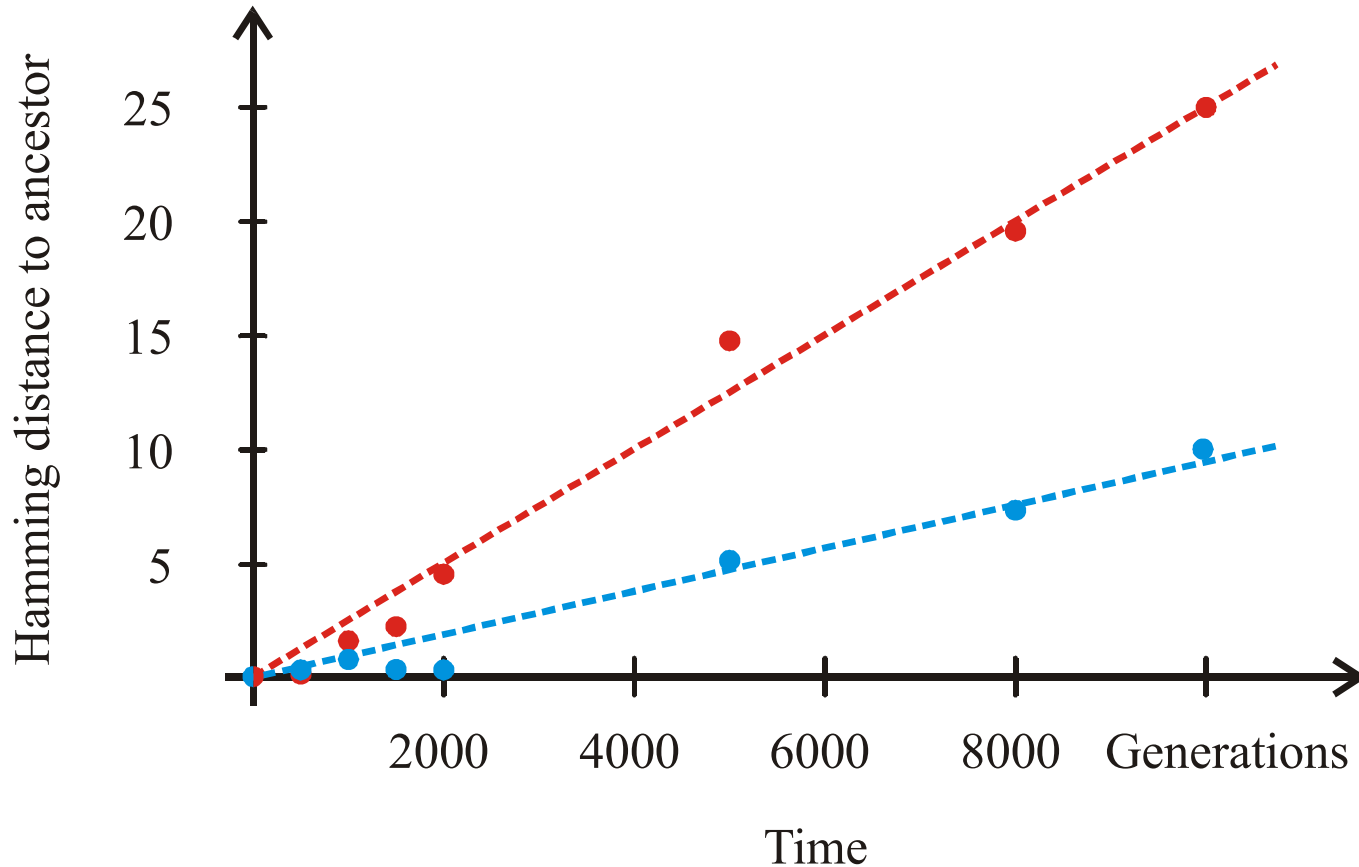


Fig. 2. Correlation between average cell size and mean fitness, each measured at 100-generation intervals for 2000 generations. Fitness is expressed relative to the ancestral genotype and was obtained from competition experiments between derived and ancestral cells (6, 7). The open symbols indicate the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

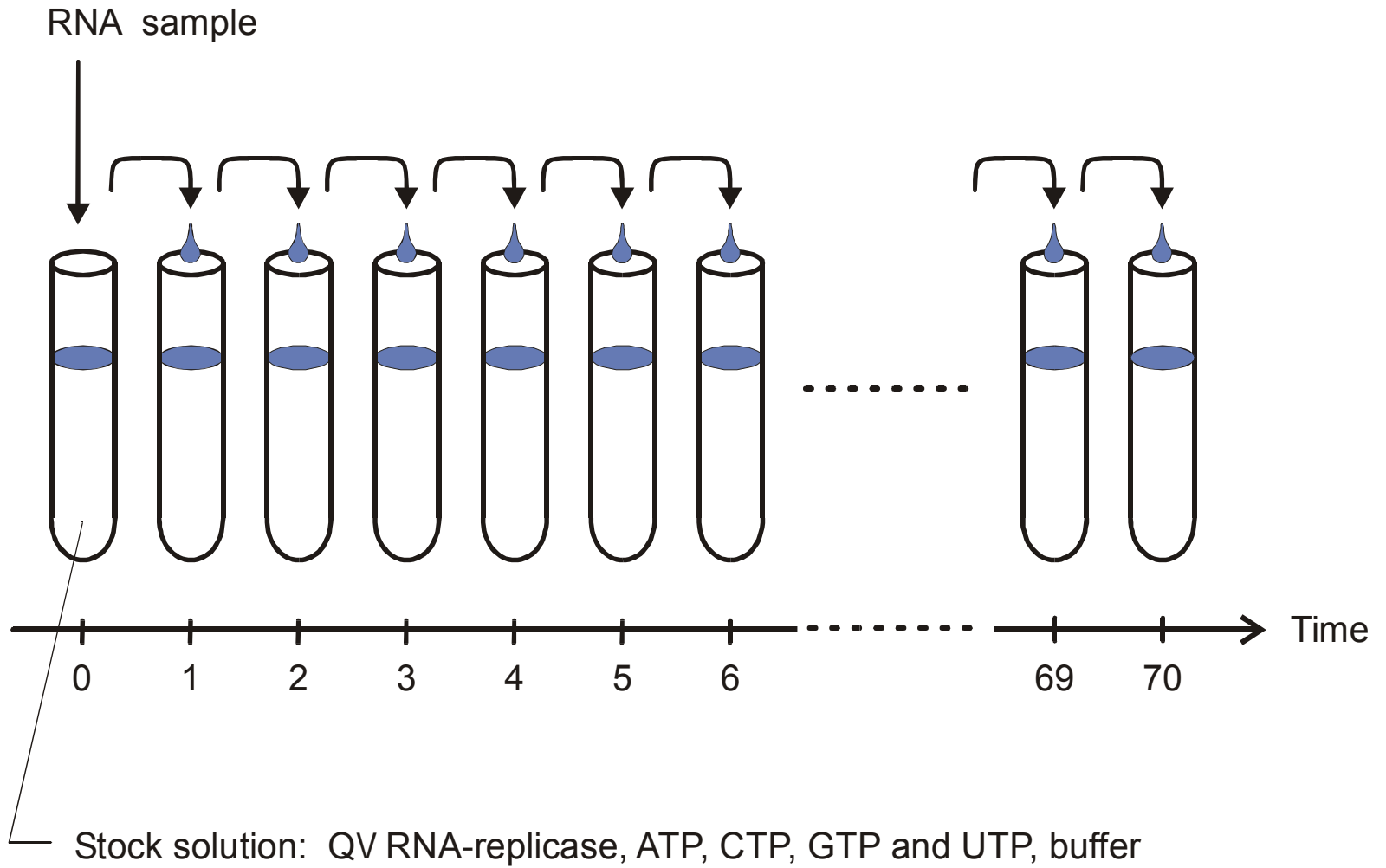
S.Spiegelman, *An approach to the experimental analysis of precellular evolution*. Quart.Rev.Biophys. **4** (1971), 213-253

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

G.Bauer, H.Otten, J.S.McCaskill, *Travelling waves of in vitro evolving RNA*. Proc.Natl.Acad.Sci.USA **86** (1989), 7937-7941

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 Q β RNA

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

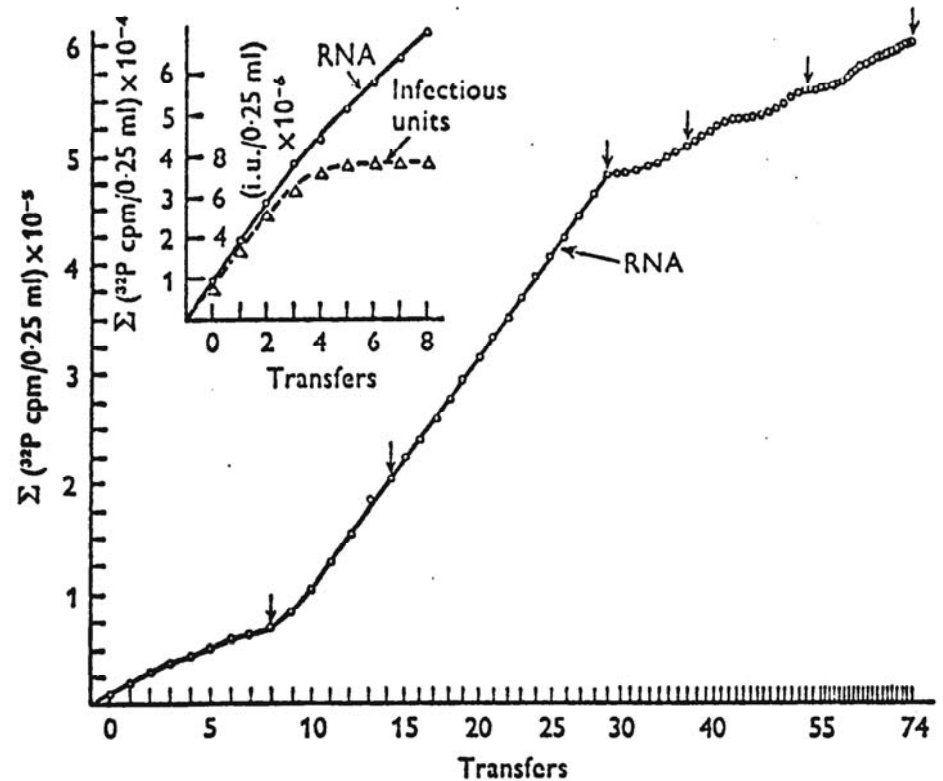
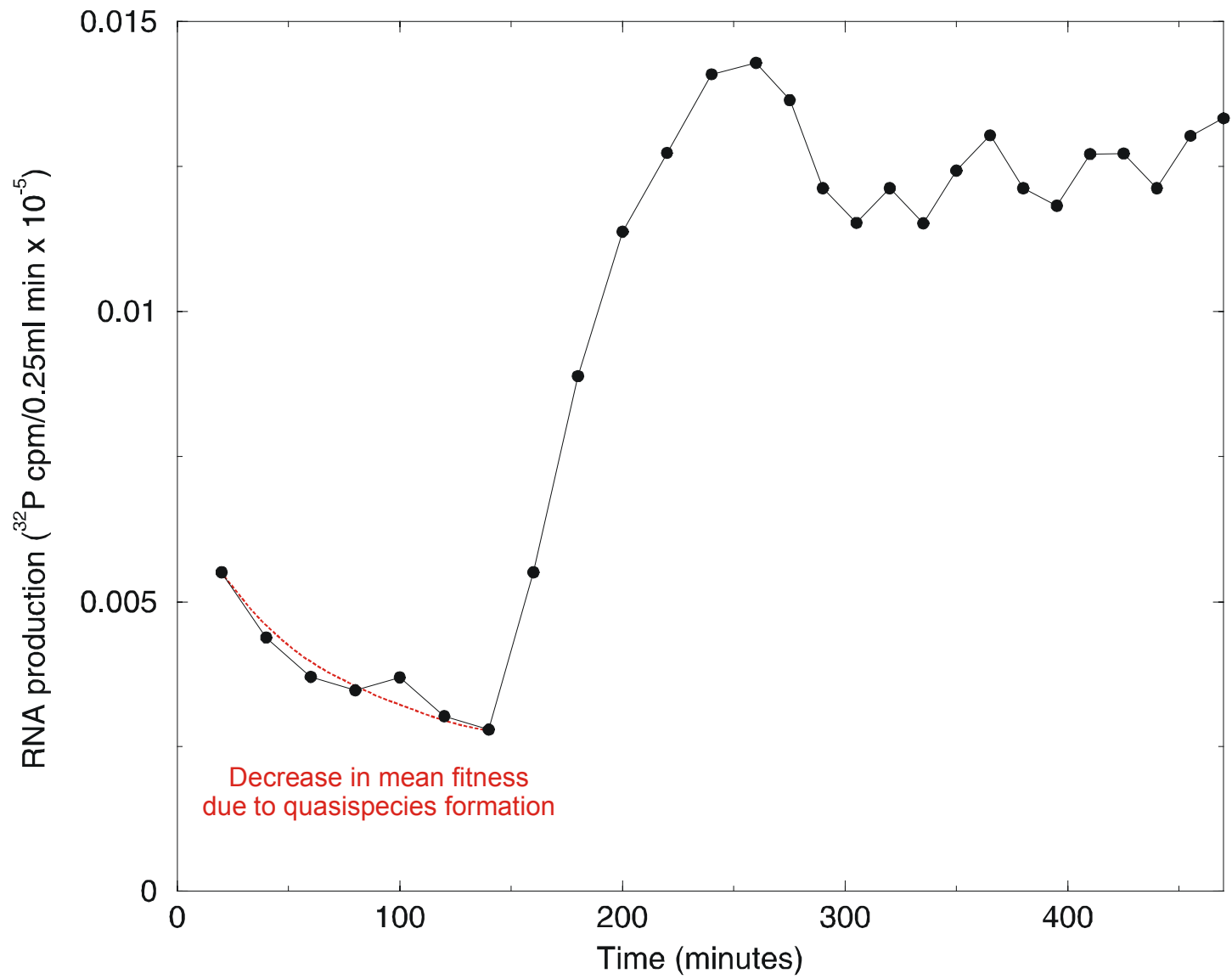


Fig. 9. Serial transfer experiment. Each 0.25 ml standard reaction mixture contained 40 μg of Q β replicase and ^{32}P -UTP. The first reaction (0 transfer) was initiated by the addition of 0.2 μg ts-1 (temperature-sensitive RNA) and incubated at 35 $^{\circ}\text{C}$ for 20 min, whereupon 0.02 ml was 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).



The increase in RNA production rate during a serial transfer experiment

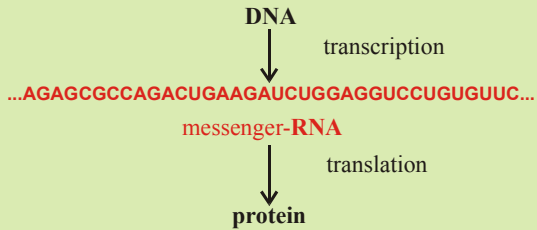
1. Controlled evolution experiments with bacteria and RNA

2. Optimization in the RNA model

3. Sequence-structure maps, neutral networks, and intersections

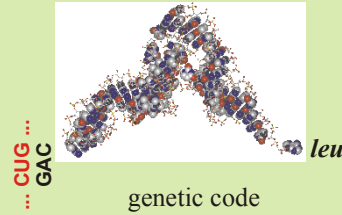
4. Selection experiments and design of RNA molecules

RNA as transmitter of genetic information

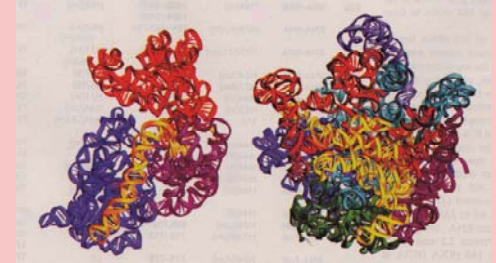


RNA as **working copy** of genetic information

RNA as adapter molecule

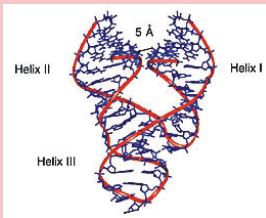


RNA is the catalytic subunit in supramolecular complexes



The ribosome is a ribozyme !

RNA as catalyst



ribozyme

RNA

RNA is modified by epigenetic control

RNA editing

Alternative splicing of messenger RNA

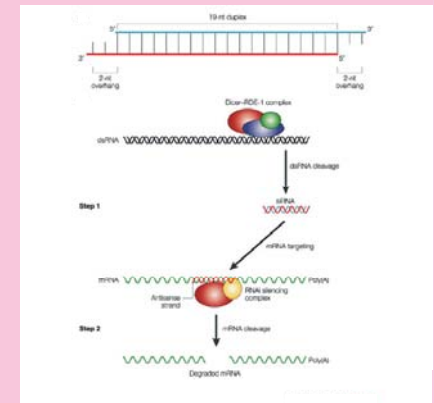
The RNA world as a precursor of the current DNA + protein biology

RNA as carrier of genetic information

RNA viruses and retroviruses

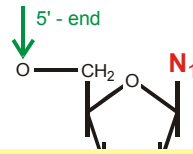
RNA as information carrier in evolution *in vitro* and evolutionary biotechnology

RNA as regulator of gene expression

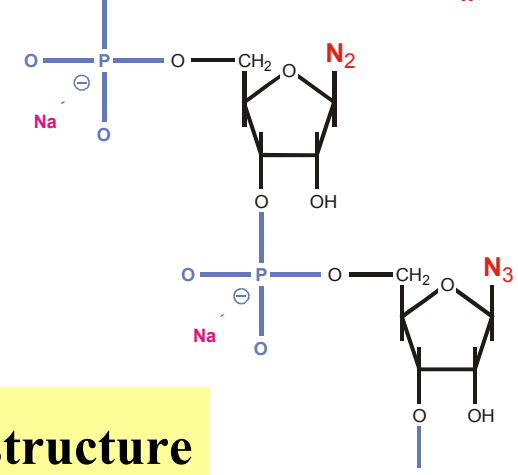


gene silencing by small interfering RNAs

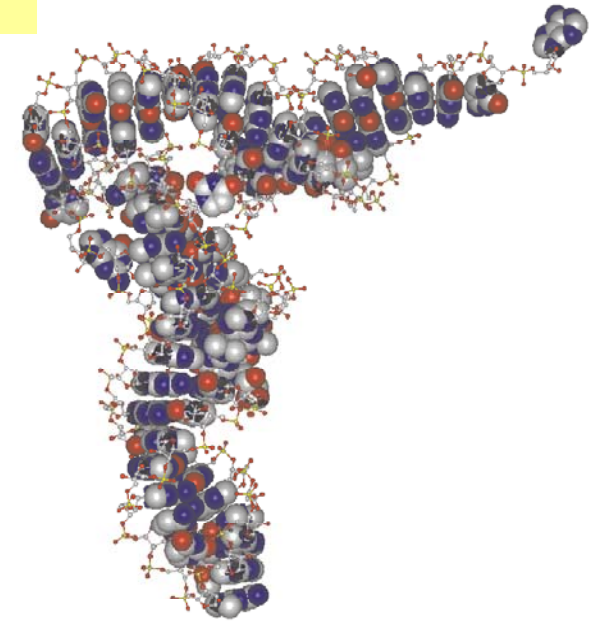
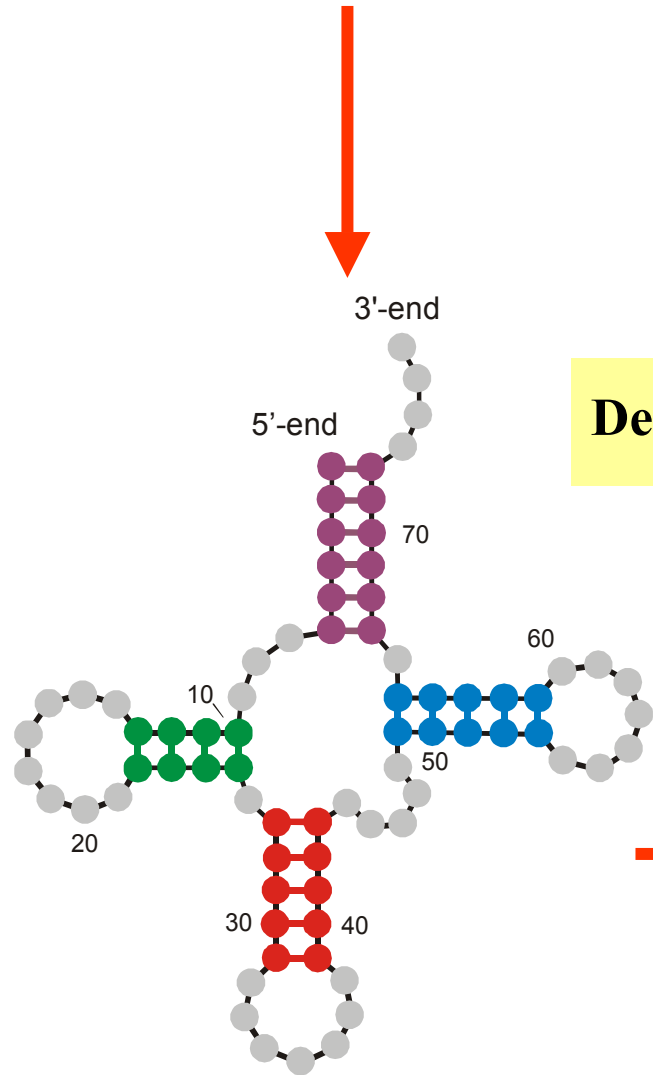
Functions of RNA molecules

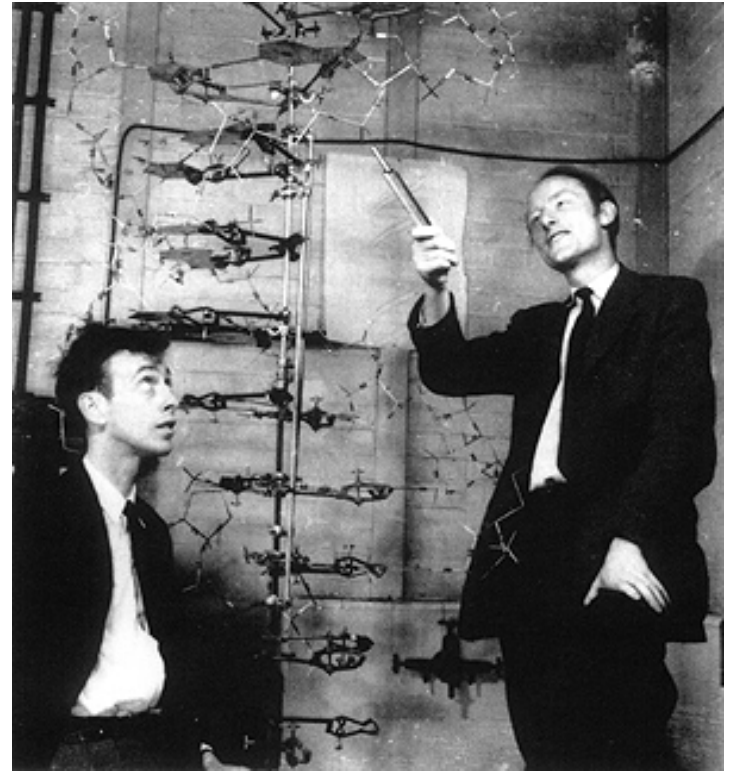
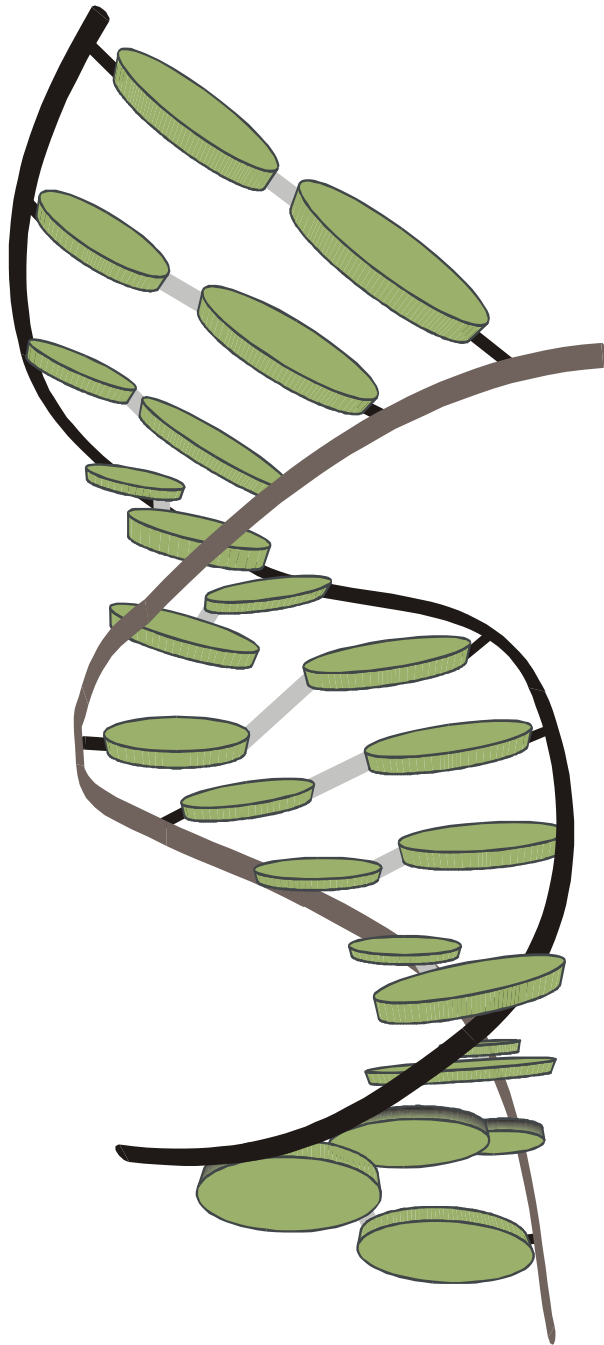


5'-end **GCGGAUUUAGCUCAGUUGGGAGAGCGCCAGACUGAAGAUCUGGAGGUCUGUGUUCGAUCCACAGAAUUCGCACCA** 3'-end



Definition of RNA structure

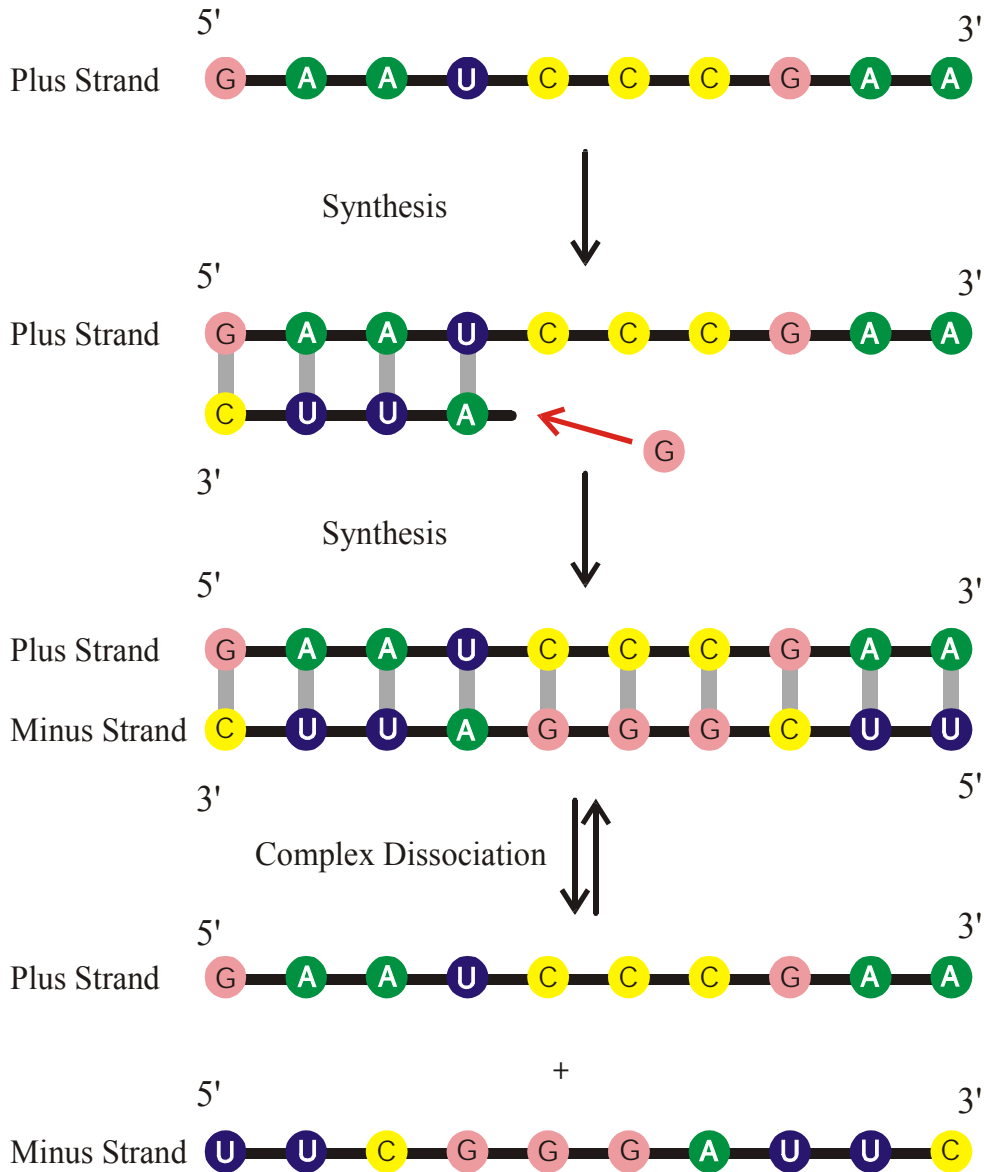




James D. Watson, 1928- , and Francis Crick, 1916- ,
Nobel Prize 1962

1953 – 2003 fifty years double helix

The three-dimensional structure of a
short double helical stack of B-DNA



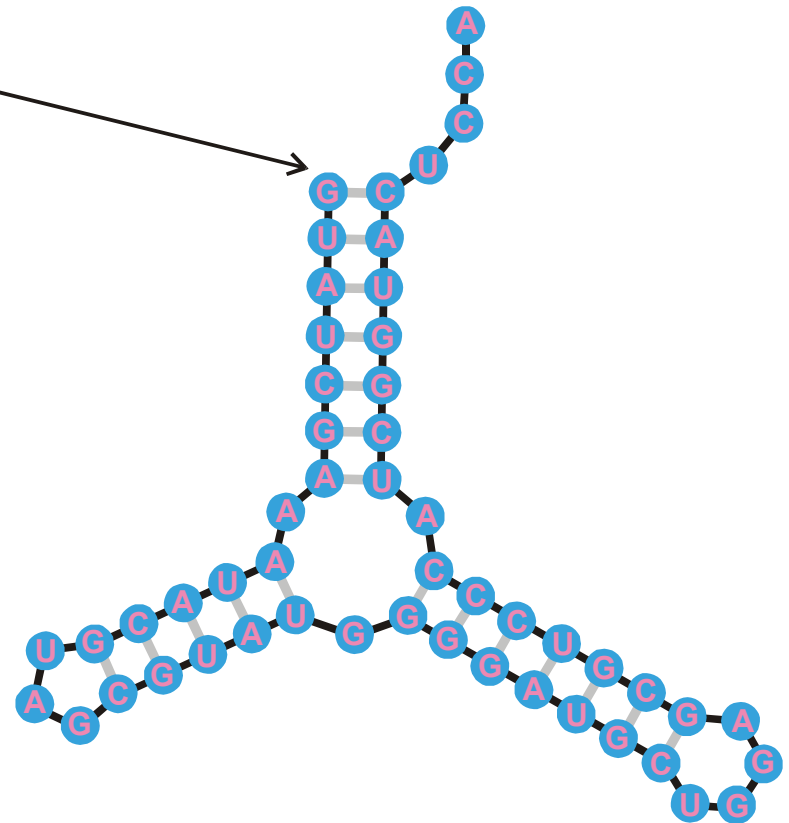
Complementary replication as the simplest copying mechanism of RNA
 Complementarity is determined by Watson-Crick base pairs:



5'-end

3'-end

GUAUCGAAAUACGUAGCGUAUGGGGAUGCUGGACGGUCCCAUCGGUACUCCA



RNAStudio.Ink

GGCGCGCCCGGCGCC

GUAUCGAAAUACGUAGCGUAUGGGGAUGCUGGACGGUCCCAUCGGUACUCCA

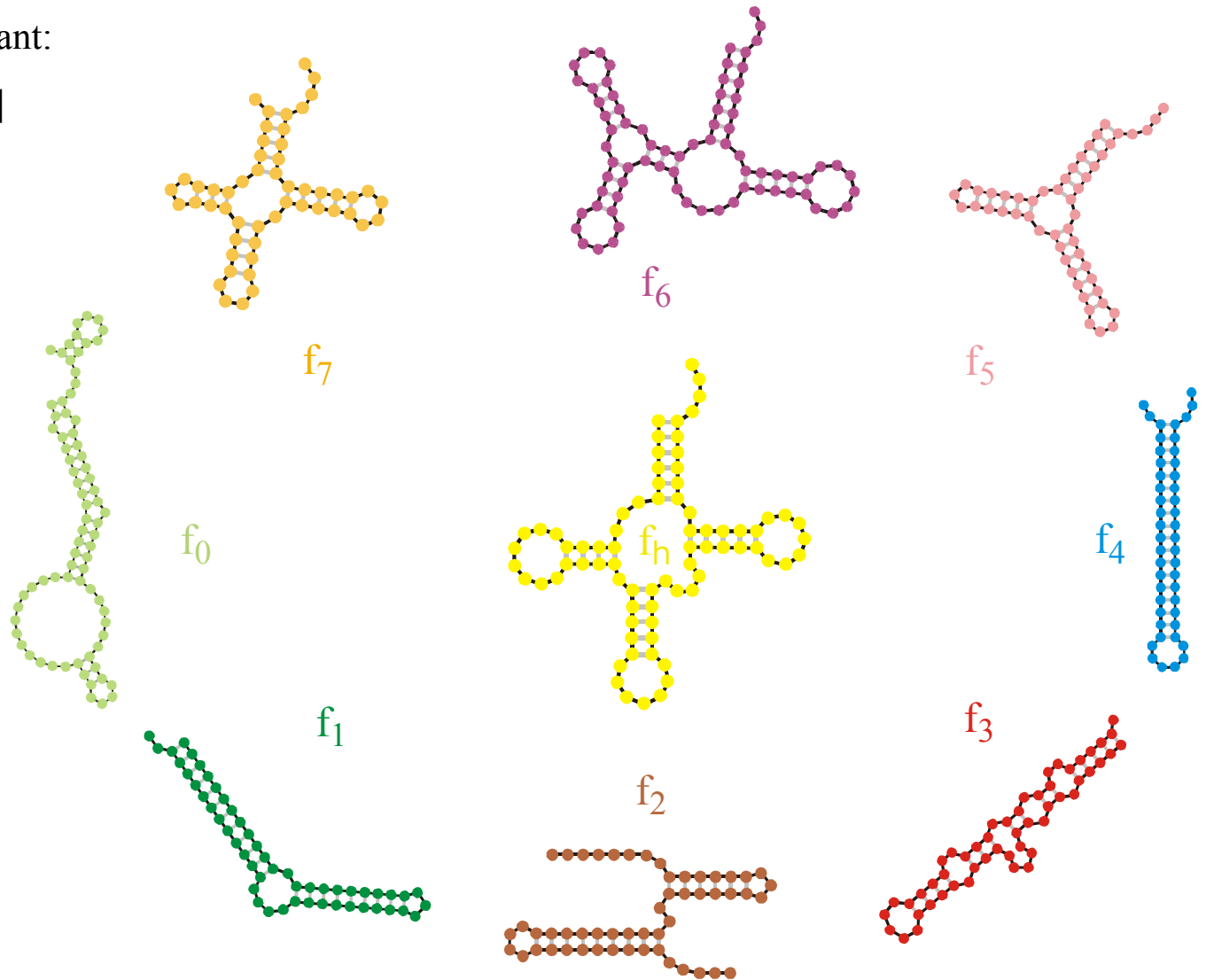
UGGUUACGCGUUGGGGUAACGAAGAUUCCGAGAGGAGUUUAGUGACUAGAGG

Folding of RNA sequences into secondary structures of minimal free energy, $8G_0^{300}$

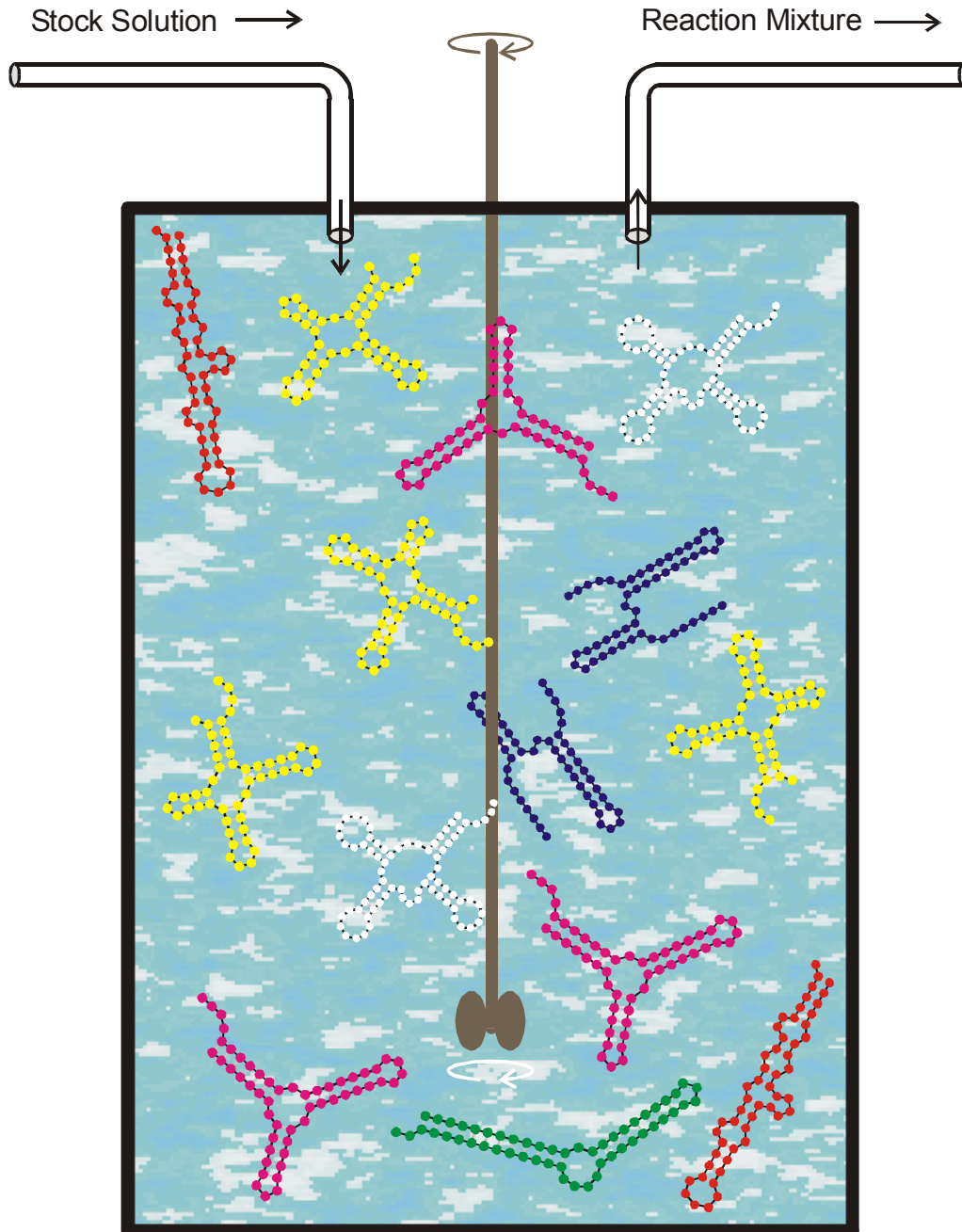
Replication rate constant:

$$f_k = \frac{[S_k]}{[U] + \sum d_S^{(k)}} \quad \text{or} \quad f_k = \frac{[S_k]}{[U] + 8d_S^{(k)}}$$

$$8d_S^{(k)} = d_H(S_k, S_h)$$



Evaluation of RNA secondary structures yields replication rate constants



Replication rate constant:

$$f_k = [/ [U + \delta d_S^{(k)}]$$

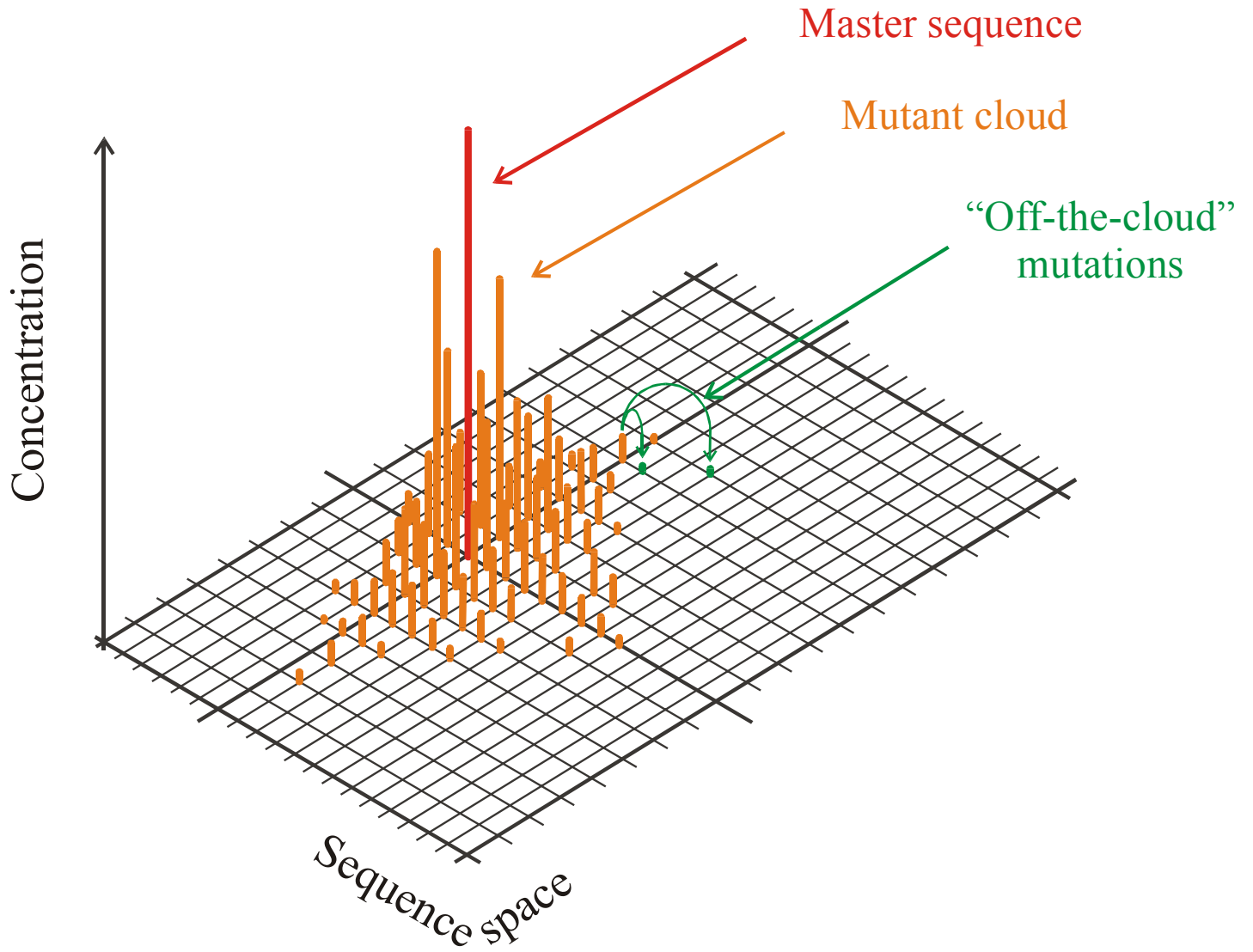
$$\delta d_S^{(k)} = d_H(S_k, S_h)$$

Selection constraint:

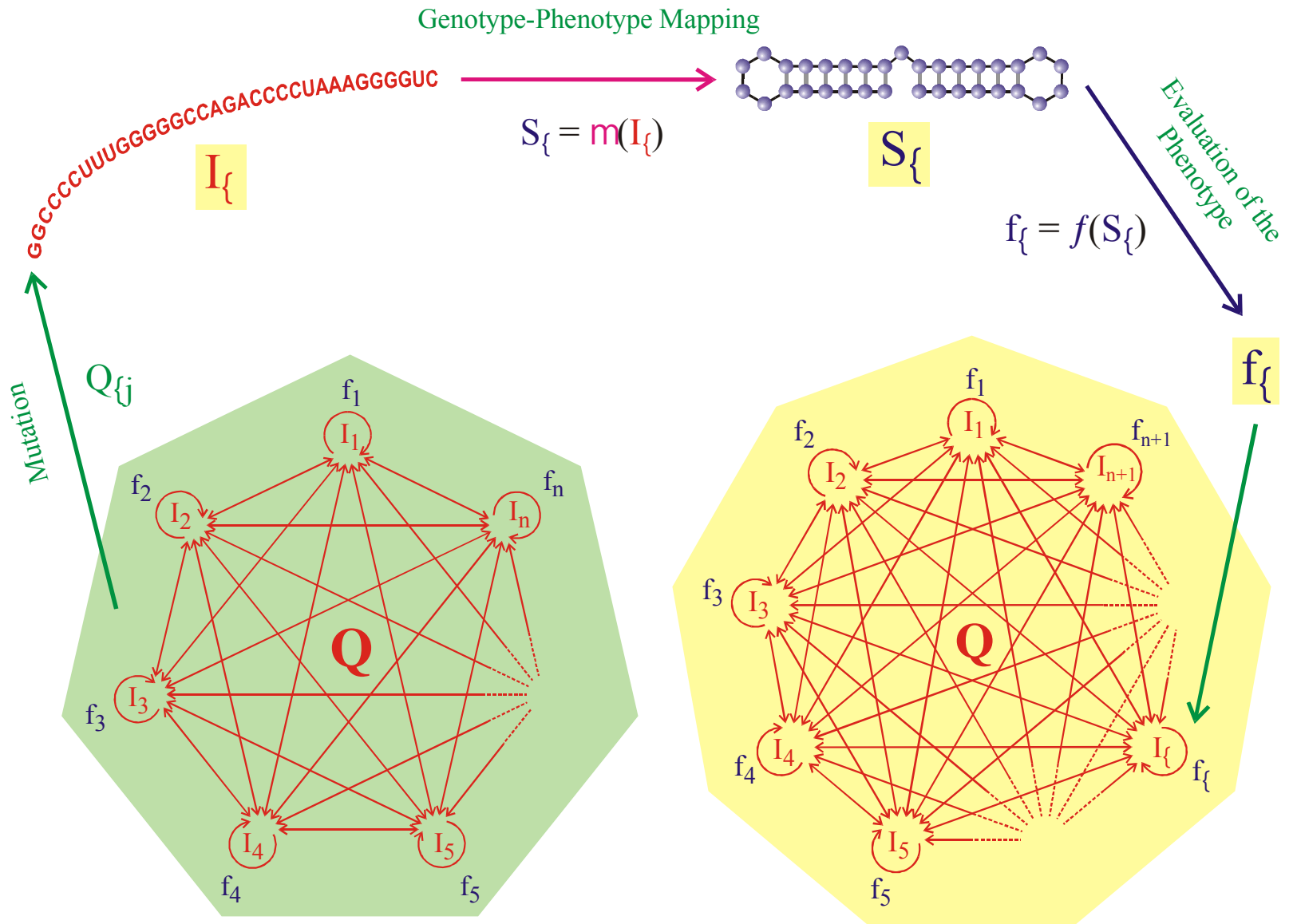
RNA molecules is controlled by the flow

$$N(t) \approx \bar{N} \pm \sqrt{\bar{N}}$$

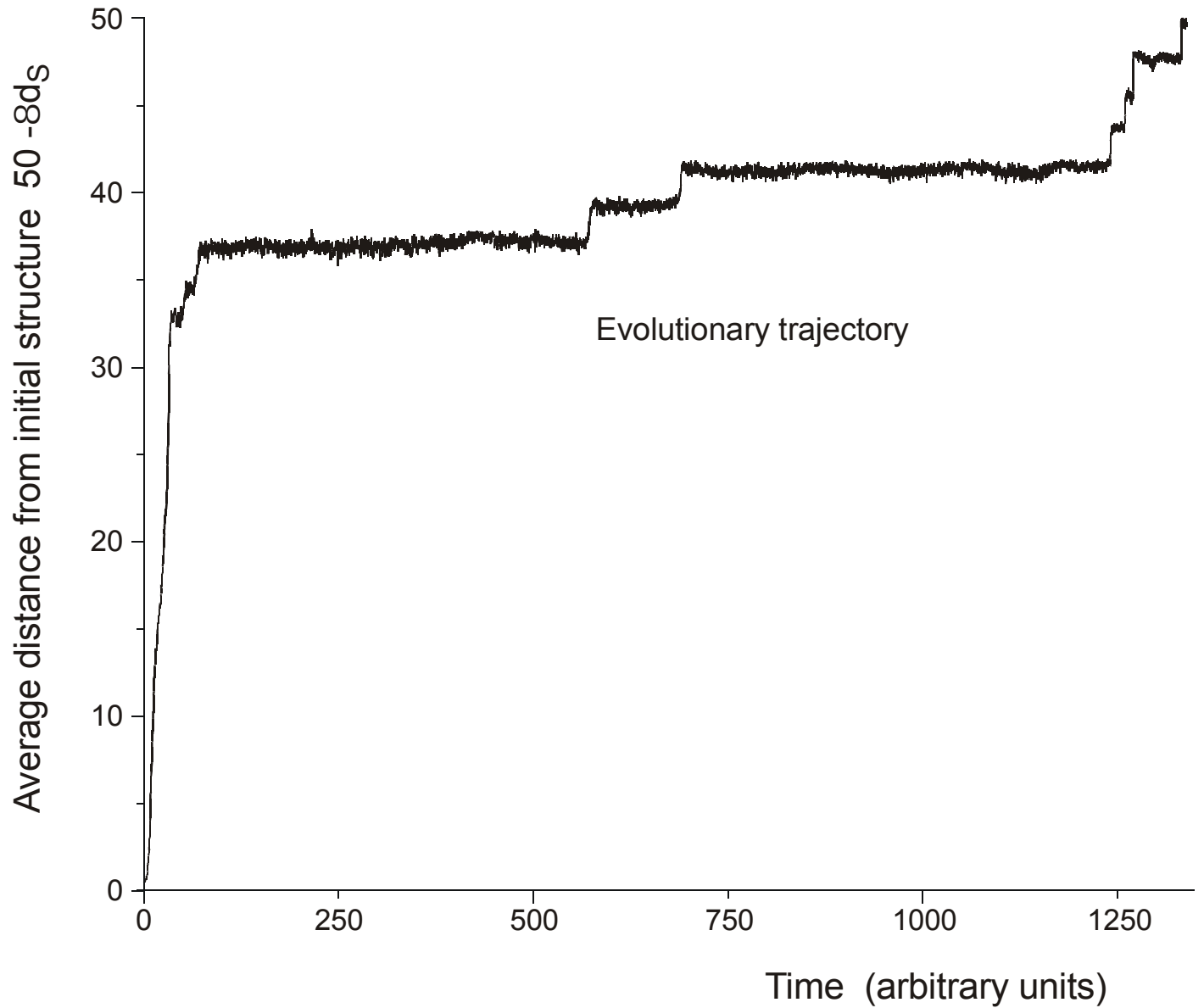
The flowreactor as a device for studies of evolution *in vitro* and *in silico*



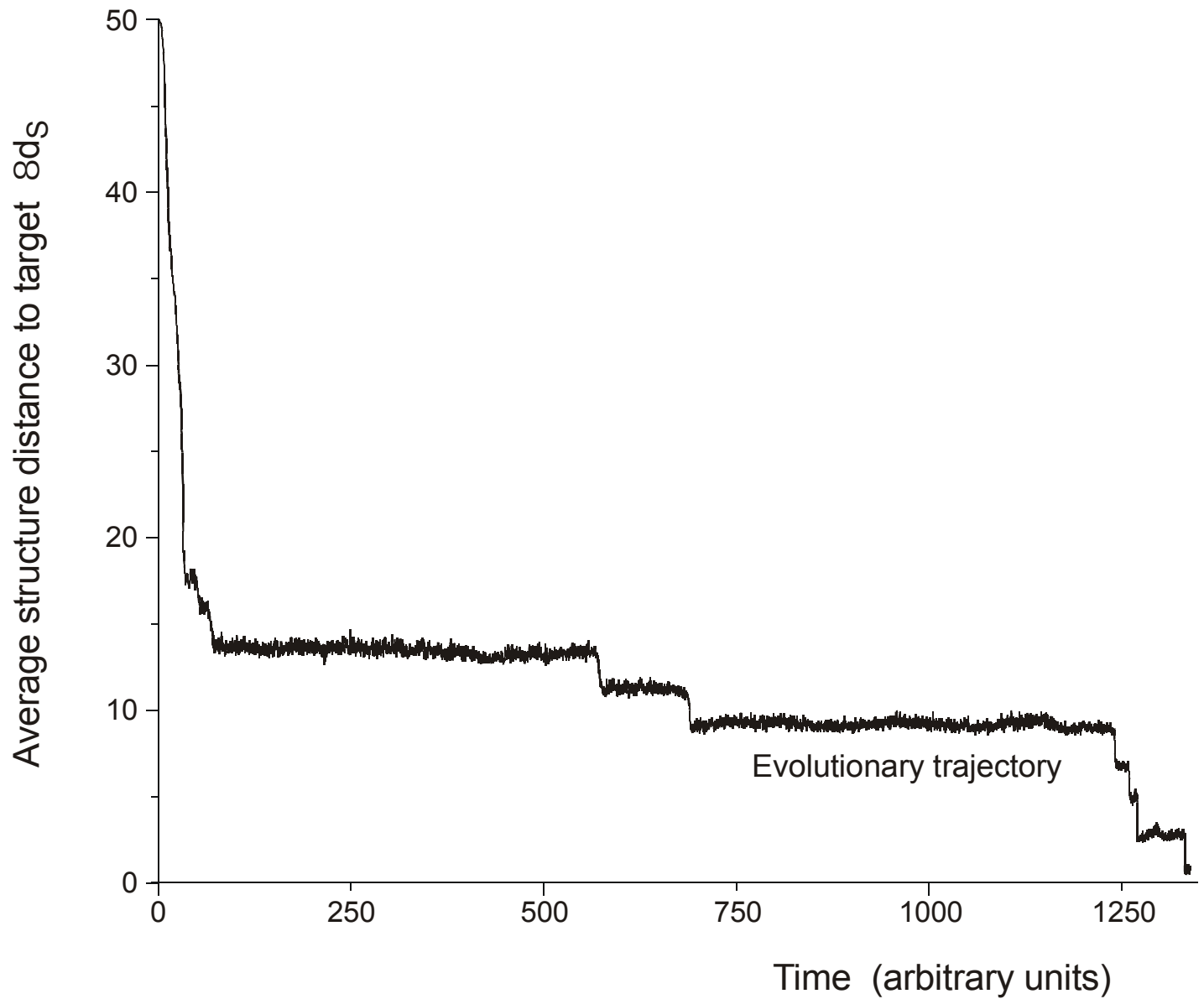
The molecular quasispecies
in sequence space



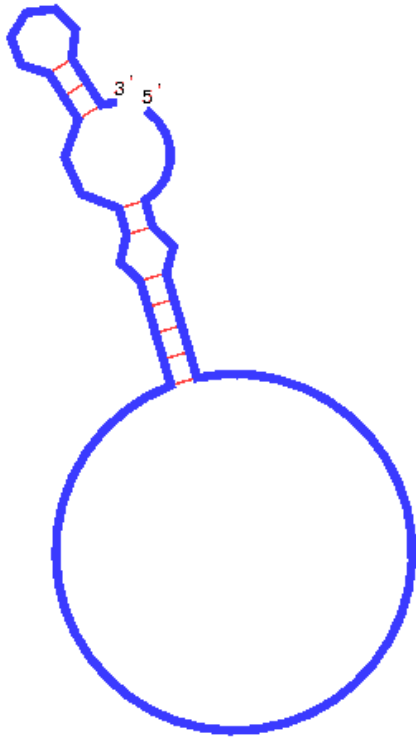
Evolutionary dynamics
including molecular phenotypes



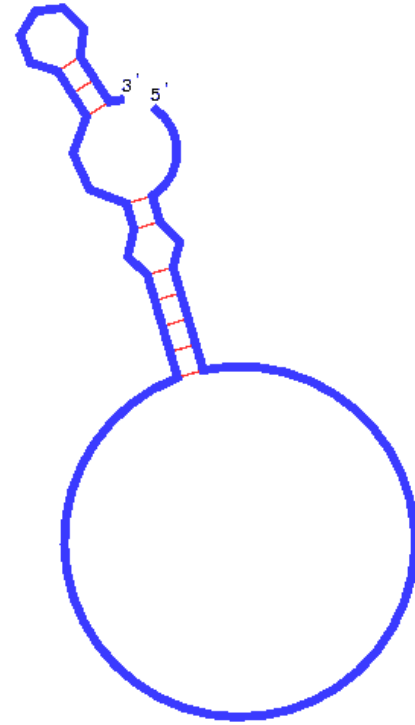
In silico optimization in the flow reactor: Trajectory (**biologists' view**)



In silico optimization in the flow reactor: Trajectory (**physicists' view**)

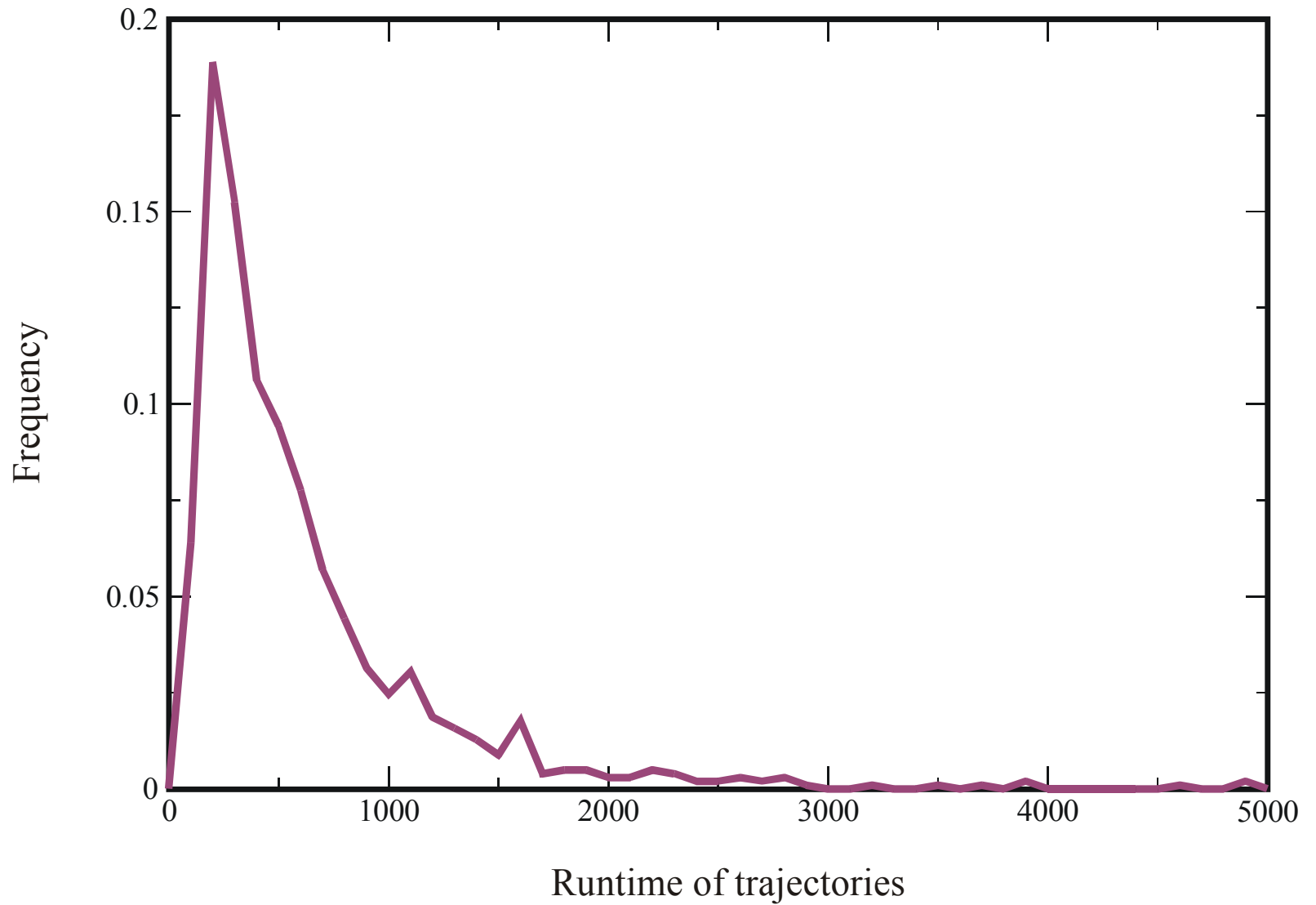


AUGC

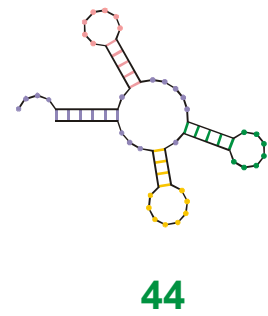
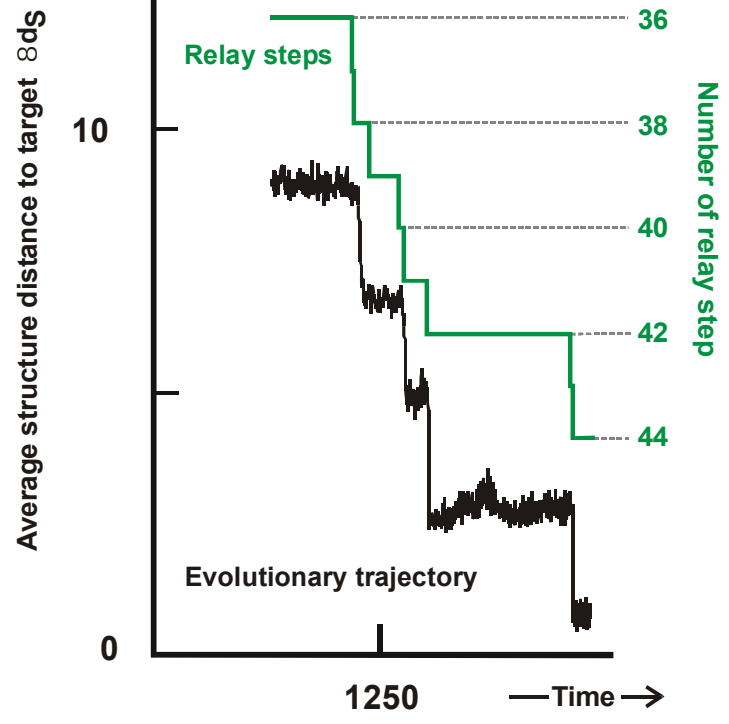


GC

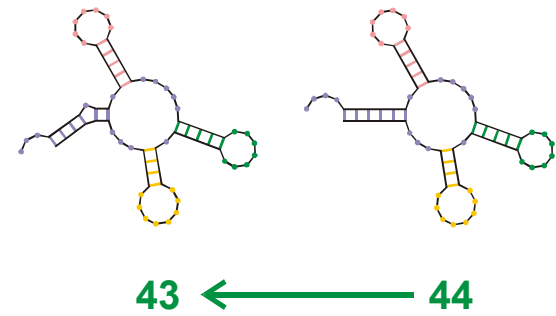
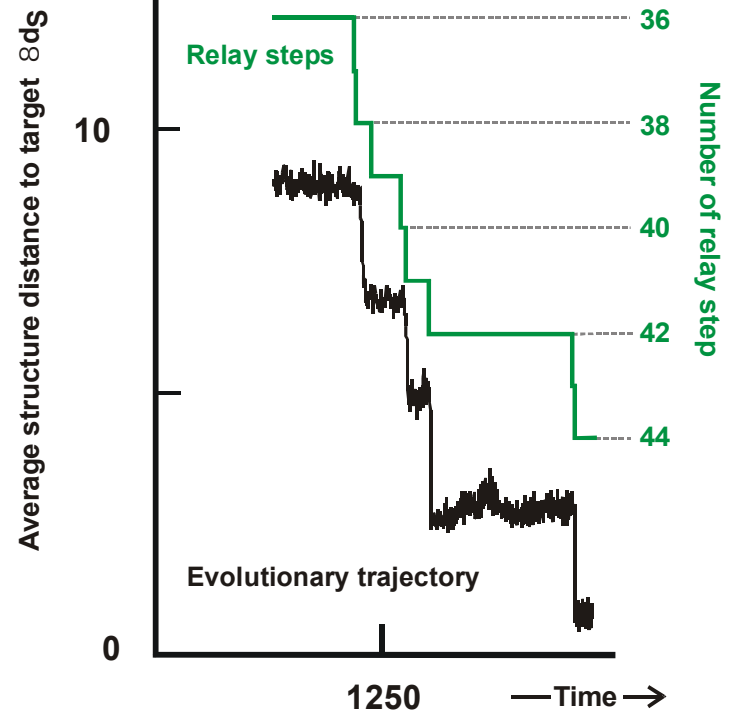
Movies of optimization trajectories over the **AUGC** and the **GC** alphabet



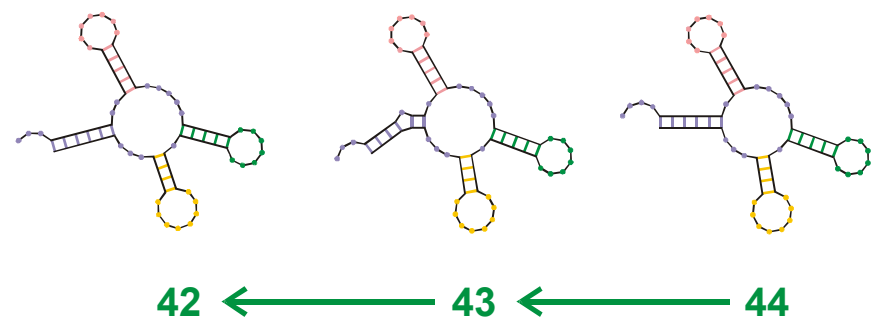
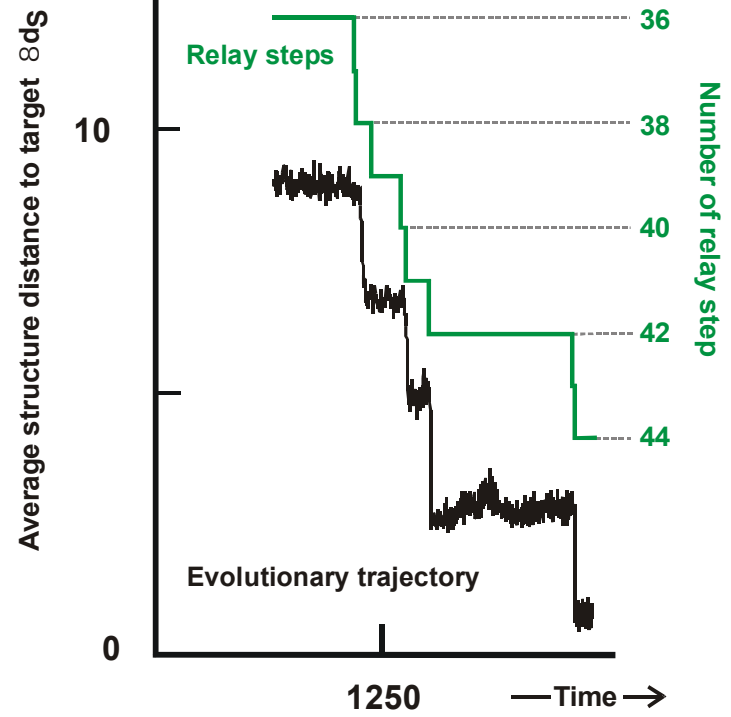
Statistics of the lengths of trajectories from initial structure to target (**AUGC**-sequences)



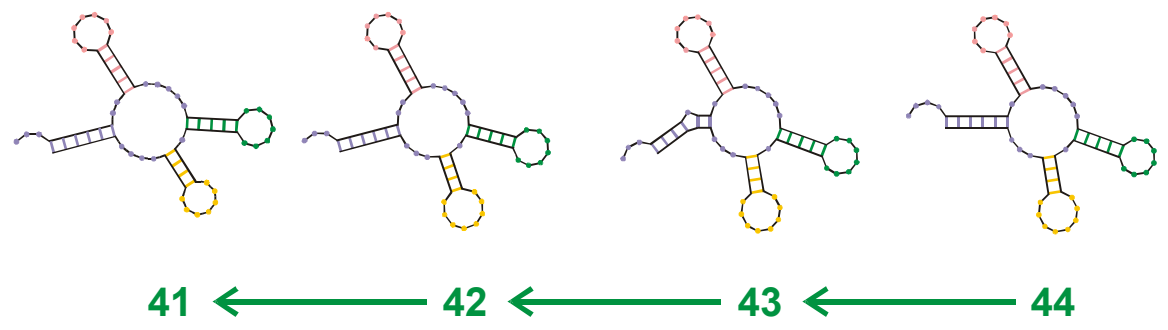
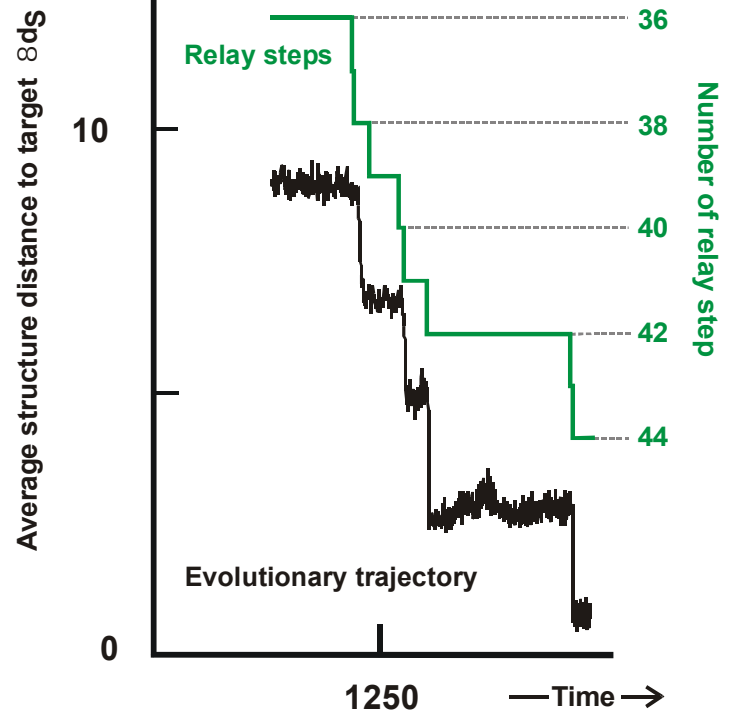
Endconformation of optimization



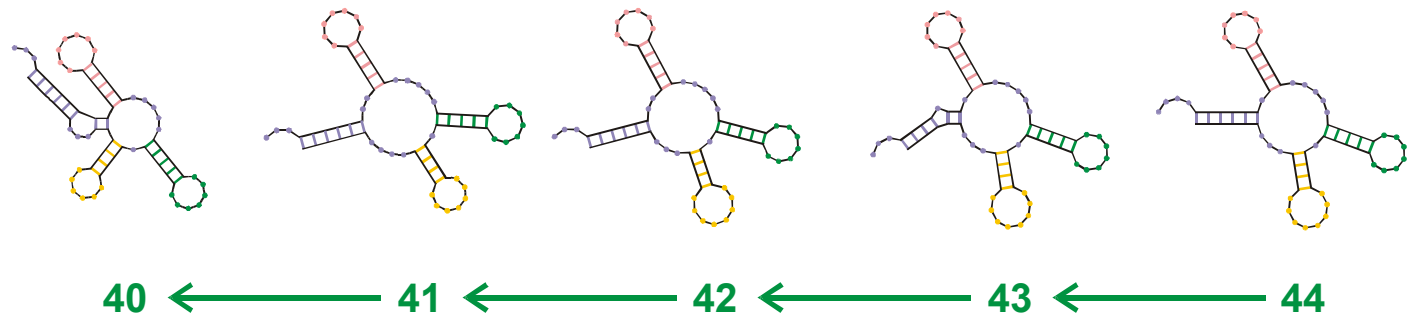
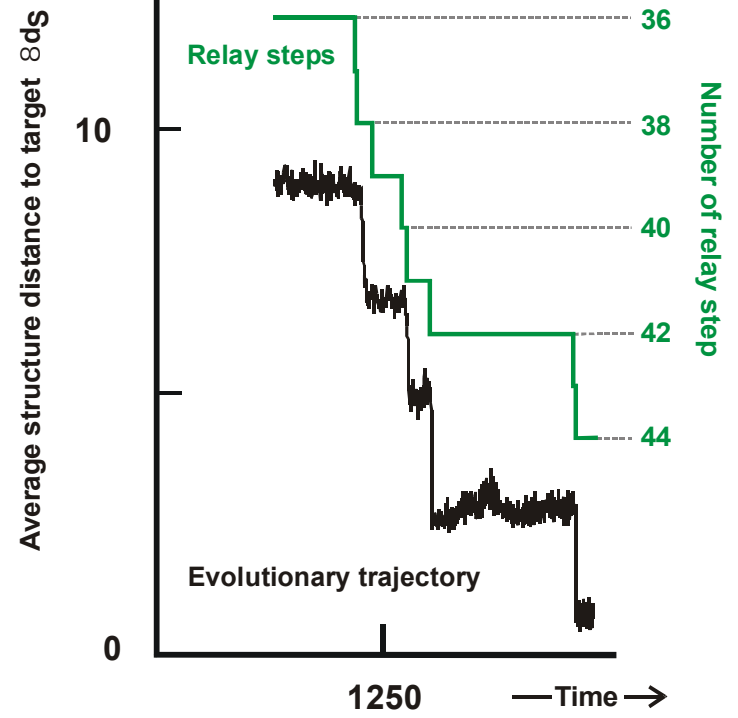
Reconstruction of the last step 43 \rightarrow 44



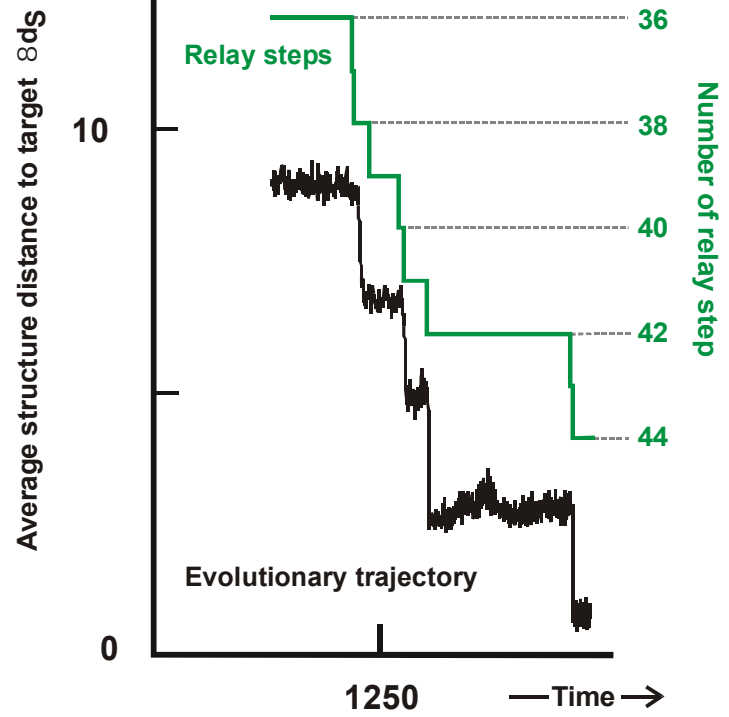
Reconstruction of last-but-one step 42 \checkmark 43 (\checkmark 44)



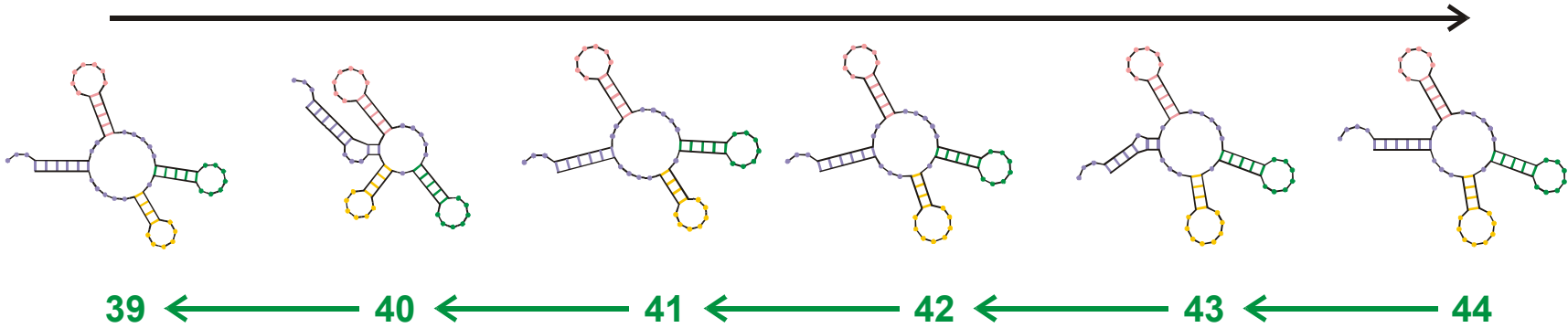
Reconstruction of step 41 š 42 (š 43 š 44)



Reconstruction of step 40 š 41 (š 42 š 43 š 44)



Evolutionary process



39 ← 40 ← 41 ← 42 ← 43 ← 44

Reconstruction

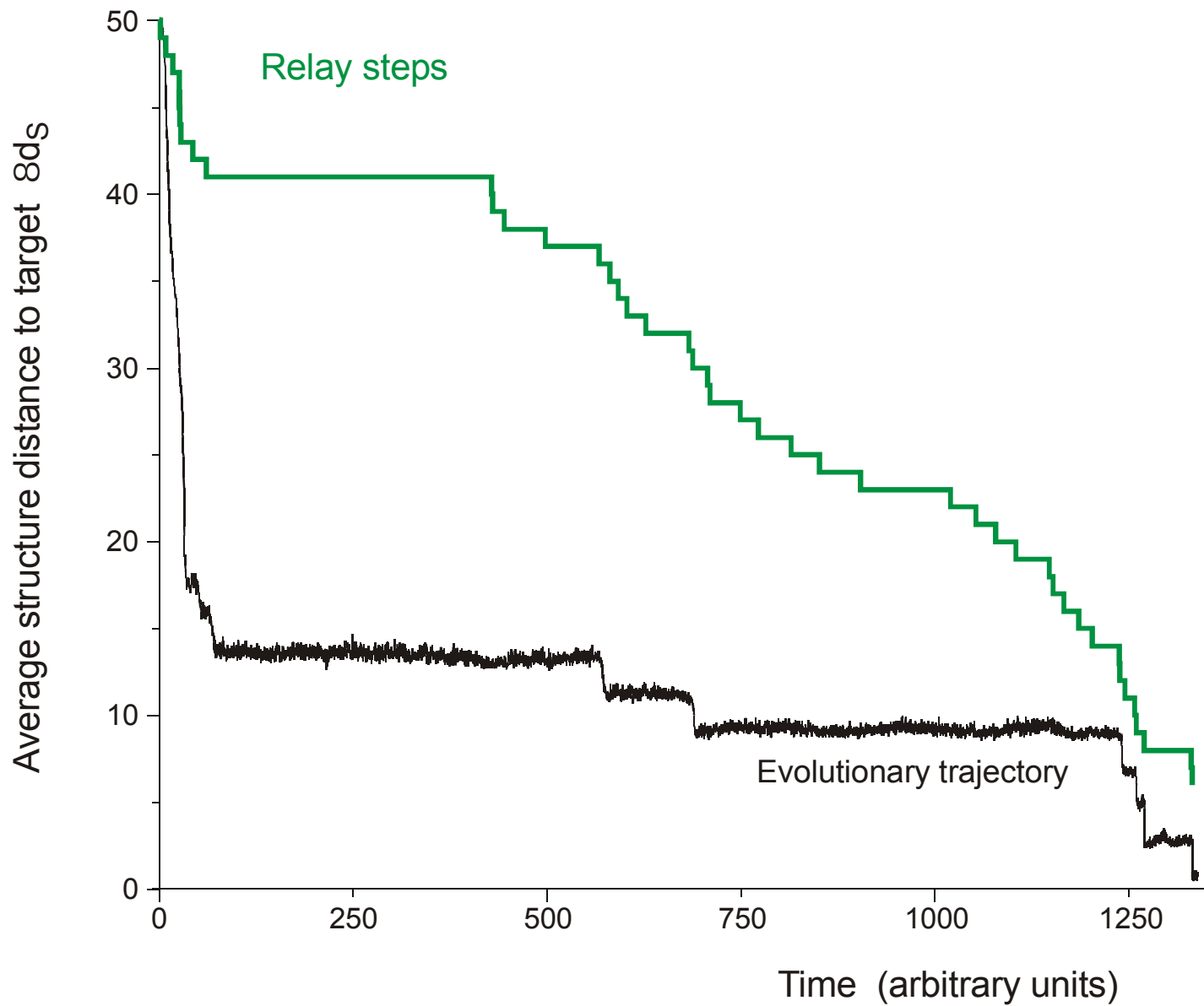
Reconstruction of the relay series

entry 39 GGGAUACAUGUGGCCCCUCAAGGCCCUAGCGAAACUGCUGCUGAAACCGUGUGAAUAAUCCGCACCCUGUCCCGA
 (((((((.....((((.....))))).((((.....))))).(((.....))))).))))))...
 exit GGGAUAUACGAGGCCCGUCAAGGCCGUAAGCGAACGACUGUUGAAACUGUGCGAAUAAUCCGCACCCUGUCCCGGG
 entry 40 GGGAUAUACGGGGGCCCGUCAAGGCCGUAAGCGAACCGACUGUUGAAACUGUGCGAAUAAUCCGCACCCUGUCCCGGG
 (((((((...((((.....))))).((((.....))))).(((.....))))).))))))...
 exit GGGAUAUACGGGGGCCCGUCAAGGCCGUAAGCGAACCGACUGUUGAGACUGUGCGAAUAAUCCGCACCCUGUCCCGGG
 entry 41 GGGAUAUACGGGGGCCCGUCAAGGCCGUAAGCGAACCGACUGUUGAGACUGUGCGAAUAAUCCGCACCCUGUCCCGGG
 (((((((.....((((.....))))).((((.....))))).(((.....))))).))))))...
 exit GGGAUAUACGGGGCCCUUCAAGGCCAUAAGCGAACCGACUGUUGAAACUGUGCGAAUAAUCCGCACCCUGUCCCGGA
 entry 42 GGGAUAUACGGGGCCCUUCAAGGCCAUAAGCGAACCGACUGUUGAAACUGUGCGAAUAAUCCGCACCCUGUCCCGGA
 (((((((...((((.....))))).((((.....))))).(((.....))))).))))))...
 exit GGGAUGAUAGGGCGUGUGAUAGCCCAUAGCGAACCCCGCUGAGCUUGUGCGACGUUUGUGCACCUGUCCCGCU
 entry 43 GGGAGAUAGGGCGUGUGAUAGCCCAUAGCGAACCCCGCUGAGCUUGUGCGACGUUUGUGCACCUGUCCCGCU
 (((((((...((((.....))))).((((.....))))).(((.....))))).))))))...
 exit GGGAGAUAGGGCGUGUGAUAGCCCAUAGCGAACCCCGCUGAGCUUGUGCGACGUUUGUGCACCUGUCCCGCU
 entry 44 GGGAGAUAGGGCGUGUGAUAGCCCAUAGCGAACCCCGCUGAGCUUGUGCGACGUUUGUGCACCUGUCCCGCU
 (((((((...((((.....))))).((((.....))))).(((.....))))).))))))...

Transition inducing point mutations

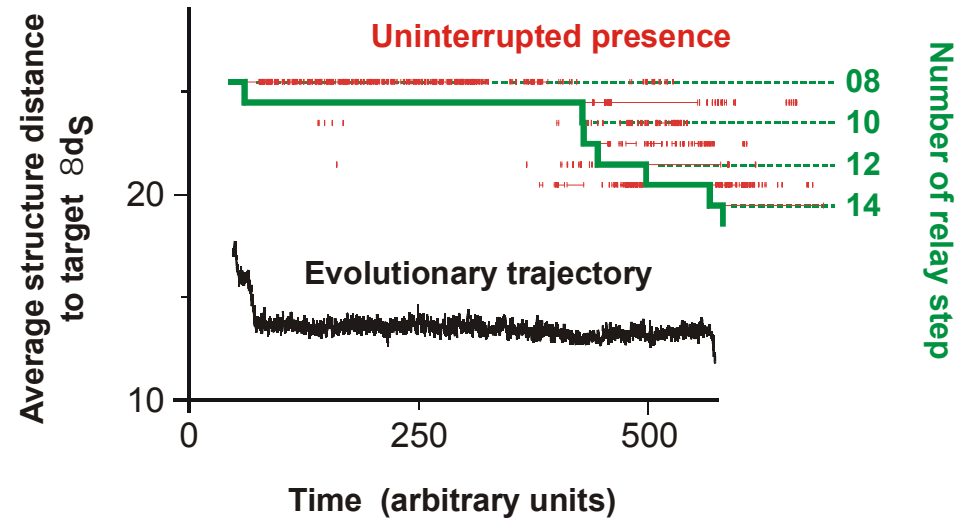
Neutral point mutations

Change in RNA sequences during the final five relay steps 39 § 44



In silico optimization in the flow reactor: Trajectory and relay steps

28 neutral point mutations during a long quasi-stationary epoch

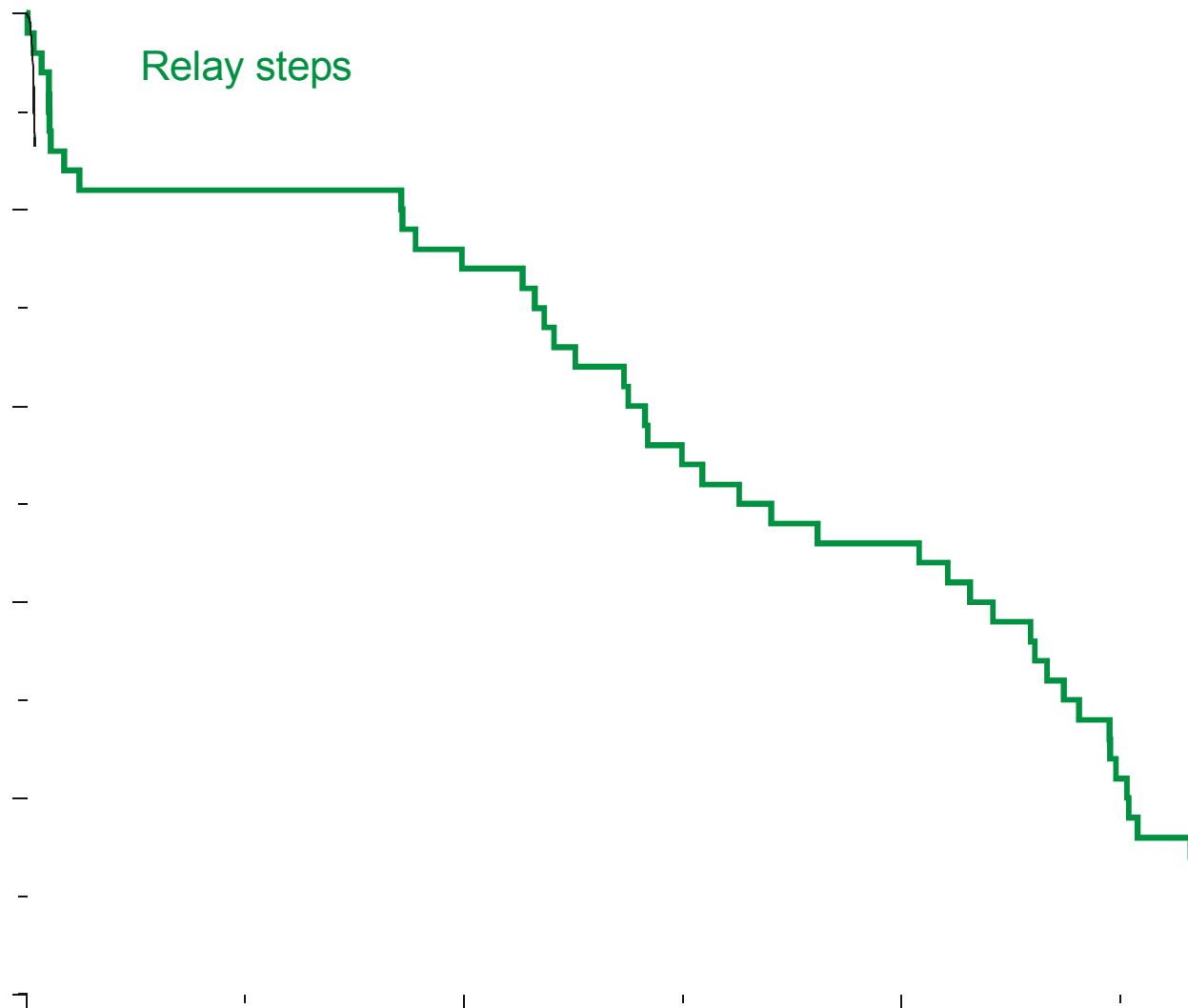


entry 8 GGUAUGGGCGUUGAAUAGUAGGGUUUAAACCAAUCGGCAACGAUCUCGUGUGCGCAUUUCAUAUCCCGUACAGAA
 .(((((((((((((. (((.))))))(((((.)))))))))
 exit 8 GGUAUGGGCGUUGAAUAUJAGGGUUUAAACCAAUCGGCCAACGAUCUCGUGUGCGCAUUUCAUAUCCAUAACAGAA
 entry 9 GGUAUGGGCGUUGAAUAAUAGGGUUUAAACCAAUCGGCCAACGAUCUCGUGUGCGCAUUUCAUAUAACCAUACAGAA
 .(((((((((. (((.))))))(((((.))))))
 exit 9 UGGAUGGACGUUGAAUAACAAGGUAUCGACCAAACAACCAACGAGUAAGUGUGUACGCCCCACACACCGUCCCAAG
 entry 10 UGGAUGGACGUUGAAUAACAAGGUAUCGACCAAACAACCAACGAGUAAGUGUGUACGCCCCACACACCGUCCCAAG
 .(((((. (((.))))))(((((.))))
 exit 10 UGGAUGGACGUUGAAUAACAAGGUAUCGACCAAACAACCAACGAGUAAGUGUGUACGCCCCACACAGCGUCCCAAG

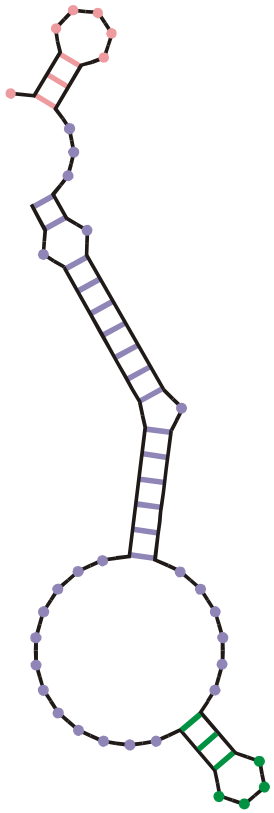
Transition inducing point mutations

Neutral point mutations

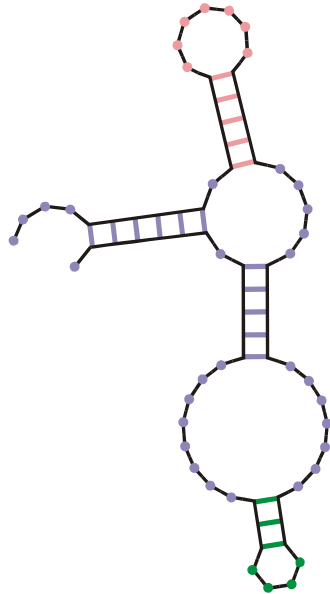
Neutral genotype evolution during phenotypic stasis



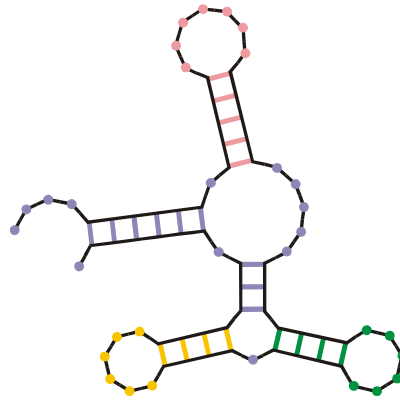
In silico optimization in the flow reactor: Main transitions



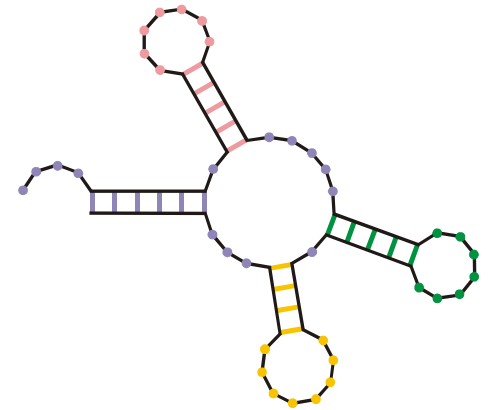
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09

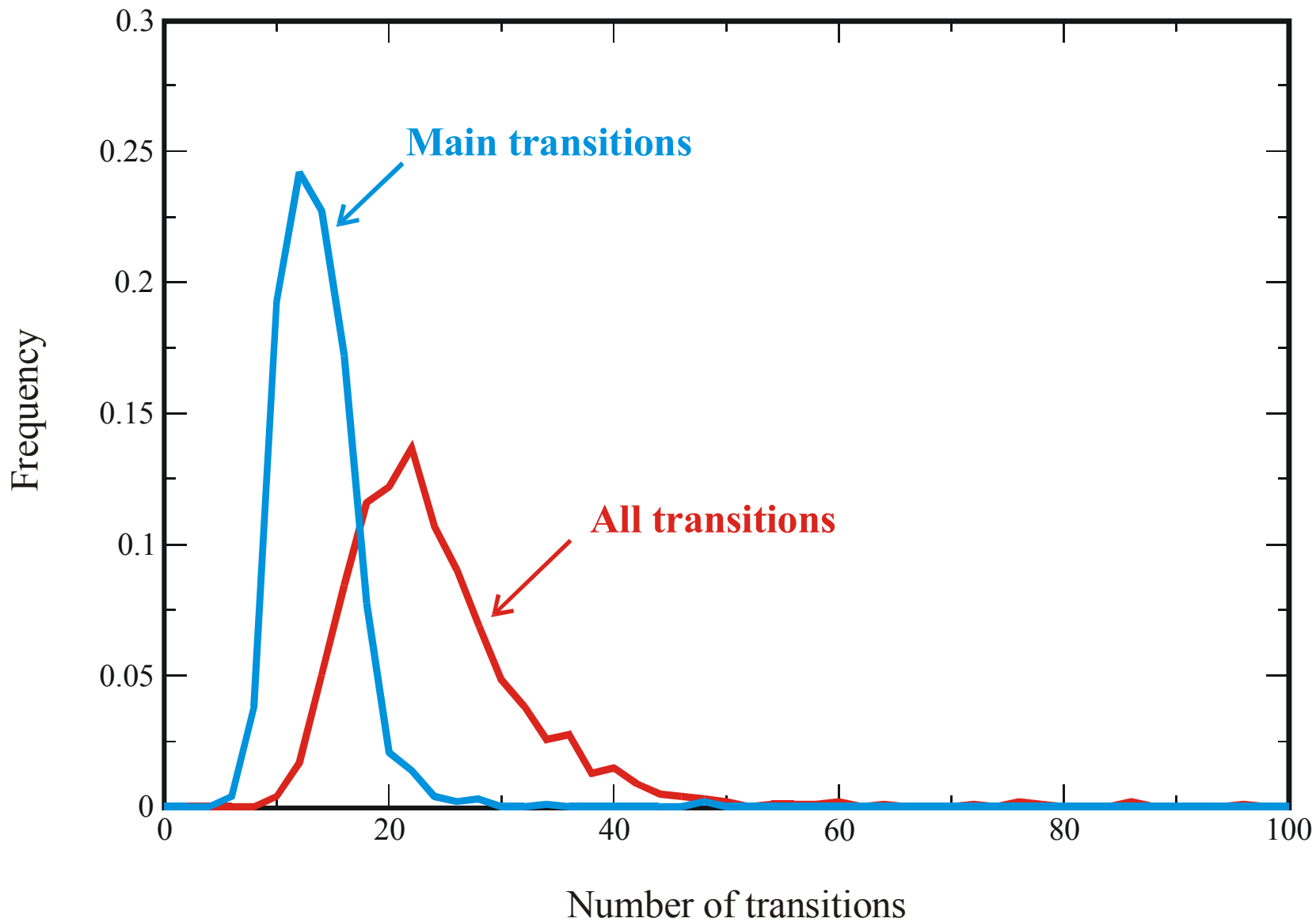


31



44

Three important steps in the formation of the tRNA clover leaf from a randomly chosen initial structure corresponding to three **main transitions**.



Statistics of the numbers of transitions from initial structure to target (**AUGC**-sequences)

Alphabet	Runtime	Transitions	Main transitions	No. of runs
AUGC	385.6	22.5	12.6	1017
GUC	448.9	30.5	16.5	611
GC	2188.3	40.0	20.6	107

Statistics of trajectories and relay series (mean values of log-normal distributions)

1. Controlled evolution experiments with bacteria and RNA

2. Optimization in the RNA model

3. Sequence-structure maps, neutral networks, and intersections

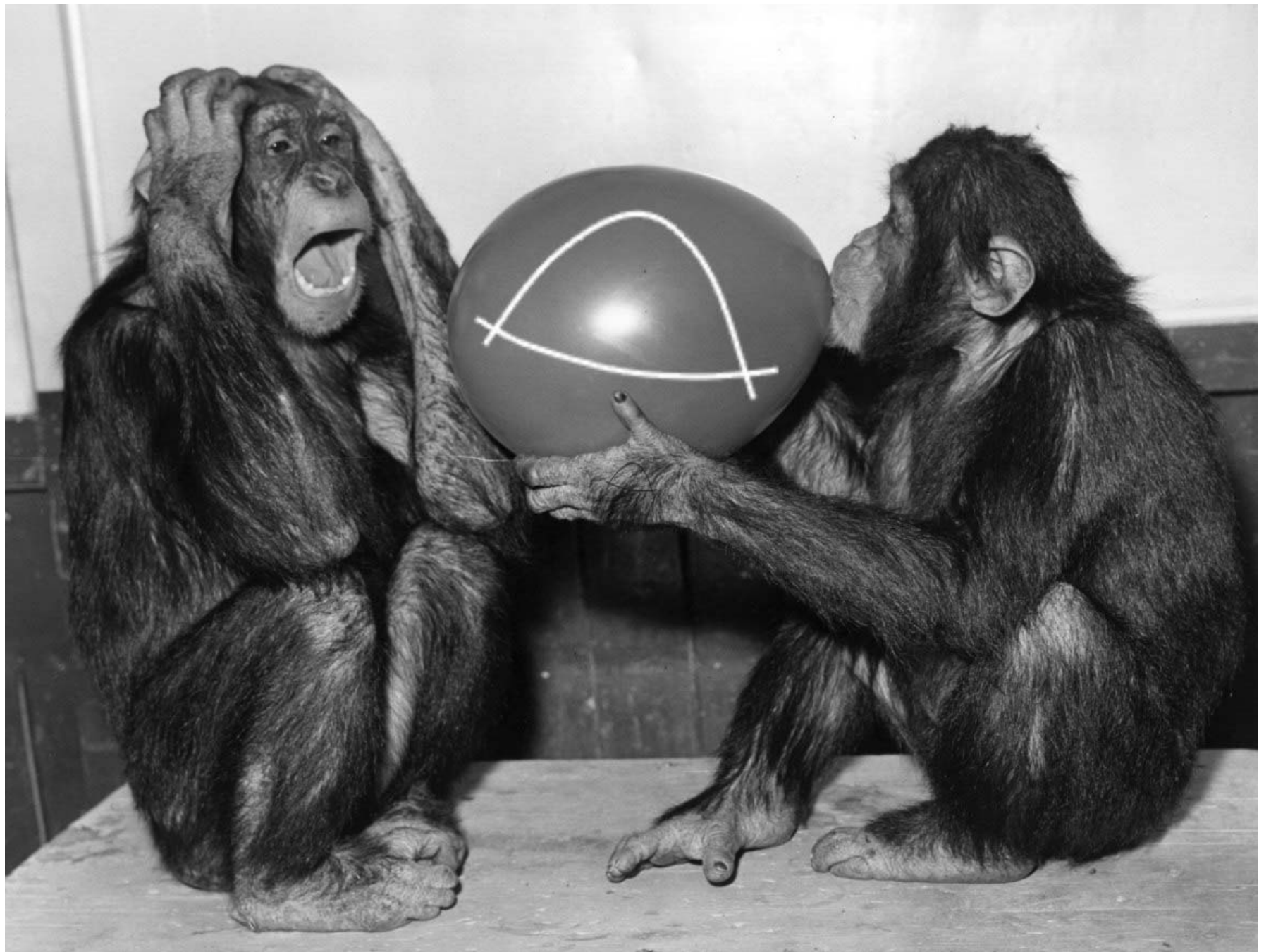
4. Selection experiments and design of RNA molecules

The inverse folding algorithm searches for sequences that form a given RNA secondary structure under the minimum free energy criterion.

RNA **sequences** as well as RNA secondary **structures** can be visualized as objects in **metric spaces**. At constant chain length the sequence space is a (generalized) hypercube.

The **mapping** from RNA **sequences** into RNA secondary **structures** is many-to-one. Hence, it is redundant and not invertible.

RNA **sequences**, which are mapped onto the same RNA secondary **structure**, are **neutral** with respect to **structure**. The pre-images of structures in sequence space are **neutral networks**. They can be represented by graphs where the edges connect sequences of Hamming distance $d_H = 1$.



Theory of sequence – structure mappings

P. Schuster, W.Fontana, P.F.Stadler, I.L.Hofacker, *From sequences to shapes and back: A case study in RNA secondary structures*. Proc.Roy.Soc.London **B 255** (1994), 279-284

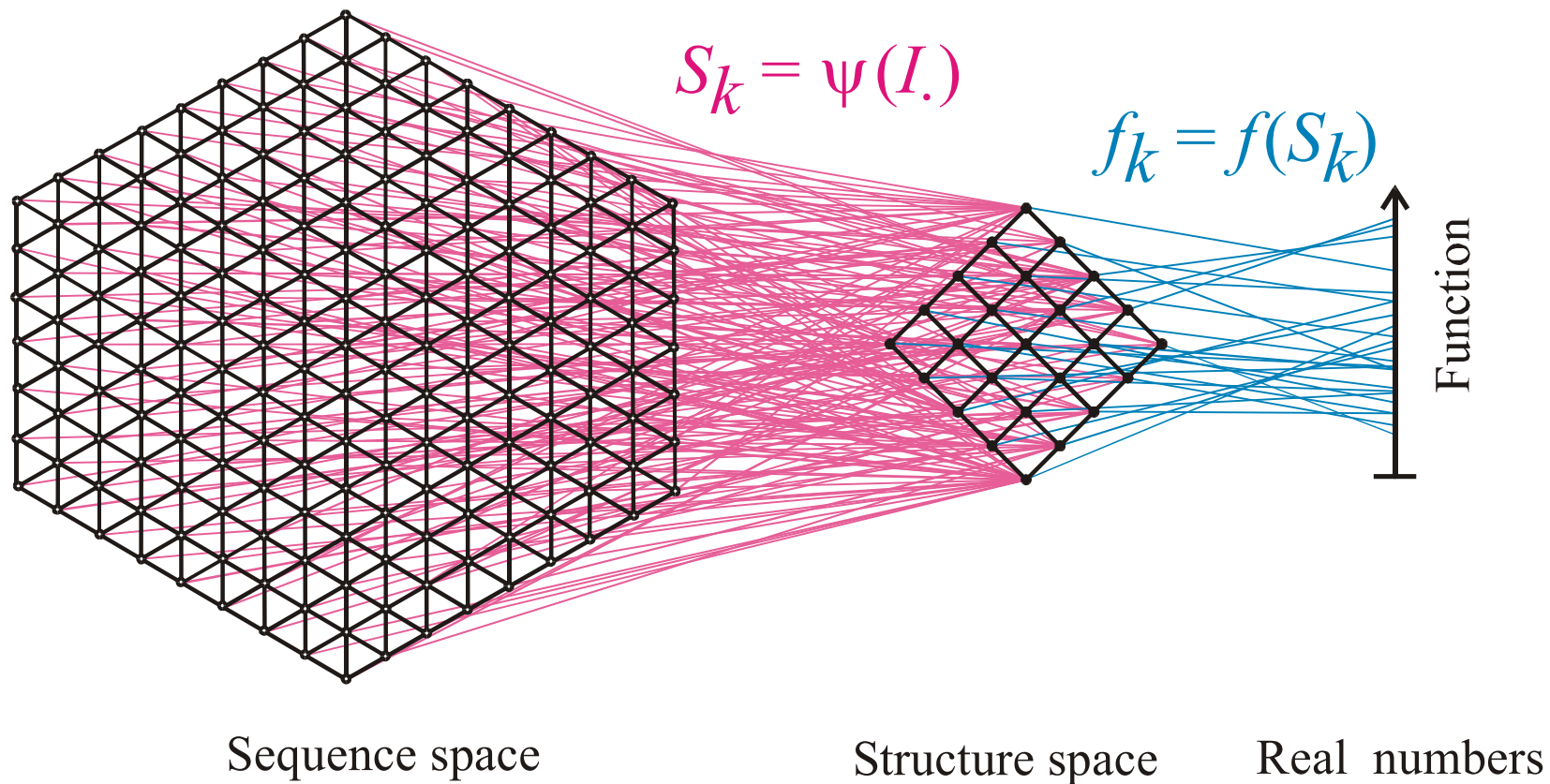
W.Grüner, R.Giegerich, D.Strothmann, C.Reidys, I.L.Hofacker, P.Schuster, *Analysis of RNA sequence structure maps by exhaustive enumeration. I. Neutral networks*. Mh.Chem. **127** (1996), 355-374

W.Grüner, R.Giegerich, D.Strothmann, C.Reidys, I.L.Hofacker, P.Schuster, *Analysis of RNA sequence structure maps by exhaustive enumeration. II. Structure of neutral networks and shape space covering*. Mh.Chem. **127** (1996), 375-389

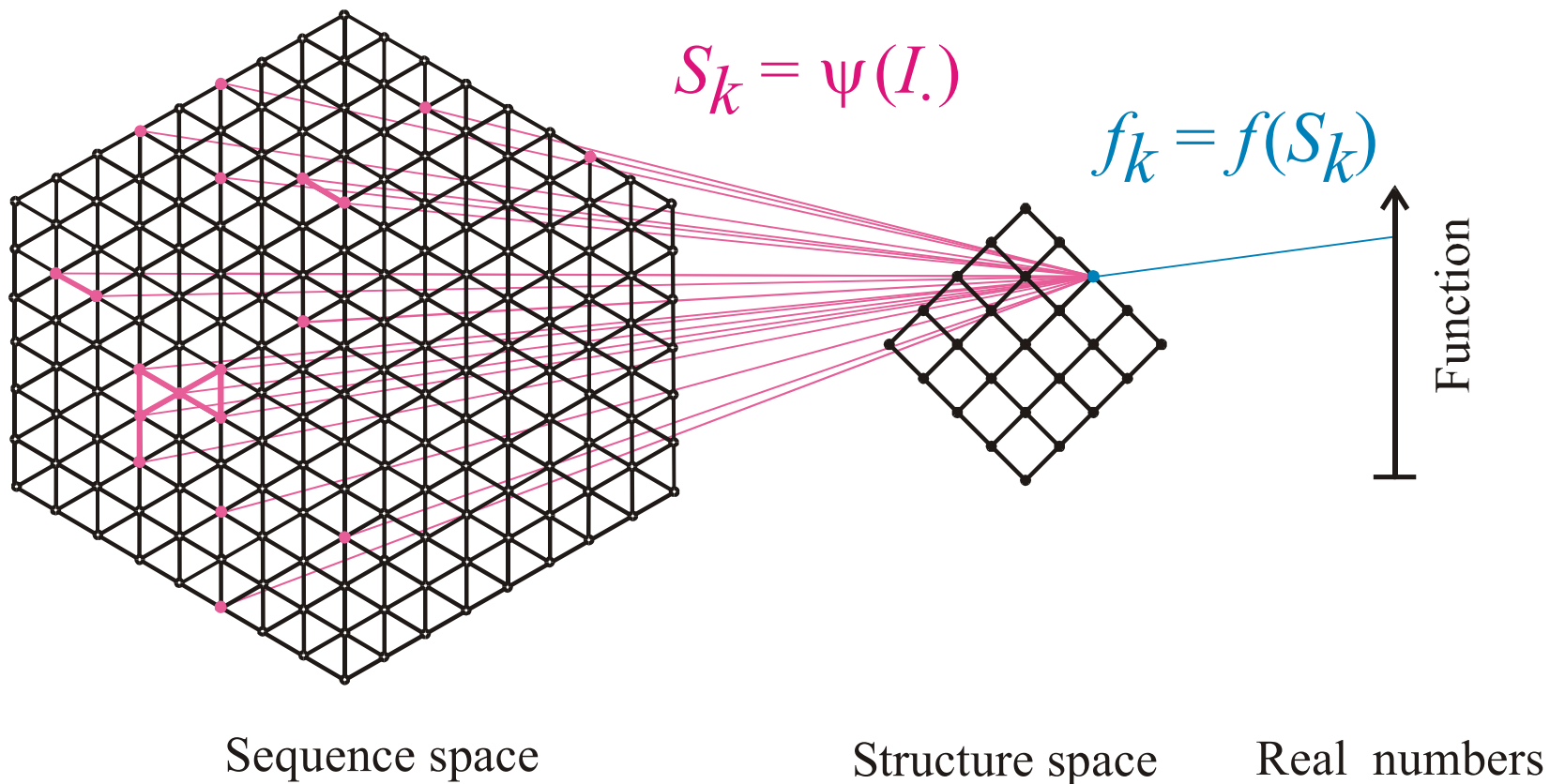
C.M.Reidys, P.F.Stadler, P.Schuster, *Generic properties of combinatory maps*. Bull.Math.Biol. **59** (1997), 339-397

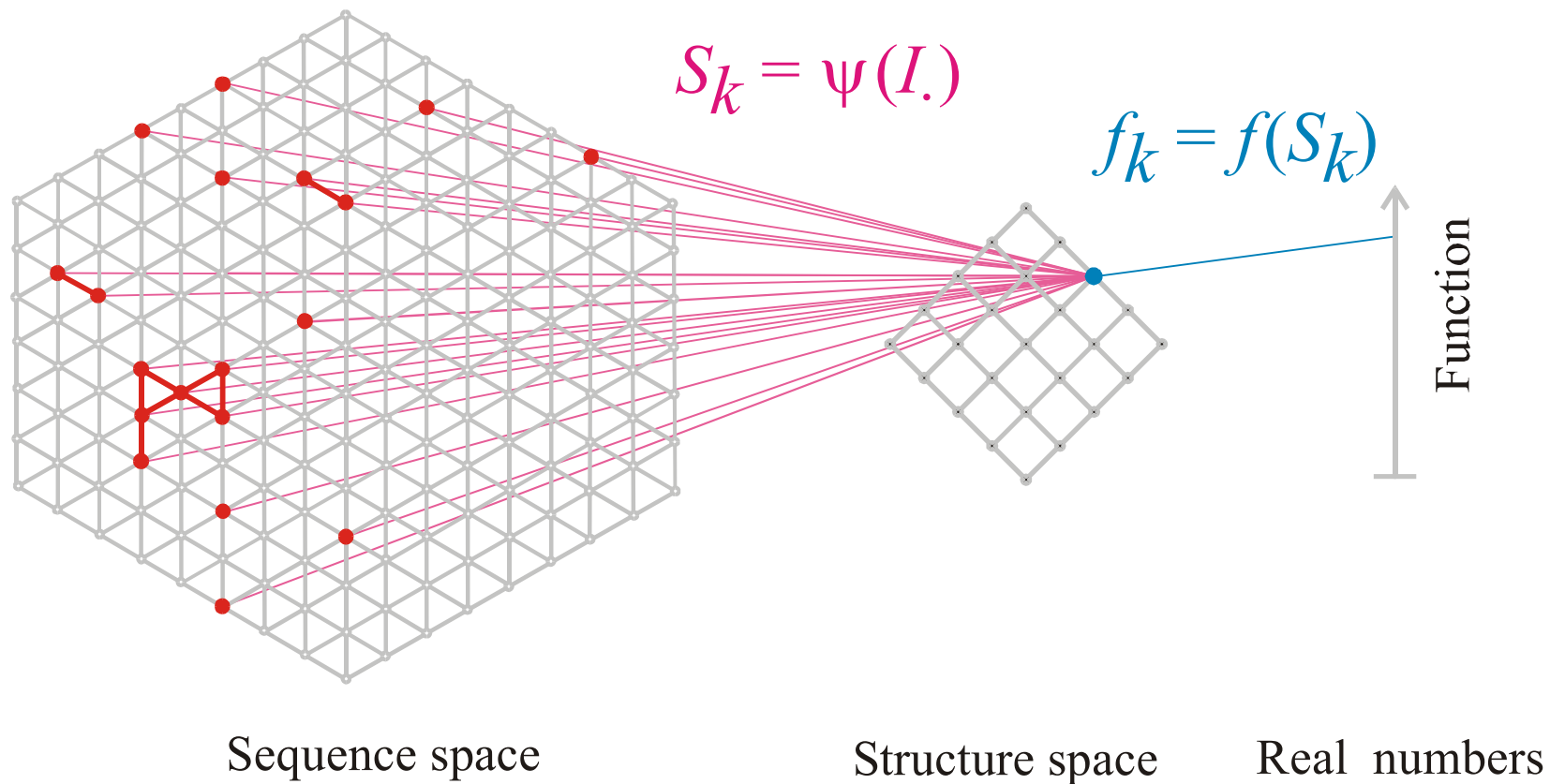
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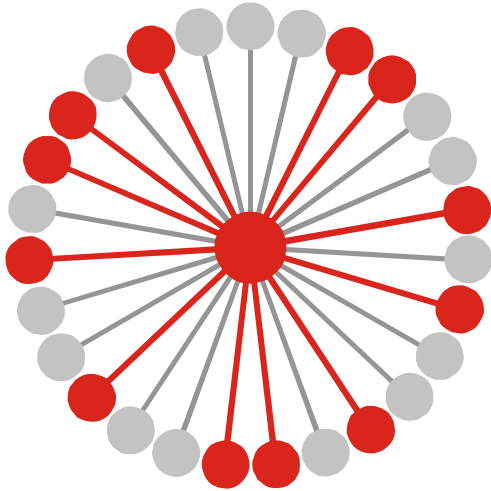


Mapping from sequence space into structure space and into function





The pre-image of the structure S_k in sequence space is the **neutral network G_k**



$$G_k = m^{-1}(S_k) \cup \{I_j \mid m(I_j) = S_k\}$$

$$\lambda_j = 12 / 27 = 0.444, \quad \bar{\lambda}_k = \frac{\sum_{j \in |G_k|} \lambda_j(k)}{|G_k|}$$

Connectivity threshold: $\lambda_{cr} = 1 - \kappa^{-1/(\kappa-1)}$

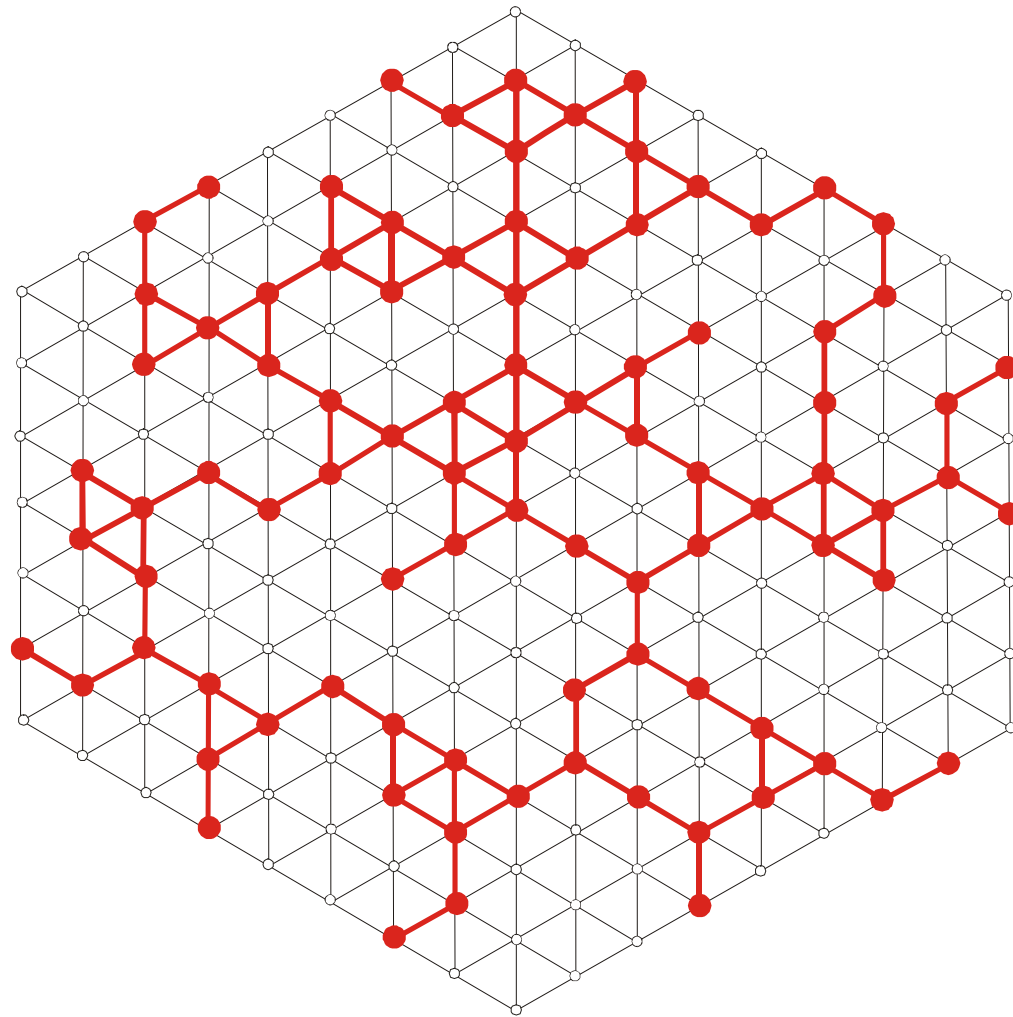
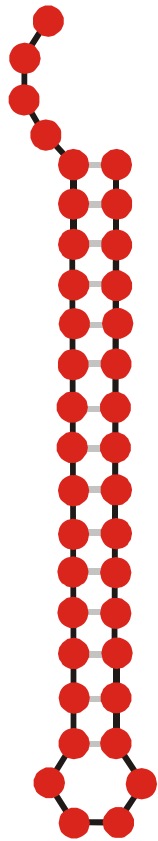
Alphabet size κ : **AUGC** | $\kappa = 4$

$\bar{\lambda}_k > \lambda_{cr}$ network **G_k** is connected

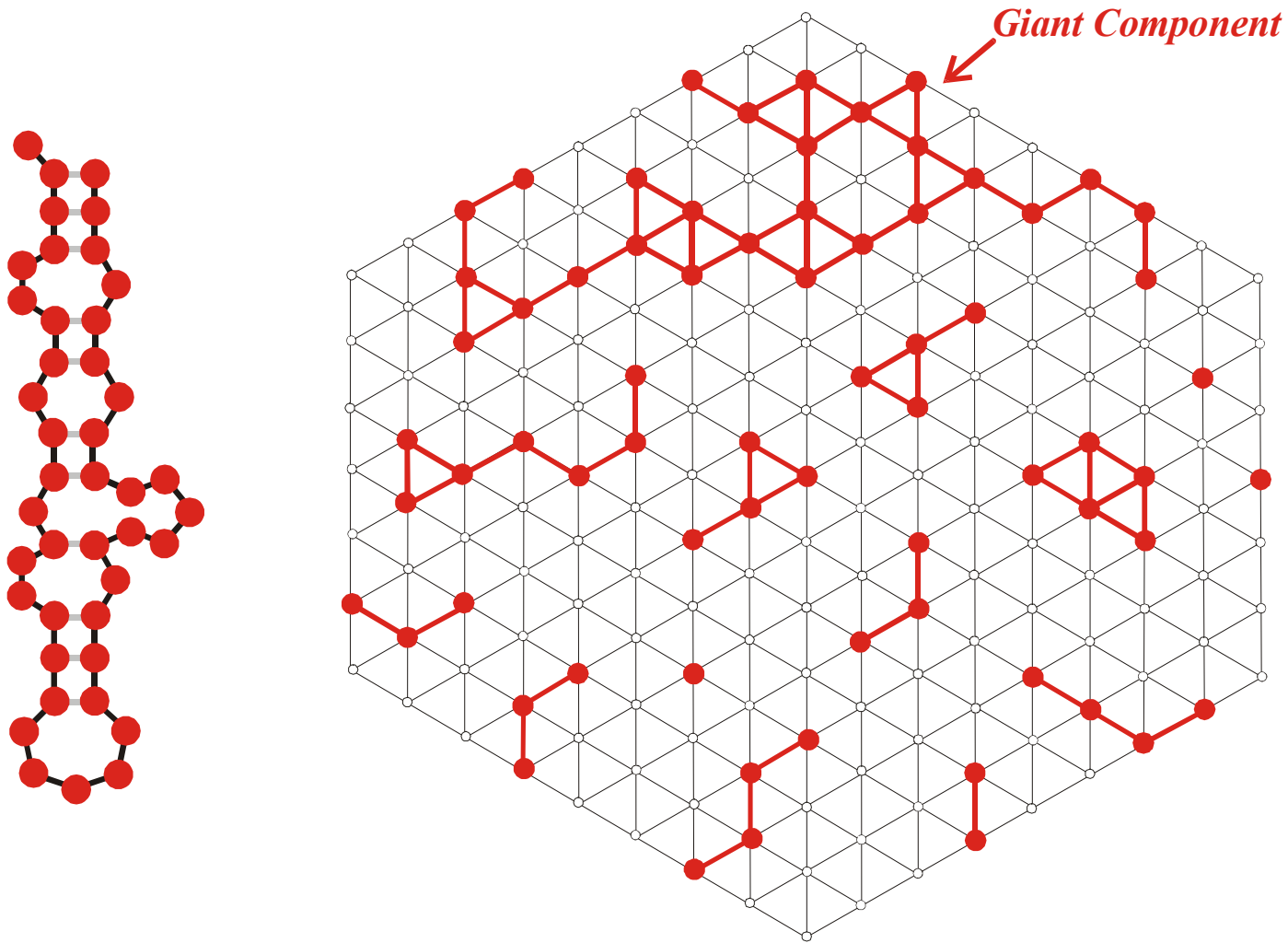
$\bar{\lambda}_k < \lambda_{cr}$ network **G_k** is **not** connected

κ	λ_{cr}	
2	0.5	GC,AU
3	0.423	GUC,AUG
4	0.370	AUGC

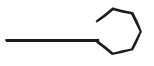
Mean degree of neutrality and connectivity of neutral networks



A connected neutral network



A multi-component neutral network



Degree of neutrality of cloverleaf RNA secondary structures over different alphabets

From sequences to shapes and back: a case study in RNA secondary structures

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AND IVO L. HOFACKER²

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² Institut für Theoretische Chemie, Universität Wien, Austria

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SUMMARY

RNA folding is viewed here as a map assigning secondary structures to sequences. At fixed chain length the number of sequences far exceeds the number of structures. Frequencies of structures are highly non-uniform and follow a generalized form of Zipf's law: we find relatively few common and many rare ones. By using an algorithm for inverse folding, we show that sequences sharing the same structure are distributed randomly over sequence space. All common structures can be accessed from an arbitrary sequence by a number of mutations much smaller than the chain length. The sequence space is percolated by extensive neutral networks connecting nearest neighbours folding into identical structures. Implications for evolutionary adaptation and for applied molecular evolution are evident: finding a particular structure by mutation and selection is much simpler than expected and, even if catalytic activity should turn out to be sparse in the space of RNA structures, it can hardly be missed by evolutionary processes.

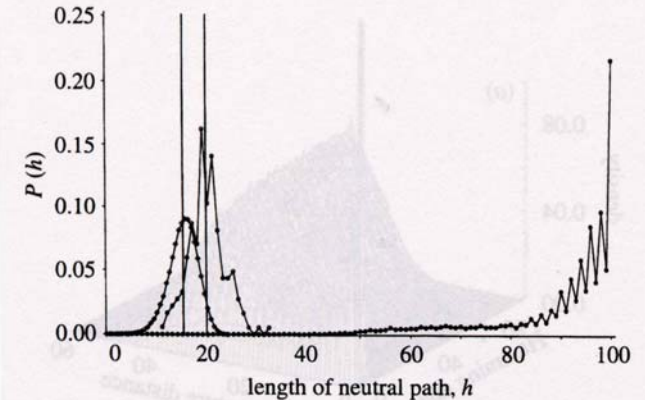
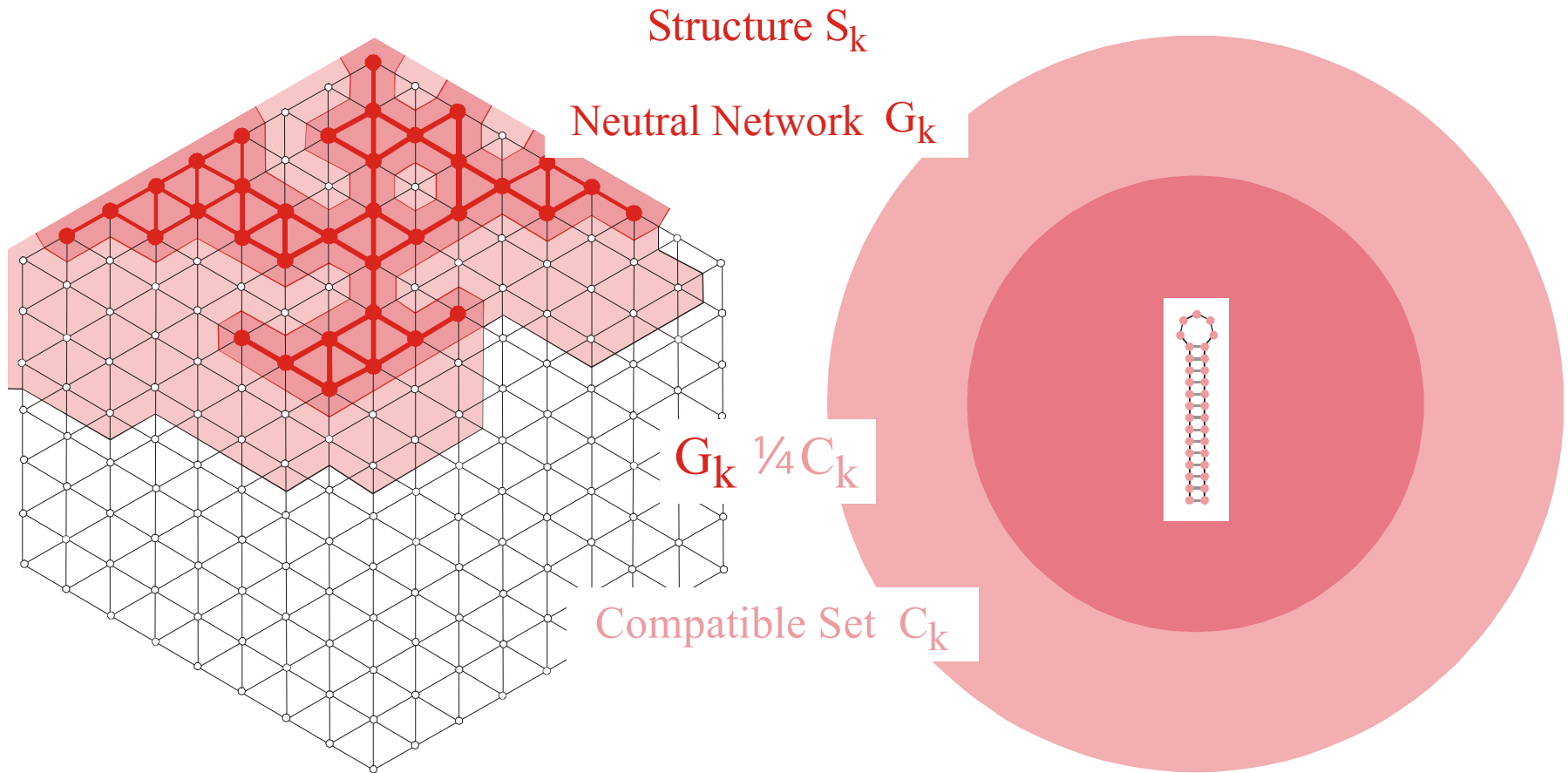
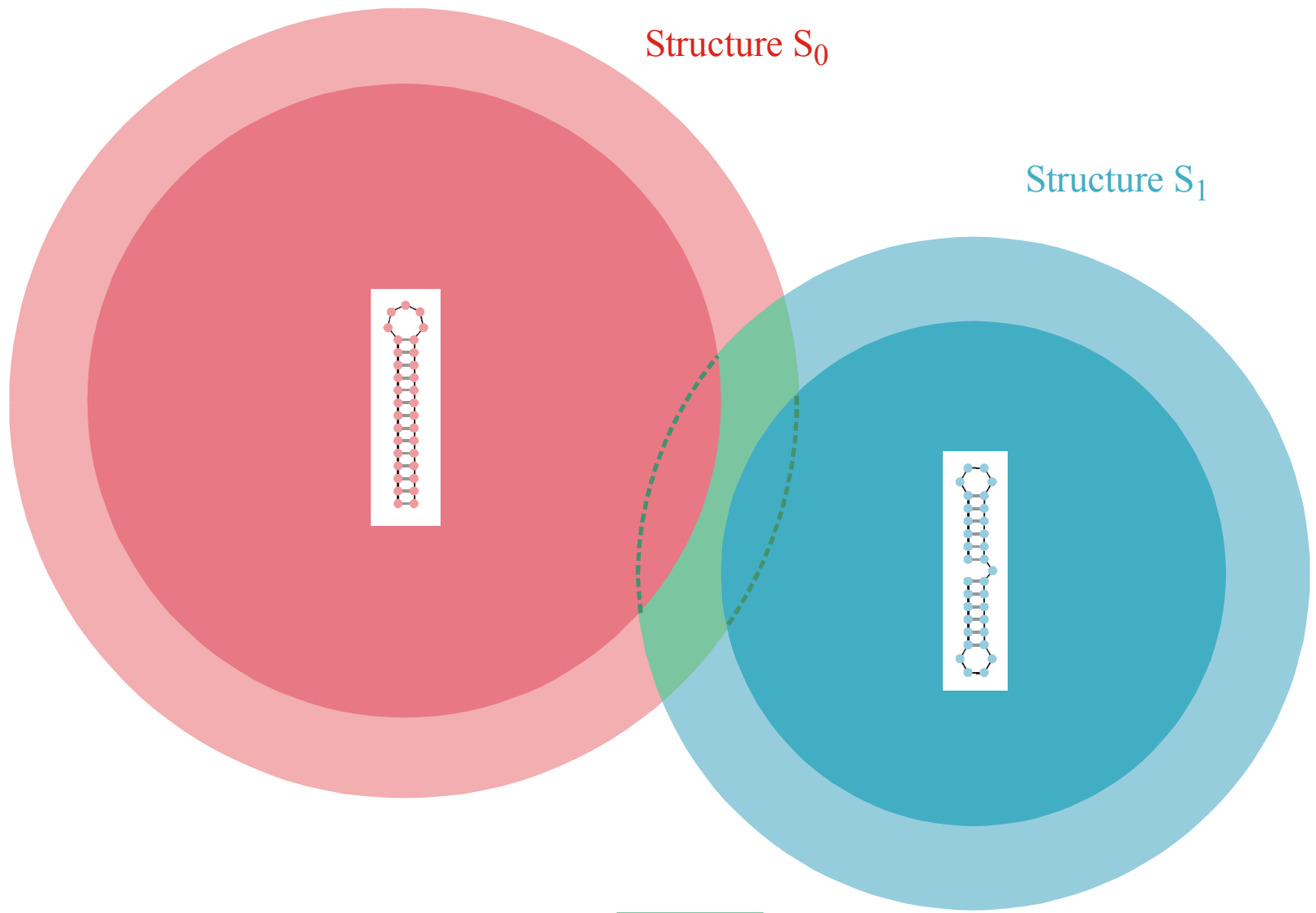


Figure 4. Neutral paths. A neutral path is defined by a series of nearest neighbour sequences that fold into identical structures. Two classes of nearest neighbours are admitted: neighbours of Hamming distance 1, which are obtained by single base exchanges in unpaired stretches of the structure, and neighbours of Hamming distance 2, resulting from base pair exchanges in stacks. Two probability densities of Hamming distances are shown that were obtained by searching for neutral paths in sequence space: (i) an upper bound for the closest approach of trial and target sequences (open circles) obtained as endpoints of neutral paths approaching the target from a random trial sequence (185 targets and 100 trials for each were used); (ii) a lower bound for the closest approach of trial and target sequences (open diamonds) derived from secondary structure statistics (Fontana *et al.* 1993a; see this paper, §4); and (iii) longest distances between the reference and the endpoints of monotonously diverging neutral paths (filled circles) (500 reference sequences were used).



The **compatible set** C_k of a structure S_k consists of all sequences which form S_k as its minimum free energy structure (the **neutral network** G_k) or one of its suboptimal structures.



Intersection of two compatible sets: $C_0 \cap C_1$

The intersection of two compatible sets is always non empty: $C_0 \cap C_1 \neq \emptyset$



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GENERIC PROPERTIES OF COMBINATORIAL MAPS: NEUTRAL NETWORKS OF RNA SECONDARY STRUCTURES¹

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Random graph theory is used to model and analyse the relationships between sequences and secondary structures of RNA molecules, which are understood as mappings from sequence space into shape space. These maps are non-invertible since there are always many orders of magnitude more sequences than structures. Sequences folding into identical structures form *neutral networks*. A neutral network is embedded in the set of sequences that are *compatible* with the given structure. Networks are modeled as graphs and constructed by random choice of vertices from the space of compatible sequences. The theory characterizes neutral networks by the mean fraction of neutral neighbors (λ). The networks are connected and percolate sequence space if the fraction of neutral nearest neighbors exceeds a threshold value ($\lambda > \lambda^*$). Below threshold ($\lambda < \lambda^*$), the networks are partitioned into a largest “giant” component and several smaller components. Structures are classified as “common” or “rare” according to the sizes of their pre-images, i.e. according to the fractions of sequences folding into them. The neutral networks of any pair of two different common structures almost touch each other, and, as expressed by the conjecture of *shape space covering* sequences folding into almost all common structures, can be found in a small ball of an arbitrary location in sequence space. The results from random graph theory are compared to data obtained by folding large samples of RNA sequences. Differences are explained in terms of specific features of RNA molecular structures. © 1997 Society for Mathematical Biology

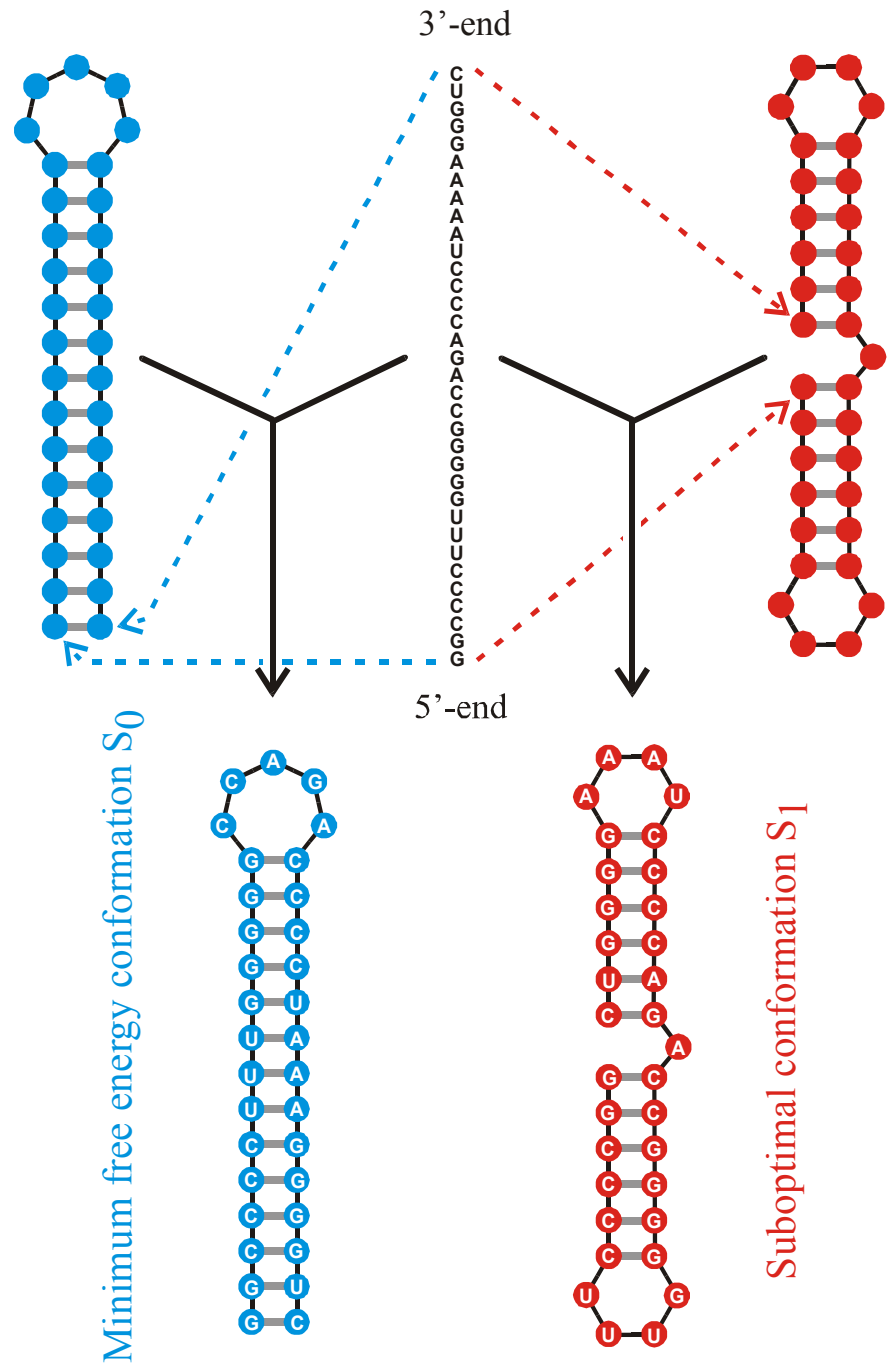
THEOREM 5. INTERSECTION-THEOREM. *Let s and s' be arbitrary secondary structures and $C[s], C[s']$ their corresponding compatible sequences. Then,*

$$C[s] \cap C[s'] \neq \emptyset.$$

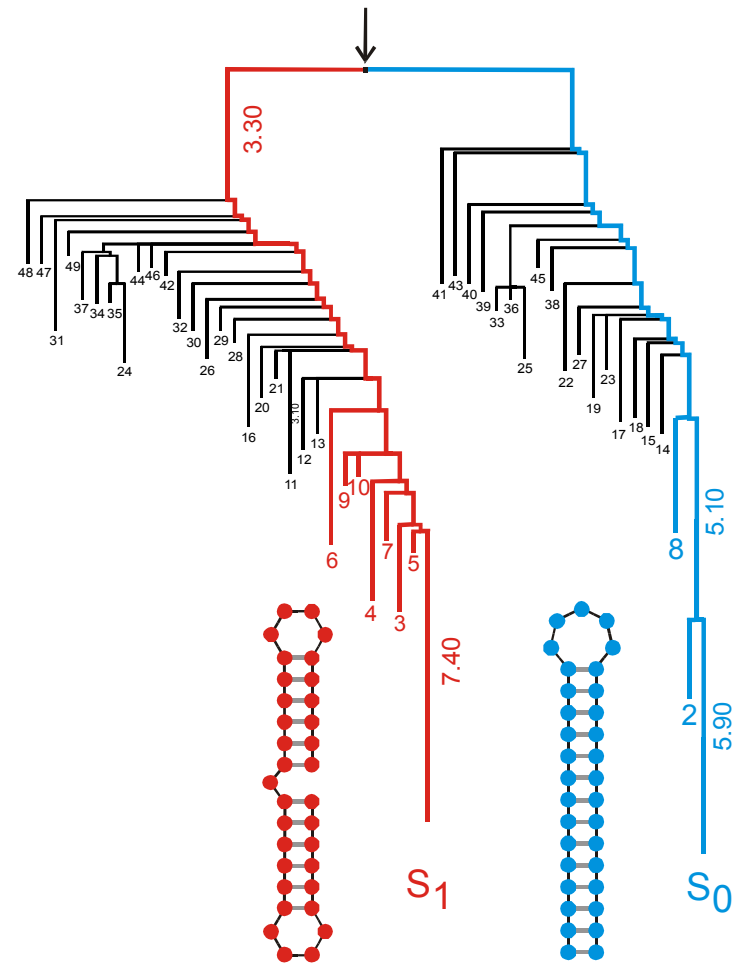
Proof. Suppose that the alphabet admits only the complementary base pair $[XY]$ and we ask for a sequence x compatible to both s and s' . Then $f(s, s') \cong D_m$ operates on the set of all positions $\{x_1, \dots, x_n\}$. Since we have the operation of a dihedral group, the orbits are either cycles or chains and the cycles have even order. A constraint for the sequence compatible to both structures appears only in the cycles where the choice of bases is not independent. It remains to be shown that there is a valid choice of bases for each cycle, which is obvious since these have even order. Therefore, it suffices to choose an alternating sequence of the pairing partners X and Y . Thus, there are at least two different choices for the first base in the orbit. ■

Remark. A generalization of the statement of theorem 5 to three different structures is false.

Reference for the definition of the intersection and the proof of the **intersection theorem**



A sequence at the **intersection** of two neutral networks is compatible with both structures



basin '1'

basin '0'

long living
metastable structure

minimum free energy
structure

Barrier tree for two
long living structures





1. Controlled evolution experiments with bacteria and RNA
2. Optimization in the RNA model
3. Sequence-structure maps, neutral networks, and intersections
- 4. Selection experiments and design of RNA molecules**

- 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 μ M) was incubated with rat liver cytosol (20 mg) or purified recombinant p115 (0.5 μ M) 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 μ l 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/CH₂Cl₂ and separated by SDS-polyacrylamide gel electrophoresis (PAGE) followed by immunoblotting using p115 mAb 13F12.
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 69. We thank G. Waters for p115 cDNA and p115 mAbs; G. Warren for p97 and p47 antibodies; R. Scheller for rbt1, membrin, and sec22 cDNAs; H. Plutner for excellent technical assistance; and P. Tan for help during the initial phase of this work. Supported by NIH grants GM 33301 and GM42336 and National Cancer Institute grant CA58689 (W.E.B.), a NIH National Research Service Award (B.D.M.), and a Wellcome Trust International Traveling Fellowship (B.B.A.).

20 March 2000; accepted 22 May 2000

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 dis-

parate 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 non-functional 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).

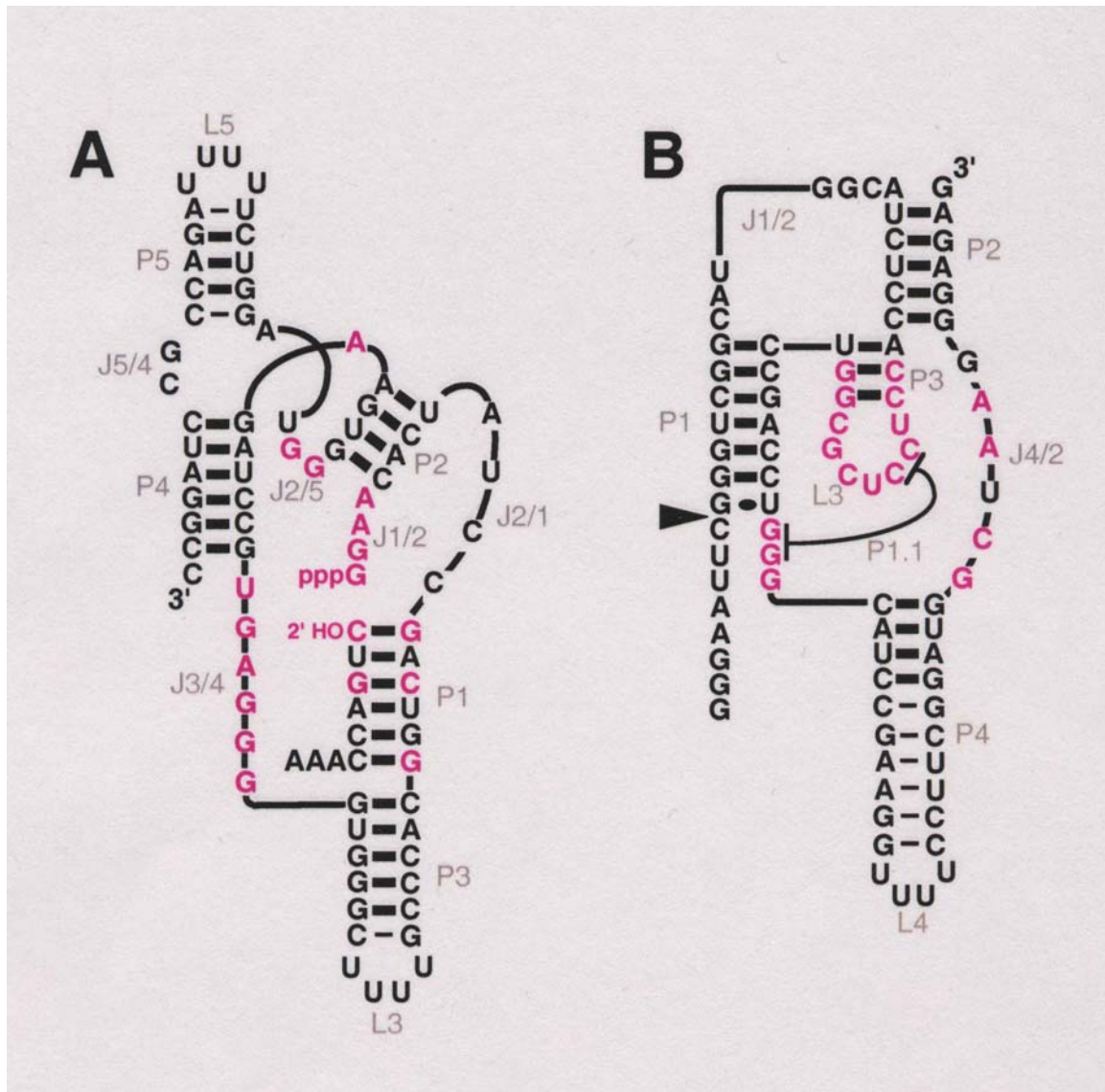
The prototype class III and HDV ribozymes have no more than the 25% sequence identity expected by chance and no fortuitous structural similarities that might favor an intersection of their two neutral networks. Nevertheless, sequences can be designed that simultaneously satisfy the base-pairing requirements

A ribozyme switch

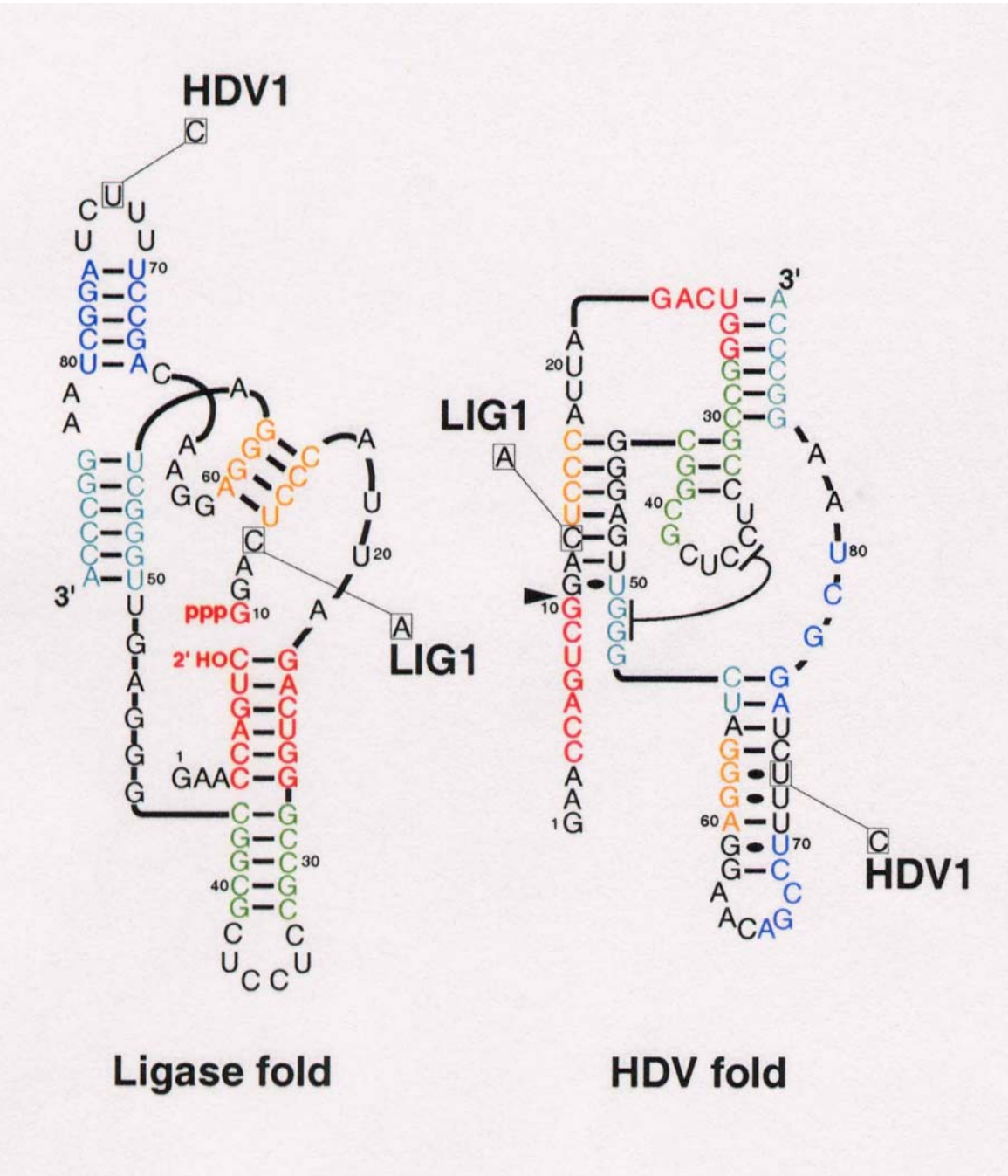
E.A.Schultes, D.B.Bartel, *Science*
289 (2000), 448-452

Whitehead Institute for Biomedical Research and Department of Biology, Massachusetts Institute of Technology, 9 Cambridge Center, Cambridge, MA 02142, USA.

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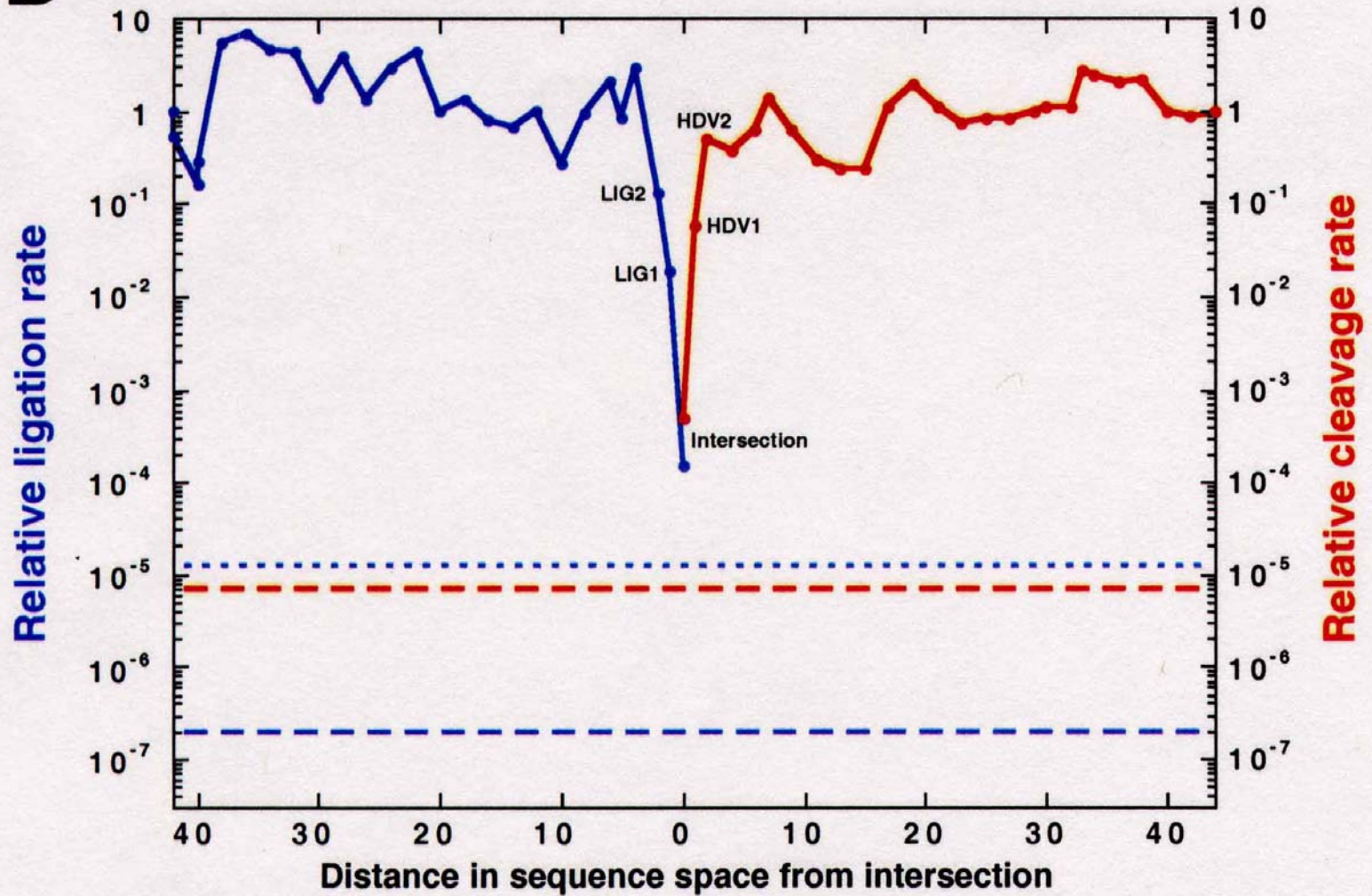


Two ribozymes of chain lengths $n = 88$ nucleotides: An artificial ligase (**A**) and a natural cleavage ribozyme of hepatitis-X-virus (**B**)

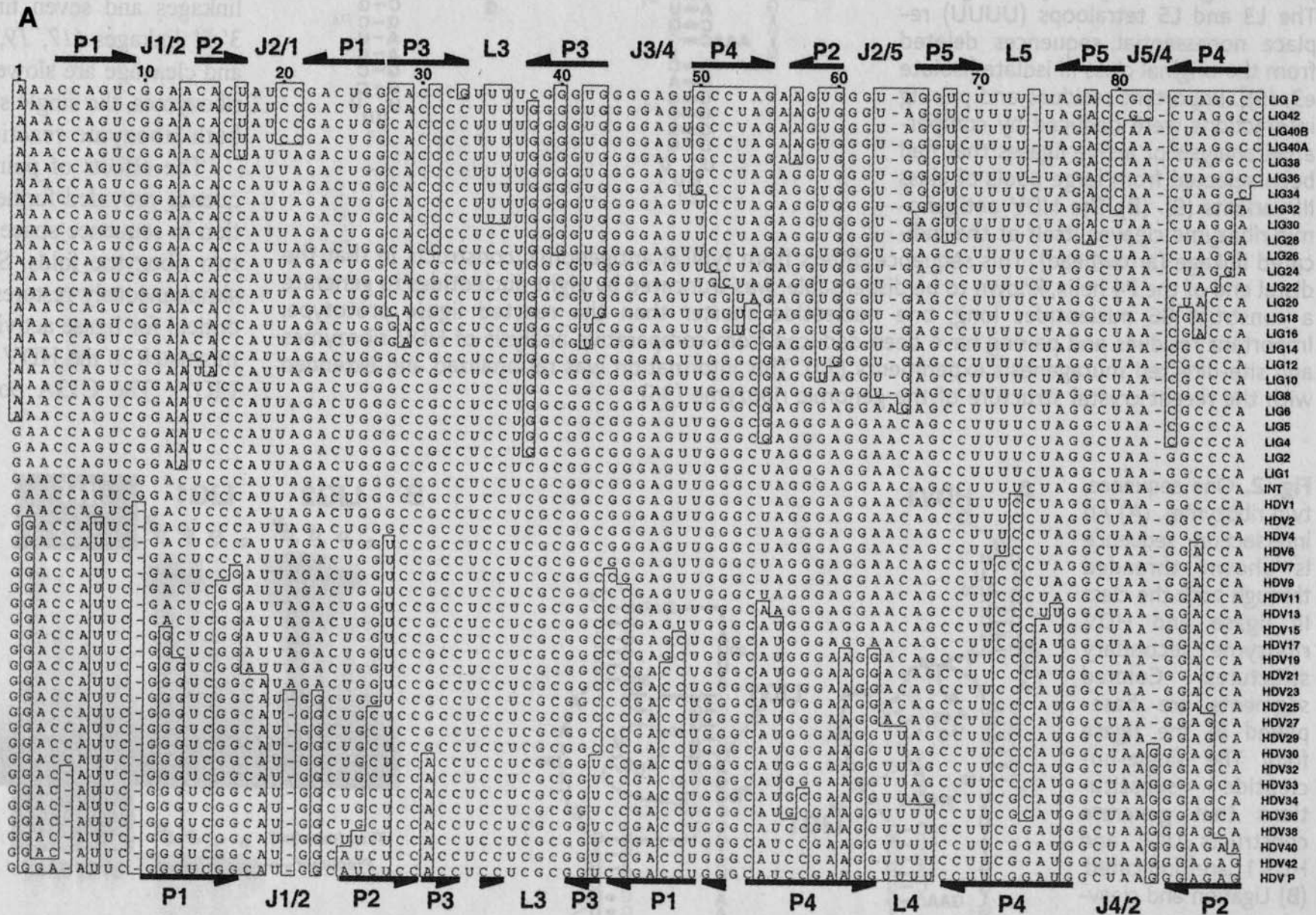


The sequence at the *intersection*:

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

B

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



Sequence of mutants from the intersection to both reference ribozymes

A ribozyme that lacks cytidine

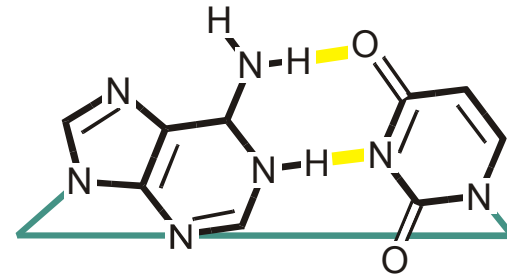
Jeff Rogers & Gerald F. Joyce

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

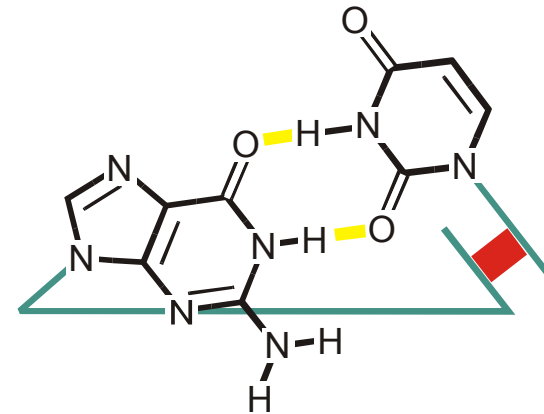
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The RNA-world hypothesis proposes that, before the advent of DNA and protein, life was based on RNA, with RNA serving as both the repository of genetic information and the chief agent of catalytic function¹. An argument against an RNA world is that 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 functions²⁻⁷. 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 10⁵-fold faster than the uncatalysed rate of template-directed RNA ligation.

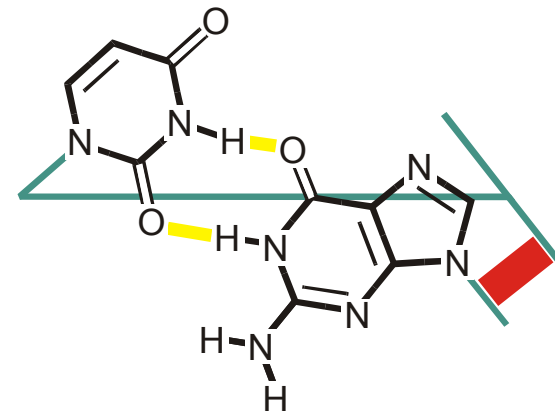
Catalytic activity in the
AUG alphabet



A=U
(U=A)



G=U



U=G

Base pairs in the **AUG** alphabet

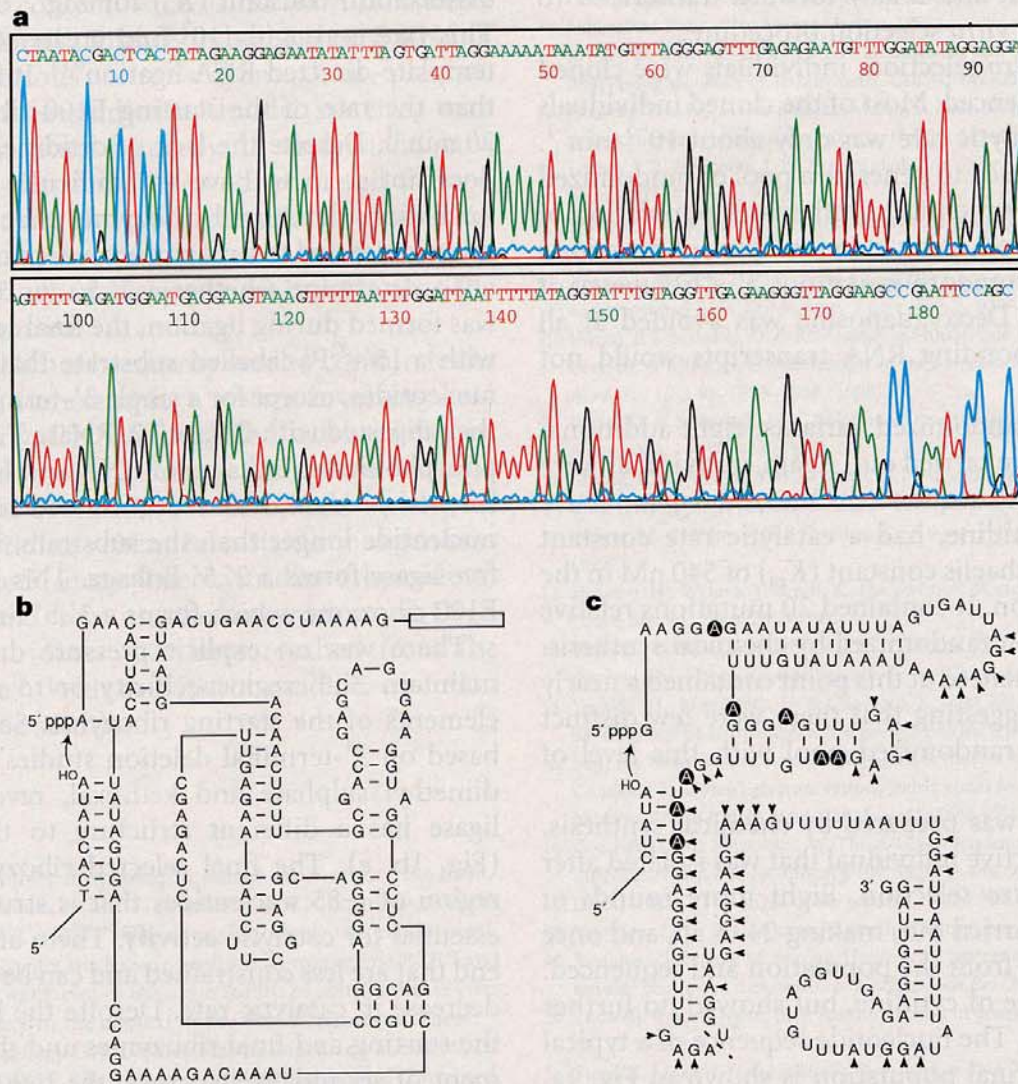


Figure 1 Composition of the final selected cytidine-free ribozyme. **a**, Sequence trace showing the lack of cytidines at nucleotide positions 19–173. Positions 2–18 correspond to the T7 promoter sequence and positions 174–188 correspond to the downstream vector sequence (pCR 2.1). Automated sequencing 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

ligase¹⁰. 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.

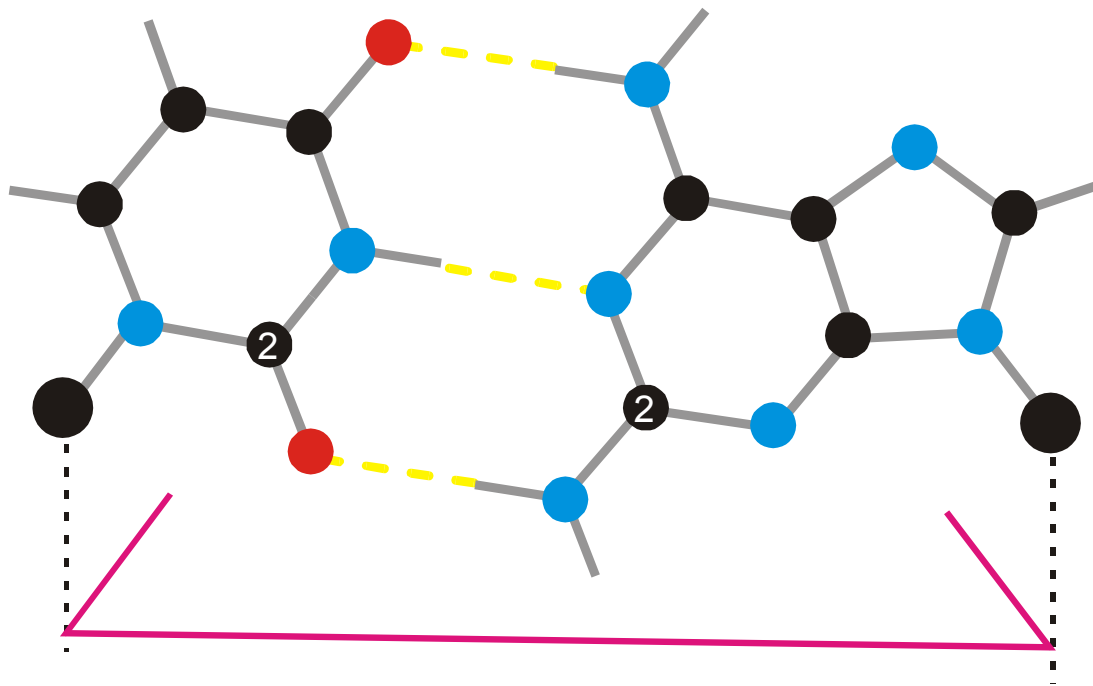
A ribozyme composed of only two different nucleotides

John S. Reader & 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

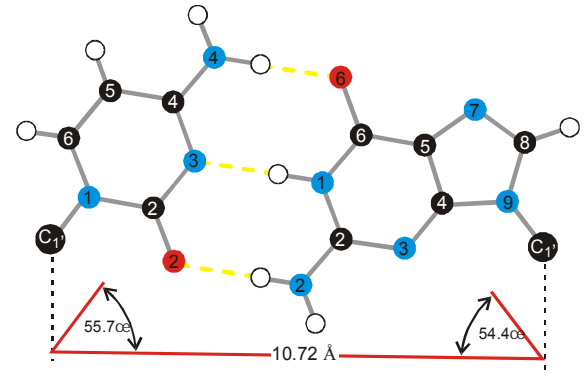
RNA molecules are thought to have been prominent in the early history of life on Earth because of their ability both to encode genetic information and to exhibit catalytic function¹. The modern genetic alphabet relies on two sets of complementary base pairs to store genetic information. However, owing to the chemical instability of cytosine, which readily deaminates to uracil², 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 unit³⁻⁷. 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

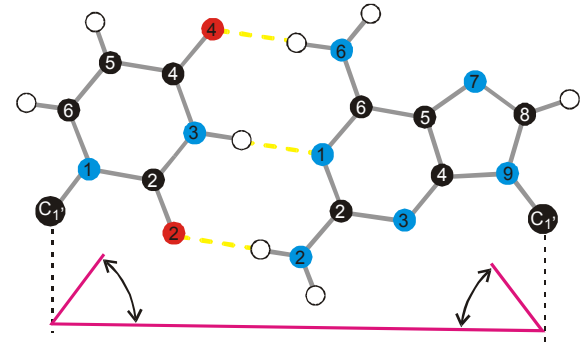


The 2,6-diamino purine – uracil, **DU**, base pair

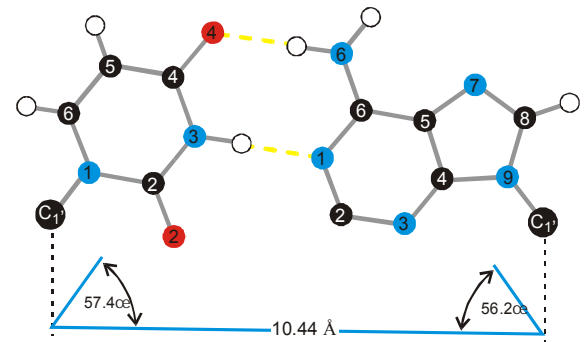
G © C



D © U



A = U



Three Watson-Crick type base pairs

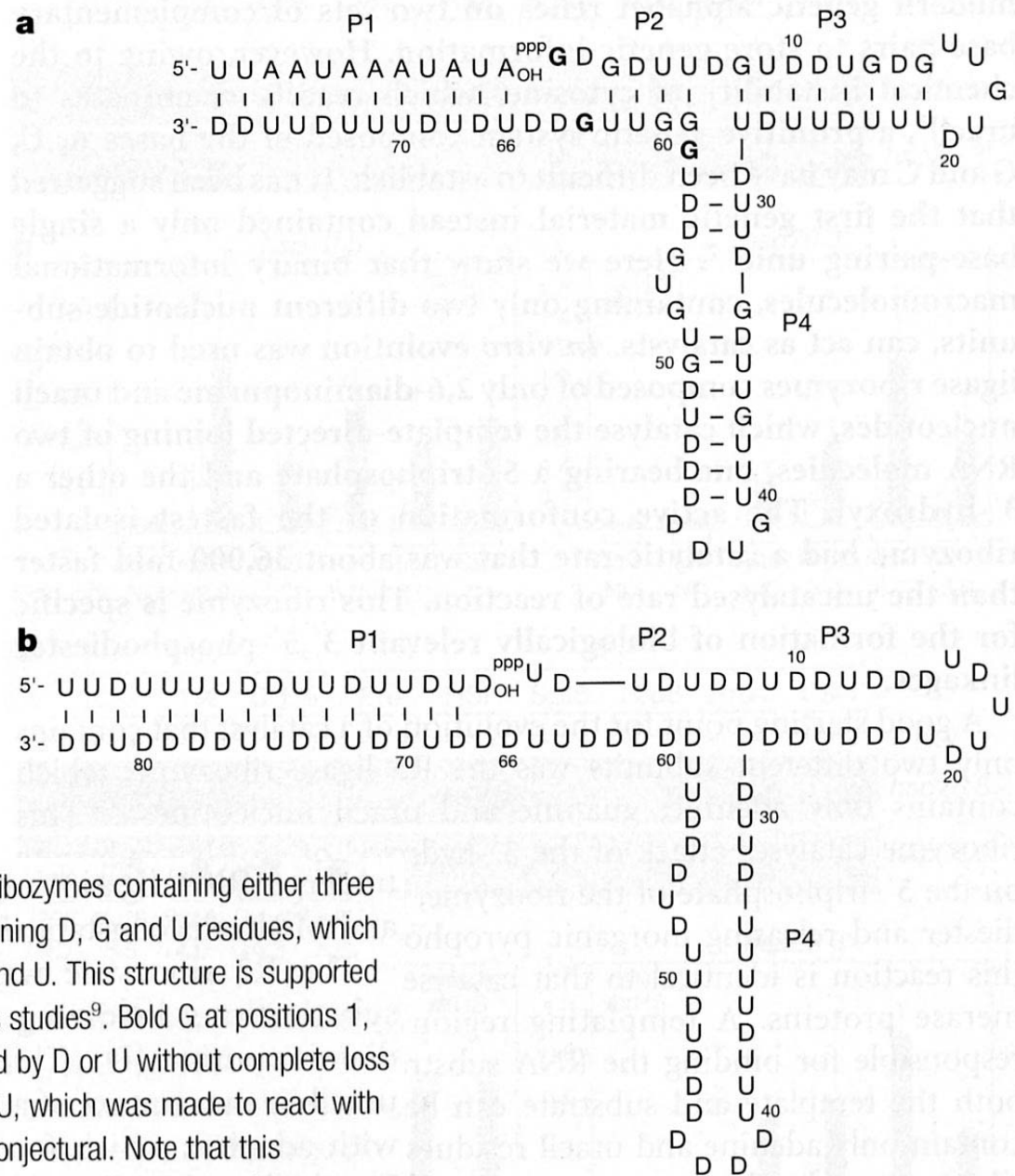


Figure 1 Sequence and secondary structure of ligase ribozymes containing either three or two different nucleotide subunits. **a**, Ribozyme containing D, G and U residues, which was made to react with a substrate containing only A and U. This structure is supported by chemical modification and site-directed mutagenesis studies⁹. 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**.

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

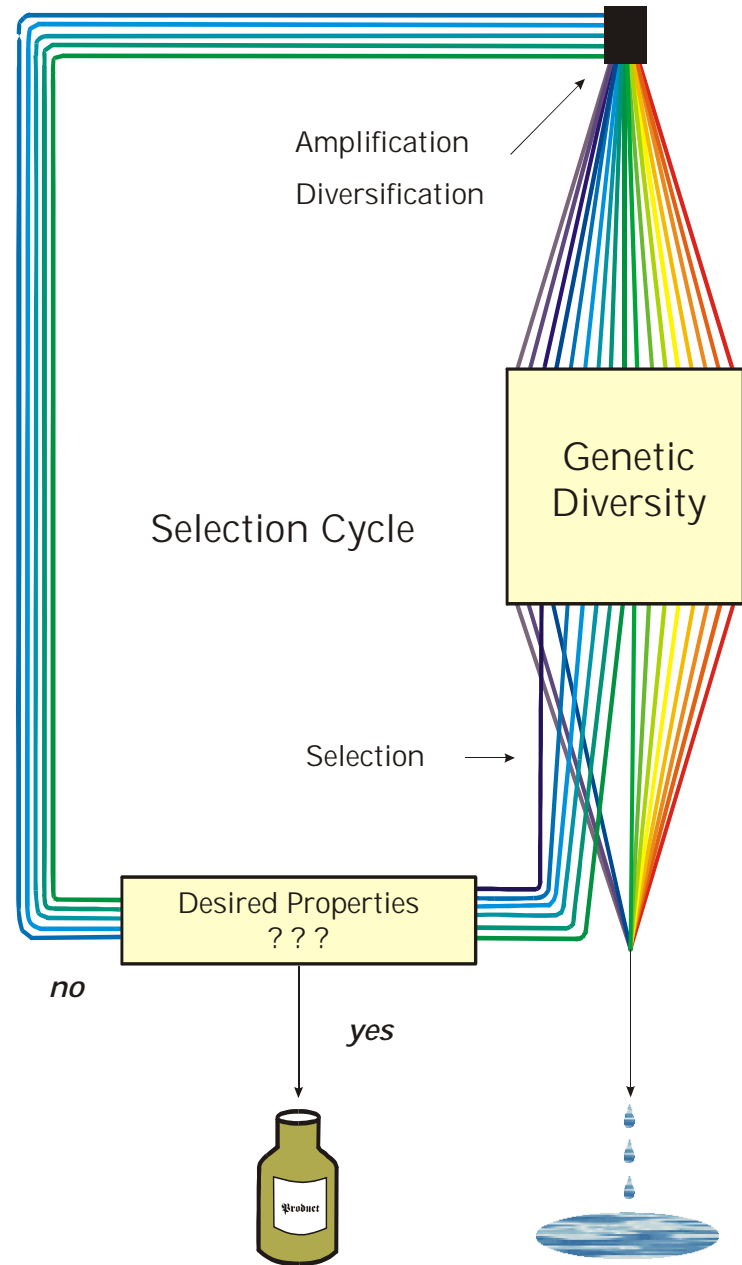
C.Tuerk, L.Gold, *SELEX - Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase.* Science **249** (1990), 505-510

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

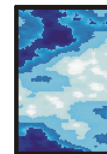
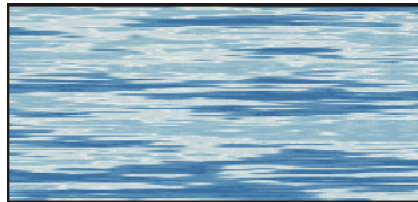
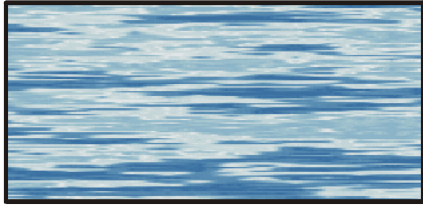
R.D.Jenison, S.C.Gill, A.Pardi, B.Poliski, *High-resolution molecular discrimination by RNA.* Science **263** (1994), 1425-1429

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

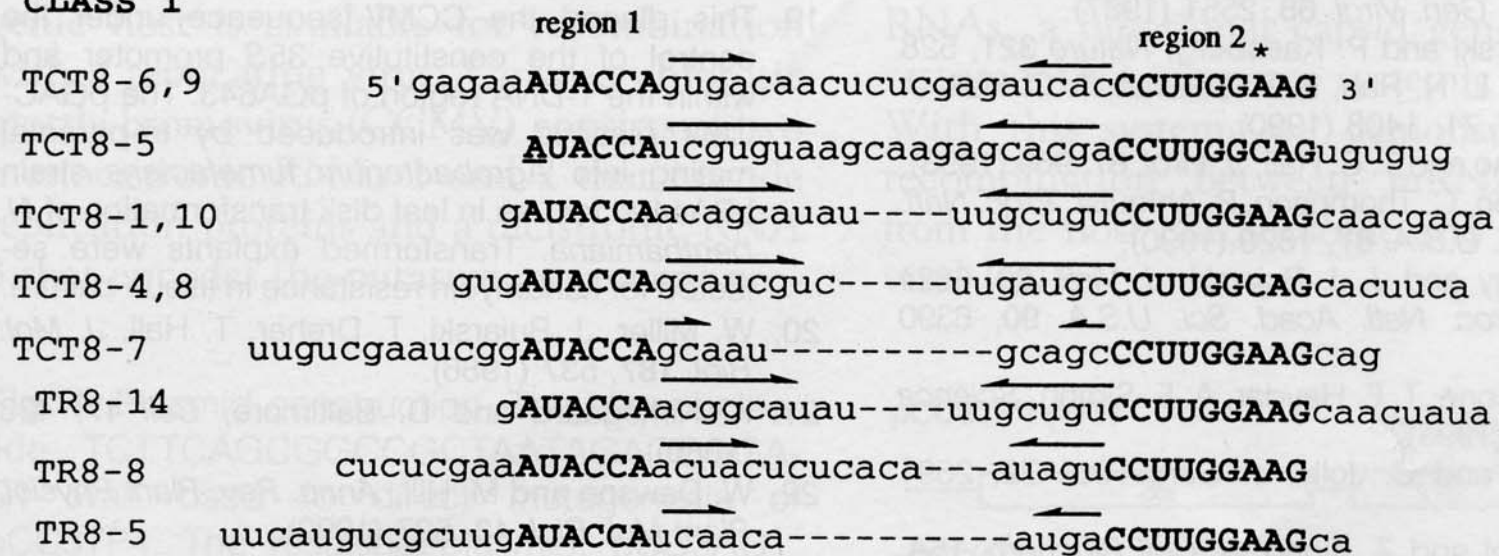
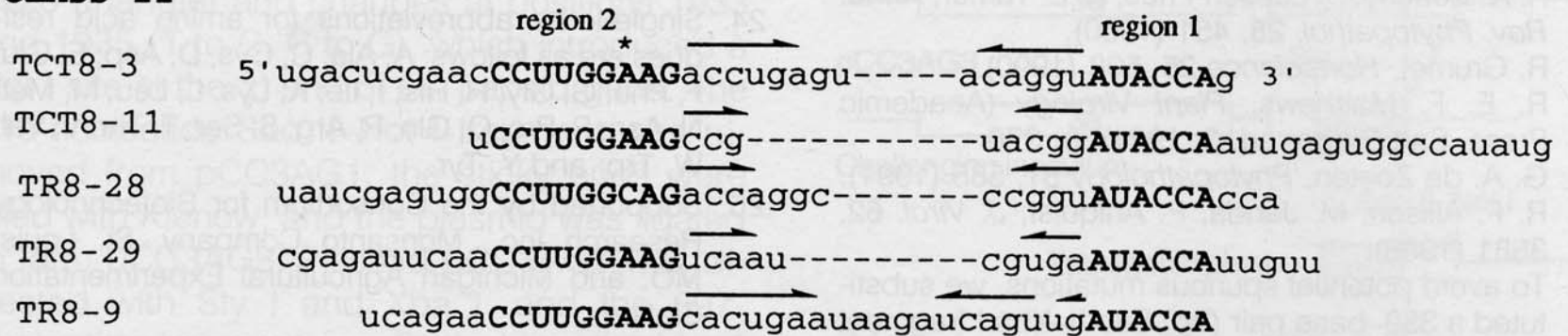
Jiang, A. K. Suri, R. Fiala, D. J. Patel, *Saccharide-RNA recognition in an aminoglycoside antibiotic-RNA aptamer complex.* Chemistry & Biology **4** (1997), 35-50



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

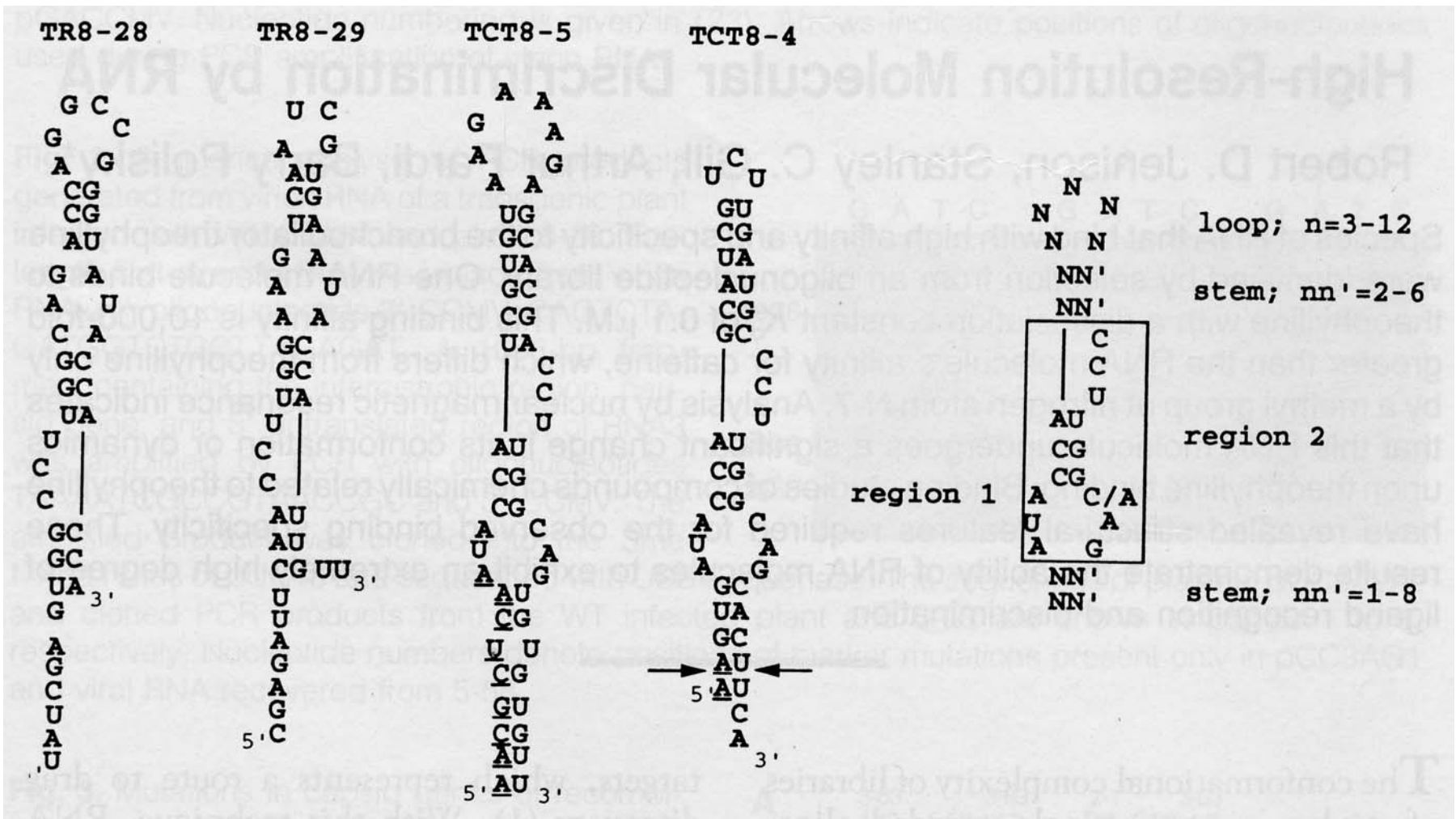


The SELEX technique for the evolutionary design of *aptamers*

CLASS I**CLASS II**

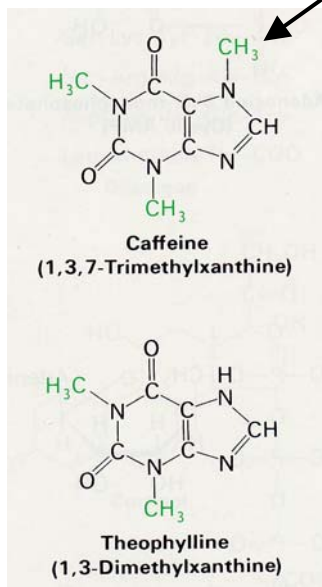
Sequences of aptamers binding theophyllin, caffeine, and related compounds

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



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

additional methyl group



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	$K_d(c)$ (μ M)	$K_d(c)/K_d(t)$
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

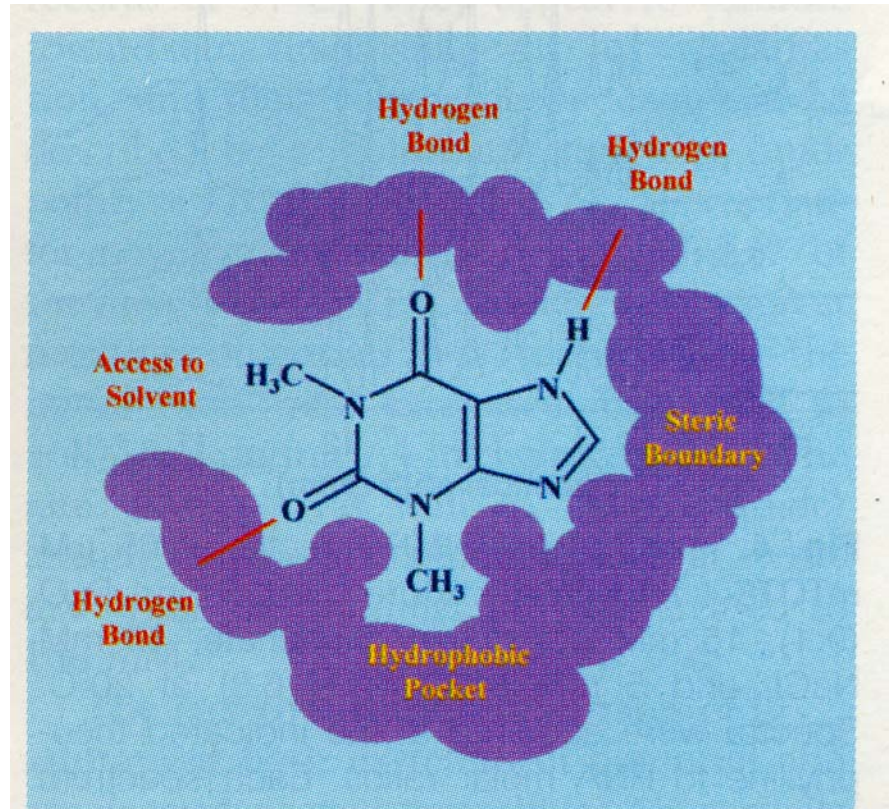
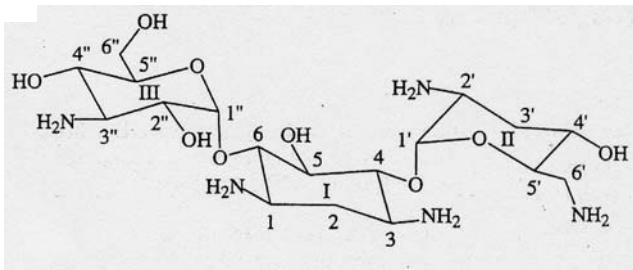
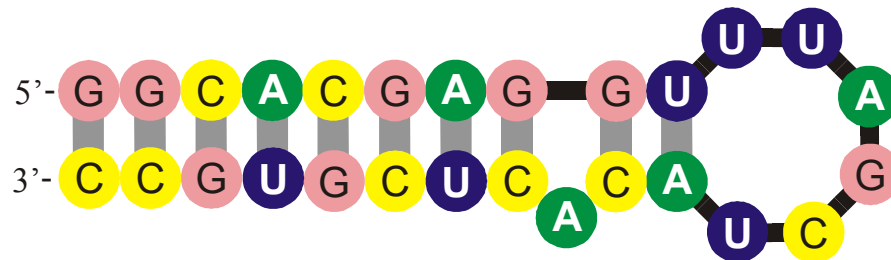


Fig. 3. Schematic representation of the RNA (purple) binding site for theophylline (blue).

Schematic drawing of the aptamer binding site for the theophylline molecule



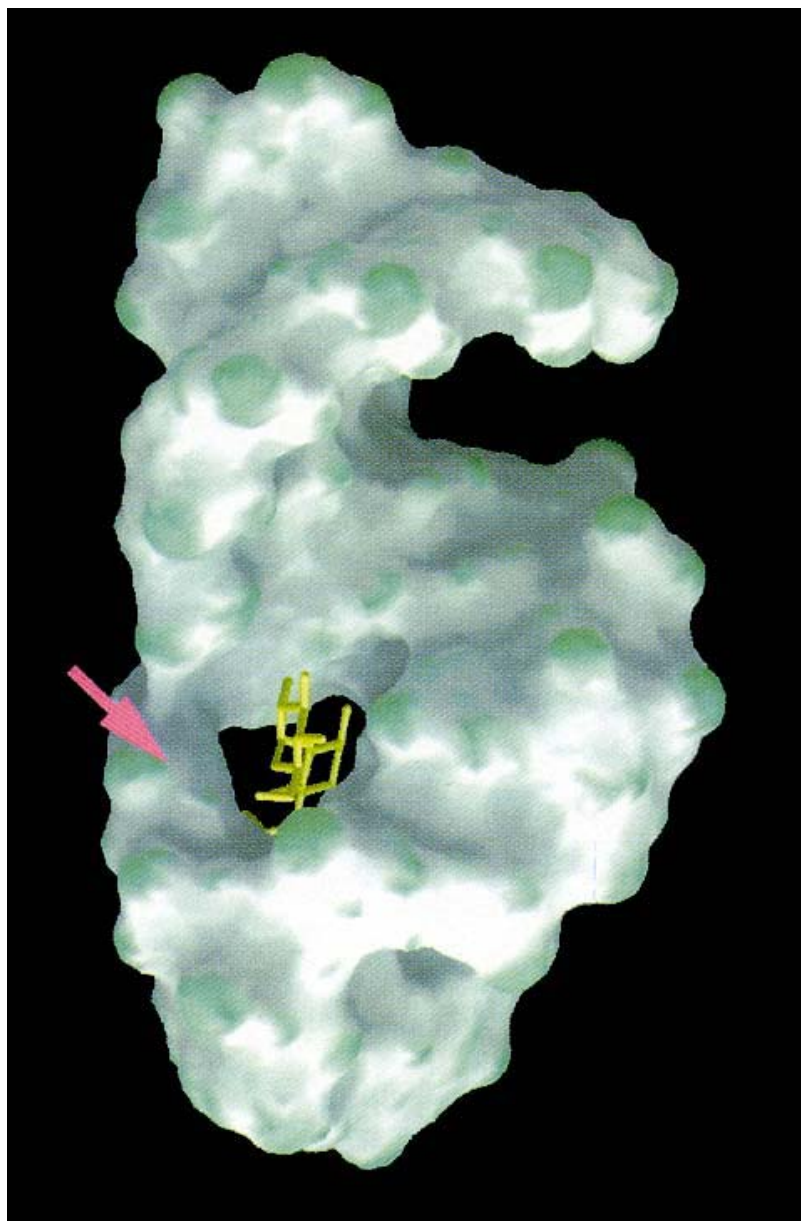
tobramycin



RNA aptamer

Formation of secondary structure of the tobramycin binding RNA aptamer

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,
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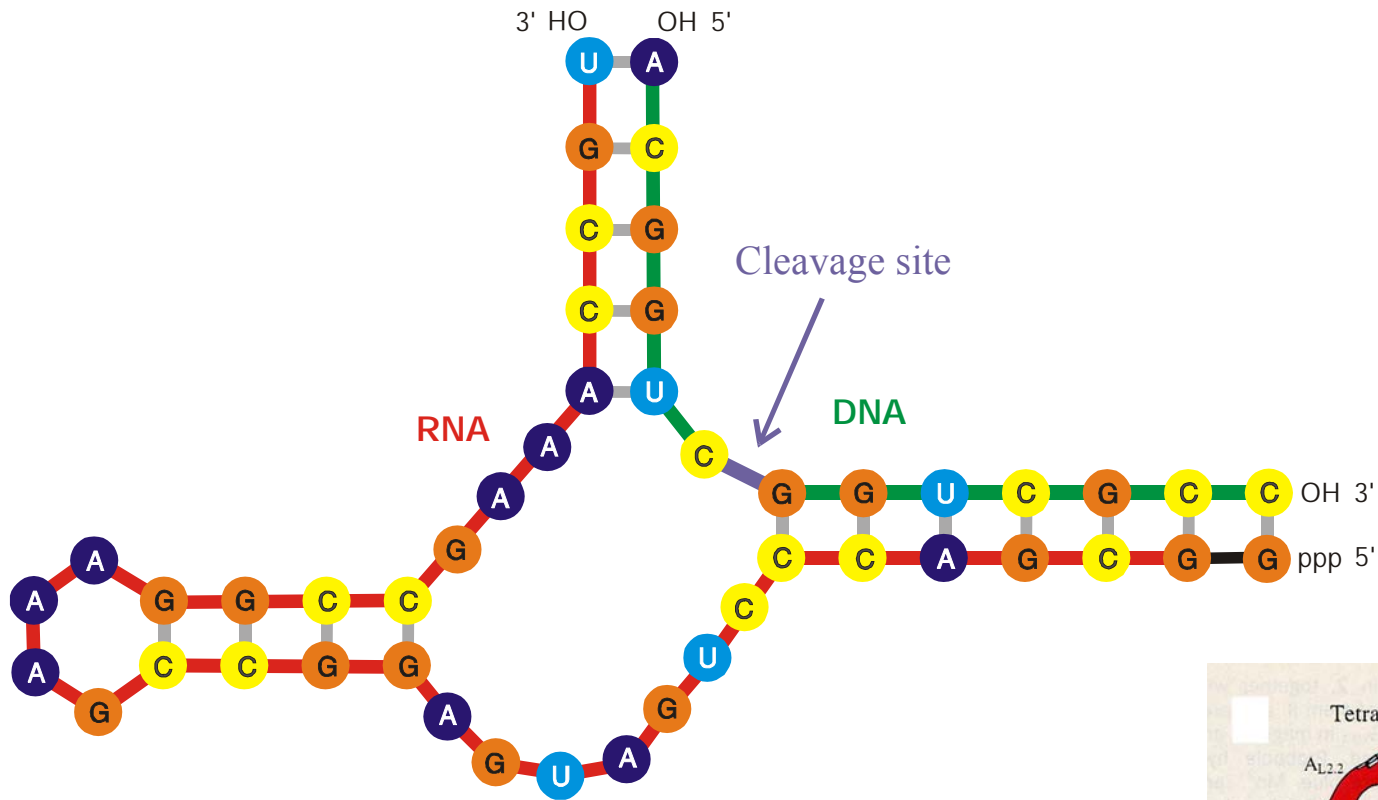
Hammerhead ribozyme – The smallest RNA based catalyst

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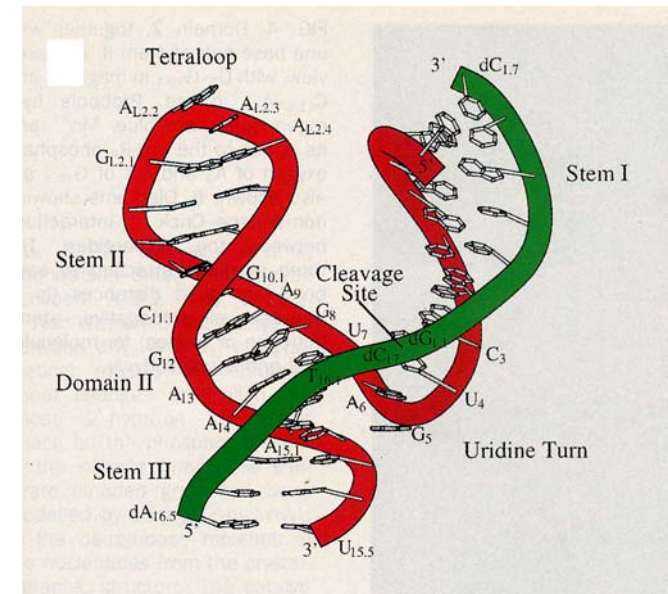
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Hammerhead ribozyme: The smallest known catalytically active RNA molecule



Allosteric effectors:

FMN = flavine mononucleotide

H10 – H12

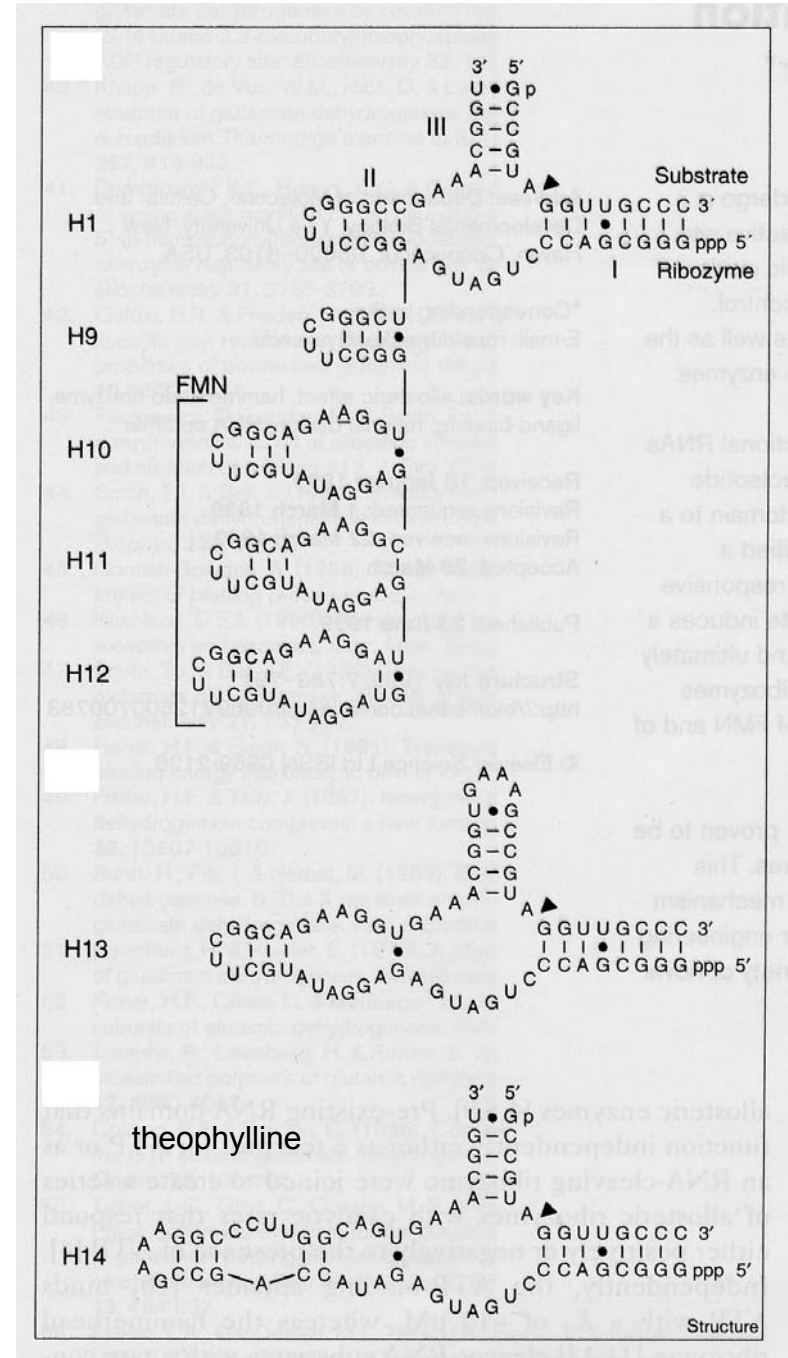
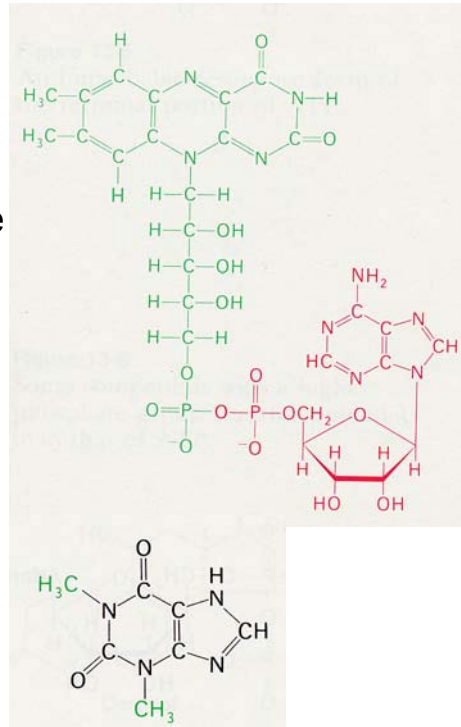
theophylline

H14

Self-splicing allosteric ribozyme

H13

Hammerhead ribozymes with allosteric effectors



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

