RNA Secondary Structures Beyond Neutral Networks

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Road to the RNA World: Intersections of Theory and Experiment

Leipzig, 09.– 11.06.2005

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

The physicist's dream is the designer's nightmare.

1. What are neutral networks ?

2. Mutations and structural stability

3. Structures from defective alphabets

4. Suboptimal conformations and structural stability

5. Metastable structures and RNA switches

6. How to handle multiple constraints

1. **What are neutral networks ?**
2. Mutations and structural stability
3. Structures from defective alphabets
4. Suboptimal conformations and structural stability
5. Metastable structures and RNA switches
6. How to handle multiple constraints

Definition and **physical relevance** of RNA secondary structures

RNA secondary structures are listings of Watson-Crick and GU wobble base pairs, which are free of knots and pseudokots. This definition allows for rigorous mathematical analysis by means of combinatorics.

D.Thirumalai, N.Lee, S.A.Woodson, and D.K.Klimov.
Annu.Rev.Phys.Chem. **52**:751-762 (2001):

„**Secondary structures are folding intermediates in the formation of full three-dimensional structures**.“

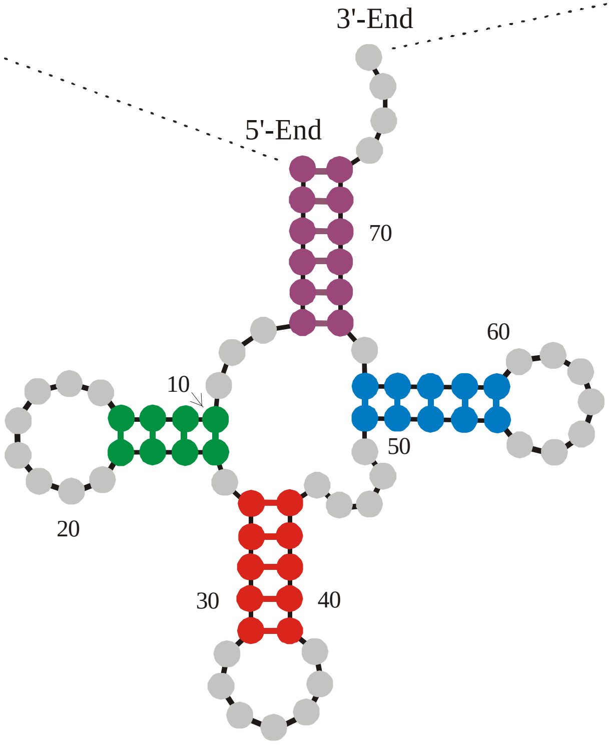
Secondary structures have been and still are frequently used to predict and discuss RNA function.

Sequence

5'-End 3'-End

GCGGAUUUAGCUCAGDDGGGAGAGCMCCAGACUGAAYAUCUGGAGMUCCUGUGTPCGAUCCACAGAAUUCGCACCA

Secondary structure



|||

Symbolic notation

((((((•••((((••••••••))))•(((((•••••••)))))•••••(((((•••••••)))))•))))))••••

5'-End 3'-End

A symbolic notation of RNA secondary structure that is equivalent to the conventional graphs

RNA sequence GUAUCGAAAUACGUAGCGUAUGGGGAUGCUGGACGGUCCCAUCGGUACUCCA

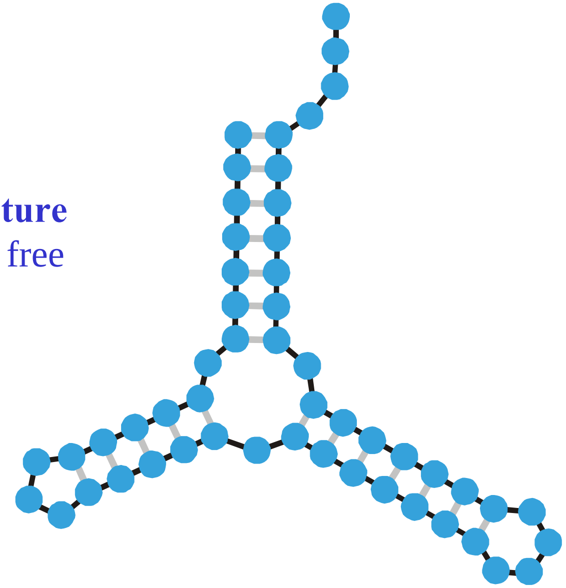
RNA folding:

Structural biology, spectroscopy of biomolecules, understanding **molecular function**

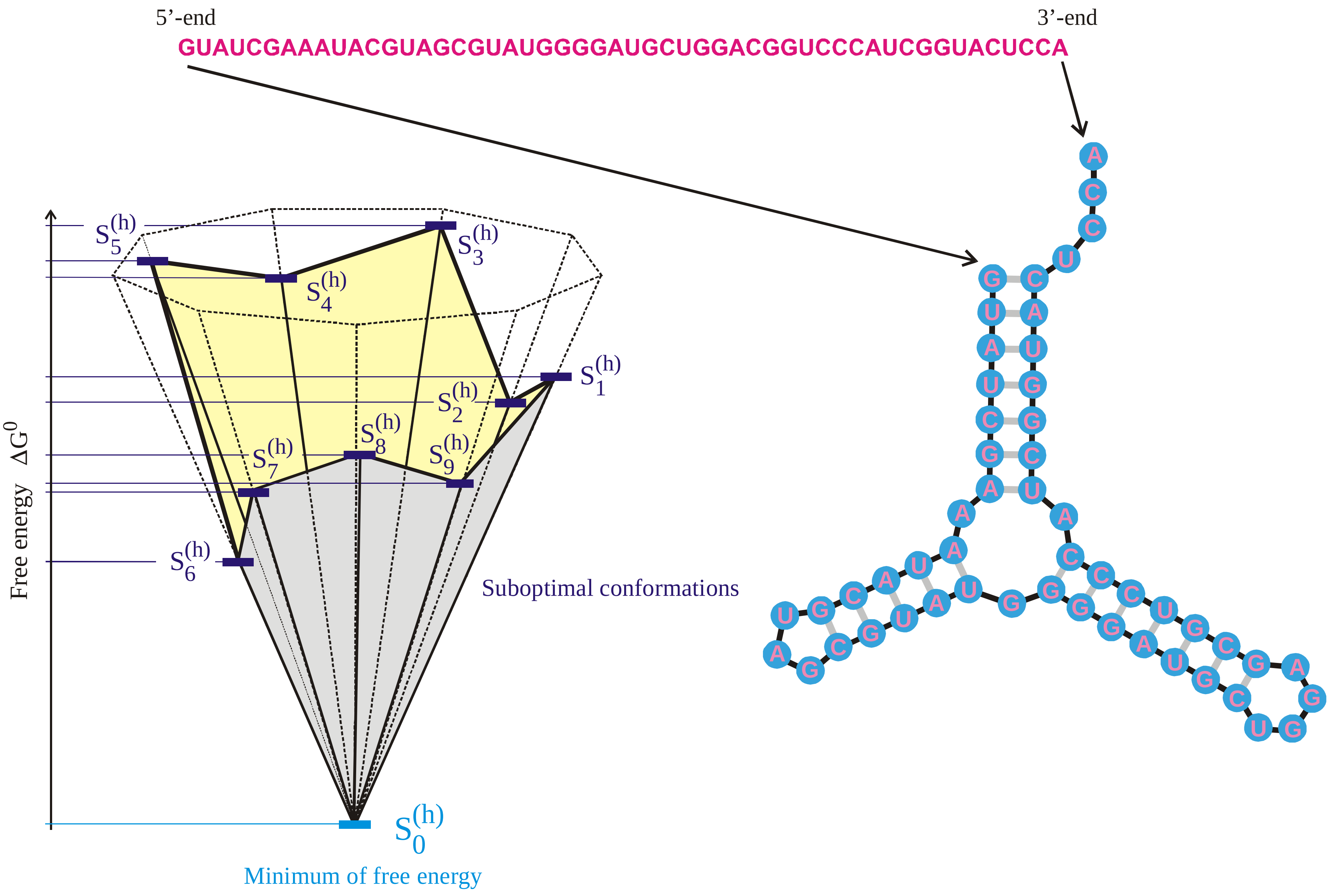
Biophysical chemistry: thermodynamics and kinetics

Empirical parameters

RNA structure of minimal free energy



Sequence, structure, and design



The minimum free energy structures on a discrete space of conformations

RNA sequence **GUAUCGAAAUACGUAGCGUAUGGGGAUGCUGGACGGUCCCAUCGGUACUCCA**

RNA folding:

Structural biology, spectroscopy of biomolecules, understanding **molecular function**

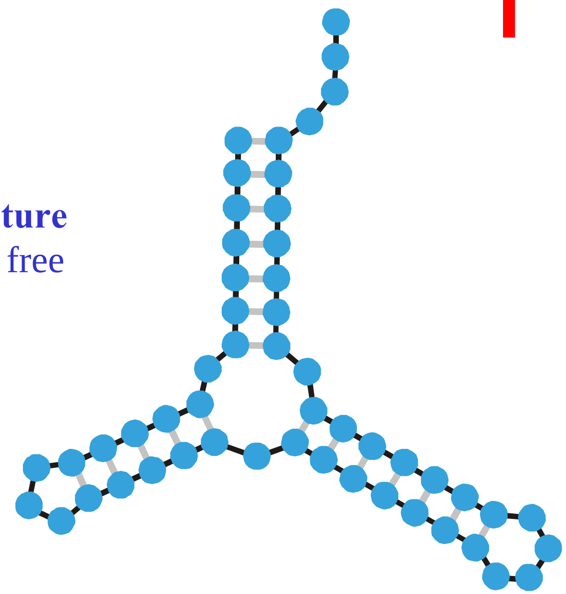
Iterative determination of a sequence for the given secondary structure

Inverse Folding Algorithm

Inverse folding of RNA:

Biotechnology, **design of biomolecules** with predefined structures and functions

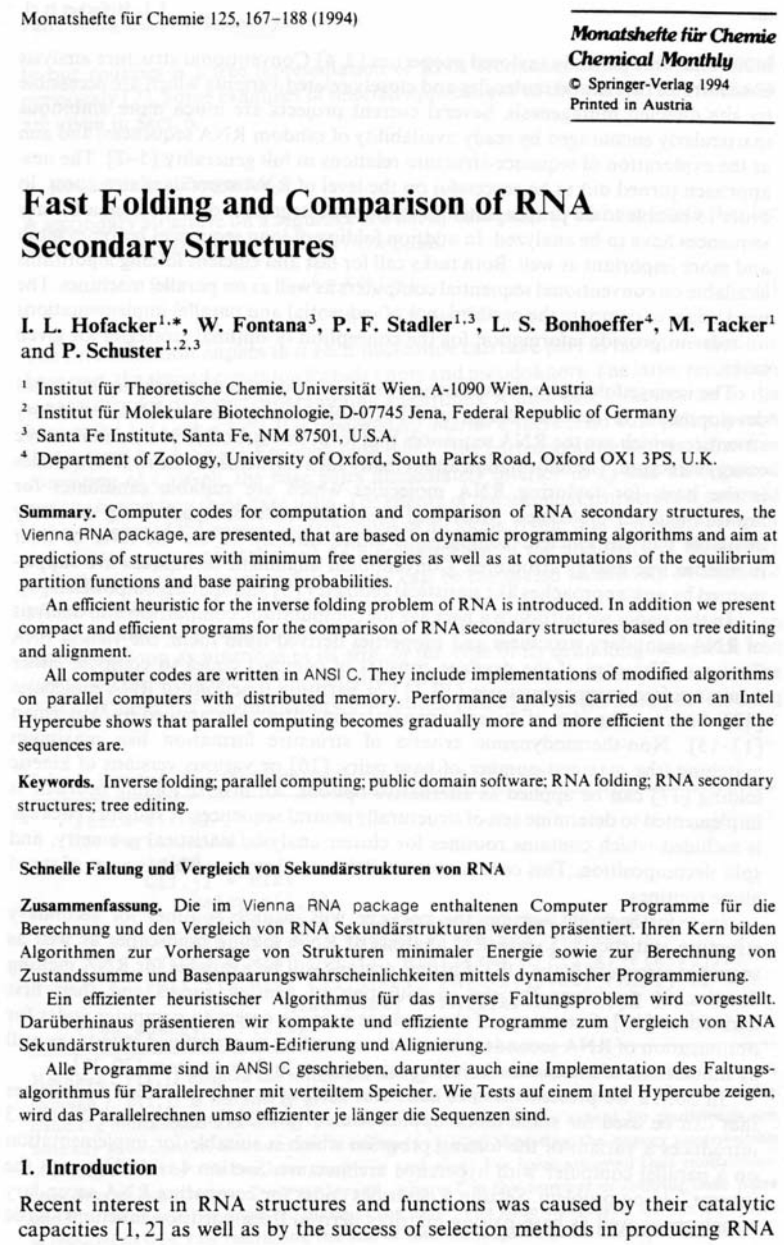
RNA structure of minimal free energy



Sequence, structure, and design

The ***Vienna RNA-Package***:

A library of routines for folding, ***inverse folding***, sequence and structure alignment, ***kinetic folding***, ***cofolding***, …



Monatshefte für Chemie 125, 167–188 (1994)

Monatshefte für Chemie
Chemical Monthly
© Springer-Verlag 1994
Printed in Austria

Fast Folding and Comparison of RNA Secondary Structures

I. L. Hofacker[1,*], W. Fontana[3], P. F. Stadler[1,3], L. S. Bonhoeffer[4], M. Tacker[1] and P. Schuster[1,2,3]

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[2] Institut für Molekulare Biotechnologie, D-07745 Jena, Federal Republic of Germany
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Summary. Computer codes for computation and comparison of RNA secondary structures, the Vienna RNA package, are presented, that are based on dynamic programming algorithms and aim at predictions of structures with minimum free energies as well as at computations of the equilibrium partition functions and base pairing probabilities.

An efficient heuristic for the inverse folding problem of RNA is introduced. In addition we present compact and efficient programs for the comparison of RNA secondary structures based on tree editing and alignment.

All computer codes are written in ANSI C. They include implementations of modified algorithms on parallel computers with distributed memory. Performance analysis carried out on an Intel Hypercube shows that parallel computing becomes gradually more and more efficient the longer the sequences are.

Keywords. Inverse folding; parallel computing; public domain software; RNA folding; RNA secondary structures; tree editing.

Schnelle Faltung und Vergleich von Sekundärstrukturen von RNA

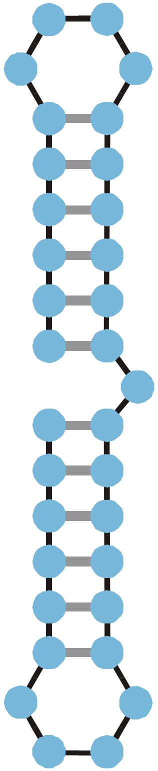
Zusammenfassung. Die im Vienna RNA package enthaltenen Computer Programme für die Berechnung und den Vergleich von RNA Sekundärstrukturen werden präsentiert. Ihren Kern bilden Algorithmen zur Vorhersage von Strukturen minimaler Energie sowie zur Berechnung von Zustandssumme und Basenpaarungswahrscheinlichkeiten mittels dynamischer Programmierung.

Ein effizienter heuristischer Algorithmus für das inverse Faltungsproblem wird vorgestellt. Darüberhinaus präsentieren wir kompakte und effiziente Programme zum Vergleich von RNA Sekundärstrukturen durch Baum-Editierung und Alignierung.

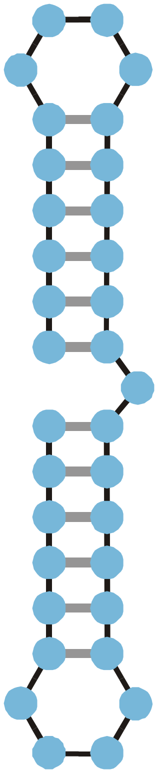
Alle Programme sind in ANSI C geschrieben, darunter auch eine Implementation des Faltungsalgorithmus für Parallelrechner mit verteiltem Speicher. Wie Tests auf einem Intel Hypercube zeigen, wird das Parallelrechnen umso effizienter je länger die Sequenzen sind.

1. Introduction

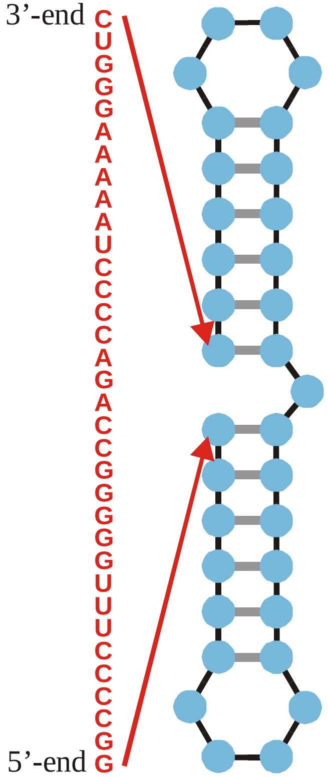
Recent interest in RNA structures and functions was caused by their catalytic capacities [1, 2] as well as by the success of selection methods in producing RNA



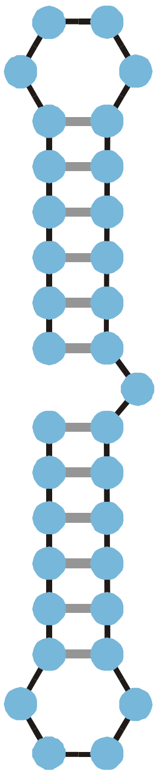
Structure



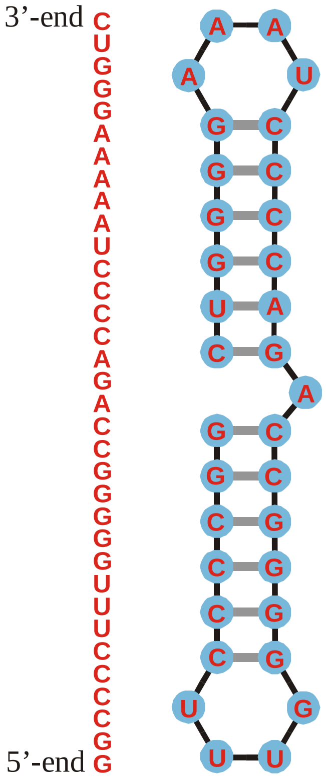
Structure



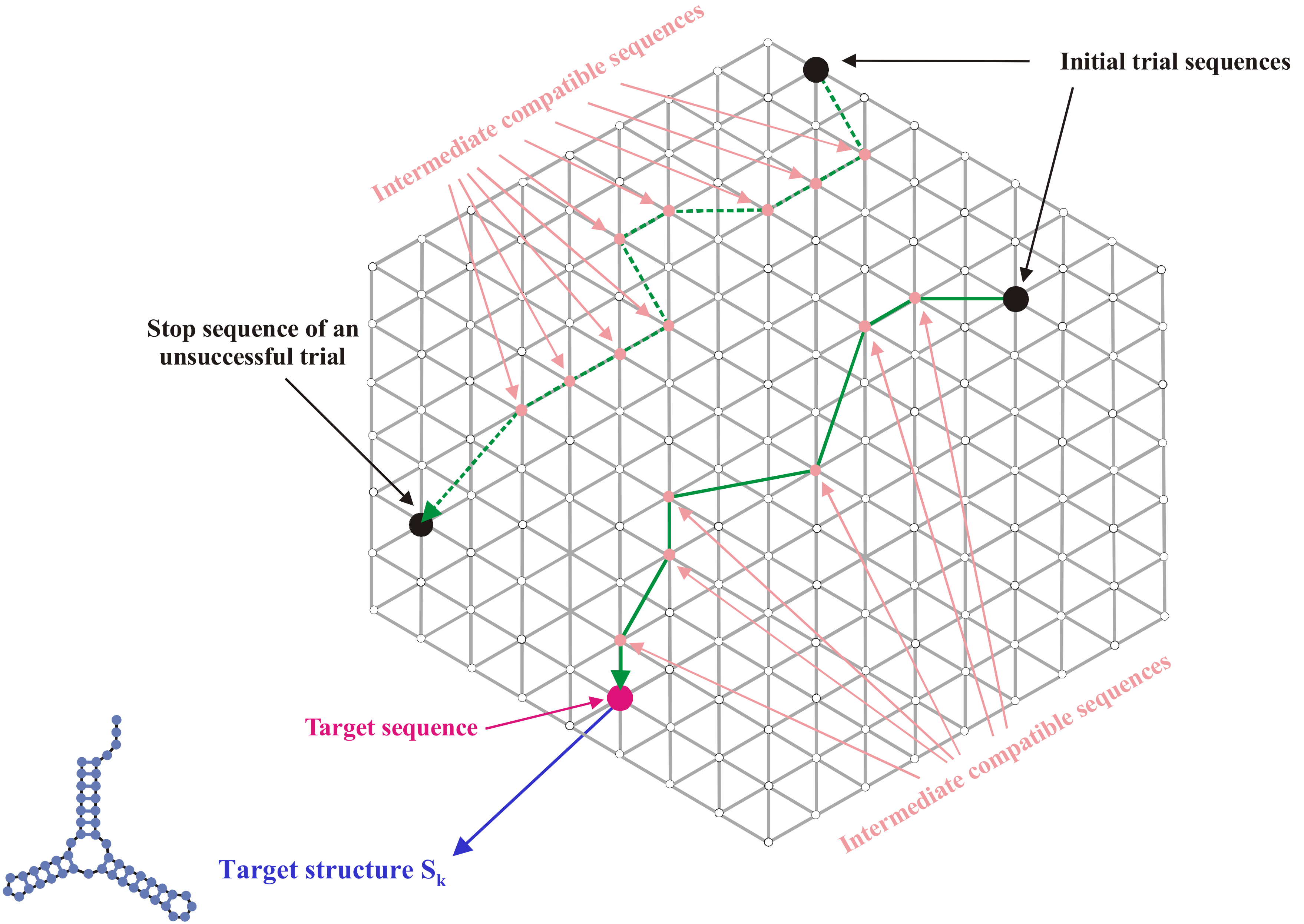
Compatible sequence



Structure



Compatible sequence



Intermediate compatible sequences

Initial trial sequences

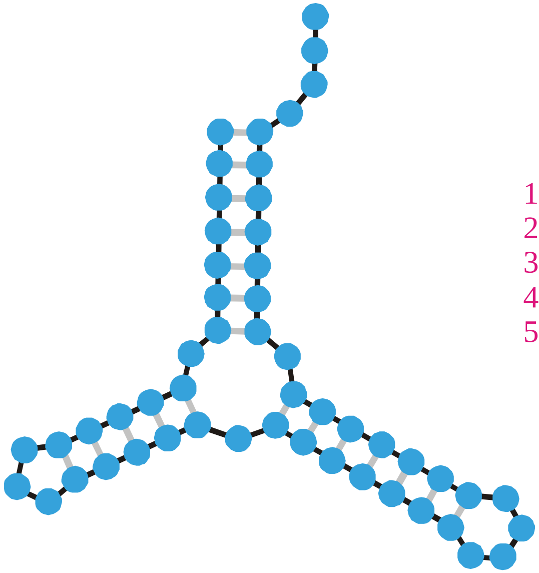
Stop sequence of an unsuccessful trial

Target sequence

Target structure S_k

Intermediate compatible sequences

Approach to the **target structure S_k** in the inverse folding algorithm



Minimum free energy
criterion

1st
2nd
3rd trial
4th
5th

GUAUCGAAAUACGUAGCGUAUGGGGAUGCUGGACGGUCCCAUCGGUACUCCA

UGGUUACGCGUUGGGGUAACGAAGAUUCCGAGAGGAGUUUAGUGACUAGAGG

CUUCUUGAGCUAGUACCUAGUCGGAUAGGAUUUCCUAUCUCCAGGGAGGAUG

CUUUUCUUCACGUUAGAUGUGUAAUGGACAUGUGUUUAUUUAGGAAAGGCGC

AUAACGUGAGUGUCUAAUACUGAUCGCUCCGGAGGGUGGUGGCGUUGUUAAU

Inverse folding of RNA secondary structures

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

Space of genotypes: $\mathcal{I} = \{I_1, I_2, I_3, I_4, \ldots , I_N\}$; Hamming metric

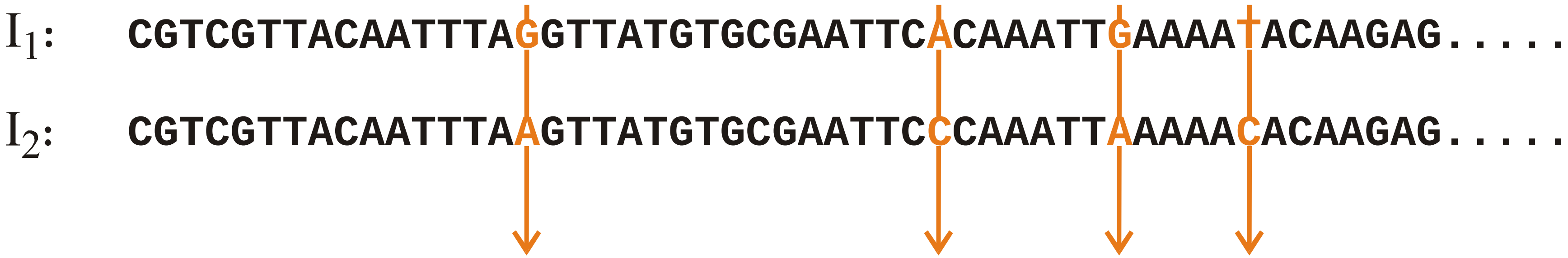
Space of phenotypes: $\mathcal{S} = \{S_1, S_2, S_3, S_4, \ldots , S_M\}$; metric (not required)

$$N >> M$$

$$\psi(I_j) = S_k$$

$$\mathbf{G}_k = \psi^{-1}(S_k) \cup \{\ \mathrm{I}_j \mid \psi(I_j) = S_k\ \}$$

A mapping ψ and its inversion



I_1: CGTCGTTACAATTTAGGTTATGTGCGAATTCACAAATTGAAAATACAAGAG.....

I_2: CGTCGTTACAATTTAAGTTATGTGCGAATTCCCAAATTAAAAACACAAGAG.....

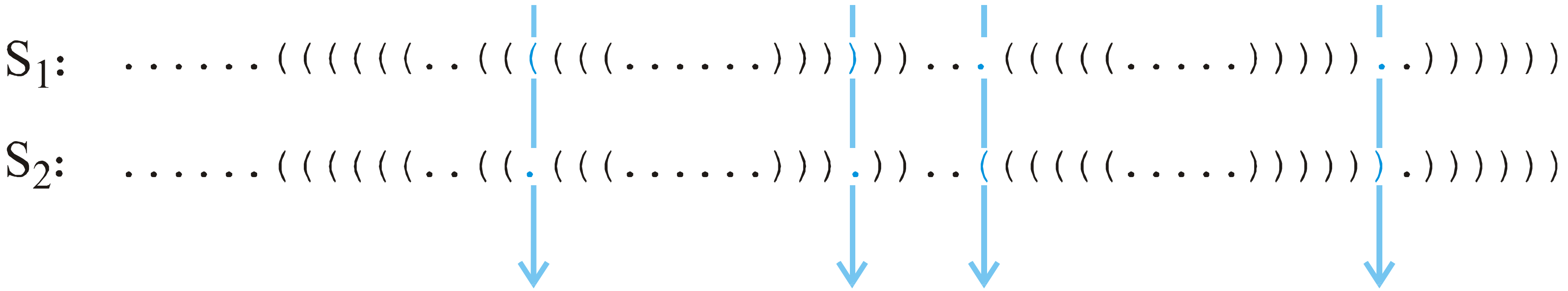
Hamming distance $d_H(I_1,I_2) = 4$

(i) $d_H(I_1,I_1) = 0$

(ii) $d_H(I_1,I_2) = d_H(I_2,I_1)$

(iii) $d_H(I_1,I_3) \leq d_H(I_1,I_2) + d_H(I_2,I_3)$

The Hamming distance between sequences induces a metric in sequence space



S_1: ......((((((..((((((......))))))))...((((((.....)))))..))))))

S_2: ......((((((..((.(((......))).))..((((((.....)))))).))))))

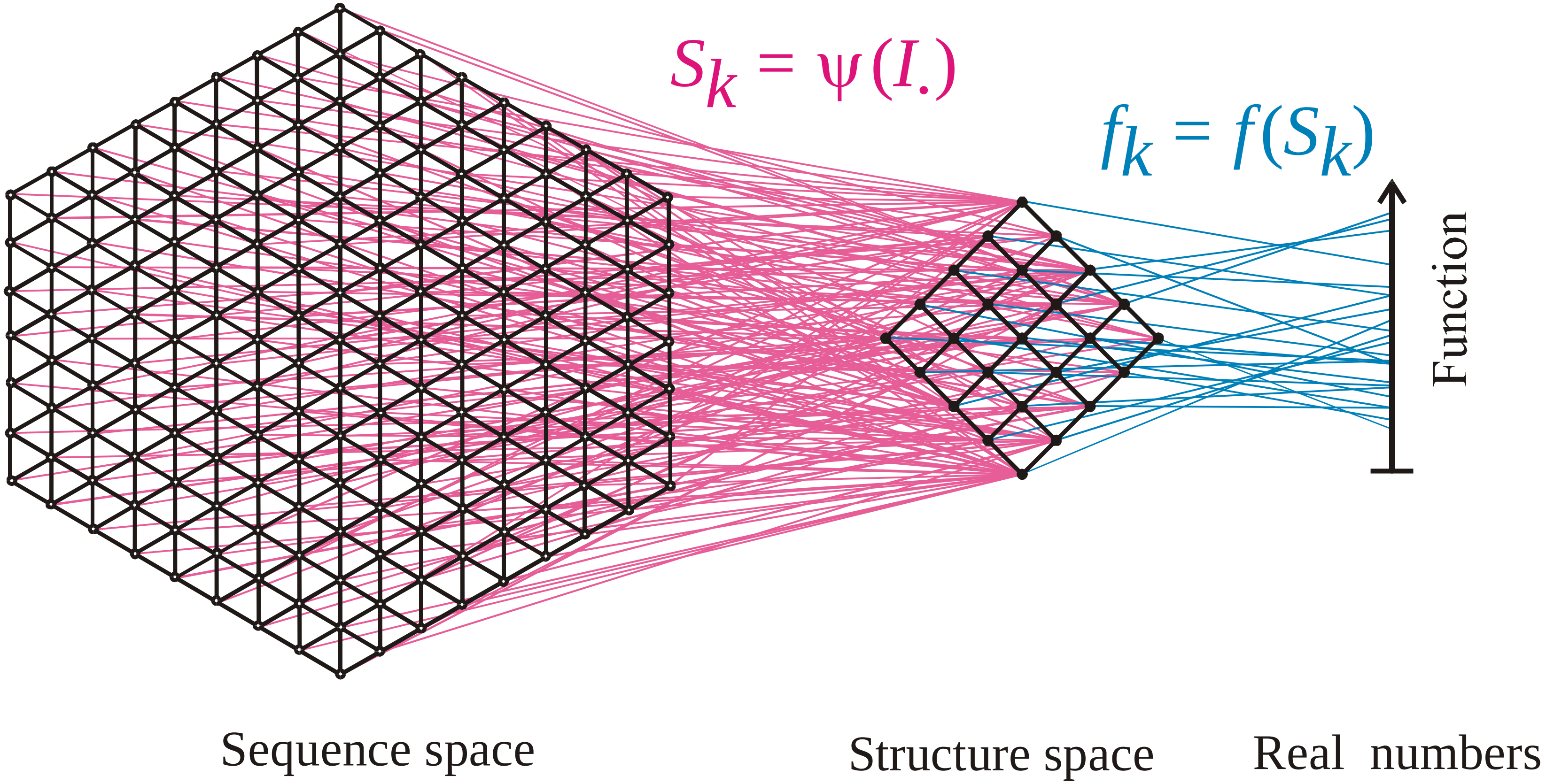
Hamming distance $d_H(S_1,S_2) = 4$

(i) $d_H(S_1,S_1) = 0$

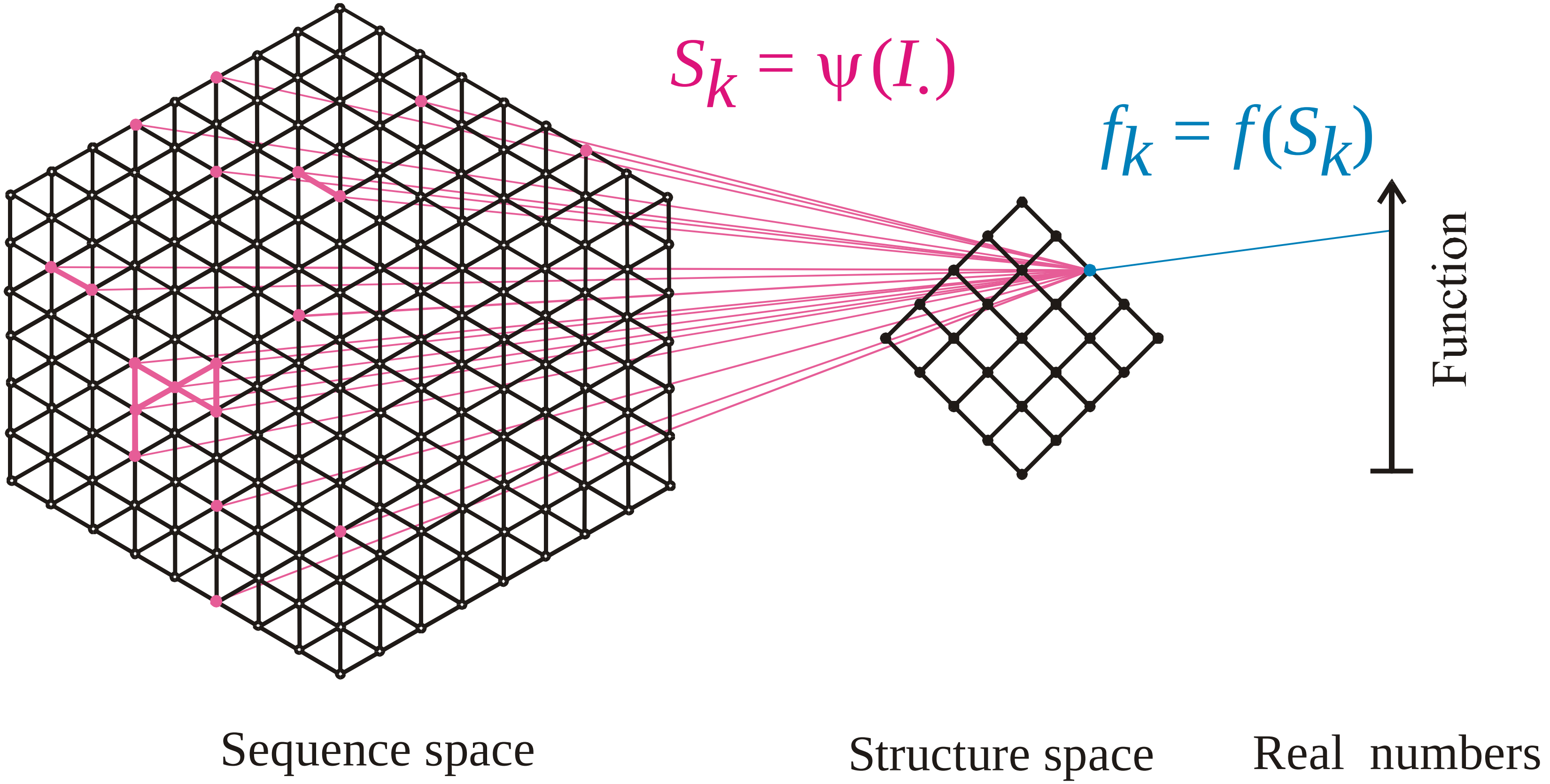
(ii) $d_H(S_1,S_2) = d_H(S_2,S_1)$

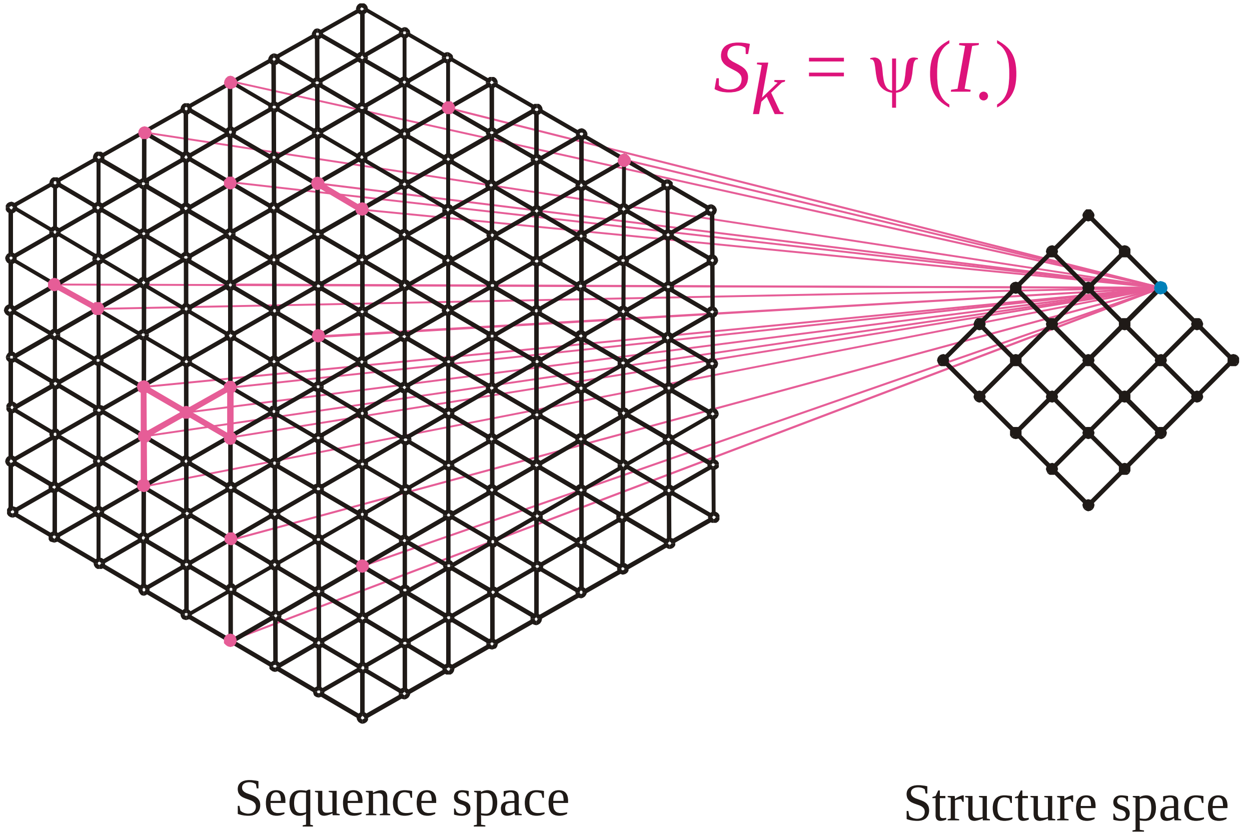
(iii) $d_H(S_1,S_3) \leq d_H(S_1,S_2) + d_H(S_2,S_3)$

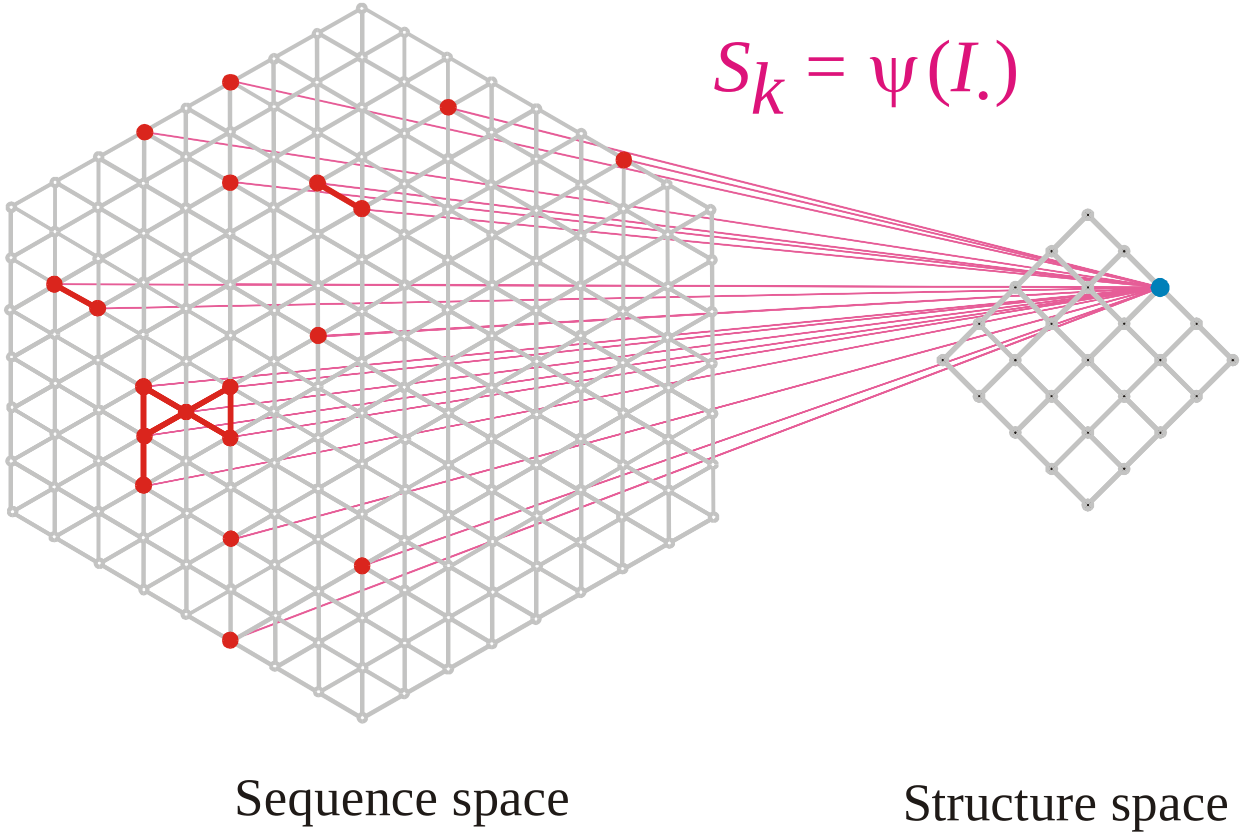
The Hamming distance between structures in parentheses notation forms a metric in structure space



Mapping from sequence space into structure space and into function

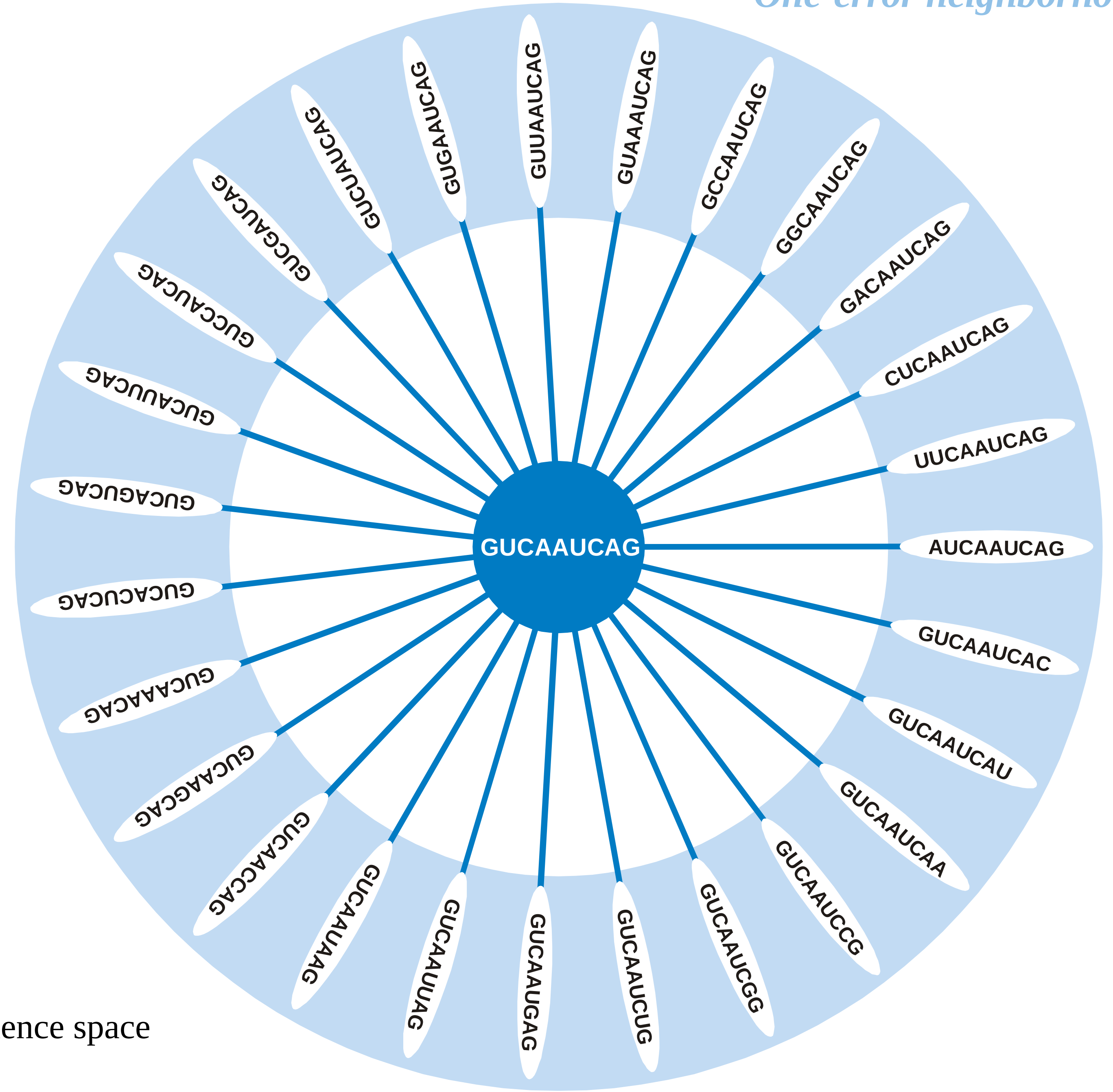

$S_k = \psi(I.)$
$f_k = f(S_k)$
Function
Sequence space
Structure space
Real numbers


$S_k = \psi(I.)$
Sequence space
Structure space

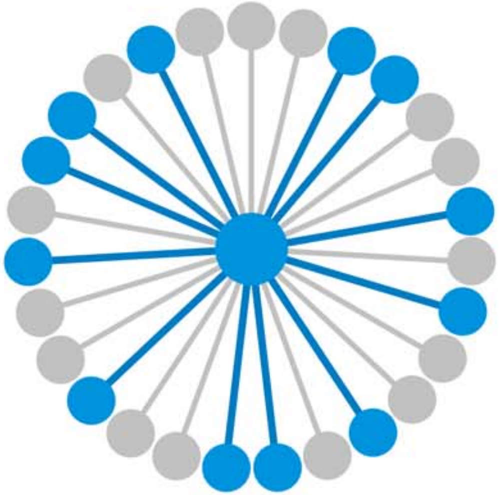


The pre-image of the structure S_k in sequence space is the **neutral network G_k**

One-error neighborhood



The surrounding of
GUCAAUCAG in sequence space



$$G_k = \psi^{-1}(S_k) \doteq \{ I_j \mid \psi(I_j) = S_k \}$$

$$\bar{\lambda}_k = \frac{\sum_{j \in |G_k|} \lambda_j(k)}{|G_k|}$$

$\lambda_j = 12 / 27 = 0.444$

Alphabet size κ :

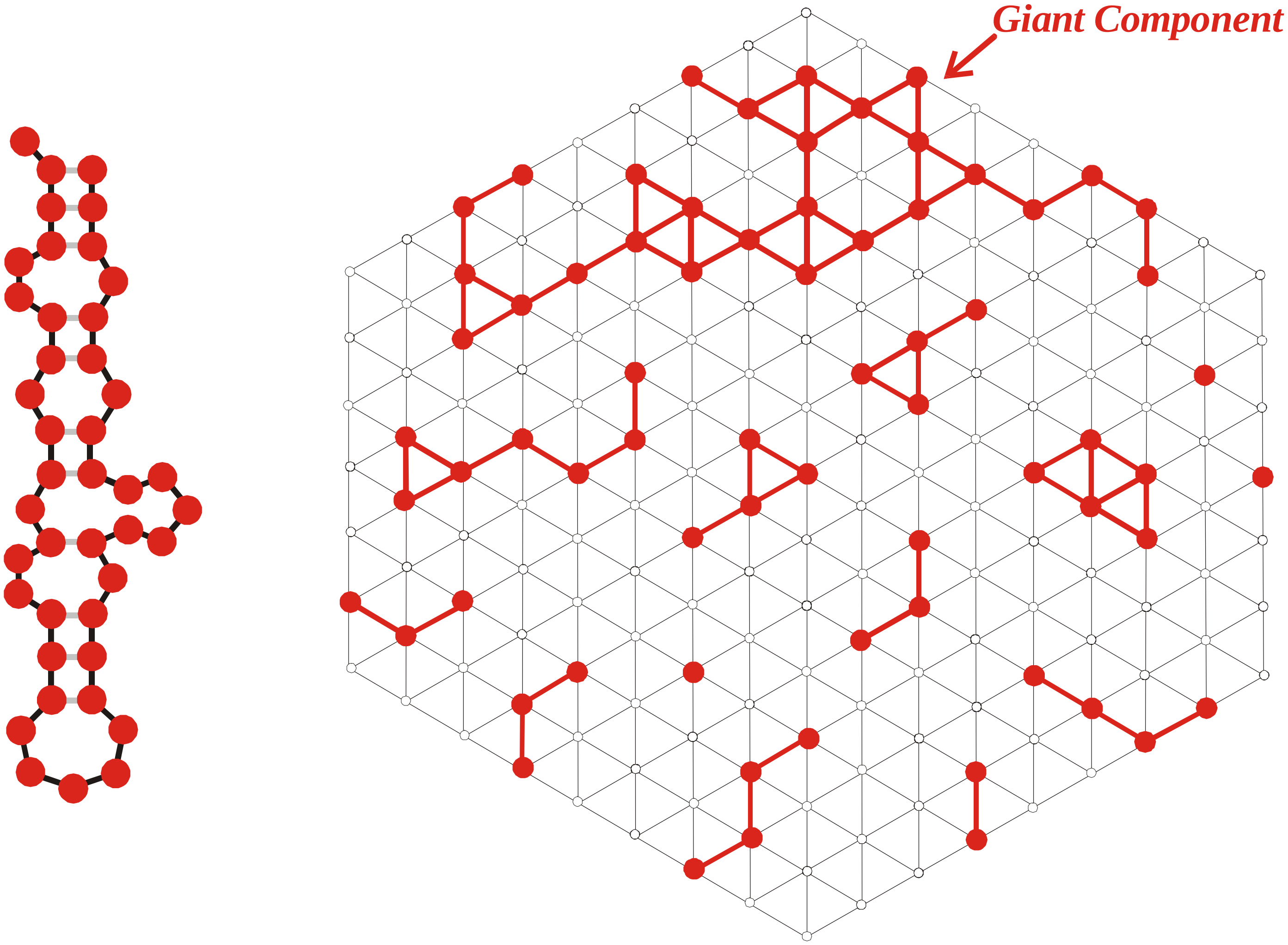
κ	λ_{cr}	
2	0.5	**AU,GC,DU**
3	0.423	**AUG , UGC**
4	0.370	**AUGC**

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

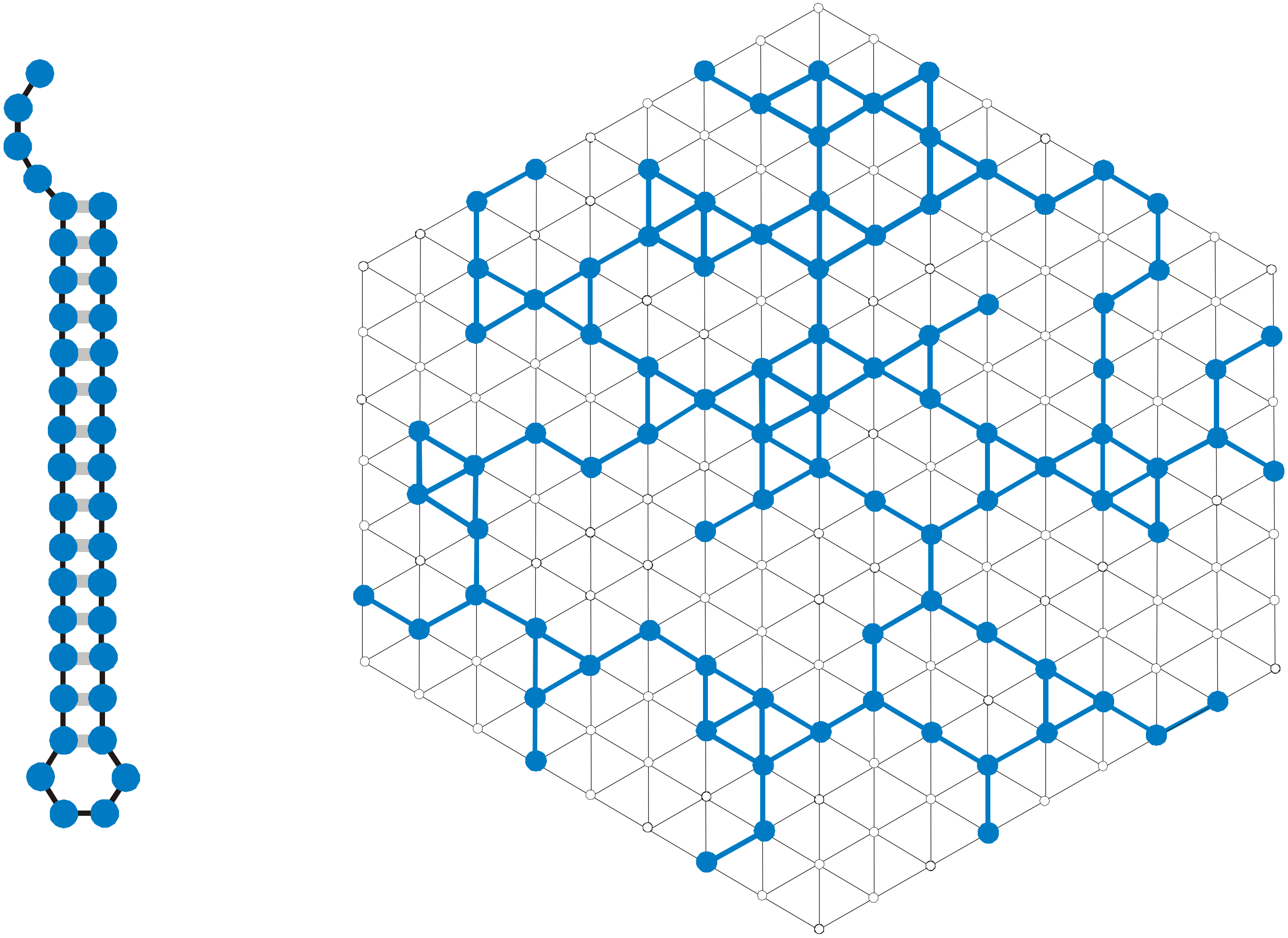
$\bar{\lambda}_k < \lambda_{cr}$ . . . . network G_k is **not** connected

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

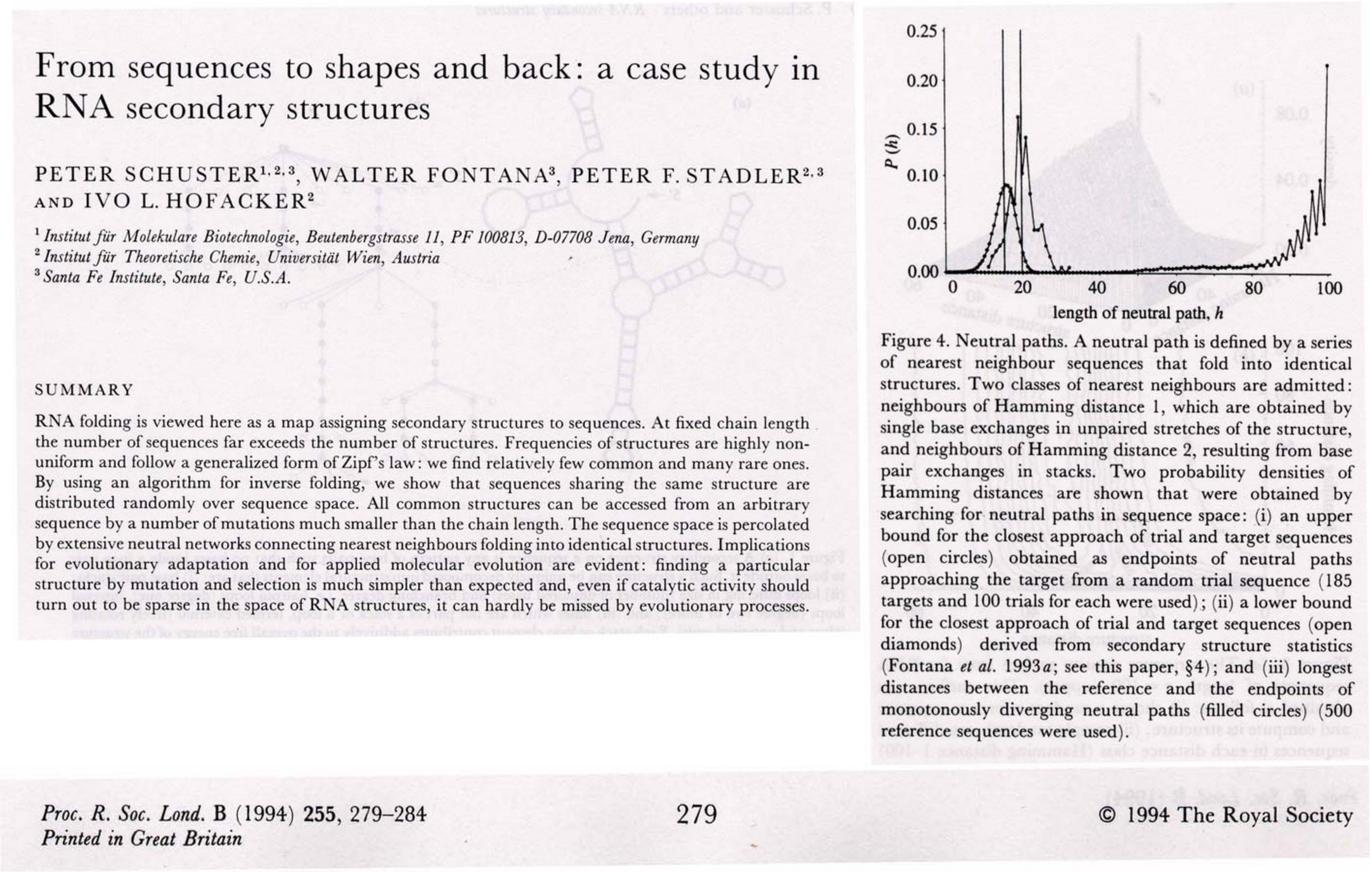
Degree of neutrality of neutral networks and the connectivity threshold



A multi-component neutral network formed by a rare structure: $\lambda < \lambda_{cr}$



A connected neutral network formed by a common structure: $\lambda > \lambda_{cr}$



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

PETER SCHUSTER[1,2,3], WALTER FONTANA[3], PETER F. STADLER[2,3] AND IVO L. HOFACKER[2]

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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.* 1993*a*; 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).

Proc. R. Soc. Lond. B (1994) **255**, 279–284
Printed in Great Britain

Reference for postulation and ***in silico*** verification of ***neutral networks***

Properties of RNA sequence to secondary structure mapping

1. More sequences than structures

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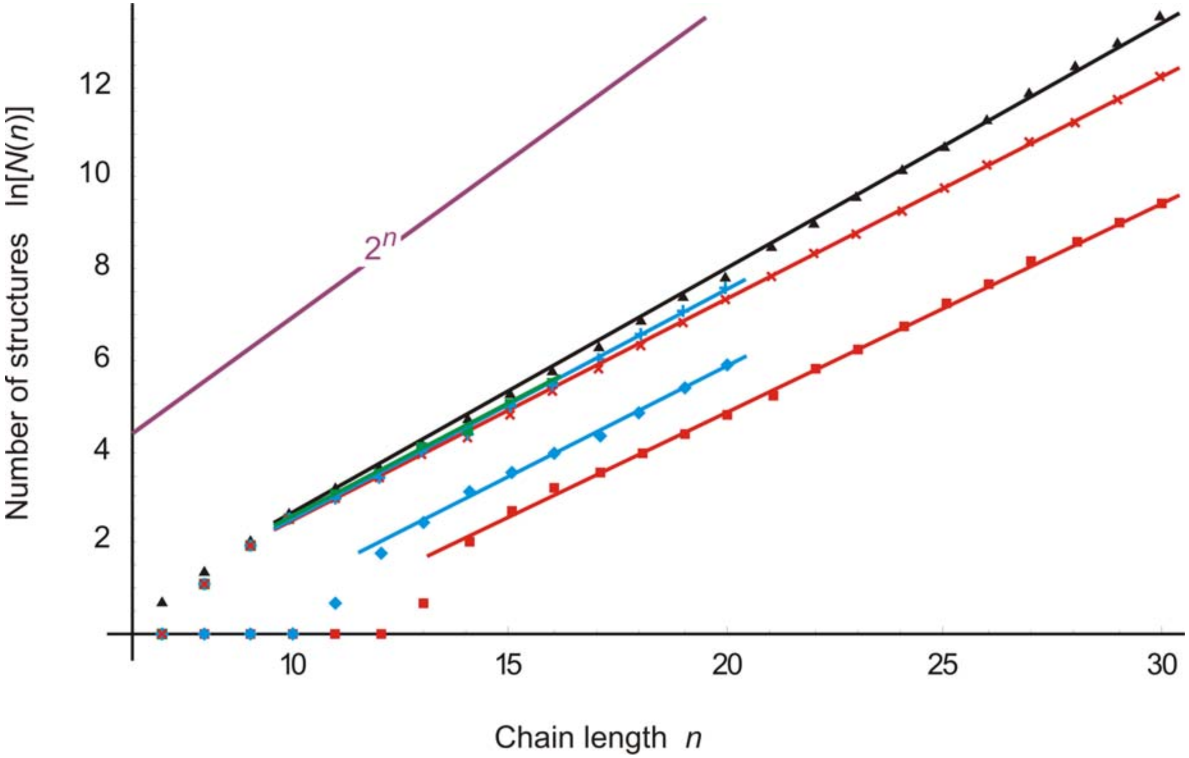

Properties of RNA sequence to secondary structure mapping

1. More sequences than structures
2. Few common versus many rare structures

Properties of RNA sequence to secondary structure mapping

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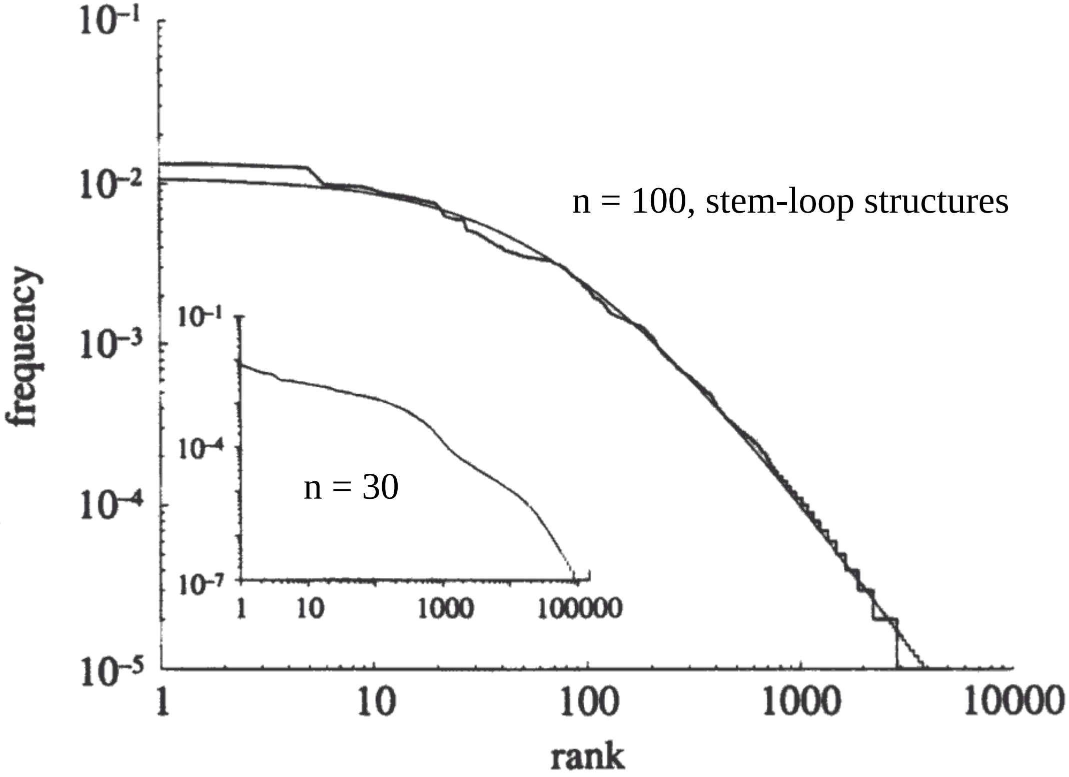
2. Few common versus many rare structures



RNA secondary structures and Zipf’s law

Properties of RNA sequence to secondary structure mapping

1. More sequences than structures
2. Few common versus many rare structures
3. Shape space covering of common structures

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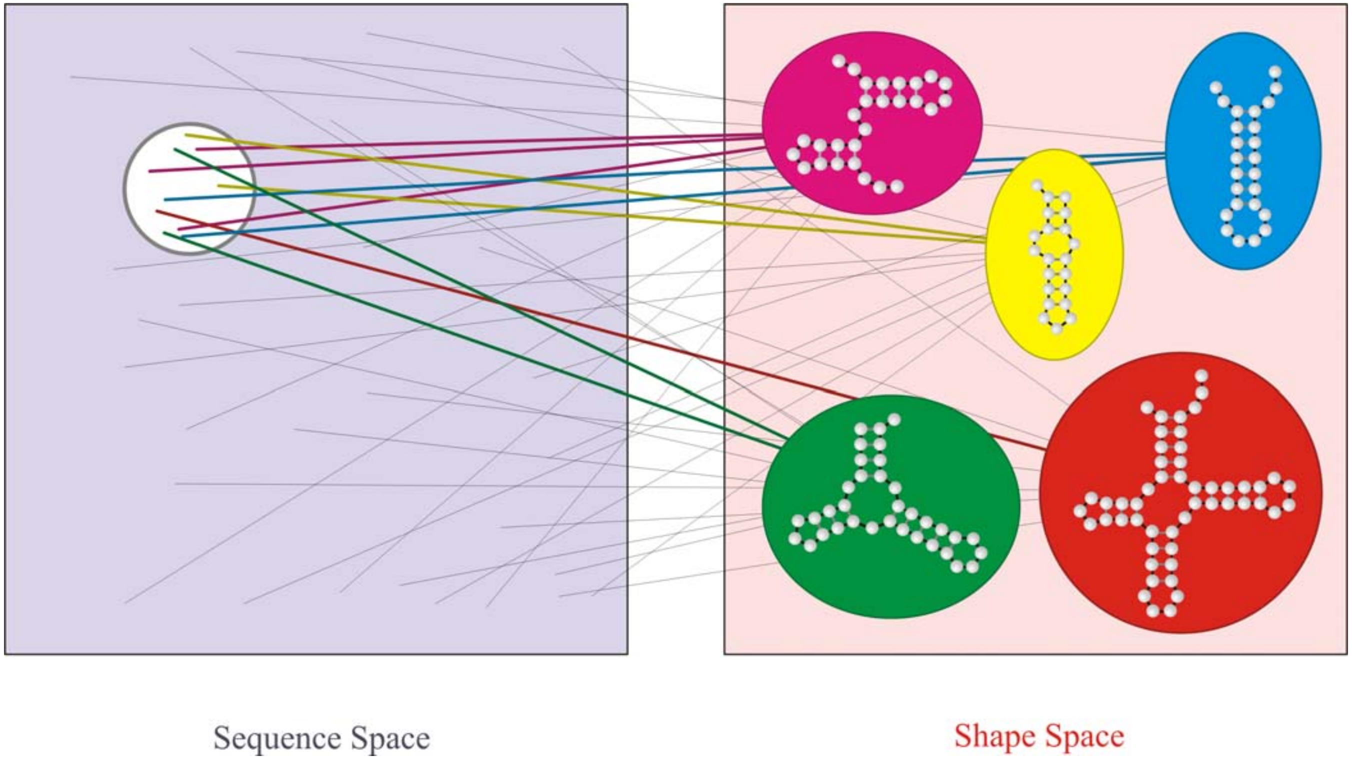

Properties of RNA sequence to secondary structure mapping

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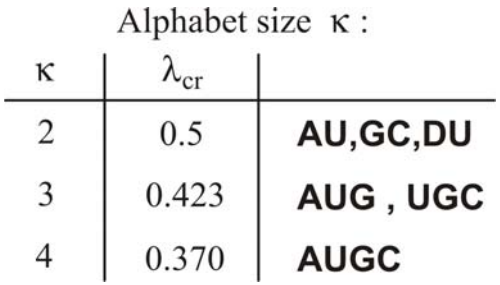

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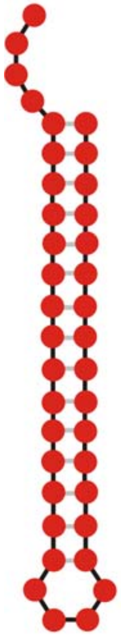

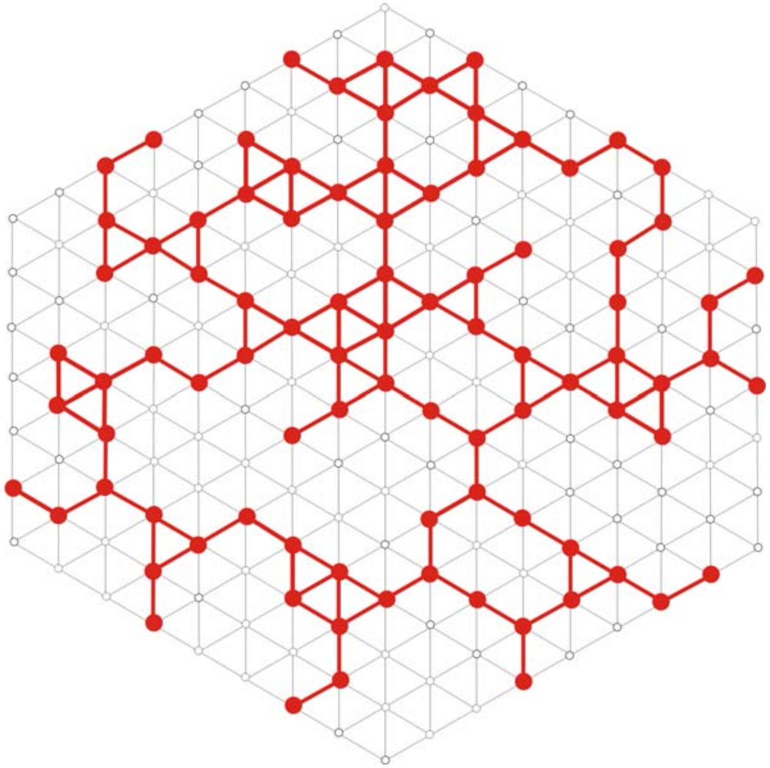

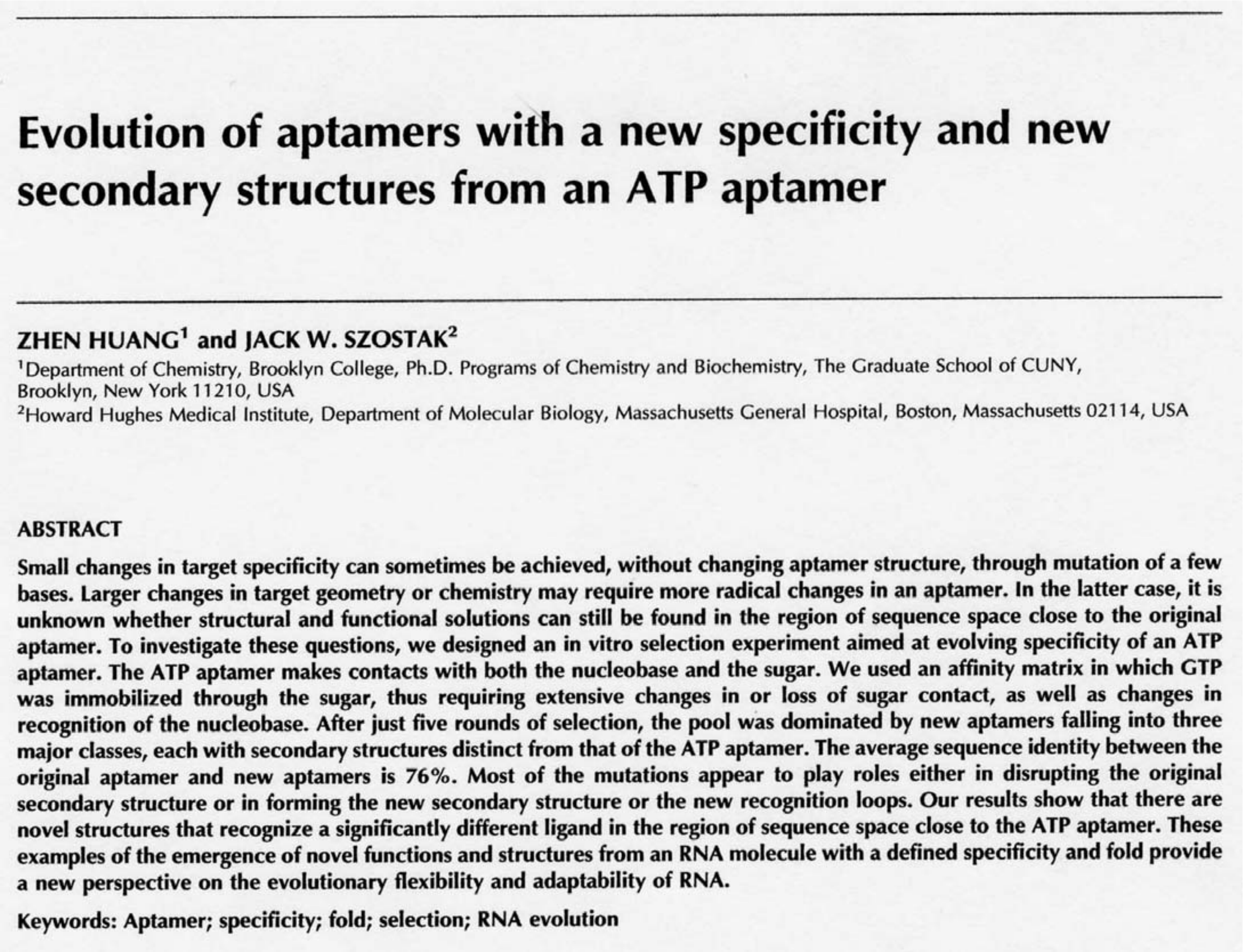

Evolution of aptamers with a new specificity and new secondary structures from an ATP aptamer

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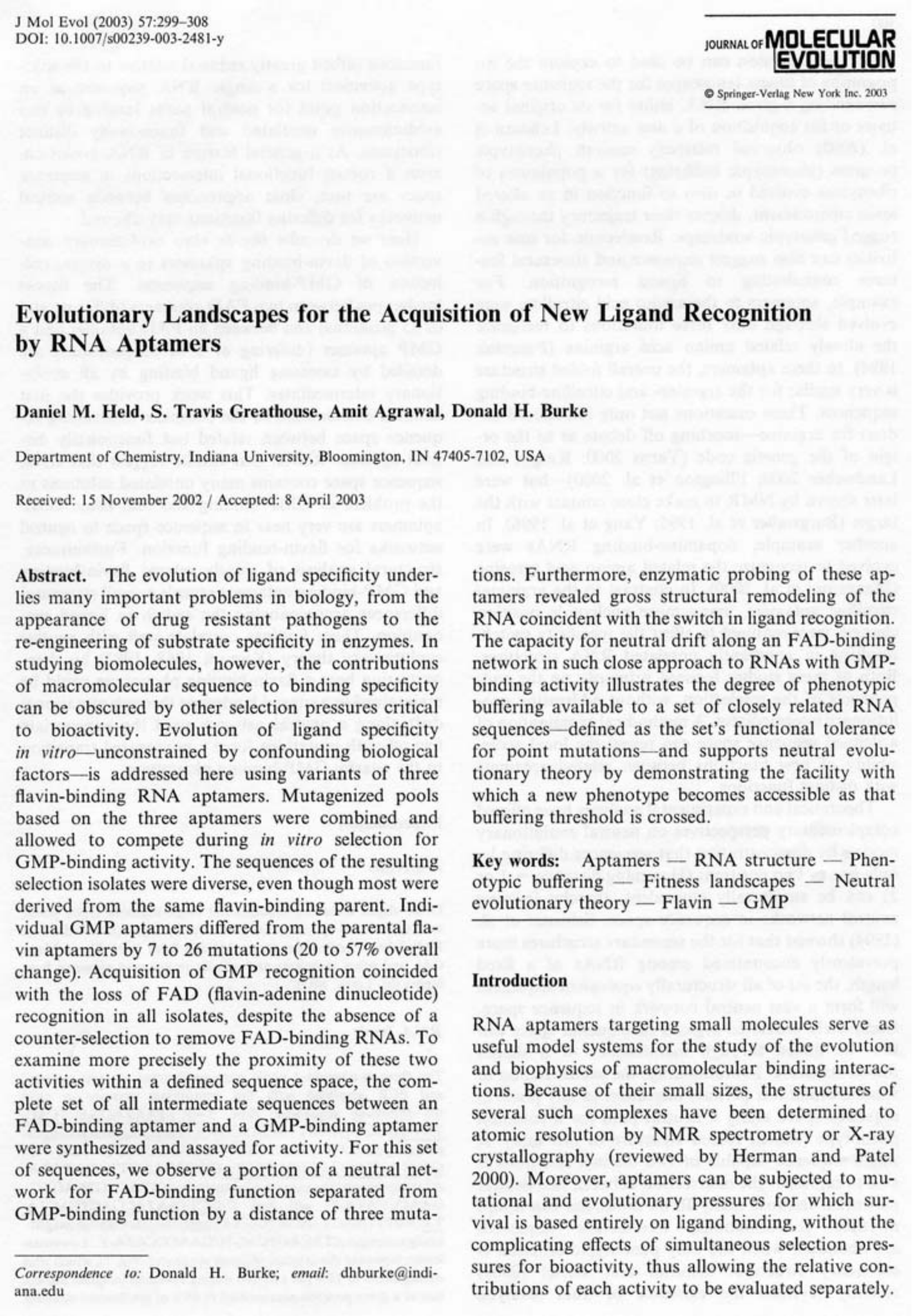
ABSTRACT

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

RNA **9**:1456-1463, 2003

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



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

JOURNAL OF MOLECULAR EVOLUTION
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Evolutionary Landscapes for the Acquisition of New Ligand Recognition by RNA Aptamers

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Received: 15 November 2002 / Accepted: 8 April 2003

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

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.

Correspondence to: Donald H. Burke; *email:* dhburke@indiana.edu

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

1. What are neutral networks ?
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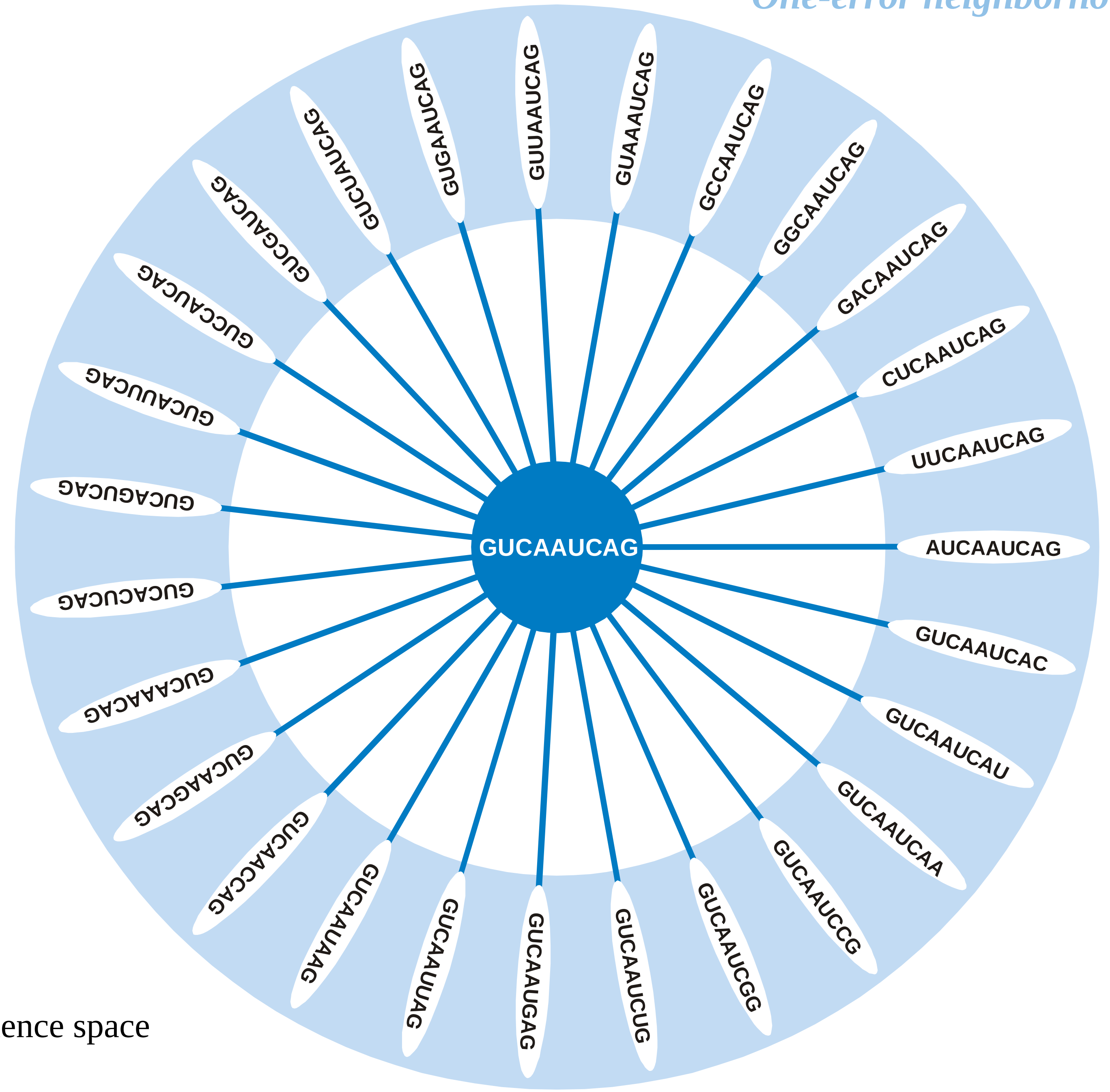
6. How to handle multiple constraints

One-error neighborhood



The surrounding of
GUCAAUCAG in sequence space

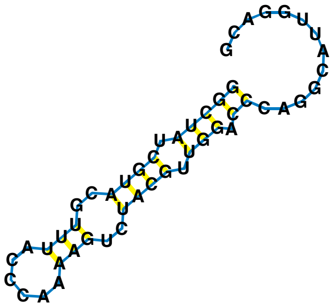
GGCUAUCGUACGUUUACCCAAAAGUCUACGUUGGACCCAGGCAUUGGACG

One error neighborhood – Surrounding of an RNA molecule in sequence and shape space

GGCUAUCGUACGUUUACCCAAAAGUCUACGUUGGACCCAGGCAUUGGACG

One error neighborhood – Surrounding of an RNA molecule in sequence and shape space

GGCUAUCGUACGUUUACCCAAAAGUCUACGUUGGACCCAGGCAUUGGACG



One error neighborhood – Surrounding of an RNA molecule in sequence and shape space

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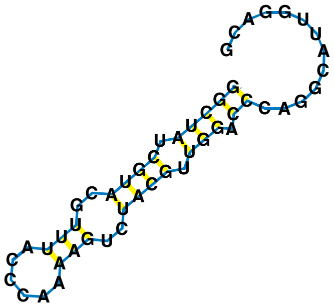
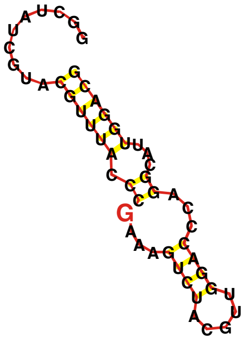


One error neighborhood – Surrounding of an RNA molecule in sequence and shape space



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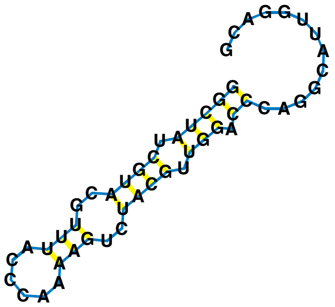
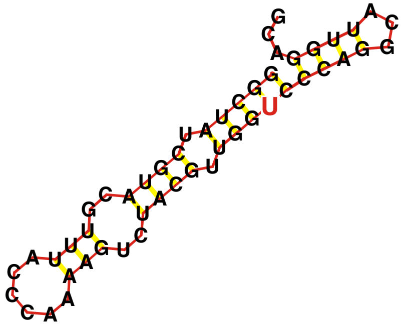
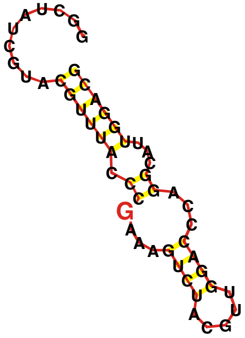


One error neighborhood – Surrounding of an RNA molecule in sequence and shape space





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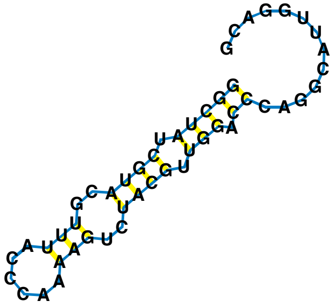
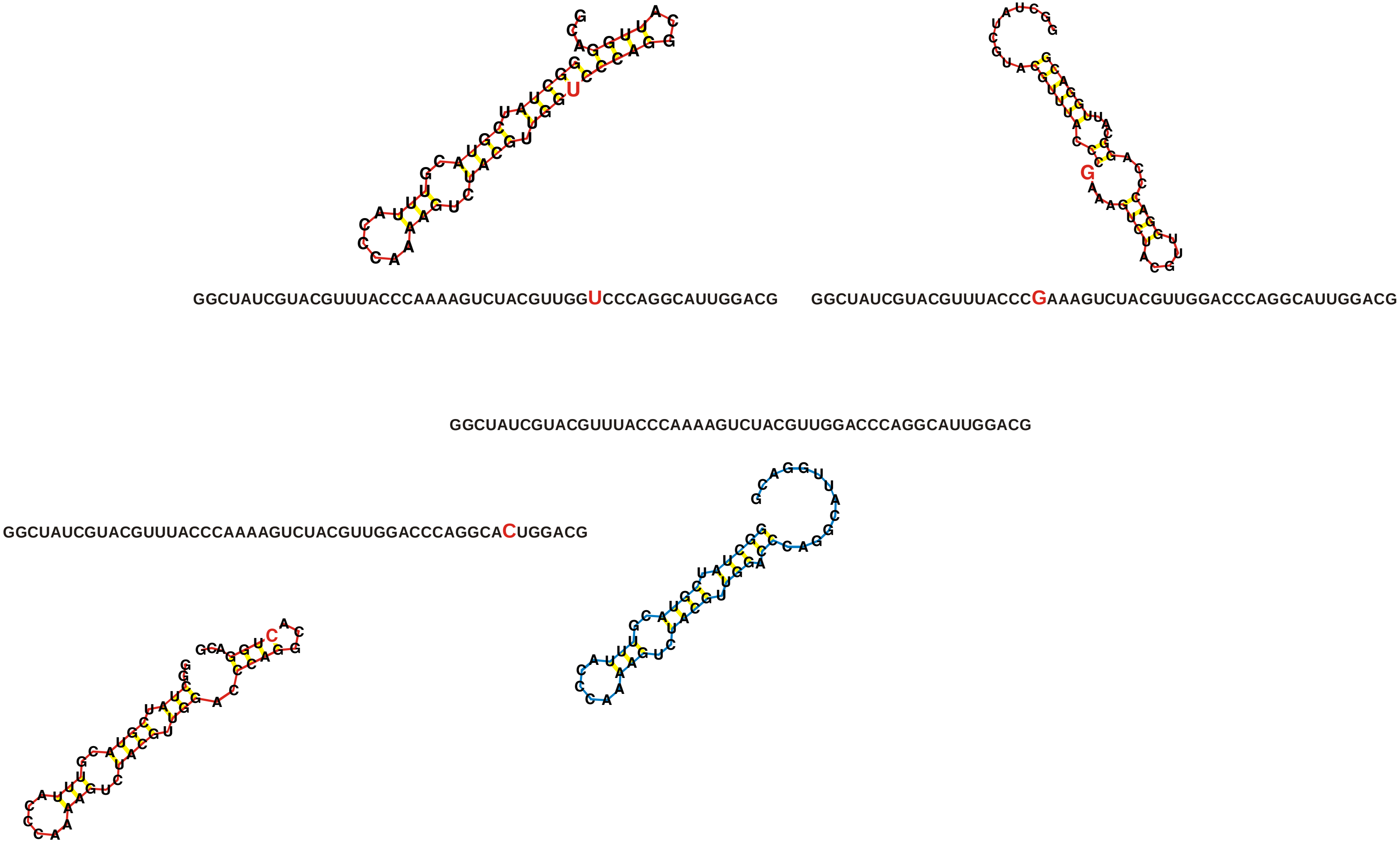


One error neighborhood – Surrounding of an RNA molecule in sequence and shape space



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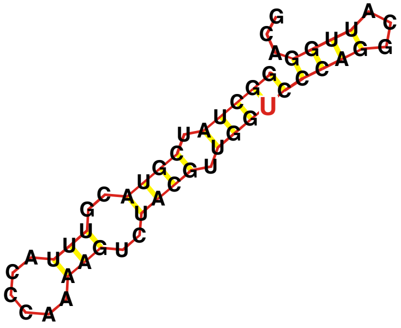
One error neighborhood – Surrounding of an RNA molecule in sequence and shape space



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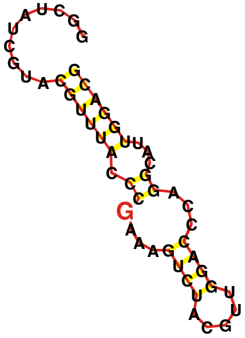

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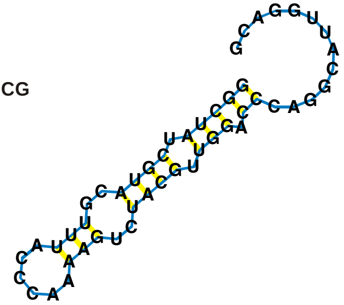

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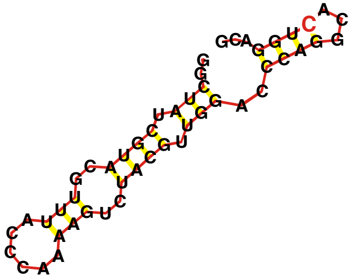

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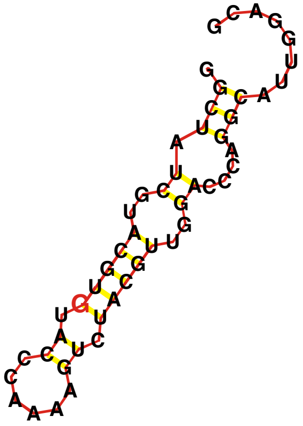

One error neighborhood – Surrounding of an RNA molecule in sequence and shape space

GGCUAUCGUAUGUUUACCCAAAAGUCUACGUUGGACCCAGGCAUUGGACG

GGCUAUCGUACGUUUACCCAAAAGUCUACGUUGGACCCAGGCAUUAGACG

GGCUAUCGUACGUUUACUCAAAAGUCUACGUUGGACCCAGGCAUUGGACG

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GGCUAUCGUACGUGUACCCAAAAGUCUACGUUGGACCCAGGCAUUGGACG

GGCUAACGUACGUUUACCCAAAAGUCUACGUUGGACCCAGGCAUUGGACG

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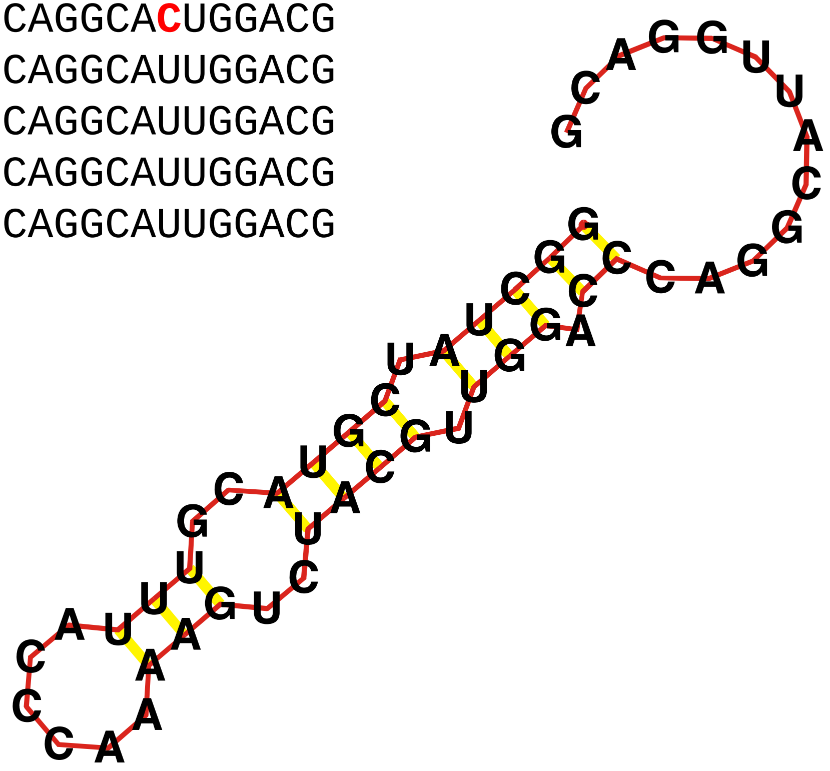

One error neighborhood – Surrounding of an RNA molecule
in sequence and shape space

```
GCAGCUUGCCCAAUGCAACCCCAUGUGGCGCUAGCUAACACCAUCCCC
 1 (((((.((((..(((......)))..)))).))).)).............       65 0.433333
 2 ..(((((((((((((......))).))).)))..))))............        9 0.060000
 3 (((((.((((....(((......))))))).))).)).............        5 0.033333
 4 ..(((.((((..(((......)))..)))).)))................        5 0.033333
 5 ..(((((((((((((......))).)))...)))))))............        4 0.026667
 6 (((((.((((((.((.....)).)).)))).))).)).............        3 0.020000
 7 (((((.((((.((((......)))).)))).))).)).............        3 0.020000
 8 (((((.(((((.(((......))).))))).))).)).............        3 0.020000
 9 ((((((((((..(((......)))..)))))))).)).............        3 0.020000
10 (((((.((((((...........)).)))).))).)).............        3 0.020000
11 (((((..(((..(((......)))..)))..))).)).............        2 0.013333
12 (((((.((((..(((......)))..)))).)).))).............        2 0.013333
13 ..((((.((.(..((((......))))..).)).))))............        2 0.013333
14 (((((.((.((((((......))).))))).))).)).............        2 0.013333
15 .((((((((((((((......))).))).)))..)))))...........        2 0.013333
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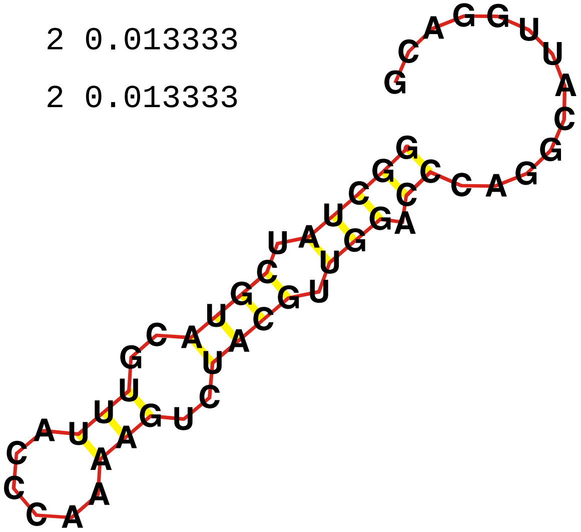

```
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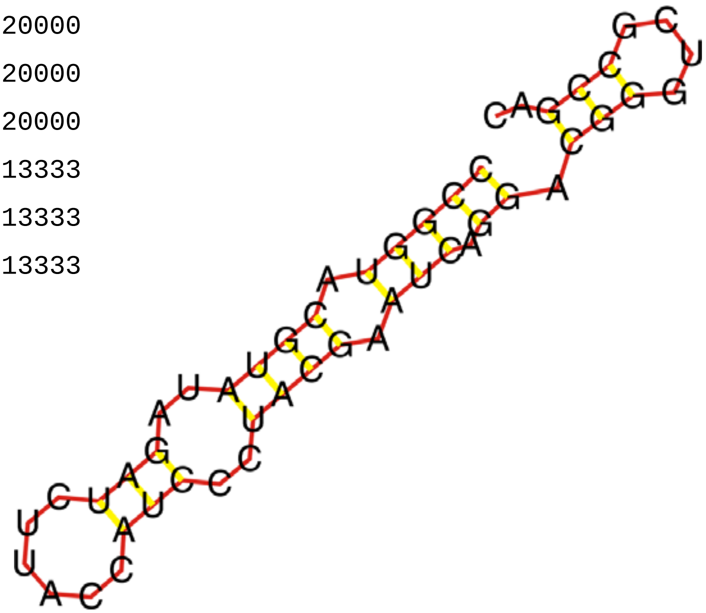
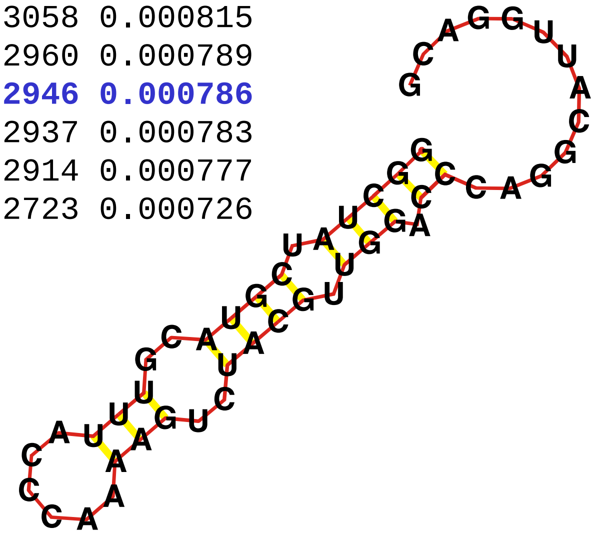
 1 (((((.((((..(((......)))..)))).))).)).(((....)))..        49 0.326667
 2 (((((.((((..(((......)))..)))).))).)).............         7 0.046667
 3 ..(((.((((..(((......)))..)))).)))....(((....)))..         6 0.040000
 4 (((((.((((..((........))..)))).))).)).(((....)))..         5 0.033333
 5 ((.((((((((...(((.((((....)).).).))).)))))..))))).         5 0.033333
 6 (((((.((((...((......))...)))).))).)).(((....)))..         5 0.033333
 7 (((((.((((..(((......)))..)))).))).))..((....))...         4 0.026667
 8 (((((.((((..(((......)))..)))).)))))..(((....)))..         4 0.026667
 9 (((((.(((...(((......)))...))).))).)).(((....)))..         3 0.020000
10 ((((((((((..(((......)))..)))))))).)).(((....)))..         3 0.020000
11 ((.(((.((((..(((..(.....)..)))..))))..))).))......         3 0.020000
12 (((((...((..(((......)))..))...))).)).(((....)))..         3 0.020000
13 (.(((.((((..(((......)))..)))).))).)..(((....)))..         3 0.020000
14 ((..(.((((..(((......)))..)))).)...)).(((....)))..         3 0.020000
15 (((((.(((((.(((......))).))))).))).)).(((....)))..         3 0.020000
16 (((((.((((.((((......)))).)))).))).)).(((....)))..         3 0.020000
17 (((((..(((..(((......)))..)))..))).)).(((....)))..         3 0.020000
18 ((.((((((((...(((.(.(........).).))).)))))..))))).         2 0.013333
19 (((((.((((..(((......)))..)))).)).))).(((....)))..         2 0.013333
20 ((.((((((((...((((((((....)).).))))).)))))..))))).         2 0.013333
```



```
                                   Number   Mean Value     Variance     Std.Dev.
Total Hamming Distance:           3750000    11.608372    22.628558     4.756948
Nonzero Hamming Distance:         2493088    16.921998    30.500616     5.522736
Degree of Neutrality:             1256912     0.335177     0.006850     0.082764
Number of Structures:               25000        52.15        84.61         9.20

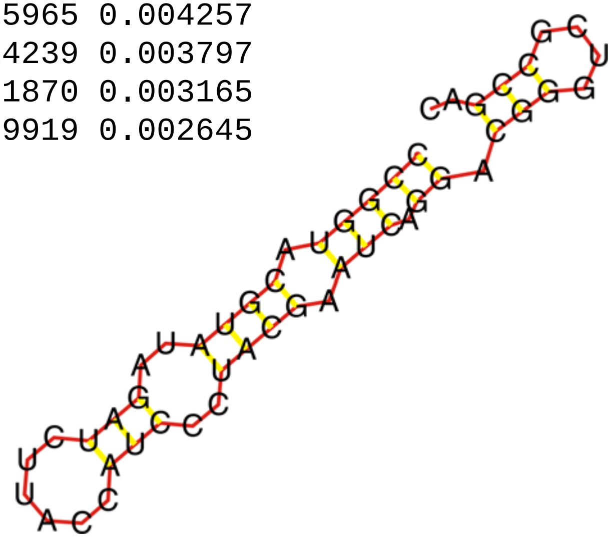
   1 (((((.((((..(((......)))..)))).))).))............. 1256912 0.335177
   2 ((((((((((..(((......)))..)))))))).)).............   69647 0.018573
   3 ..(((.((((..(((......)))..)))).)))................   69194 0.018452
   4 (((((.((((..((((....))))..)))).))).)).............   61825 0.016487
   5 (((((.((((.((((......)))).)))).))).)).............   56398 0.015039
   6 (((((.(((((.(((......))).))))).))).)).............   55423 0.014779
   7 (((((..(((..(((......)))..)))..))).)).............   34871 0.009299
   8 (((((.((((..((........))..)))).))).)).............   29201 0.007787
   9 ((((..((((..(((......)))..))))..)).)).............   25844 0.006892
  10 (((((.((((..(((......)))..)))).)))))..............   25459 0.006789

  28 (((((.((((..(((......)))..)))).))).))..(((....))).     3629 0.000968
  29 (((((...((..(((......)))..))...))).)).............     3519 0.000938
  30 ...((.((((..(((......)))..)))).)).................     3138 0.000837
  31 (((((.((....(((......)))....)).))).)).............     3067 0.000818
  32 ......((((..(((......)))..))))....................     3058 0.000815
  33 (((((.((((..(((.....)))...)))).))).)).............     2960 0.000789
  34 (((((.((((..(((......)))..)))).))).)).(((....)))..     2946 0.000786
  35 (((((.((((..(((......)))..)))).))).))...(((....)))     2937 0.000783
  36 (((...((((..(((......)))..))))....))).............     2914 0.000777
  37 ..(((.((((..(((......)))..)))).))).(((....))).....     2723 0.000726
```



Shadow – Surrounding of RNA structure I in shape space – **AUGC** alphabet

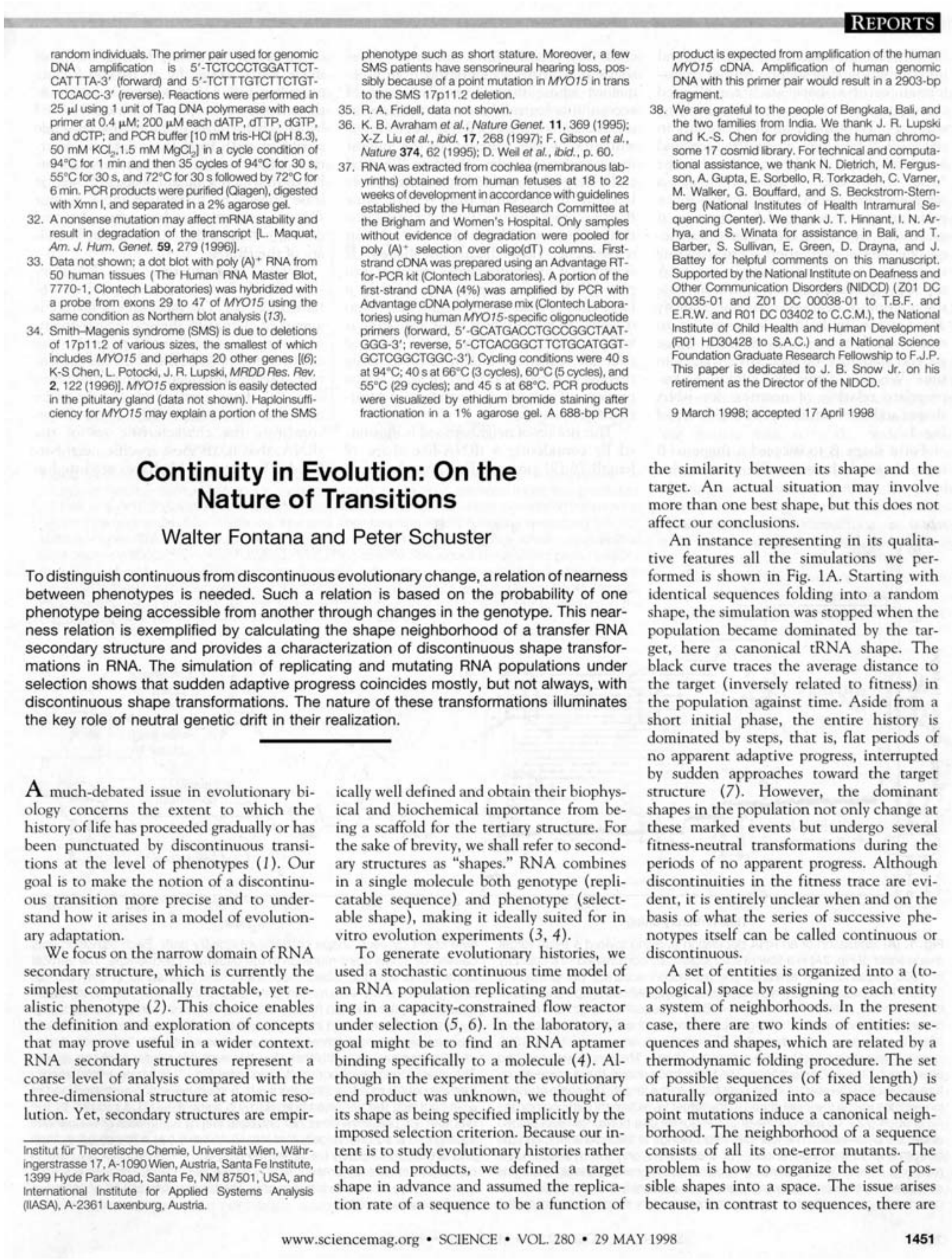
```
                                    Number     Mean Value      Variance      Std.Dev.
Total Hamming Distance:            3750000      12.498761     23.352188      4.832410
Nonzero Hamming Distance:          2807992      16.350987     29.476615      5.429237
Degree of Neutrality:               942008       0.251202      0.003690      0.060747
Number of Structures:                25000          54.16         73.46          8.57
    1 (((((.((((..(((......)))..)))).))).)).(((....)))..   942008 0.251202
    2 (((((.((((..(((......)))..)))).))).))..............   166946 0.044519
    3 ..(((.((((..(((......)))..)))).)))....(((....)))..   103673 0.027646
    4 ((((((((((..(((......)))..)))))))).)).(((....)))..    69658 0.018575
    5 (((((.((((..((((....))))..)))).))).)).(((....)))..    62183 0.016582
    6 (((((.((((.((((......)))).)))).))).)).(((....)))..    56510 0.015069
    7 (((((.(((((.(((......))).))))).))).)).(((....)))..    55902 0.014907
    8 (((((..(((..(((......)))..)))..))).)).(((....)))..    35249 0.009400
    9 .((((.((((..(((......)))..)))).))))...(((....)))..    32042 0.008545
   10 (((((.((((..((........))..)))).))).)).(((....)))..    29725 0.007927
   11 (((((.((((..(((......)))..)))).)))))..(((....)))..    27114 0.007230
   12 ((((..((((..(((......)))..))))..)).)).(((....)))..    25820 0.006885
   13 (((((.((((..(((......)))..)))).)).))).(((....)))..    22513 0.006003
   14 (((((.(((...(((......)))...))).))).)).(((....)))..    21640 0.005771
   15 ..(((.((((..(((......)))..)))).)))...((((....)))).    20394 0.005438
   16 ..(((.((((..(((......)))..)))).)))..(((((....)))))    16983 0.004529
   17 (((((.((((...((......))...)))).))).)).(((....)))..    15965 0.004257
   18 (((((.((((..(((......)))..)))).))).))..((....))...    14239 0.003797
   19 (((((.((((..(((......)))..)))).))).)).((......))..    11870 0.003165
   20 (((((.((((..(((......)))..)))).))).))((((....)))).     9919 0.002645
```



Shadow – Surrounding of RNA structure II in shape space – **AUGC** alphabet

Evolution *in silico*

W. Fontana, P. Schuster,
Science **280** (1998), 1451-1455



random individuals. The primer pair used for genomic DNA amplification is 5′-TCTCCCTGGATTCT-CATTTA-3′ (forward) and 5′-TCTTTGTCTTCTGT-TCCACC-3′ (reverse). Reactions were performed in 25 μl using 1 unit of Taq DNA polymerase with each primer at 0.4 μM; 200 μM each dATP, dTTP, dGTP, and dCTP; and PCR buffer [10 mM tris-HCl (pH 8.3), 50 mM KCl_2,1.5 mM $MgCl_2$] in a cycle condition of 94°C for 1 min and then 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s followed by 72°C for 6 min. PCR products were purified (Qiagen), digested with Xmn I, and separated in a 2% agarose gel.

32. A nonsense mutation may affect mRNA stability and result in degradation of the transcript [L. Maquat, *Am. J. Hum. Genet.* **59**, 279 (1996)].
33. Data not shown; a dot blot with poly $(A)^+$ RNA from 50 human tissues (The Human RNA Master Blot, 7770-1, Clontech Laboratories) was hybridized with a probe from exons 29 to 47 of *MYO15* using the same condition as Northern blot analysis (*13*).
34. Smith–Magenis syndrome (SMS) is due to deletions of 17p11.2 of various sizes, the smallest of which includes *MYO15* and perhaps 20 other genes [(*6*); K-S Chen, L. Potocki, J. R. Lupski, *MRDD Res. Rev.* **2**, 122 (1996)]. *MYO15* expression is easily detected in the pituitary gland (data not shown). Haploinsufficiency for *MYO15* may explain a portion of the SMS phenotype such as short stature. Moreover, a few SMS patients have sensorineural hearing loss, possibly because of a point mutation in *MYO15* in trans to the SMS 17p11.2 deletion.
35. R. A. Fridell, data not shown.
36. K. B. Avraham *et al.*, *Nature Genet.* **11**, 369 (1995); X-Z. Liu *et al.*, *ibid.* **17**, 268 (1997); F. Gibson *et al.*, *Nature* **374**, 62 (1995); D. Weil *et al.*, *ibid.*, p. 60.
37. RNA was extracted from cochlea (membranous labyrinths) obtained from human fetuses at 18 to 22 weeks of development in accordance with guidelines established by the Human Research Committee at the Brigham and Women's Hospital. Only samples without evidence of degradation were pooled for poly $(A)^+$ selection over oligo(dT) columns. First-strand cDNA was prepared using an Advantage RT-for-PCR kit (Clontech Laboratories). A portion of the first-strand cDNA (4%) was amplified by PCR with Advantage cDNA polymerase mix (Clontech Laboratories) using human *MYO15*-specific oligonucleotide primers (forward, 5′-GCATGACCTGCCGGCTAAT-GGG-3′; reverse, 5′-CTCACGGCTTCTGCATGGT-GCTCGGCTGGC-3′). Cycling conditions were 40 s at 94°C; 40 s at 66°C (3 cycles), 60°C (5 cycles), and 55°C (29 cycles); and 45 s at 68°C. PCR products were visualized by ethidium bromide staining after fractionation in a 1% agarose gel. A 688-bp PCR product is expected from amplification of the human *MYO15* cDNA. Amplification of human genomic DNA with this primer pair would result in a 2903-bp fragment.

38. We are grateful to the people of Bengkala, Bali, and the two families from India. We thank J. R. Lupski and K.-S. Chen for providing the human chromosome 17 cosmid library. For technical and computational assistance, we thank N. Dietrich, M. Fergusson, A. Gupta, E. Sorbello, R. Torkzadeh, C. Varner, M. Walker, G. Bouffard, and S. Beckstrom-Sternberg (National Institutes of Health Intramural Sequencing Center). We thank J. T. Hinnant, I. N. Arhya, and S. Winata for assistance in Bali, and T. Barber, S. Sullivan, E. Green, D. Drayna, and J. Battey for helpful comments on this manuscript. Supported by the National Institute on Deafness and Other Communication Disorders (NIDCD) (Z01 DC 00035-01 and Z01 DC 00038-01 to T.B.F. and E.R.W. and R01 DC 03402 to C.C.M.), the National Institute of Child Health and Human Development (R01 HD30428 to S.A.C.) and a National Science Foundation Graduate Research Fellowship to F.J.P. This paper is dedicated to J. B. Snow Jr. on his retirement as the Director of the NIDCD.

9 March 1998; accepted 17 April 1998

Continuity in Evolution: On the Nature of Transitions

Walter Fontana and Peter Schuster

To distinguish continuous from discontinuous evolutionary change, a relation of nearness between phenotypes is needed. Such a relation is based on the probability of one phenotype being accessible from another through changes in the genotype. This nearness relation is exemplified by calculating the shape neighborhood of a transfer RNA secondary structure and provides a characterization of discontinuous shape transformations in RNA. The simulation of replicating and mutating RNA populations under selection shows that sudden adaptive progress coincides mostly, but not always, with discontinuous shape transformations. The nature of these transformations illuminates the key role of neutral genetic drift in their realization.

A much-debated issue in evolutionary biology concerns the extent to which the history of life has proceeded gradually or has been punctuated by discontinuous transitions at the level of phenotypes (*1*). Our goal is to make the notion of a discontinuous transition more precise and to understand how it arises in a model of evolutionary adaptation.

We focus on the narrow domain of RNA secondary structure, which is currently the simplest computationally tractable, yet realistic phenotype (*2*). This choice enables the definition and exploration of concepts that may prove useful in a wider context. RNA secondary structures represent a coarse level of analysis compared with the three-dimensional structure at atomic resolution. Yet, secondary structures are empirically well defined and obtain their biophysical and biochemical importance from being a scaffold for the tertiary structure. For the sake of brevity, we shall refer to secondary structures as "shapes." RNA combines in a single molecule both genotype (replicatable sequence) and phenotype (selectable shape), making it ideally suited for in vitro evolution experiments (*3*, *4*).

To generate evolutionary histories, we used a stochastic continuous time model of an RNA population replicating and mutating in a capacity-constrained flow reactor under selection (*5*, *6*). In the laboratory, a goal might be to find an RNA aptamer binding specifically to a molecule (*4*). Although in the experiment the evolutionary end product was unknown, we thought of its shape as being specified implicitly by the imposed selection criterion. Because our intent is to study evolutionary histories rather than end products, we defined a target shape in advance and assumed the replication rate of a sequence to be a function of the similarity between its shape and the target. An actual situation may involve more than one best shape, but this does not affect our conclusions.

An instance representing in its qualitative features all the simulations we performed is shown in Fig. 1A. Starting with identical sequences folding into a random shape, the simulation was stopped when the population became dominated by the target, here a canonical tRNA shape. The black curve traces the average distance to the target (inversely related to fitness) in the population against time. Aside from a short initial phase, the entire history is dominated by steps, that is, flat periods of no apparent adaptive progress, interrupted by sudden approaches toward the target structure (*7*). However, the dominant shapes in the population not only change at these marked events but undergo several fitness-neutral transformations during the periods of no apparent progress. Although discontinuities in the fitness trace are evident, it is entirely unclear when and on the basis of what the series of successive phenotypes itself can be called continuous or discontinuous.

A set of entities is organized into a (topological) space by assigning to each entity a system of neighborhoods. In the present case, there are two kinds of entities: sequences and shapes, which are related by a thermodynamic folding procedure. The set of possible sequences (of fixed length) is naturally organized into a space because point mutations induce a canonical neighborhood. The neighborhood of a sequence consists of all its one-error mutants. The problem is how to organize the set of possible shapes into a space. The issue arises because, in contrast to sequences, there are

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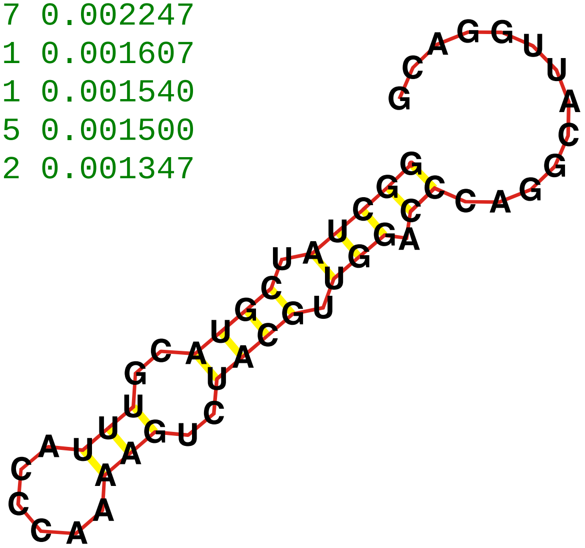
1. What are neutral networks ?
2. Mutations and structural stability
3. **Structures from defective alphabets**
4. Suboptimal conformations and structural stability
5. Metastable structures and RNA switches
6. How to handle multiple constraints

```
                              Number     Mean Value      Variance      Std.Dev.
Total Hamming Distance:       150000      11.647973     23.140715      4.810480
Nonzero Hamming Distance:      99875      16.949991     30.757651      5.545958
Degree of Neutrality:          50125       0.334167      0.006961      0.083434
Number of Structures:           1000          52.31         85.30          9.24

   1 (((((.((((..(((......)))..)))).))).)).............    50125 0.334167
   2 ..(((.((((..(((......)))..)))).)))................     2856 0.019040
   3 ((((((((((..(((......)))..)))))))).)).............     2799 0.018660
   4 (((((.((((..((((....))))..)))).))).)).............     2417 0.016113
   5 (((((.((((.((((......)))).)))).))).)).............     2265 0.015100
   6 (((((.(((((.(((......))).))))).))).)).............     2233 0.014887
   7 (((((..(((..(((......)))..)))..))).)).............     1442 0.009613
   8 (((((.((((..((........))..)))).))).)).............     1081 0.007207
   9 ((((..((((..(((......)))..))))..)).)).............     1025 0.006833
  10 (((((.((((..(((......)))..)))).)))))..............     1003 0.006687
  11 .((((.((((..(((......)))..)))).))))...............      963 0.006420
  12 (((((.(((...(((......)))...))).))).)).............      860 0.005733
  13 (((((.((((..(((......)))..)))).)).))).............      800 0.005333
  14 (((((.((((...((......))...)))).))).)).............      548 0.003653
  15 (((((.((((................)))).))).)).............      362 0.002413
  16 ((.((.((((..(((......)))..)))).))..)).............      337 0.002247
  17 (.(((.((((..(((......)))..)))).))).)..............      241 0.001607
  18 (((((.(((((((((......))))))))).))).)).............      231 0.001540
  19 ((((..((((..(((......)))..))))...)))).............      225 0.001500
  20 ((....((((..(((......)))..)))).....)).............      202 0.001347
```

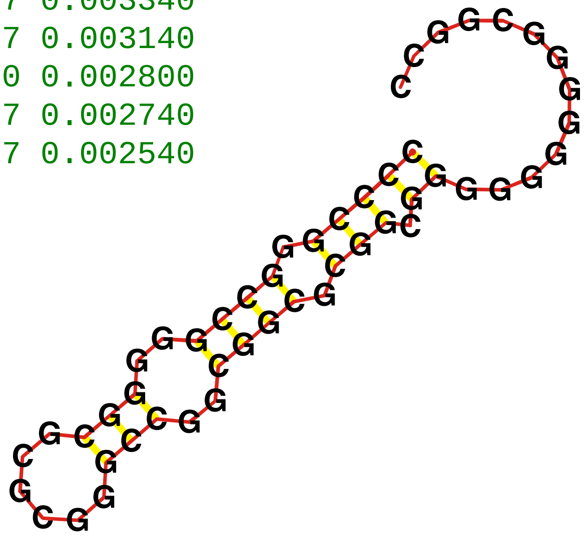


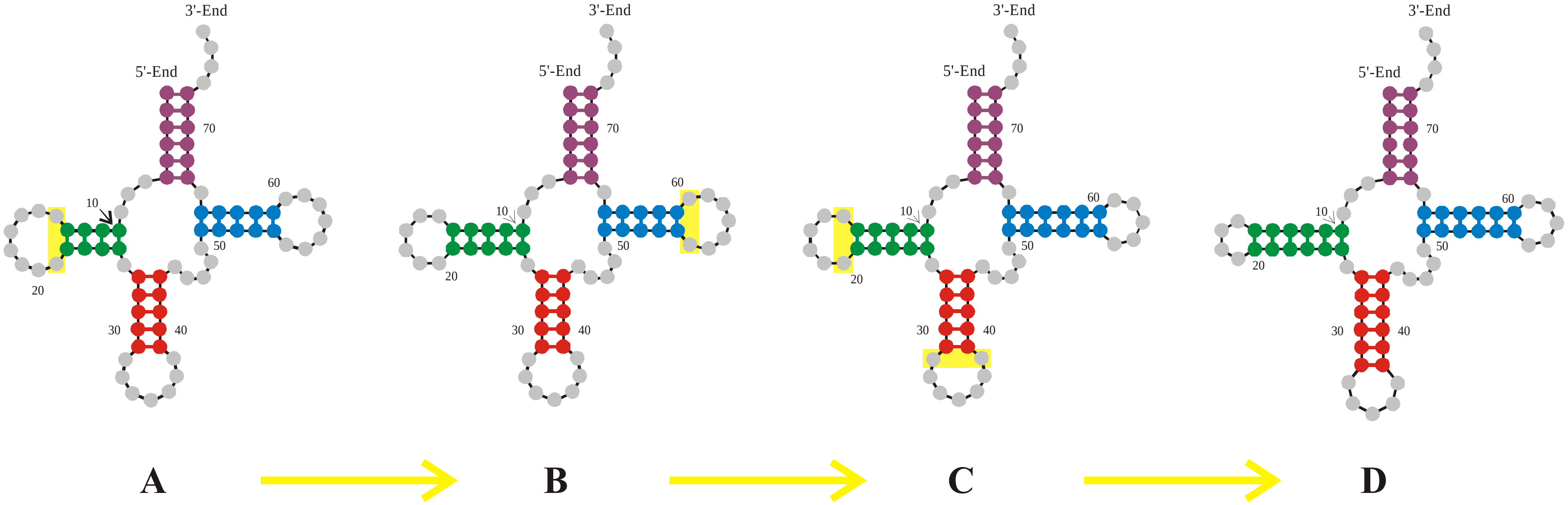
Shadow – Surrounding of an RNA structure in shape space – **AUGC** alphabet

```
                             Number     Mean Value      Variance      Std.Dev.
Total Hamming Distance:       50000      13.673580     10.795762      3.285691
Nonzero Hamming Distance:     45738      14.872054     10.821236      3.289565
Degree of Neutrality:          4262       0.085240      0.001824      0.042708
Number of Structures:          1000          36.24          6.27          2.50

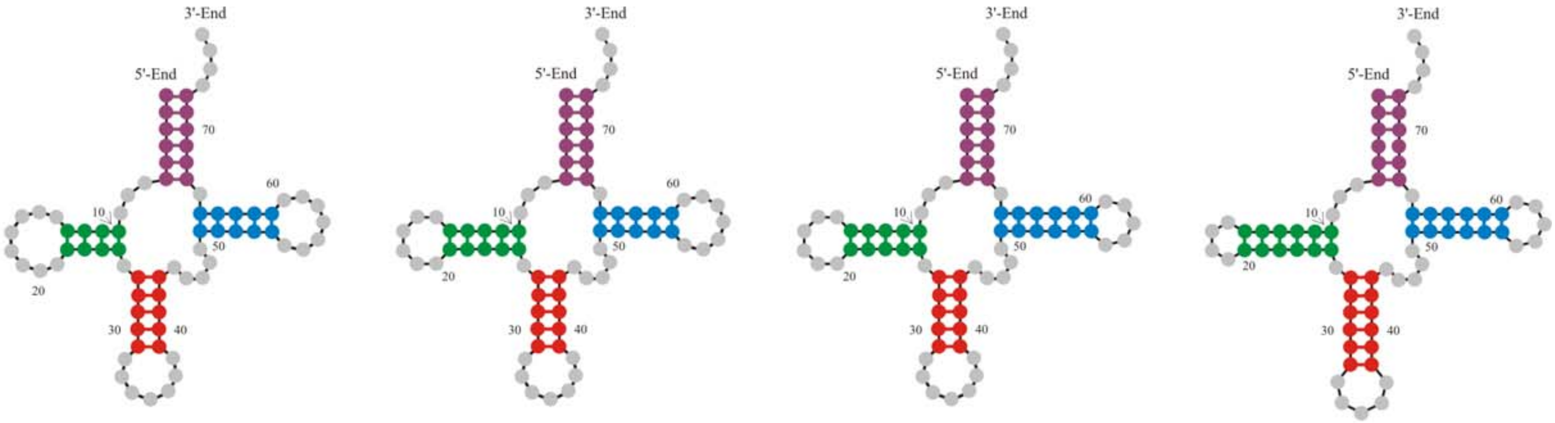
   1 (((((.((((..(((......)))..)))).))).)).............     4262 0.085240
   2 ((((((((((..(((......)))..)))))))).)).............     1940 0.038800
   3 (((((.(((((.(((......))).))))).))).)).............     1791 0.035820
   4 (((((.((((.((((......)))).)))).))).)).............     1752 0.035040
   5 (((((.((((..((((....))))..)))).))).)).............     1423 0.028460
   6 (.(((.((((..(((......)))..)))).))).)..............      665 0.013300
   7 (((((.((((..((........))..)))).))).)).............      308 0.006160
   8 (((((.((((..(((......)))..)))).))))).............. ..   280 0.005600
   9 (((((.((((..(((......)))..)))).))).))...(((....)))      278 0.005560
  10 (((((.(((...(((......)))...))).))).)).............      209 0.004180
  11 (((((.((((..(((......)))..)))).))).)).(((......)))      193 0.003860
  12 (((((.((((..(((......)))..)))).))).))..(((.....)))      180 0.003600
  13 (((((.((((..((((.....)))).)))).))).)).............      180 0.003600
  14 ..(((.((((..(((......)))..)))).)))................      176 0.003520
  15 (((((.((((.((((.....))))..)))).))).)).............      175 0.003500
  16 (((((.((((..(((......)))..)))))))))...............      167 0.003340
  17 (((((.((((...((......))...)))).))).)).............      157 0.003140
  18 (((((.(.((..(((......)))..)).).))).)).............      140 0.002800
  19 (((((..(((..(((......)))..)))..))).)).............      137 0.002740
  20 .((((.((((..(((......)))..)))).))))...............      127 0.002540
```

Shadow – Surrounding of an RNA structure in shape space – **GC** alphabet



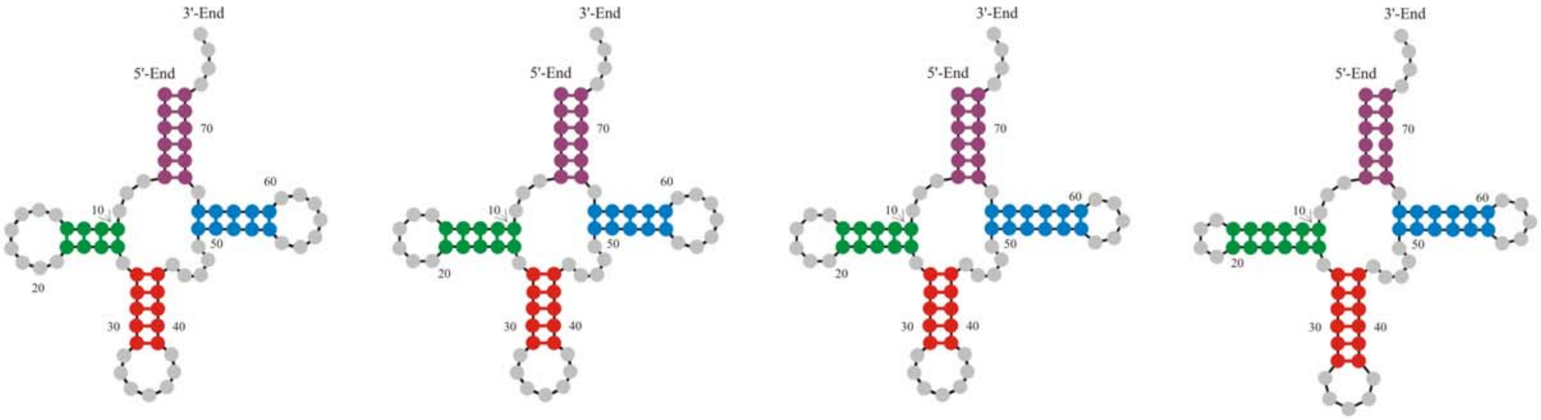


RNA clover-leaf secondary structures of sequences with chain length n=76



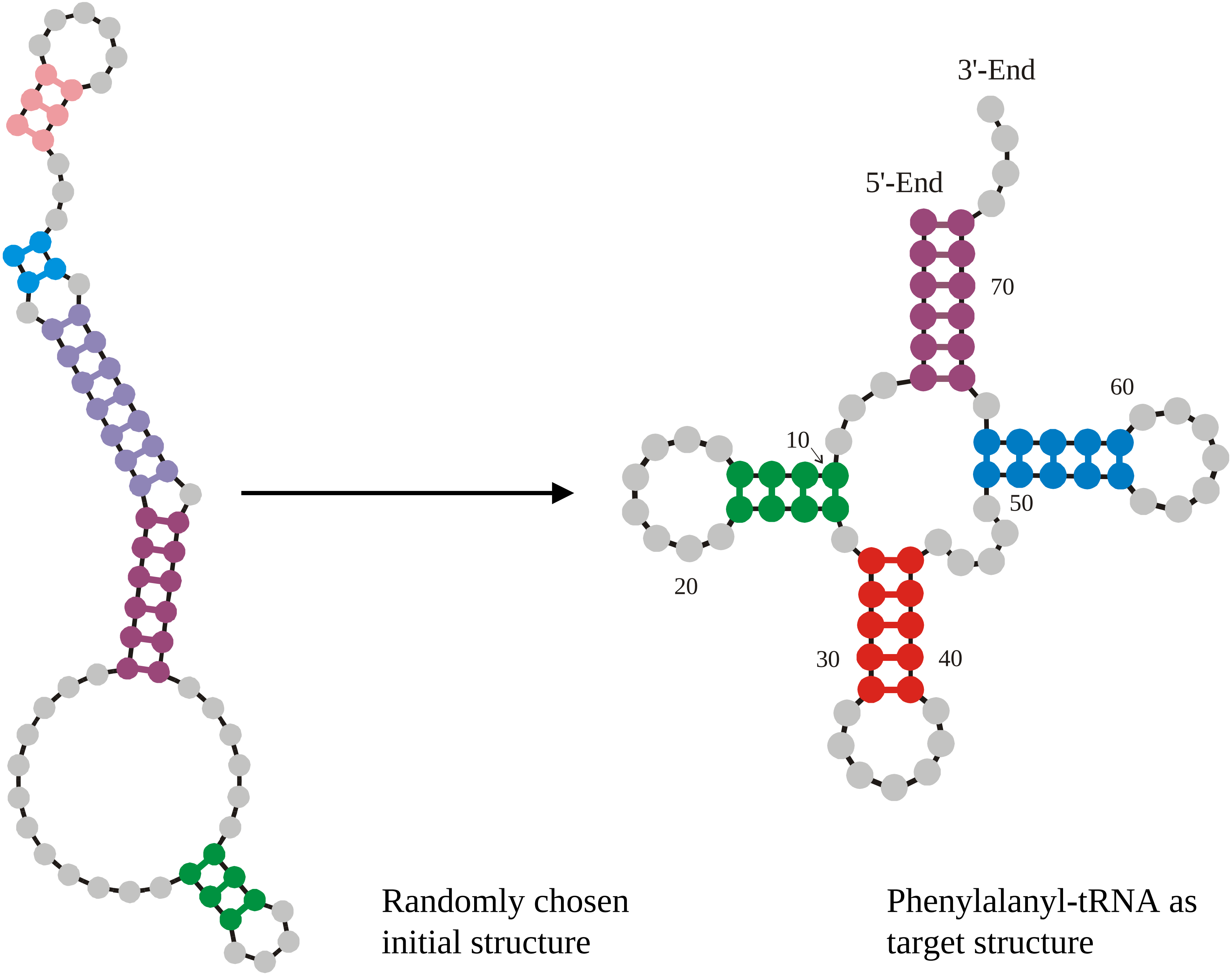
Alphabet	Probability of successful trials in inverse folding			
AU	- -	- -	- -	0.051 ± 0.006
AUG	- -	0.003 ± 0.001	0.026 ± 0.006	0.374 ± 0.016
AUGC	0.794 ± 0.007	0.884 ± 0.008	0.934 ± 0.009	0.982 ± 0.004
UGC	0.548 ± 0.011	0.628 ± 0.012	0.697 ± 0.020	0.818 ± 0.012
GC	0.067 ± 0.007	0.086 ± 0.008	0.087 ± 0.008	0.127 ± 0.006

Probability of finding cloverleaf RNA secondary structures from different alphabets



Alphabet	Degree of neutrality $\bar{\lambda}$			
AU	- -	- -	- -	0.073 ± 0.032
AUG	- -	0.217 ± 0.051	0.207 ± 0.055	0.201 ± 0.056
AUGC	0.275 ± 0.064	0.279 ± 0.063	0.289 ± 0.062	0.313 ± 0.058
UGC	0.263 ± 0.071	0.257 ± 0.070	0.251 ± 0.068	0.250 ± 0.064
GC	0.052 ± 0.033	0.057 ± 0.034	0.060 ± 0.033	0.068 ± 0.034

Degree of neutrality of cloverleaf RNA secondary structures over different alphabets



Randomly chosen initial structure

Phenylalanyl-tRNA as target structure

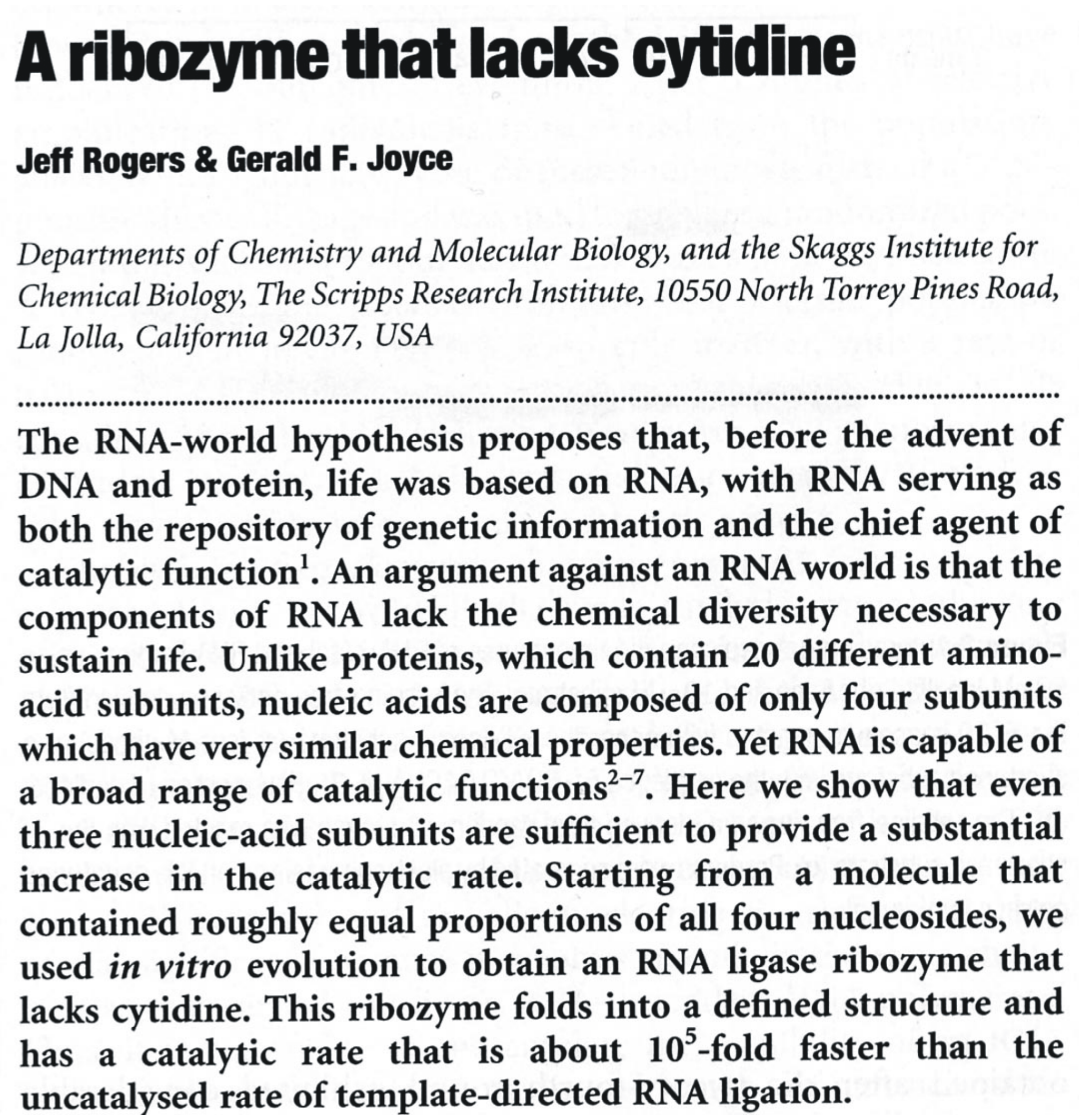
Alphabet	**Real time**	Transitions	Major transitions	Sample size
AUGC	**398.3**	22.8	12.7	1199
GUC	**448.9**	30.5	16.5	611
GC	**1908.7**	38.7	20.1	278

Mean population size: $N = 3000$;   mutation rate: p = 0.001

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

AUGC neutral networks of tRNAs are near the connectivity threshold, **GC** neutral networks are way below.

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



A ribozyme that lacks cytidine

Jeff Rogers & Gerald F. Joyce

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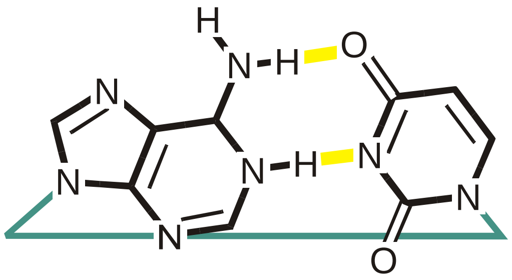
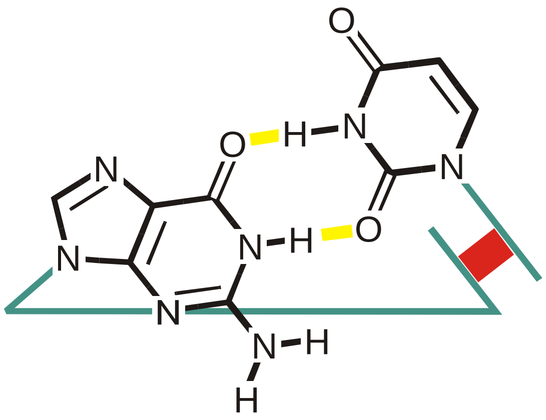
The RNA-world hypothesis proposes that, before the advent of DNA and protein, life was based on RNA, with RNA serving as both the repository of genetic information and the chief agent of catalytic function[1]. An argument against an RNA world is that the components of RNA lack the chemical diversity 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.

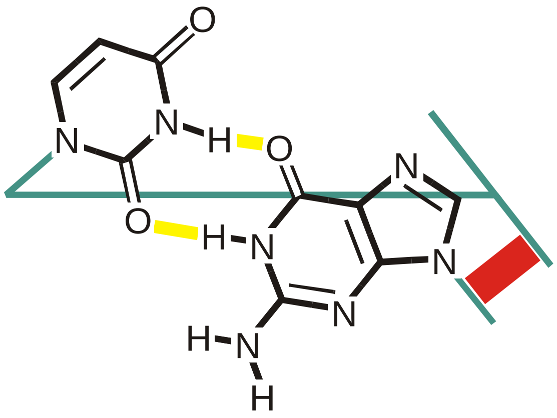
Catalytic activity in the **AUG** alphabet



A=U
(U=A)

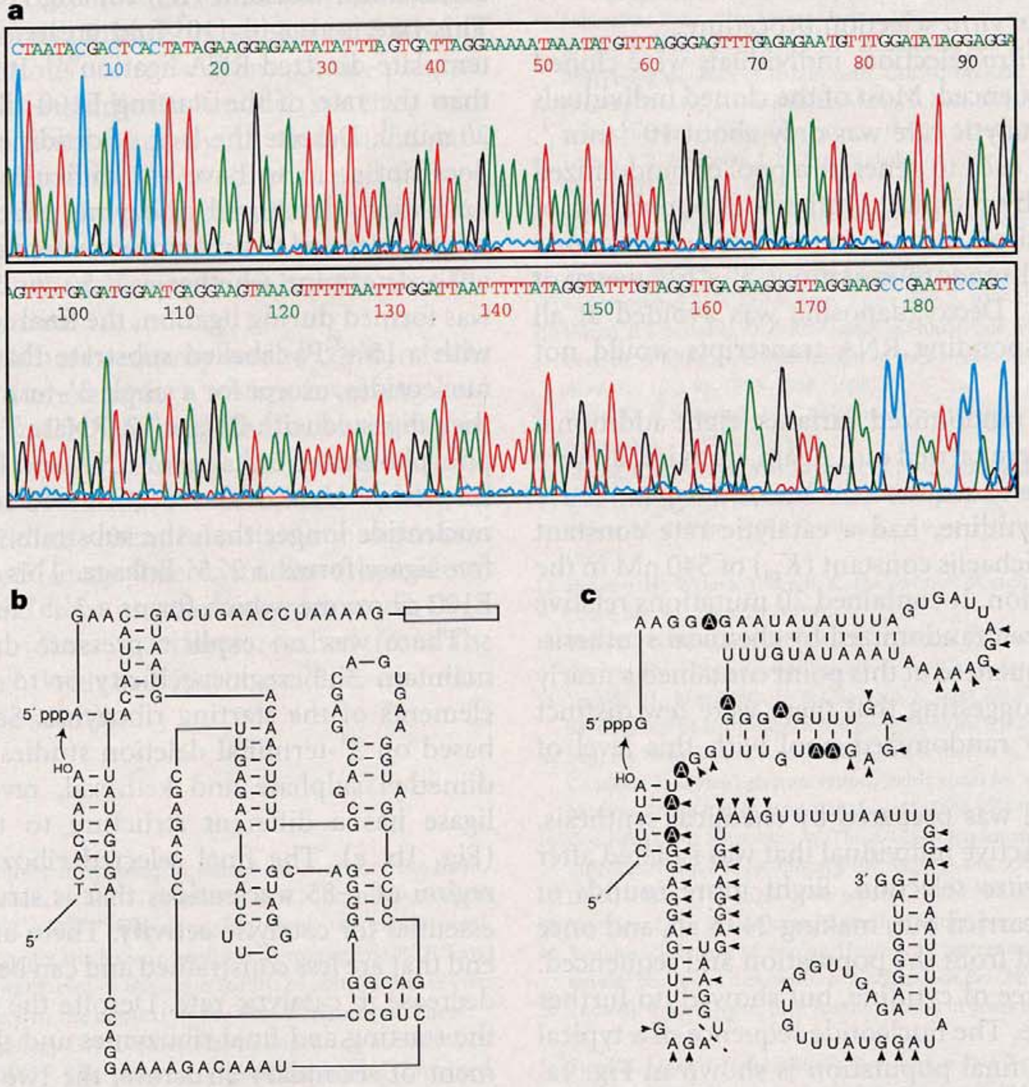


G=U



U=G

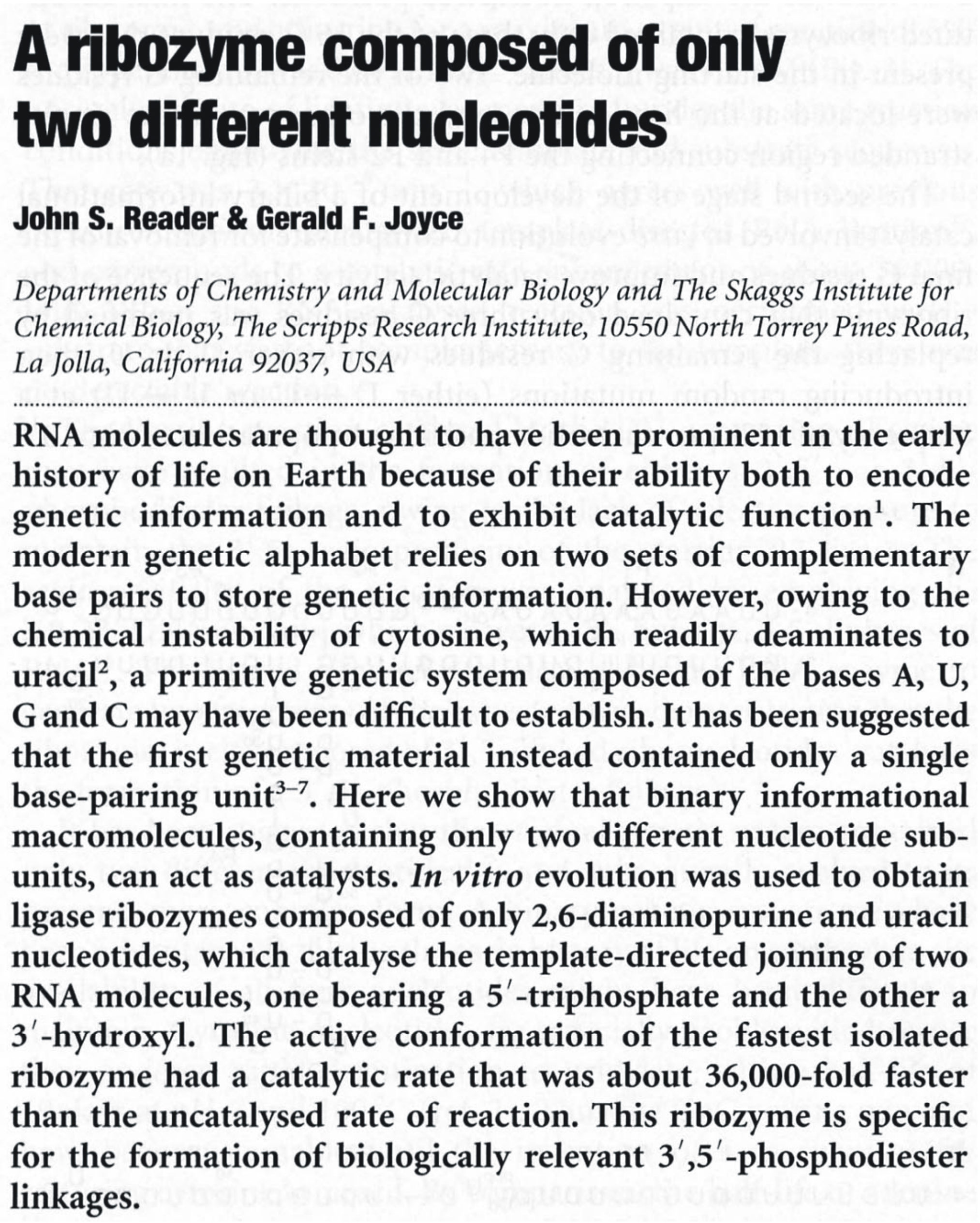
Base pairs in the **AUG** alphabet



a
CTAATACGACTCACTATAGAAGGAGAATATATTTAGTGATTAGGAAAAATAAATATGTTTAGGGAGTTTGAGAGAATGTTTGGATATAGGAGGA
10
20
30
40
50
60
70
80
90
AGTTTTGAGATGGAATGAGGAAGTAAAGTTTTTAATTTGGATTAATTTTTATAGGTATTTGTAGGTTGAGAAGGGTTAGGAAGCCGAATTCCAGC
100
110
120
130
140
150
160
170
180
b
5′ pppA
HO
5′
c
5′ pppG
HO
5′
3′

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.

Nature **420**, 841-844, 2002



A ribozyme composed of only two different nucleotides

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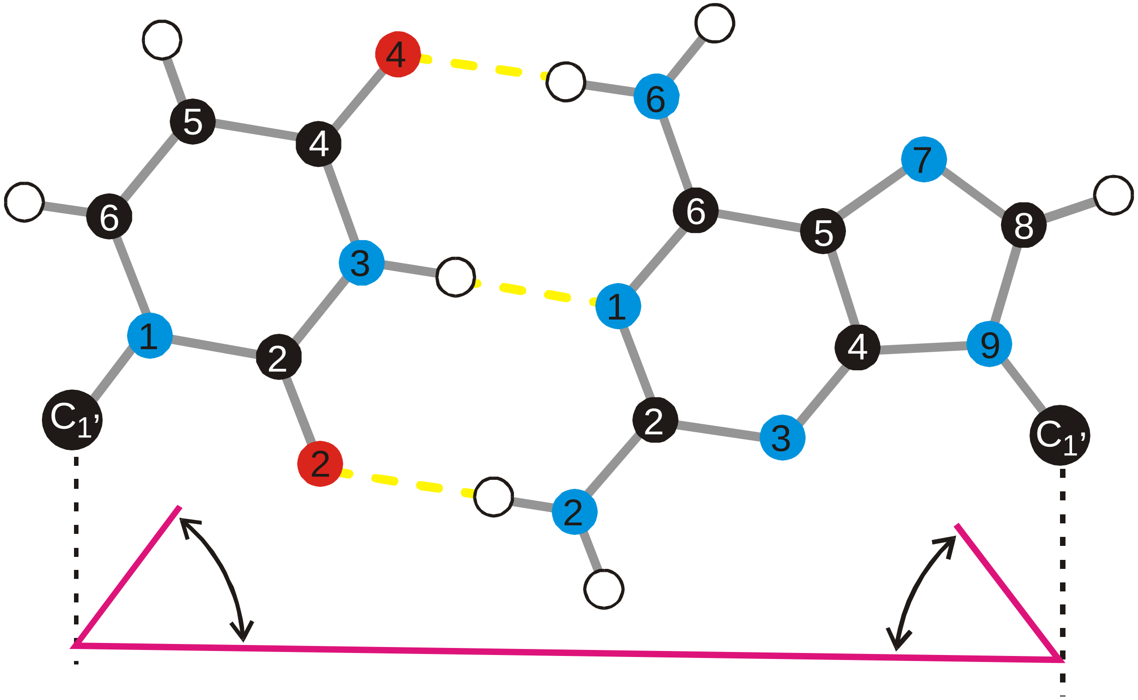
RNA molecules are thought to have been prominent in the early history of life on Earth because of their ability both to encode genetic information and to exhibit catalytic function[1]. The modern genetic alphabet relies on two sets of complementary base pairs to store genetic information. However, owing 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

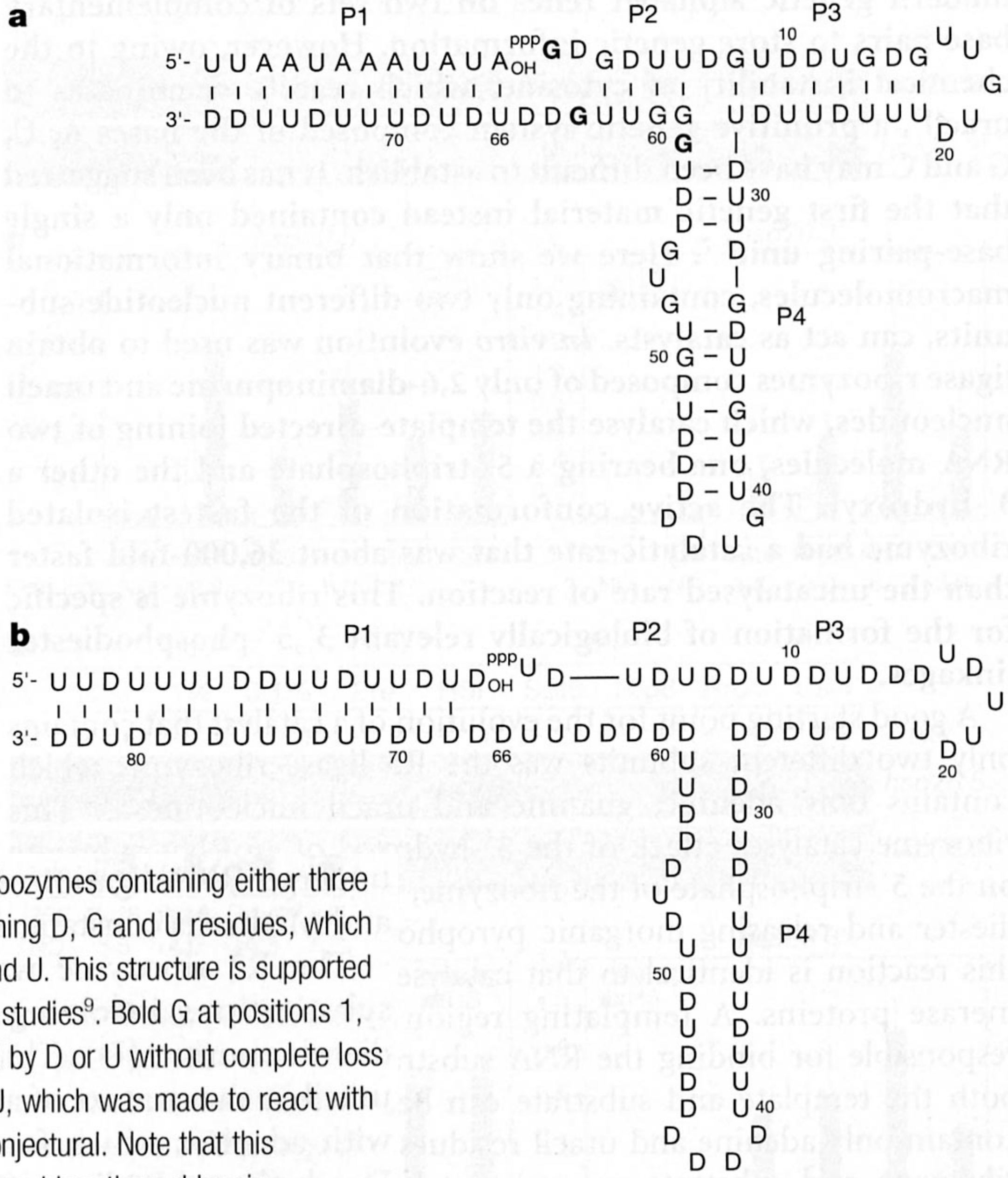
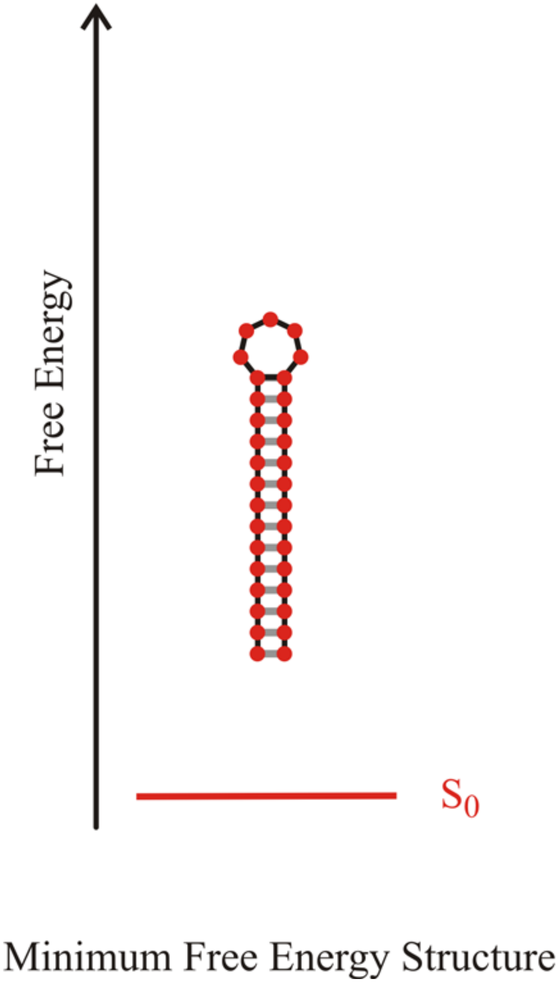
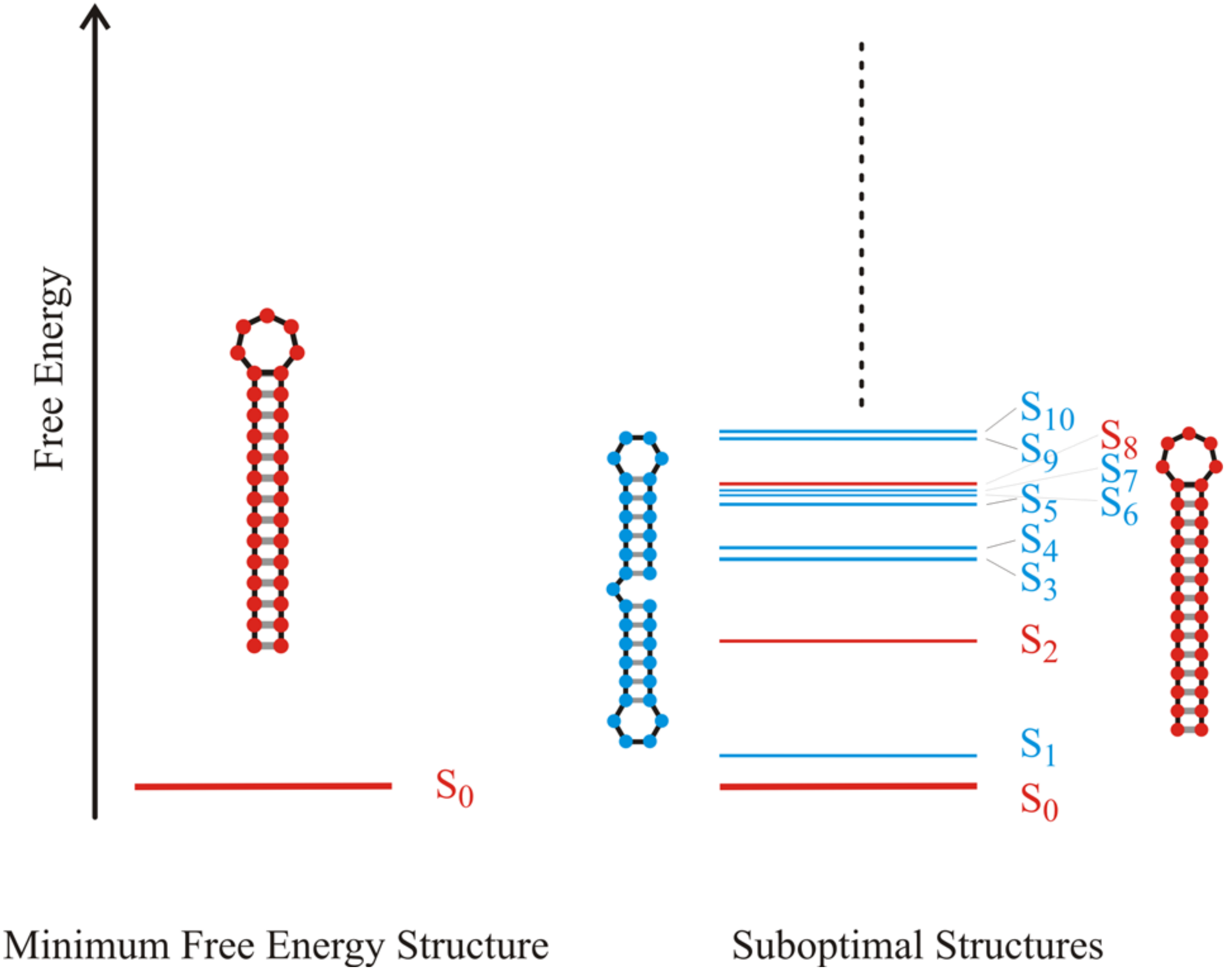

a
P1
P2
P3
P4
5'- U U A A U A A A U A U A OH ppp G D G D U U D G U D D U G D G U U G
3'- D D U U D U U U D U D U D D G U U G G U D U U D U U U D U
70
66
60
10
20
30
40
50
b
P1
P2
P3
P4
5'- U U D U U U U D D U U D U U D U D OH ppp U D — U D U D D U D D U D D D U D U
3'- D D U D D D D U U D D U D D U D D D U U D D D D D D D D U D D D U D U
80
70
66
60
10
20
30
40
50

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**.

1. What are neutral networks ?
2. Mutations and structural stability
3. Structures from defective alphabets
4. **Suboptimal conformations and structural stability**
5. Metastable structures and RNA switches
6. How to handle multiple constraints

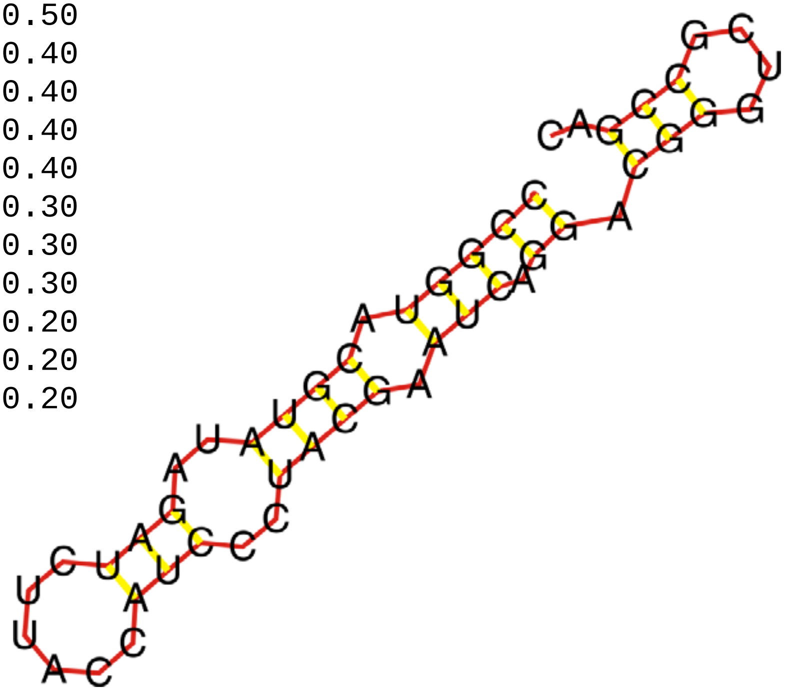


Suboptimal secondary structures of an RNA sequence

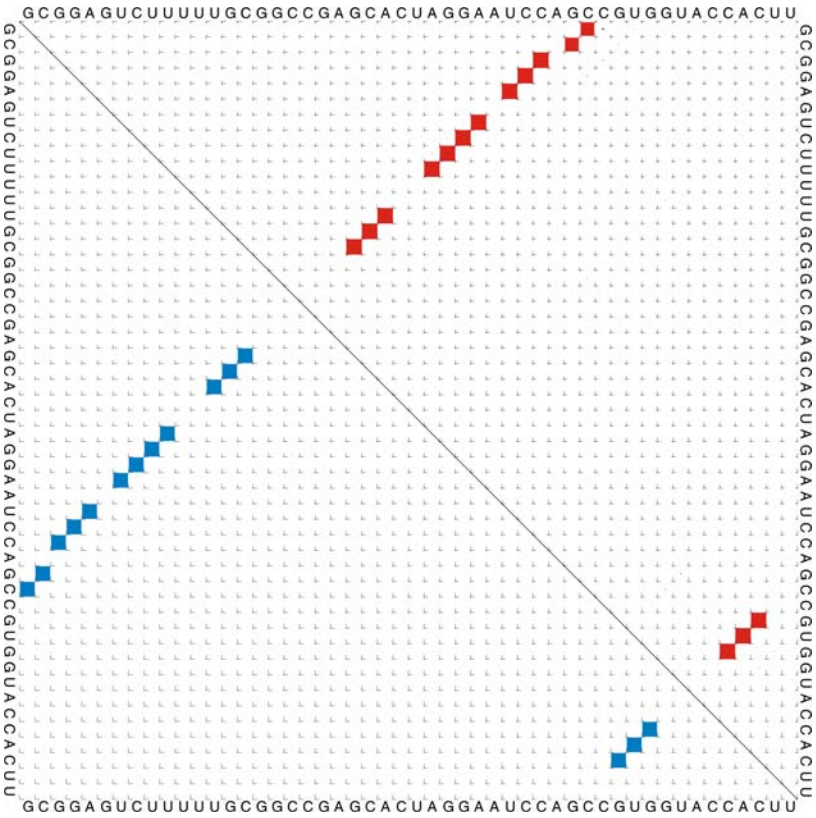


Suboptimal secondary structures of an RNA sequence

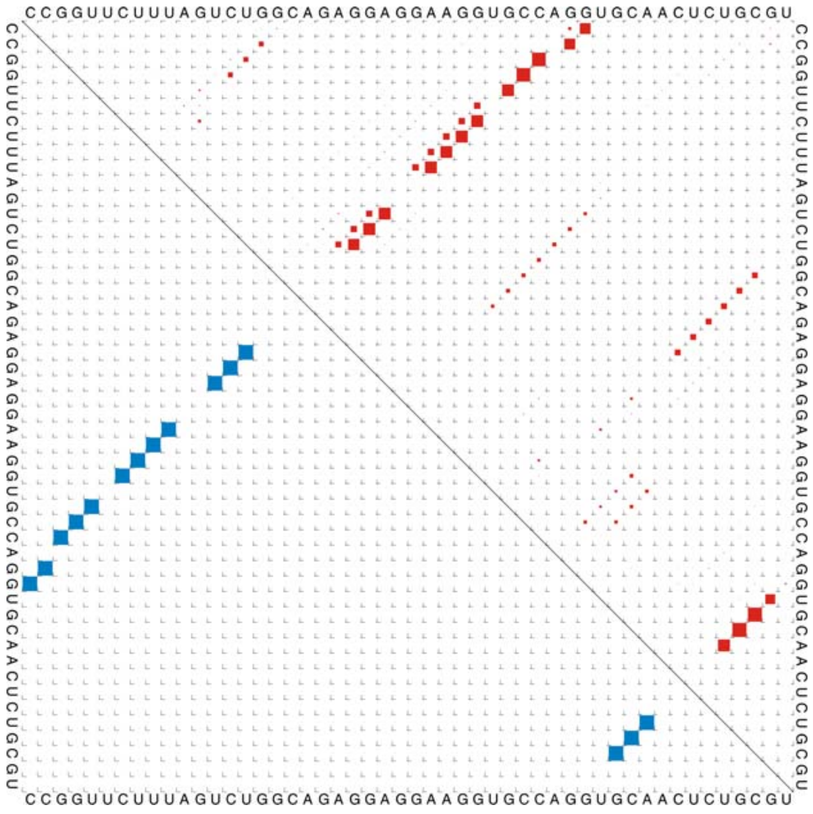
```
GCGUCGCGUGCCAUGGAGCAUCAUUACAUGAGACAGCCCCGGCCUCGGAU  -1220    200
(((((.((((..(((......)))..)))).))).)).(((....))).. -12.20
(((((.((((..((((....))))..)))).))).)).(((....))).. -12.10
..(((.((.(((..((.((.((((...))))....)))).)))..))))) -11.50
..(((.((((..(((......)))..)))).)))....(((....))).. -11.40
..(((.((((..((((....))))..)))).)))....(((....))).. -11.30
..(((.((.(((..((.((.(((.....)))....)))).)))..))))) -11.30
..(((.((.(((..((.((.((((...))))....)).)))))..))))) -11.10
...(((.(.(((..((.((.((((...))))....)))).)))).))).. -11.10
..(((.((.(((..((.((.(((.....)))....)).)))))..))))) -10.90
...(((.(.(((..((.((.(((.....)))....)))).)))).))).. -10.90
(((((.((((..(((......)))..)))).))).)).((......)).. -10.80
(((((.((((..((((....))))..)))).))).)).((......)).. -10.70
...(((.(.(((..((.((.((((...))))....)).))))))).))).. -10.70
..(((.((.(((..((....((((...)))).....))..)))..))))) -10.60
...((.((.(((..((.((.((((...))))....)))).)))..)))). -10.60
...(((.(.(((..((.((.(((.....)))....)).)))))).))).. -10.50
....((.(.(((..((.((.((((...))))....)))).)))).))... -10.50
..(((.((((..(((......)))..)))).))).((....))....... -10.40
..(((.((.(((..((.((.((.......))....)))).)))..))))) -10.40
..(((.((.(((..((....(((.....))).....))..)))..))))) -10.40
...((.((.(((..((.((.(((.....))).....)))).)))..)))). -10.40
(((((.((((...((......))...)))).))).)).(((....))).. -10.30
..(((.((((..((((....))))..)))).))).((....))....... -10.30
....((.(.(((..((.((.(((.....))).....)))).)))).))... -10.30
(((((.((((...(((....)))...)))).))).)).(((....))).. -10.20
...(((.(.(((..((....((((...)))).....))..)))).))).. -10.20
...((.((.(((..((.((.((((...))))....)).))))))..)))). -10.20
                .............................
                .............................
                .............................
```



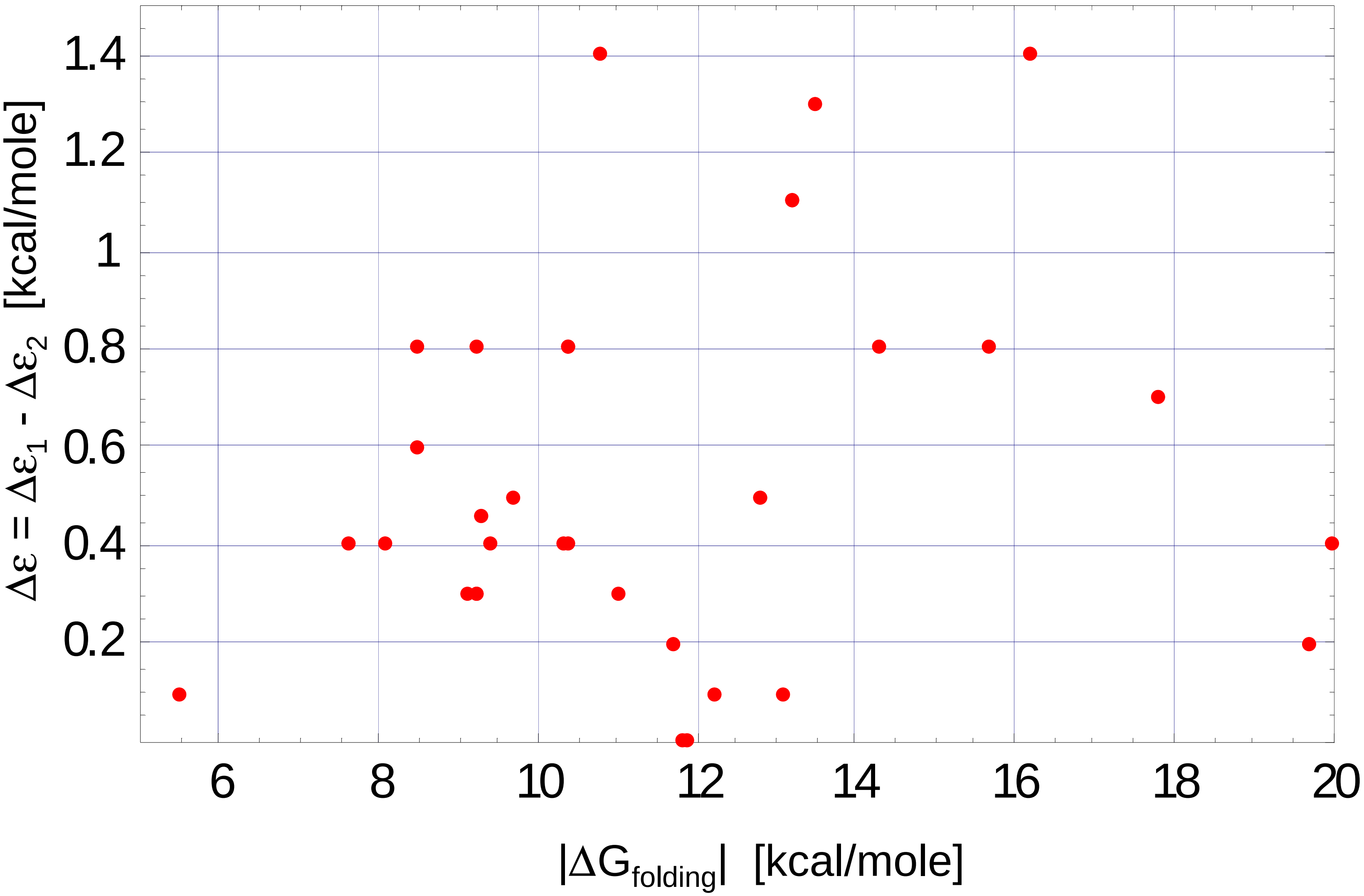
GCGGAGUCUUUUUGCGGCCGAGCACUAGGAAUCCAGCCGUGGUACCACUU

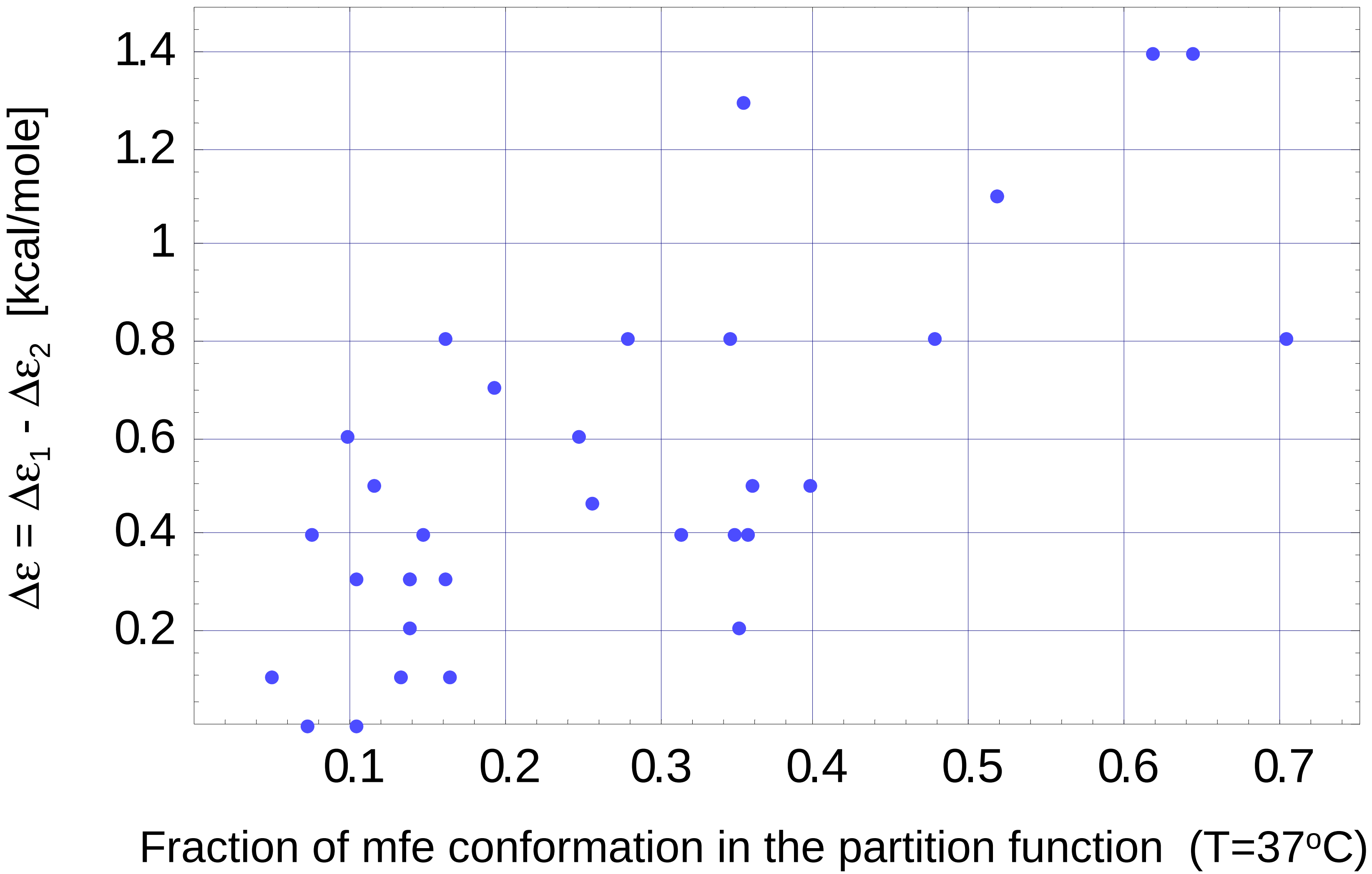


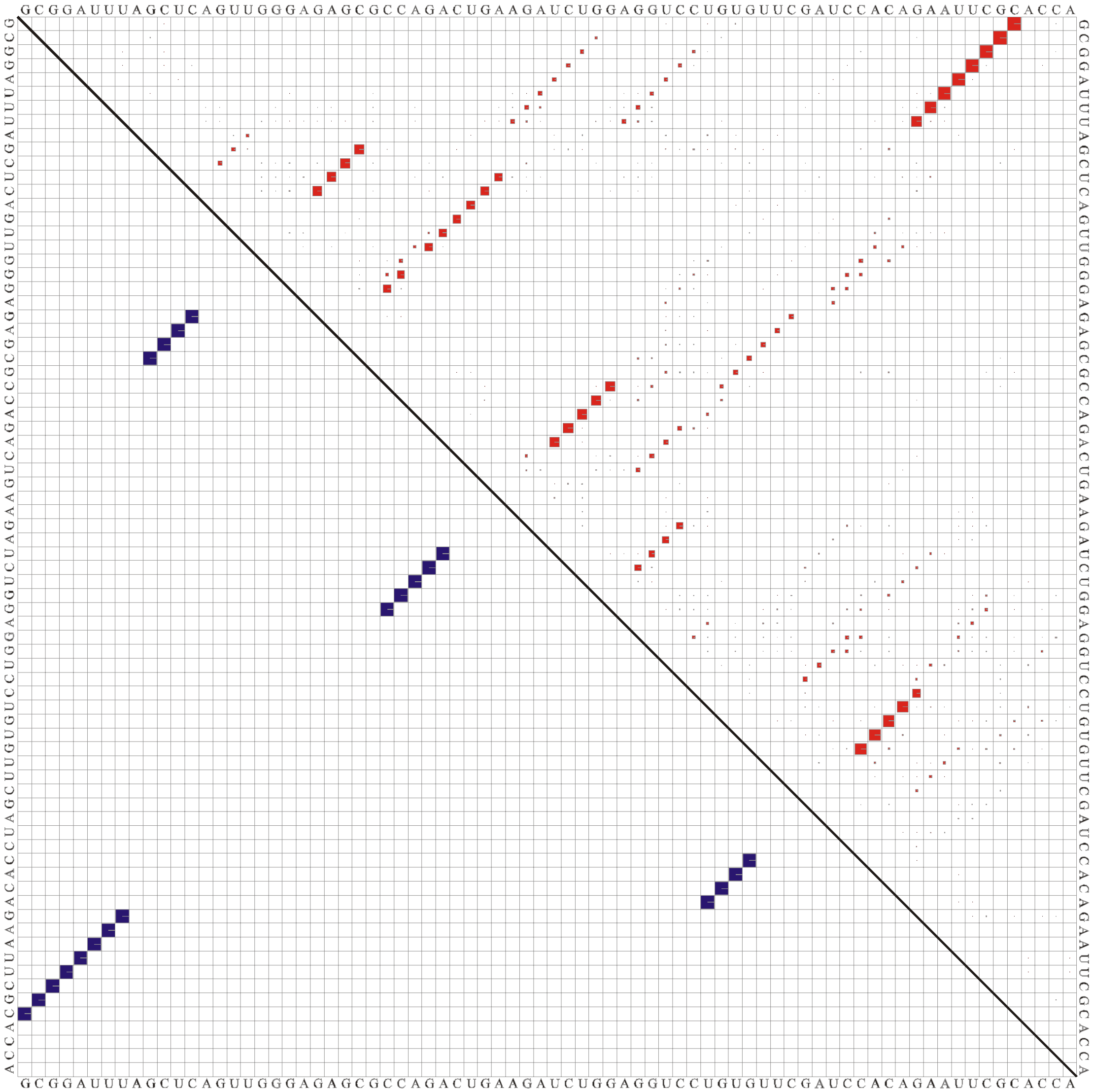
CCGGUUCUUUAGUCUGGCAGAGGAGGAAGGUGCCAGGUGCAACUCUGCGU

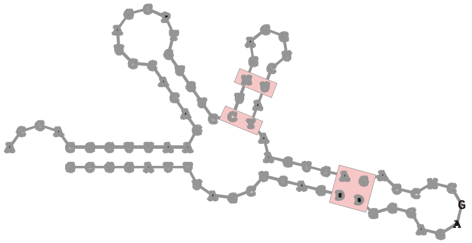


Two neutral sequences with very different contributions of suboptimal conformations


1.4
1.2
1
0.8
0.6
0.4
0.2
$\Delta\varepsilon = \Delta\varepsilon_1 - \Delta\varepsilon_2$ [kcal/mole]
6
8
10
12
14
16
18
20
$|\Delta G_{folding}|$ [kcal/mole]

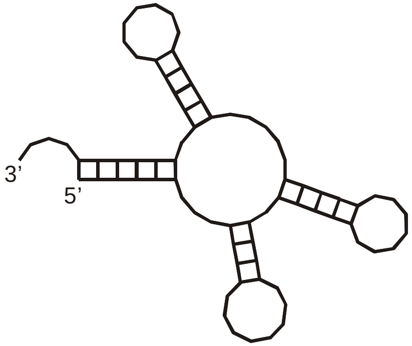

1.4
1.2
1
0.8
0.6
0.4
0.2
0.1
0.2
0.3
0.4
0.5
0.6
0.7
$\Delta\varepsilon = \Delta\varepsilon_1 - \Delta\varepsilon_2$ [kcal/mole]
Fraction of mfe conformation in the partition function (T=37^{o}C)





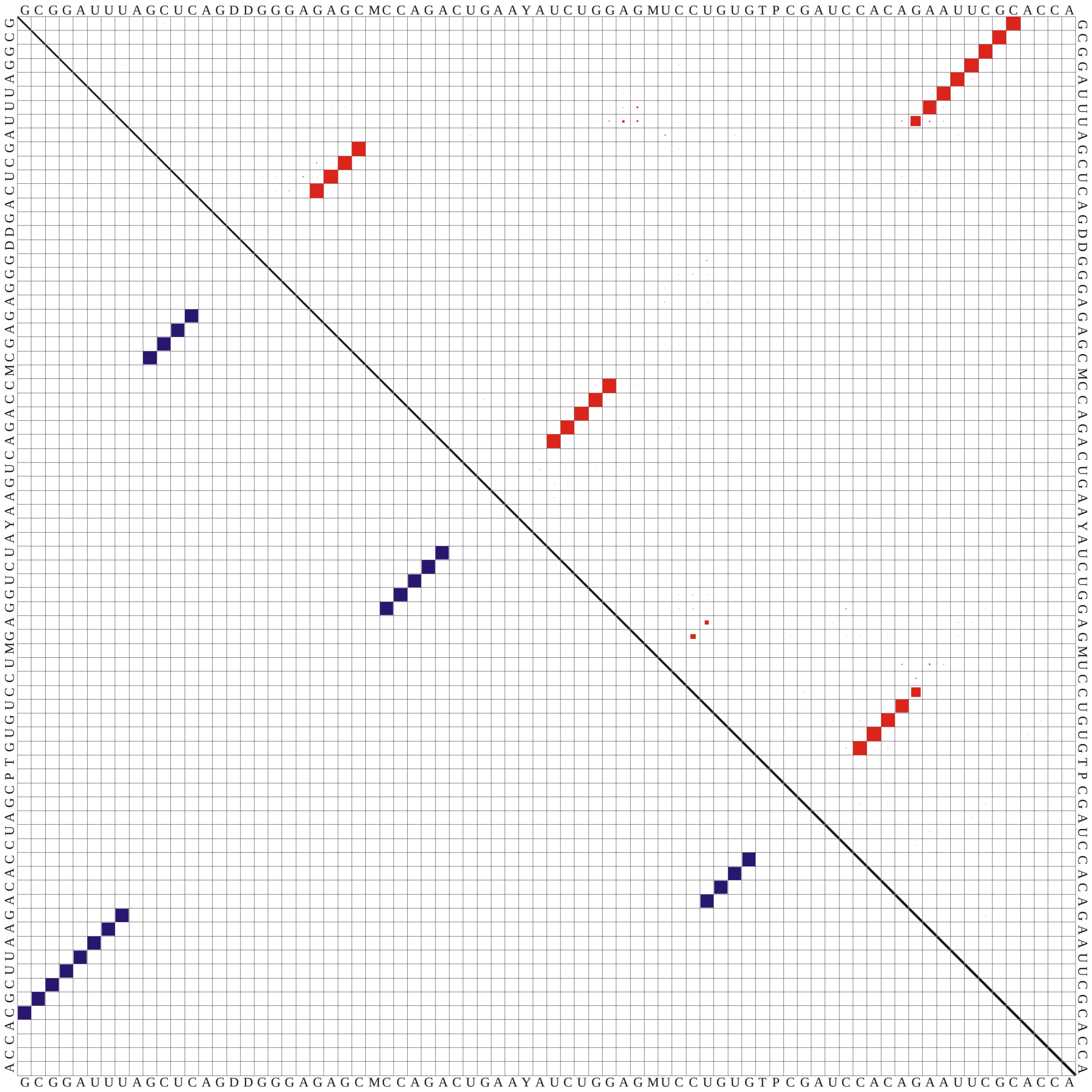
first suboptimal configuration

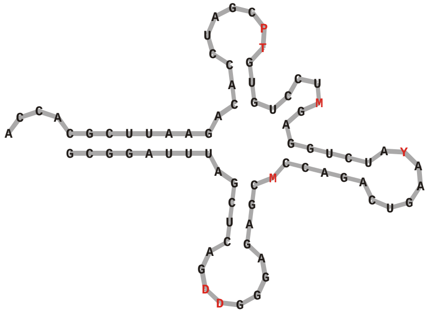
$\Delta E_{0\rightarrow1}$ = 0.43 kcal / mole



tRNA^{phe}

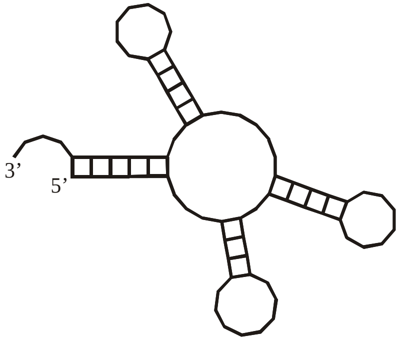
without modified bases





first suboptimal configuration

$\Delta E_{0\rightarrow 1}$ = 0.94 kcal / mole



tRNA$^{\text{phe}}$

with modified bases

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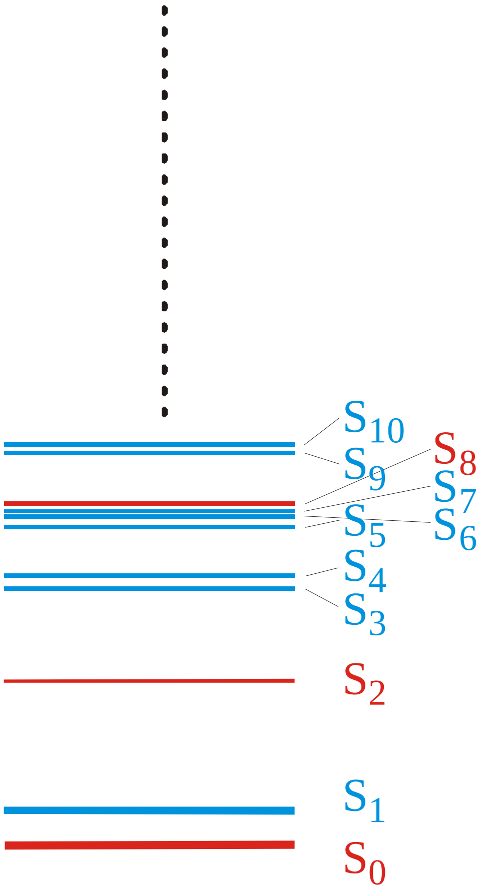
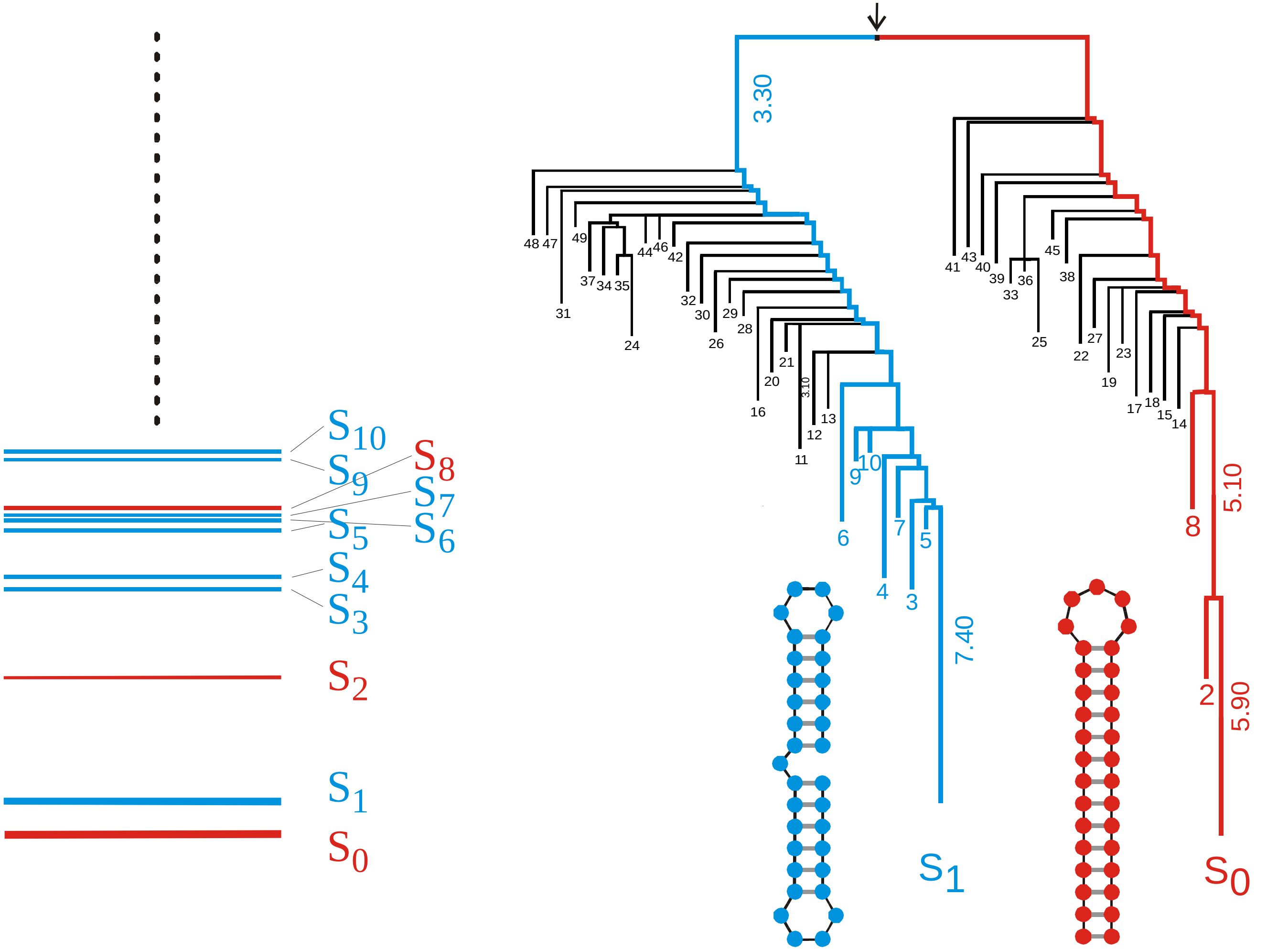


Suboptimal structures

Suboptimal secondary structures of an RNA sequence



Suboptimal structures

Metastable

Stable

structure

An RNA molecule with two (**meta**)**stable** conformations

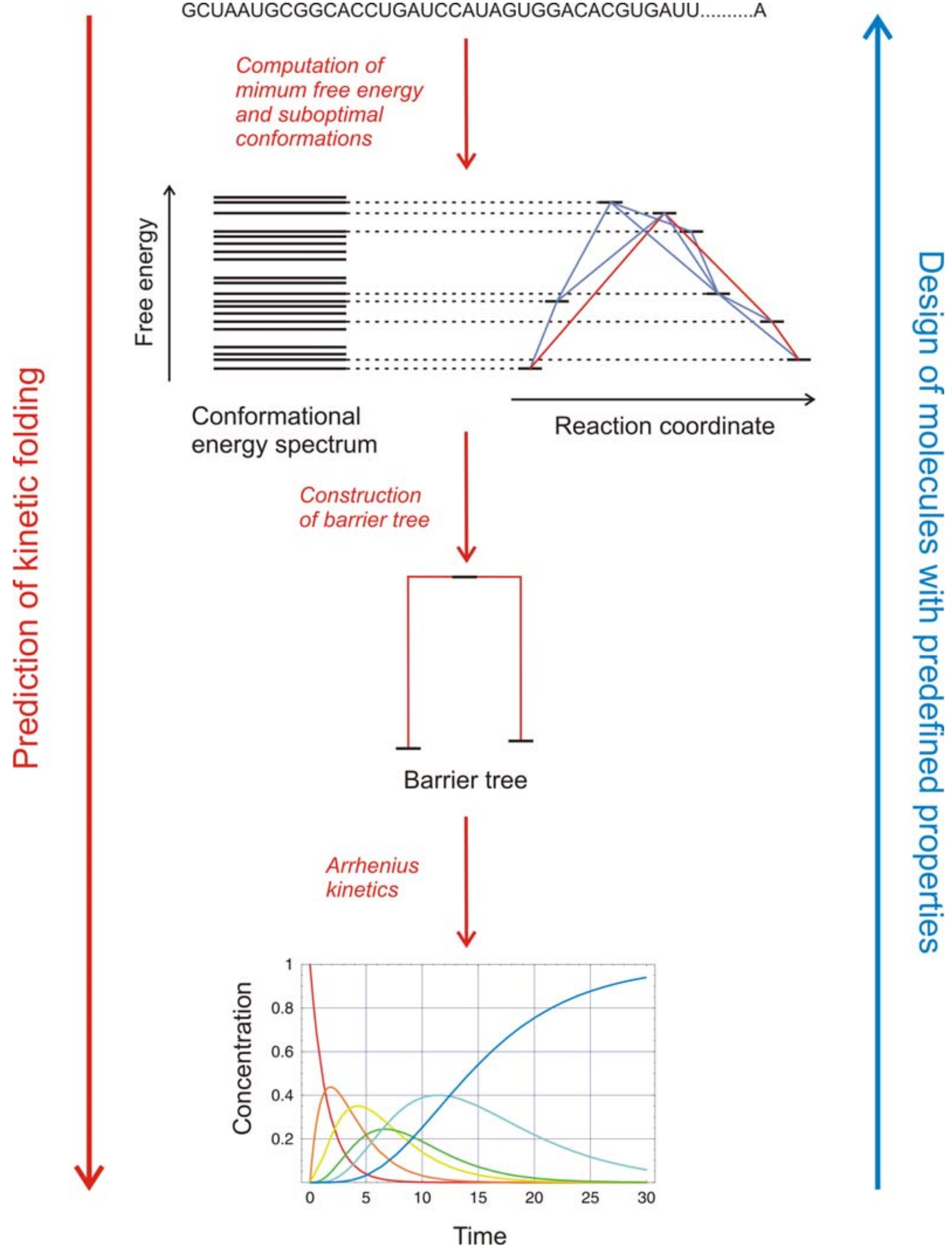
Kinetic Folding of RNA Secondary Structures

Christoph Flamm, Walter Fontana, Ivo L. Hofacker, Peter Schuster. *RNA folding kinetics at elementary step resolution.* RNA **6**:325-338, 2000

Christoph Flamm, Ivo L. Hofacker, Sebastian Maurer-Stroh, Peter F. Stadler, Martin Zehl. *Design of multistable RNA molecules.* RNA **7**:325-338, 2001

Christoph Flamm, Ivo L. Hofacker, Peter F. Stadler, Michael T. Wolfinger. *Barrier trees of degenerate landscapes*. Z.Phys.Chem. **216**:155-173, 2002

Michael T. Wolfinger, W. Andreas Svrcek-Seiler, Christoph Flamm, Ivo L. Hofacker, Peter F. Stadler. *Efficient computation of RNA folding dynamics*.
J.Phys.A: Math.Gen. **37**:4731-4741, 2004



GCUAAUGCGGCACCUGAUCCAUAGUGGACACGUGAUU..........A

Computation of mimum free energy and suboptimal conformations

Conformational energy spectrum

Reaction coordinate

Construction of barrier tree

Barrier tree

Arrhenius kinetics

Prediction of kinetic folding

Design of molecules with predefined properties

Computation of kinetic folding

The Folding Algorithm

A sequence I specifies an energy ordered set of compatible structures $\mathfrak{S}(I)$:

$$\mathfrak{S}(I) = \{S_0 , S_1 , \ldots , S_m , \mathbf{O}\}$$

A trajectory $\mathfrak{T}_k(I)$ is a time ordered series of structures in $\mathfrak{S}(I)$. A folding trajectory is defined by starting with the open chain **O** and ending with the global minimum free energy structure S_0 or a metastable structure S_k which represents a local energy minimum:

$$\mathfrak{T}_0(I) = \{\mathbf{O} , S(1) , \ldots , S(t-1) , S(t) , S(t+1) , \ldots , \mathbf{S_0}\}$$

$$\mathfrak{T}_k(I) = \{\mathbf{O} , S(1) , \ldots , S(t-1) , S(t) , S(t+1) , \ldots , \mathbf{S_k}\}$$

Transition probabilities $P_{ij}(t) = \mathscr{Prob}\{S_i \rightarrow S_j\}$ are defined by

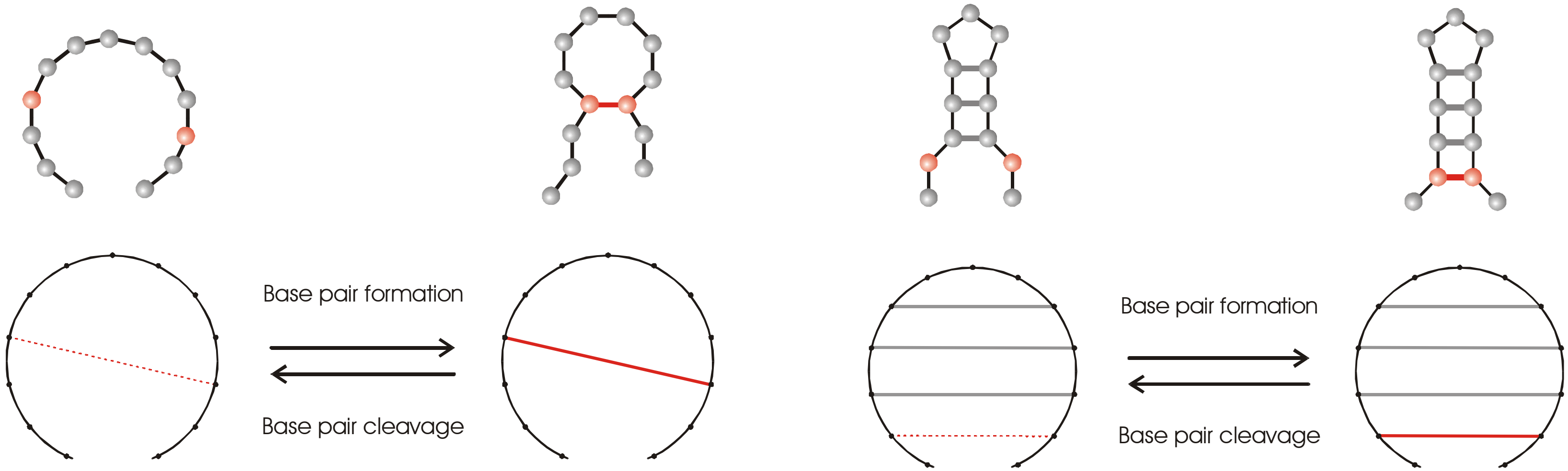
$$P_{ij}(t) = P_i(t)\, k_{ij} = P_i(t) \exp(-\Delta G_{ij}/2RT) / \Sigma_i$$

$$P_{ji}(t) = P_j(t)\, k_{ji} = P_j(t) \exp(-\Delta G_{ji}/2RT) / \Sigma_j$$

$$\Sigma_k = \sum_{k=1,k\neq i}^{m+2} \exp(-\Delta G_{ki}/2RT)$$

The symmetric rule for transition rate parameters is due to Kawasaki (K. Kawasaki, *Diffusion constants near the critical point for time depen-dent Ising models*. Phys.Rev. **145**:224-230, 1966).

Formulation of kinetic RNA folding as a stochastic process



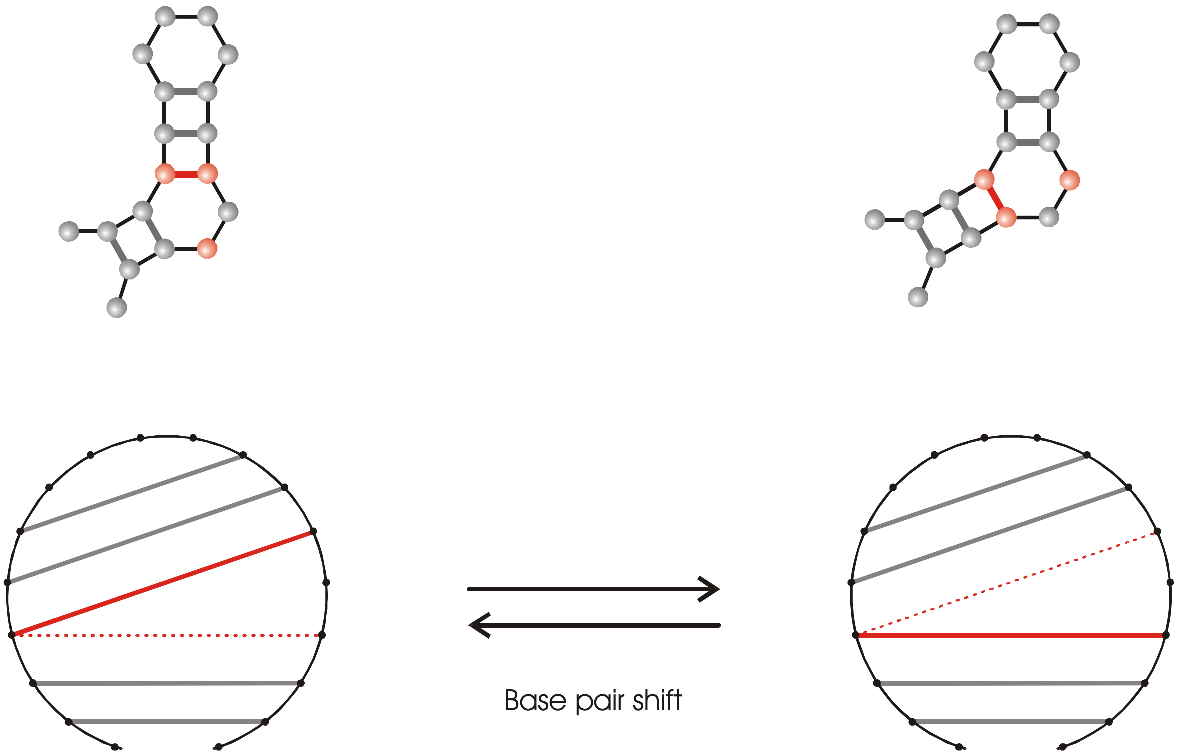
Base pair formation

Base pair cleavage

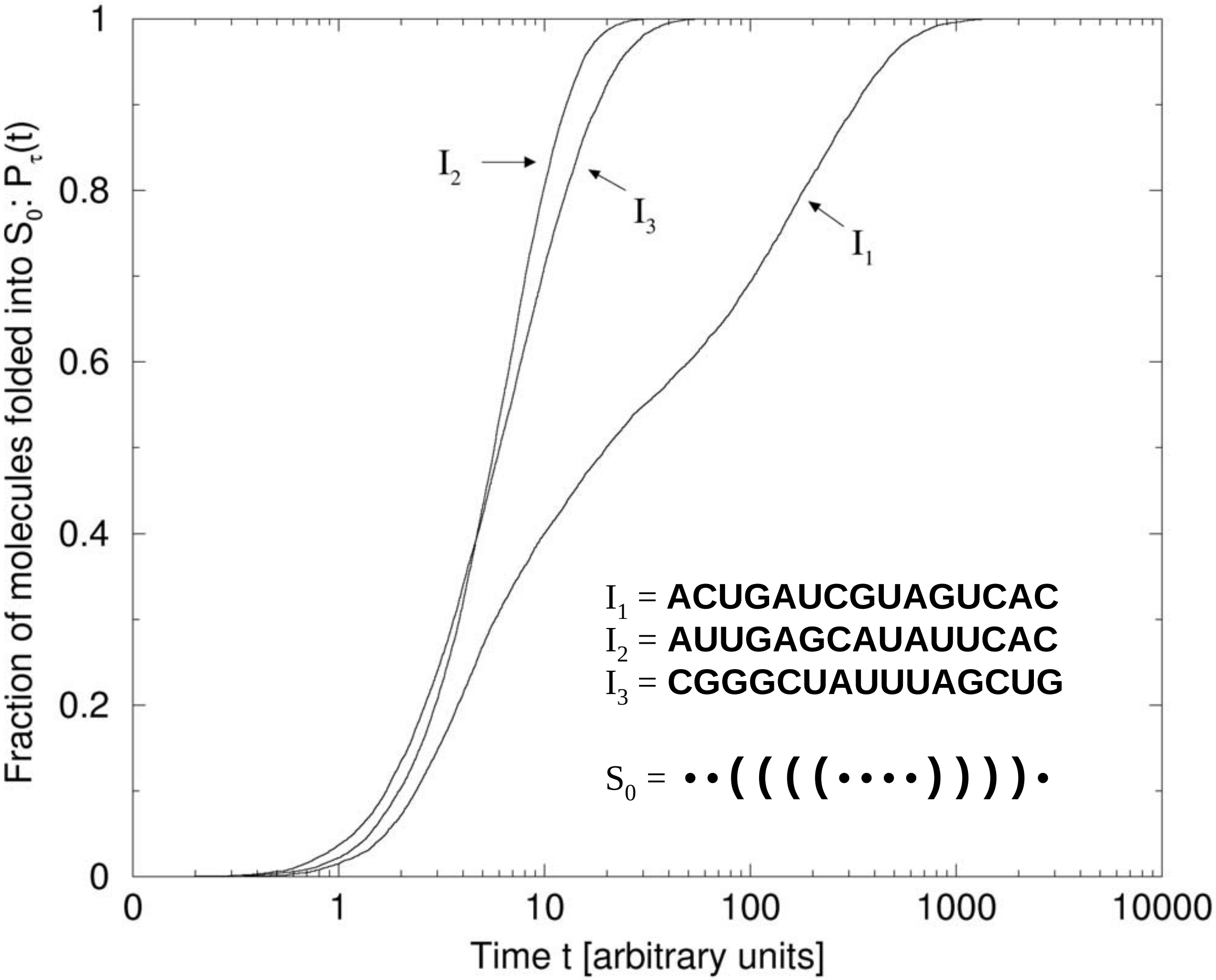
Base pair formation

Base pair cleavage

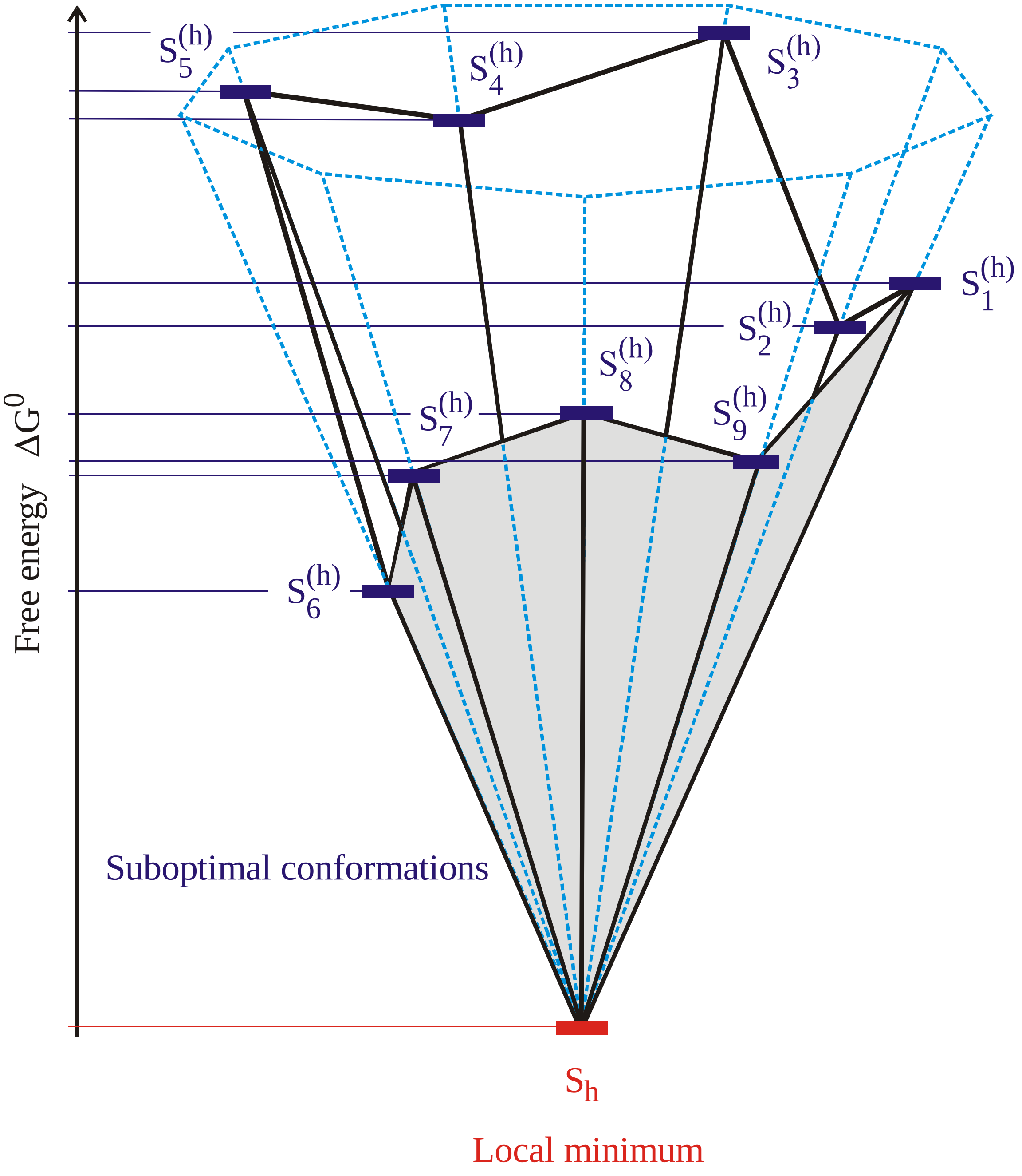
Base pair formation and base pair cleavage moves for nucleation and elongation of stacks



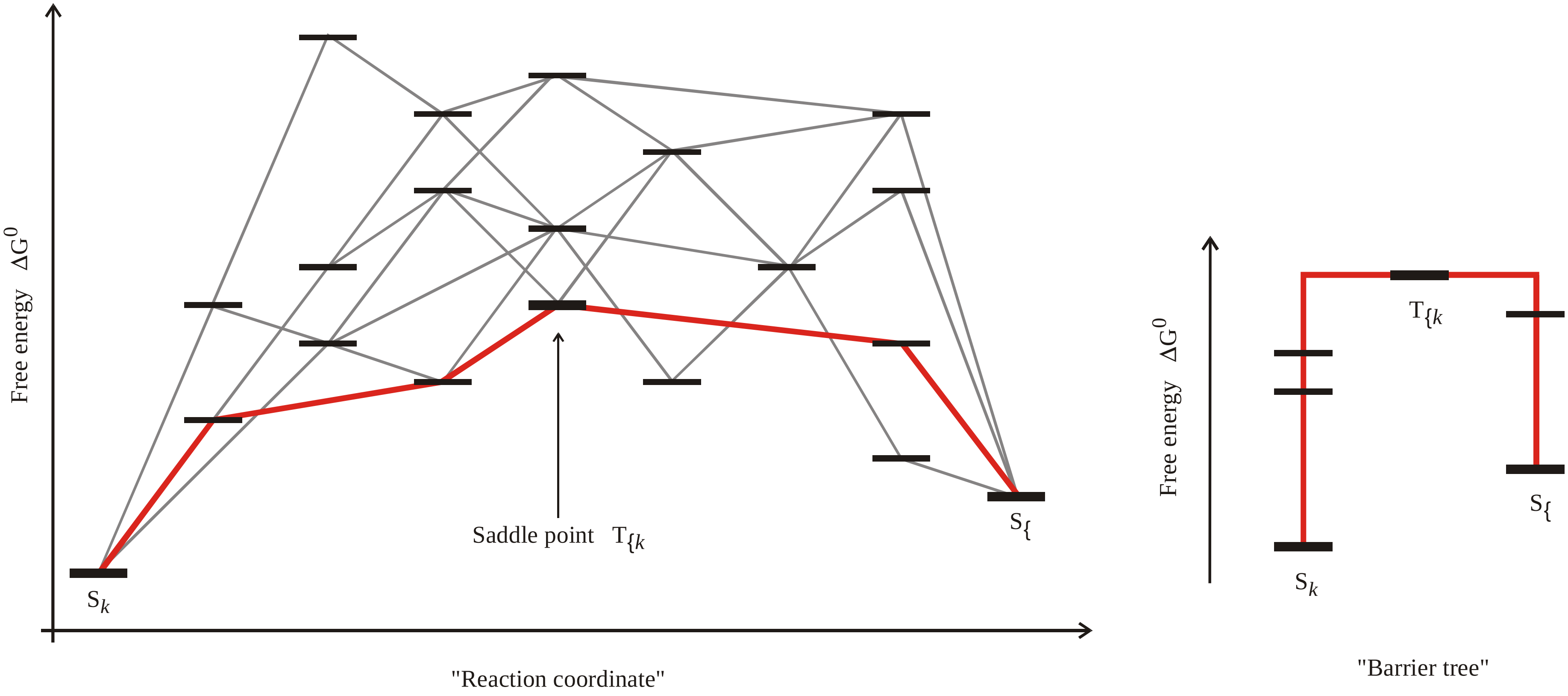
Base pair shift move of class 1: Shift inside internal loops or bulges



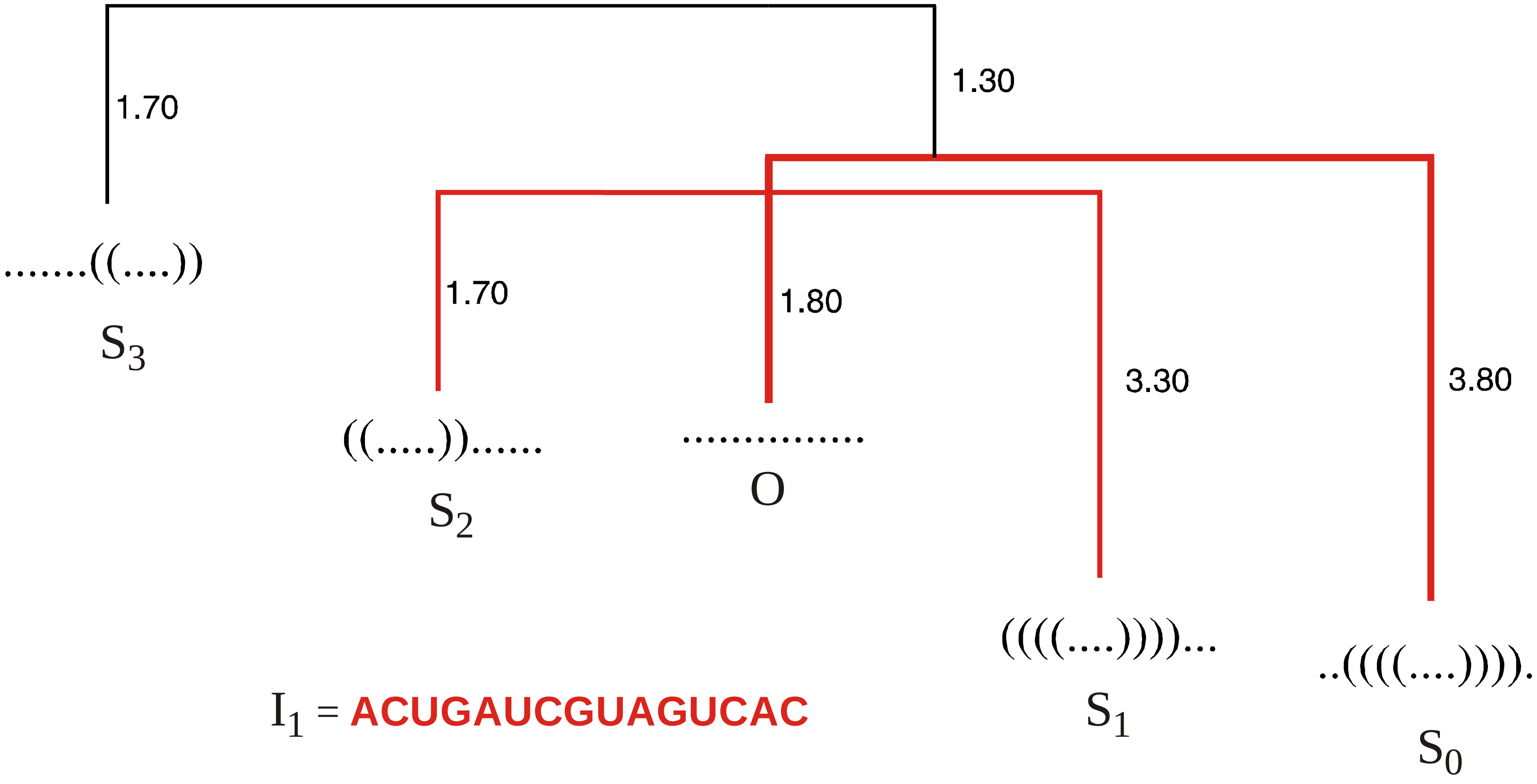
Mean folding curves for three small RNA molecules with different folding behavior



Search for local minima in conformation space



Definition of a ‚barrier tree‘



1.70

1.30

.......((....))

S_3

1.70

1.80

3.30

3.80

((.....))......

S_2

....

O

((((....)))...

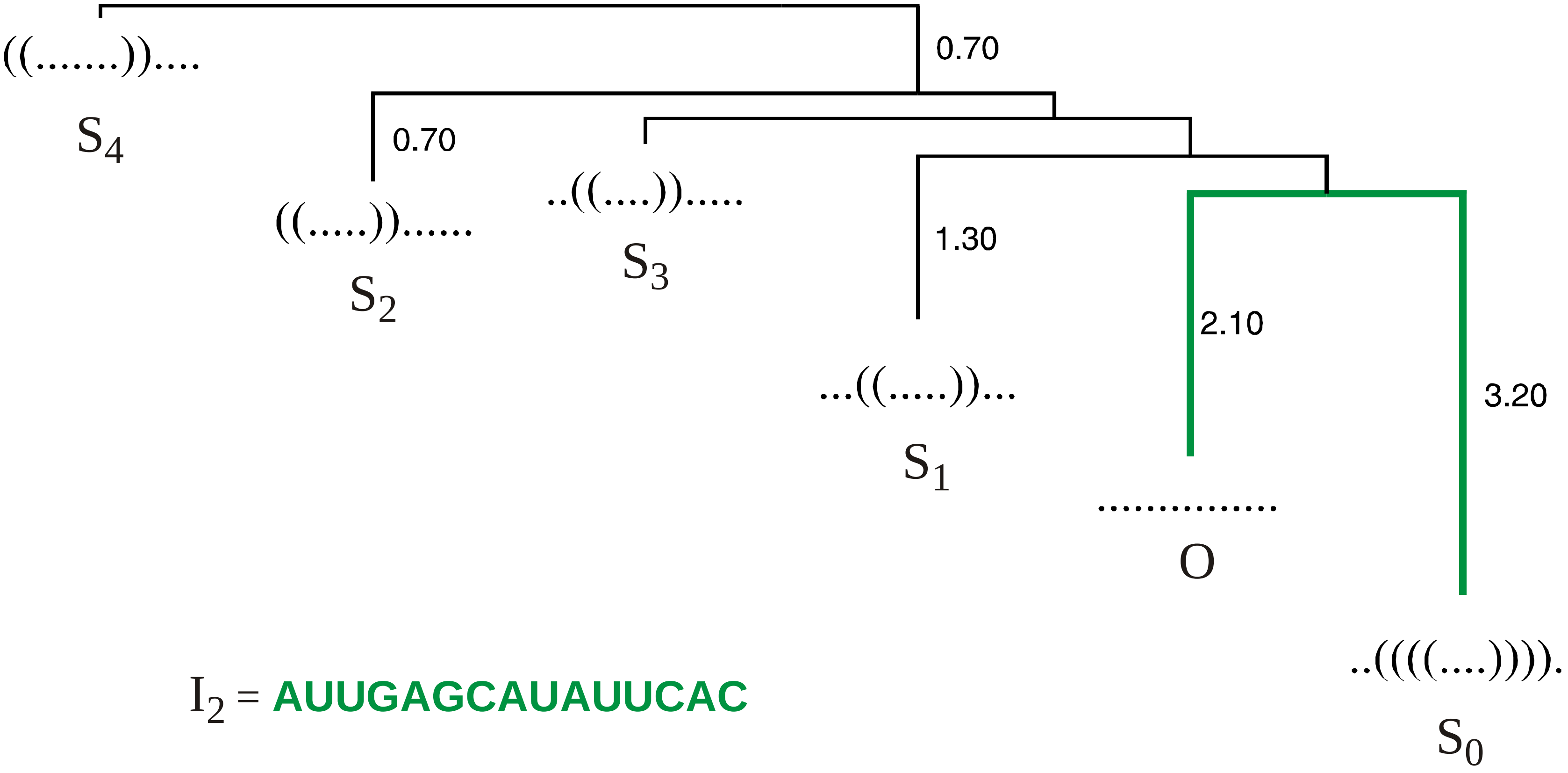
S_1

..((((....)))).

S_0

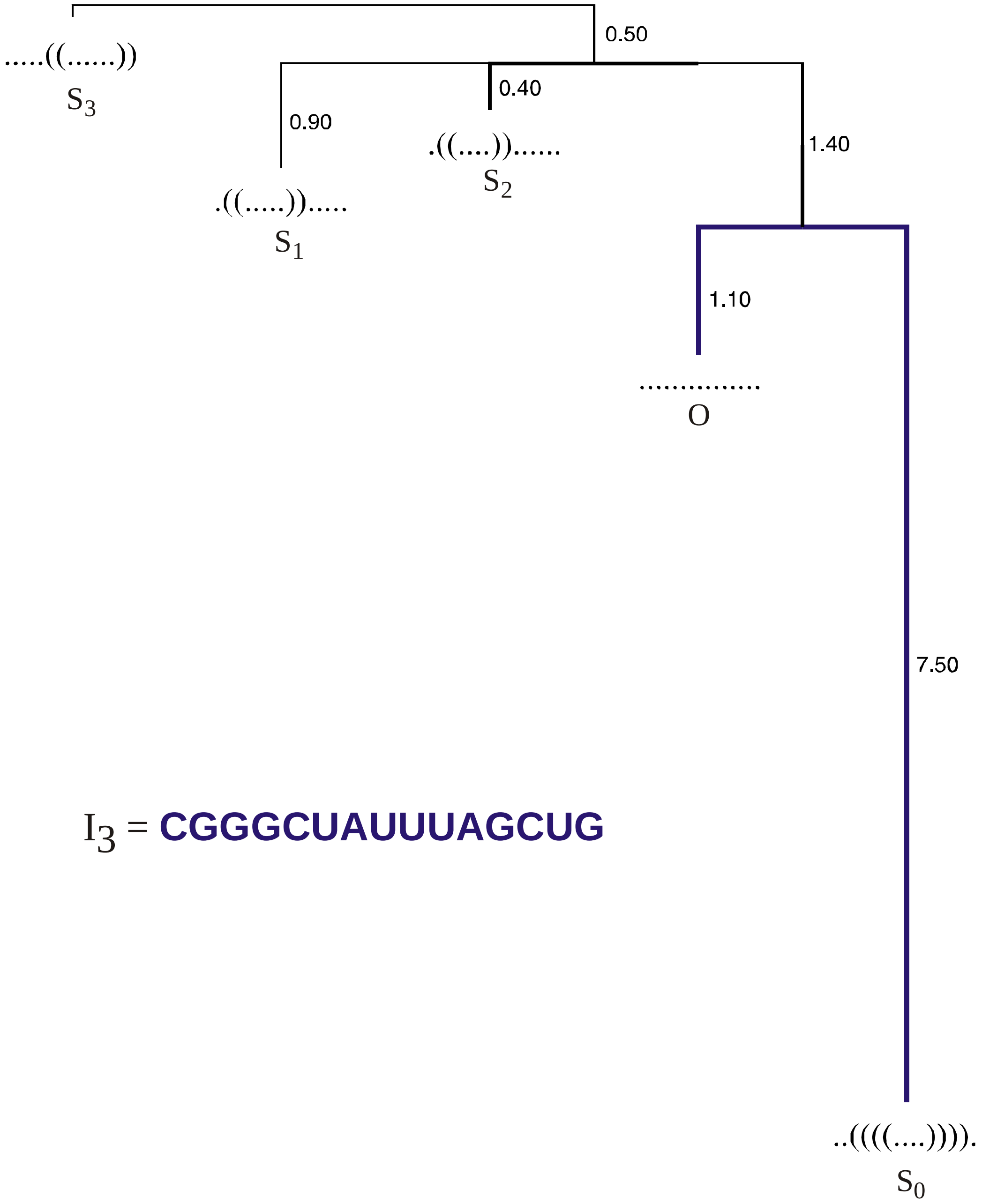
I_1 = **ACUGAUCGUAGUCAC**

Example of an unefficiently folding small RNA molecule with n = 15



I_2 = **AUUGAGCAUAUUCAC**

Example of an easily folding small RNA molecule with n = 15



0.50

.....((......))

S_3

0.90

0.40

.((.....)).....

S_1

.((....))......

S_2

1.40

1.10

....

O

7.50

..((((....)))).

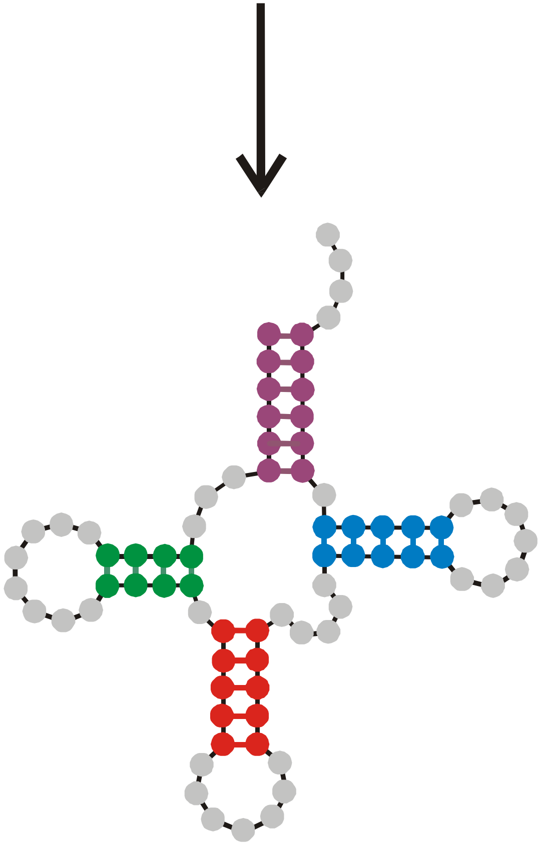
S_0

I_3 = **CGGGCUAUUUAGCUG**

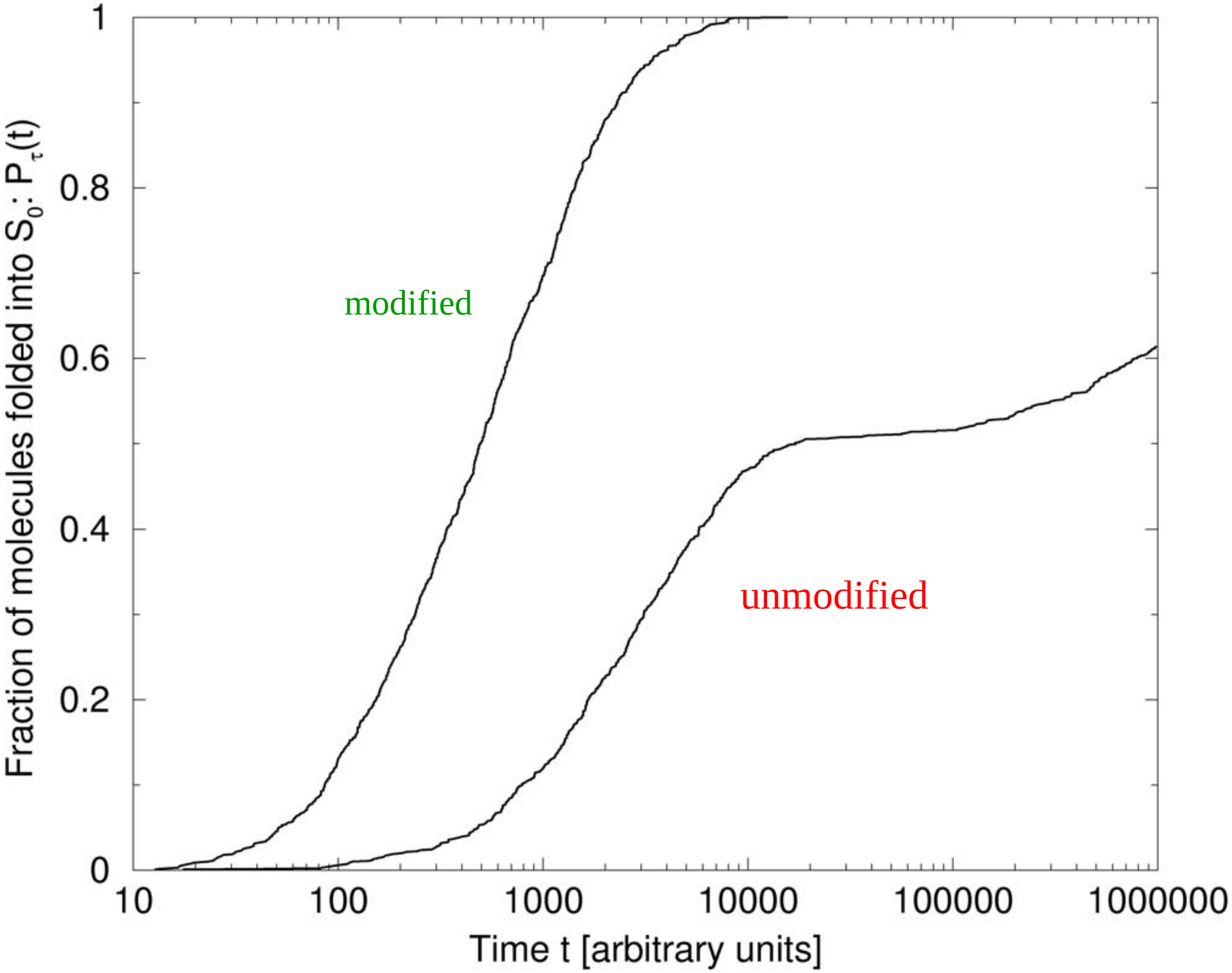
Example of an easily folding
and especially stable small
RNA molecule with n = 15

GCGGAUUUAGCUCAGUUGGGAGAGCGCCAGACUGAAGAUCUGGAGGUCCUGUGUUCGAUCCACAGAAUUCGCACCA

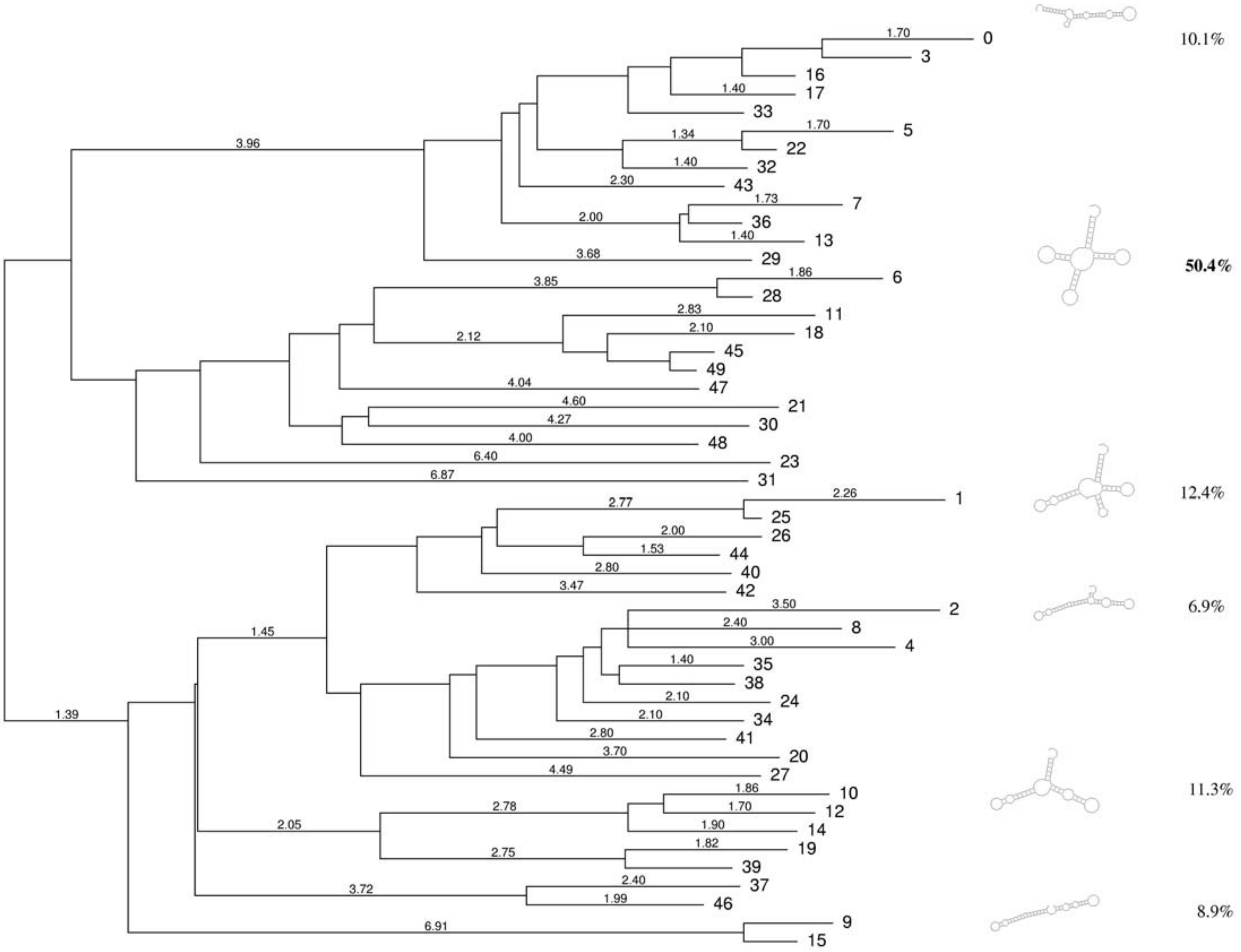
GCGGAUUUAGCUCAGDDGGGAGAGCMCCAGACUGAAYAUCUGGAGMUCCUGUGTPCGAUCCACAGAAUUCGCACCA



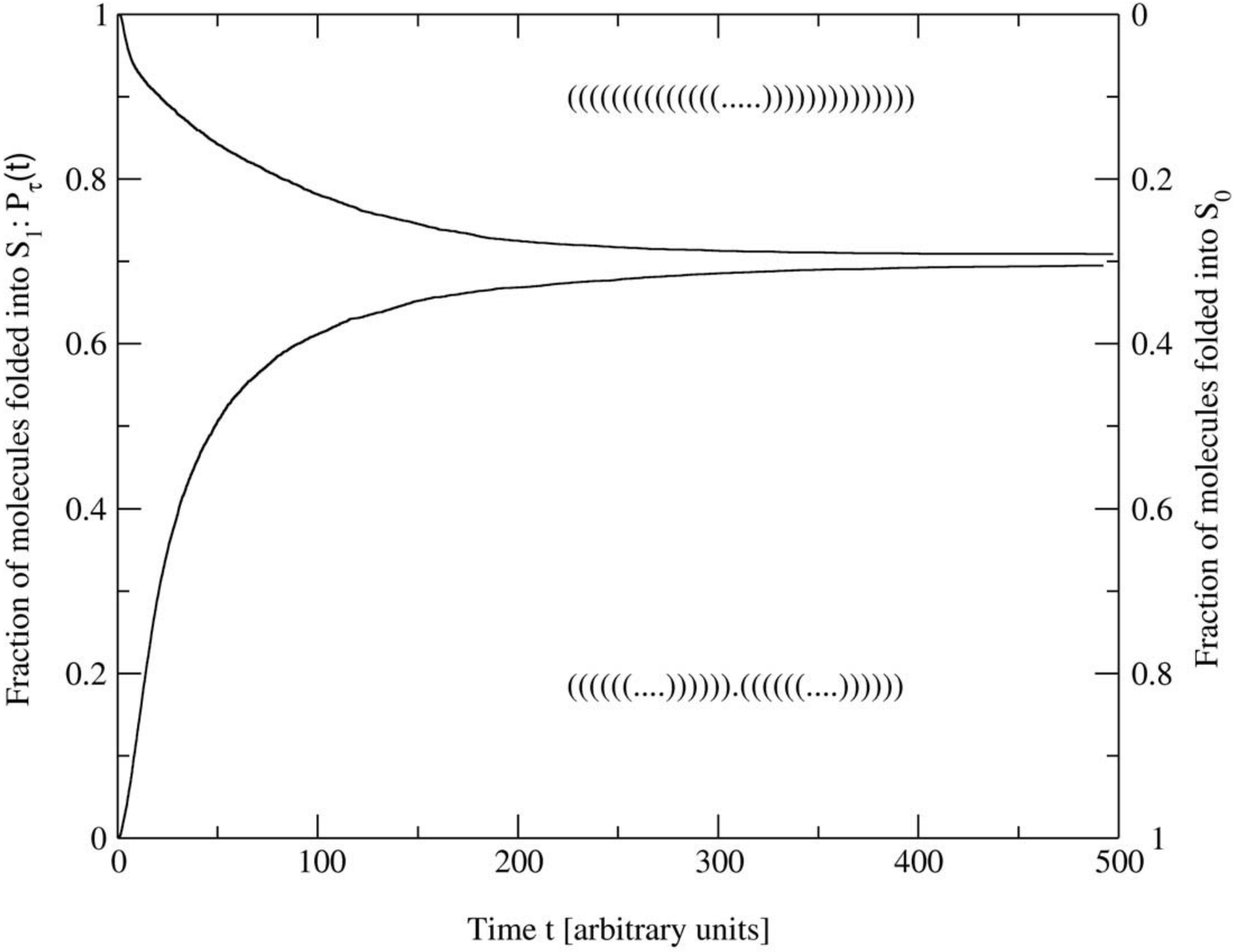
Kinetic folding of phenylalanyl-tRNA



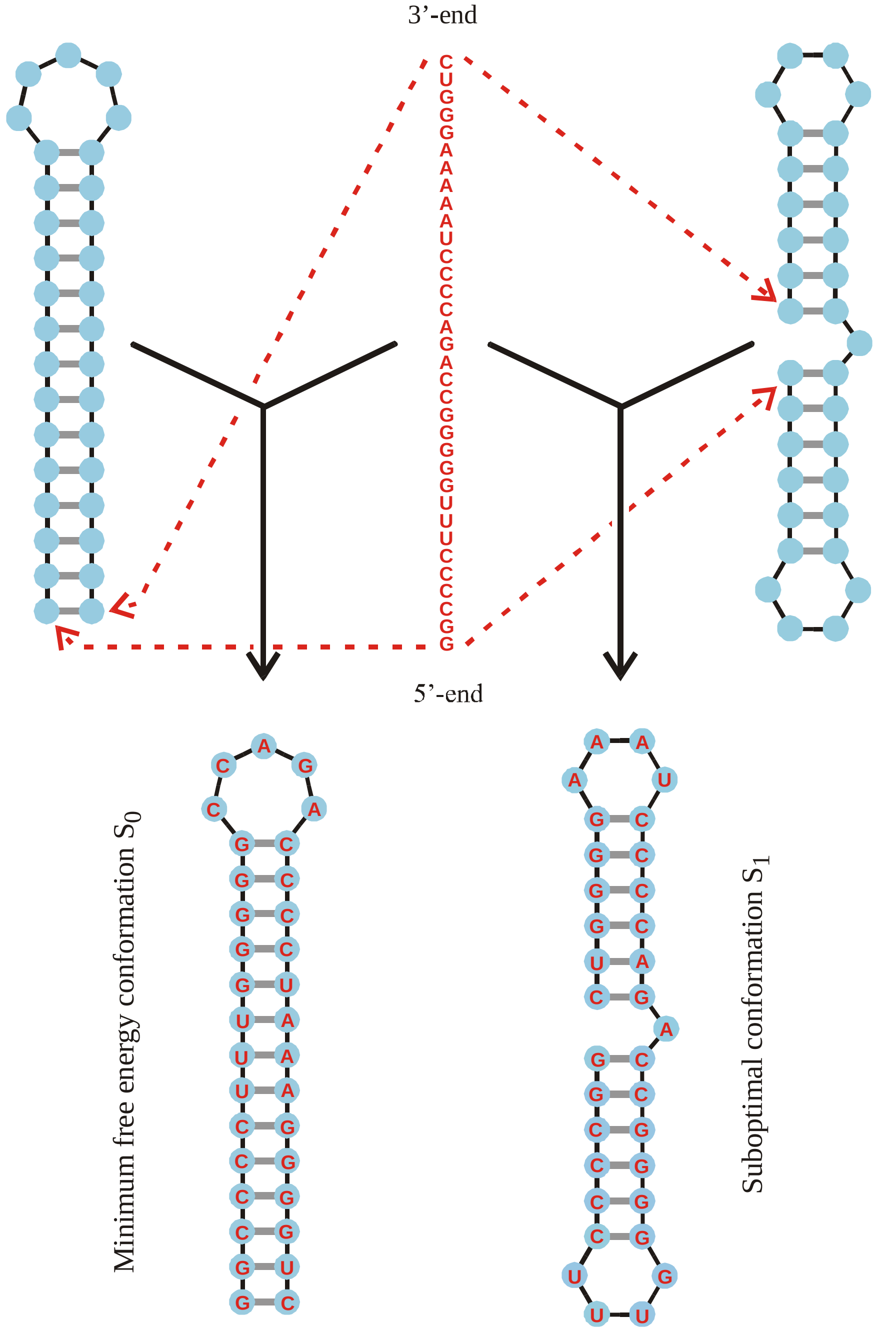
Folding dynamics of tRNA^{phe} with and without modified nucelotides



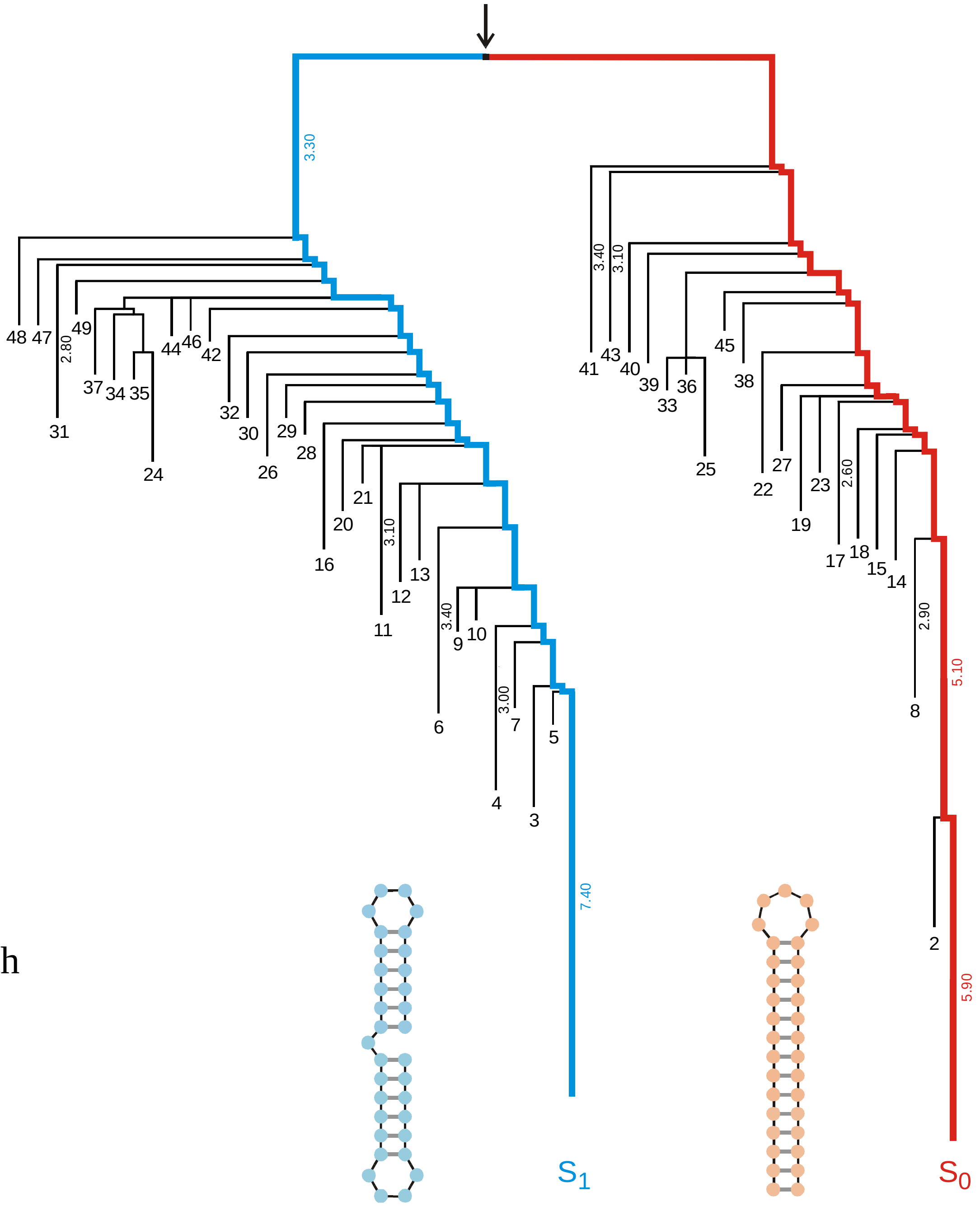
Barrier tree of tRNA^{phe} without modified nucelotides



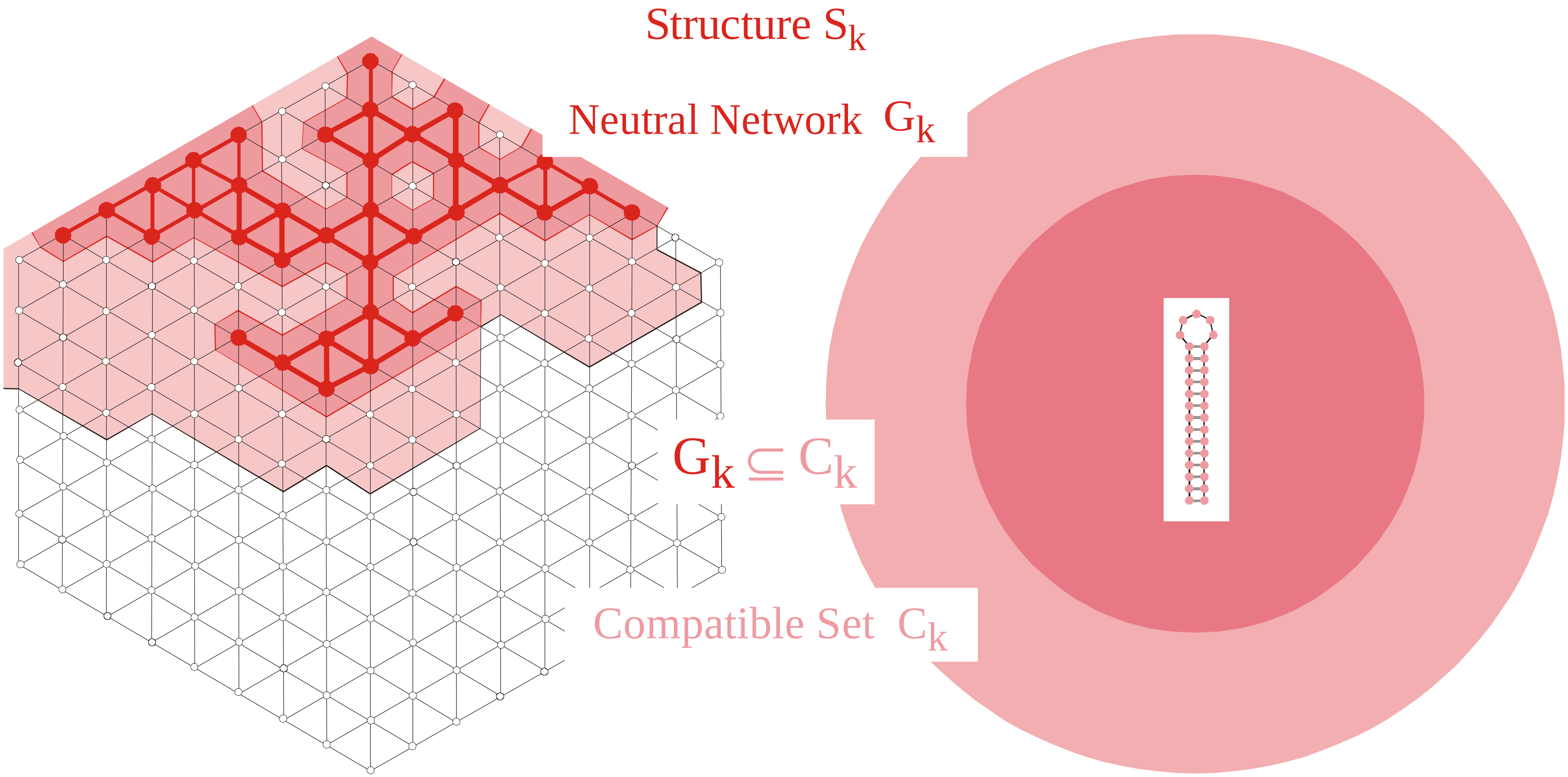
Folding dynamics of the sequence **GGCCCCUUUGGGGGCCAGACCCCUAAAAAGGGUC**


3’-end
CUGGGAAAAAUCCCCAGACCGGGGGUUUCCCCGG
5’-end
Minimum free energy conformation S_0
Suboptimal conformation S_1

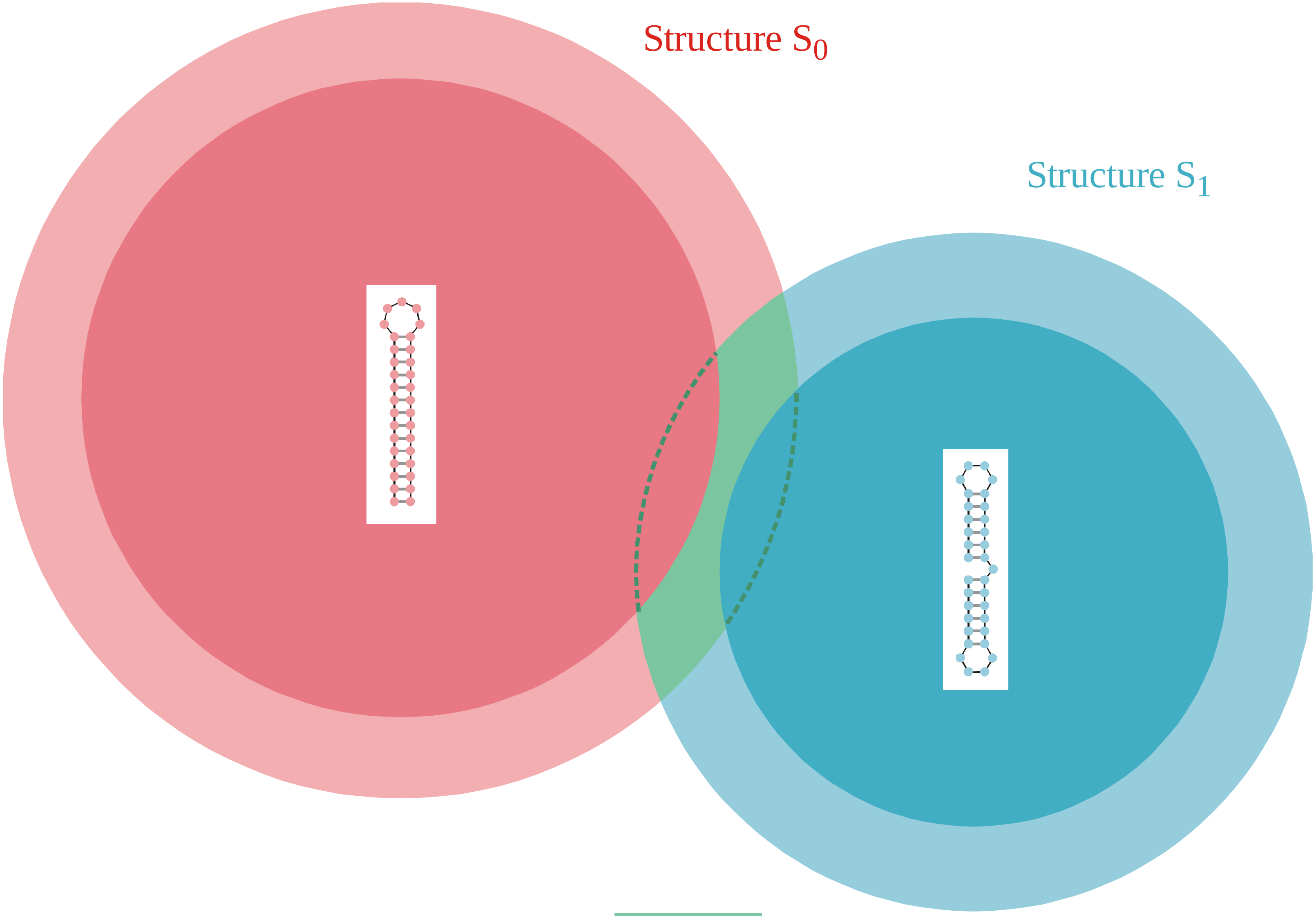
One sequence is compatible with two structures



Barrier tree of a sequence with two conformations

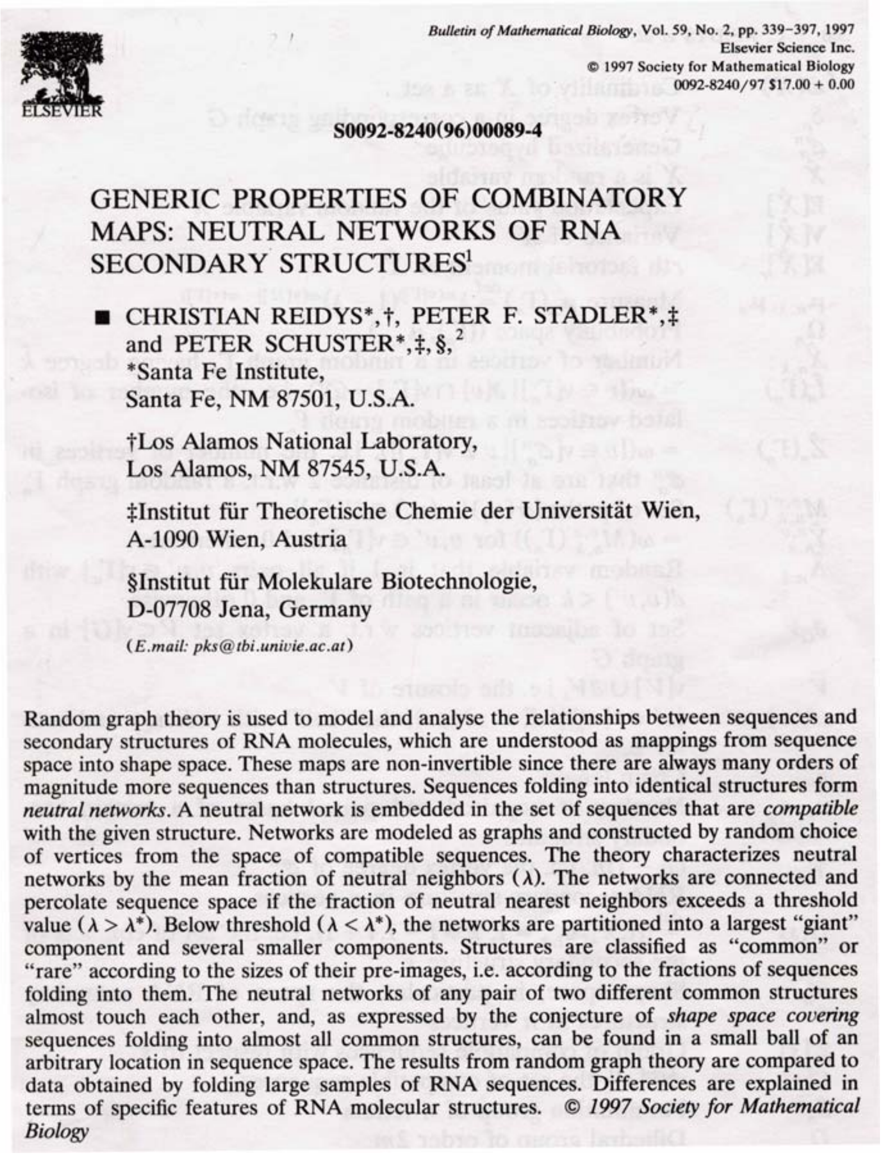


The **compatible set C_k** of a structure **S_k** consists of all sequences which form **S_k** as its minimum free energy structure (the neutral network **G_k**) or one of its suboptimal structures.



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

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



Bulletin of Mathematical Biology, Vol. 59, No. 2, pp. 339–397, 1997
Elsevier Science Inc.
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0092-8240/97 $17.00 + 0.00

S0092-8240(96)00089-4

GENERIC PROPERTIES OF COMBINATORY MAPS: NEUTRAL NETWORKS OF RNA SECONDARY STRUCTURES[1]

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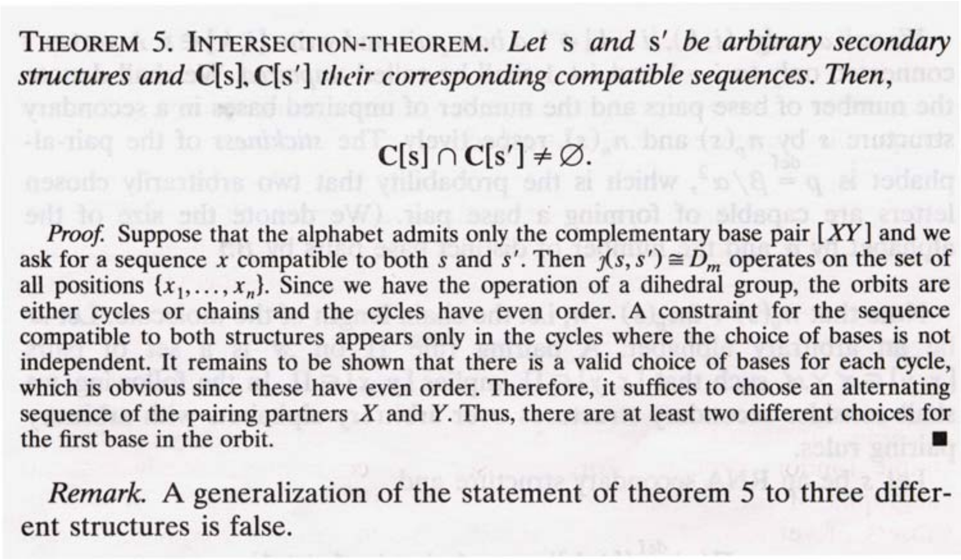
Random graph theory is used to model and analyse the relationships between sequences and secondary structures of RNA molecules, which are understood as mappings from sequence space into shape space. These maps are non-invertible since there are always many orders of magnitude more sequences than structures. 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,*

$$\mathrm{C[s]} \cap \mathrm{C[s']} \neq \varnothing.$$

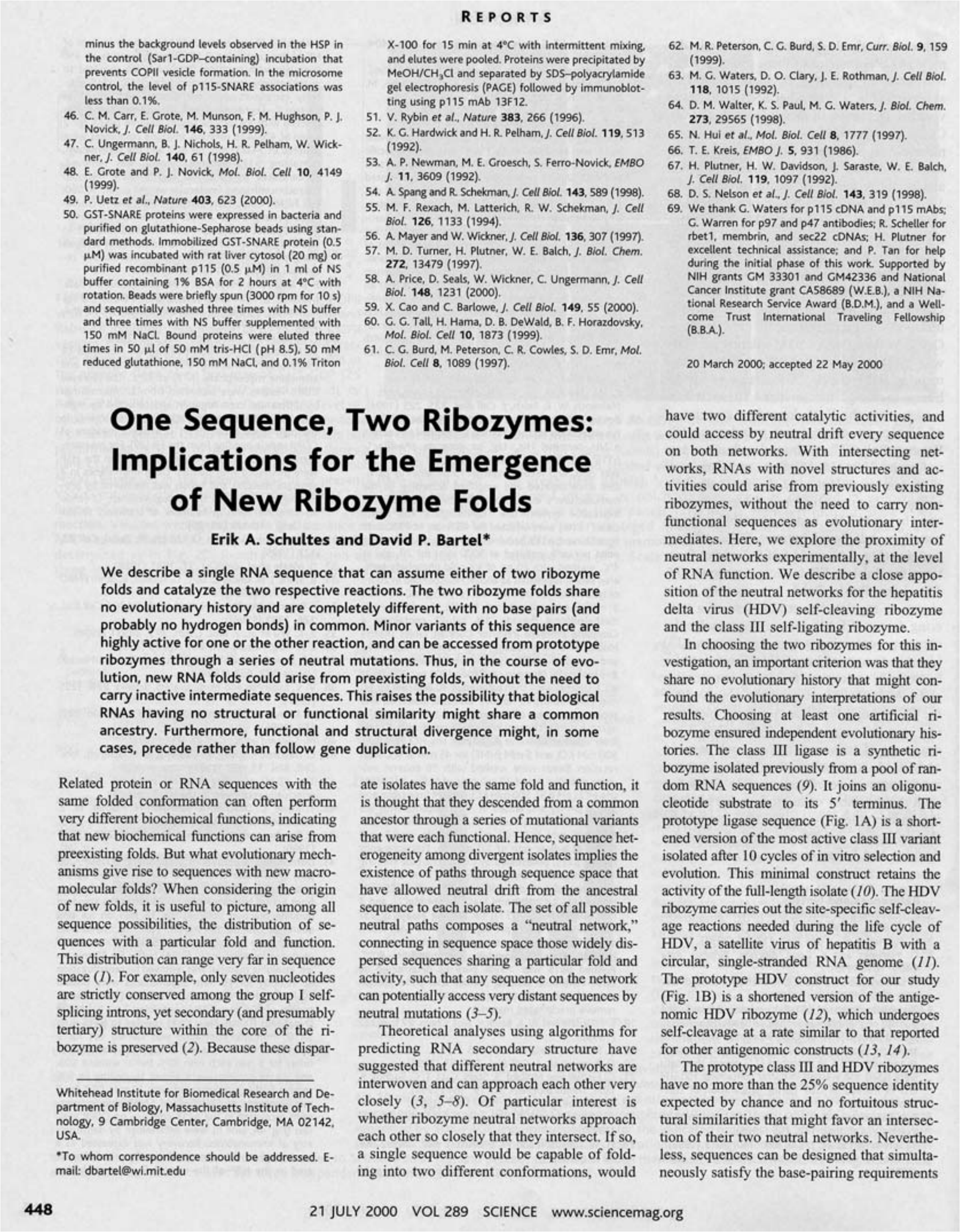
Proof. Suppose that the alphabet admits only the complementary base pair $[XY]$ and we ask for a sequence x compatible to both s and s'. Then $\mathcal{J}(s, s') \cong D_m$ operates on the set of all positions $\{x_1, \ldots, x_n\}$. Since we have the operation of a dihedral group, the orbits are either 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 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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69. We thank G. Waters for p115 cDNA and p115 mAbs; G. Warren for p97 and p47 antibodies; R. Scheller for rbet1, 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 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

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

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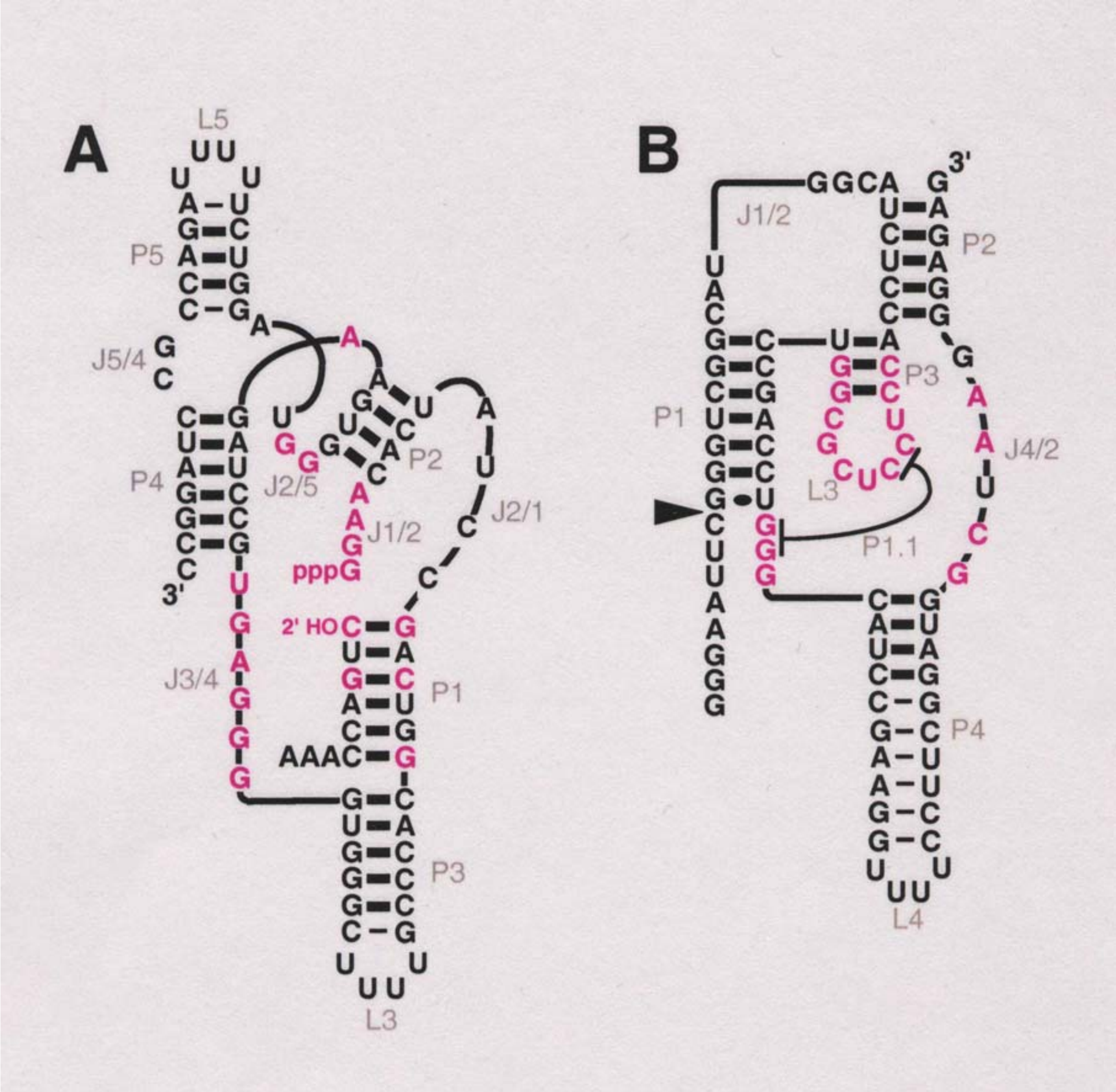
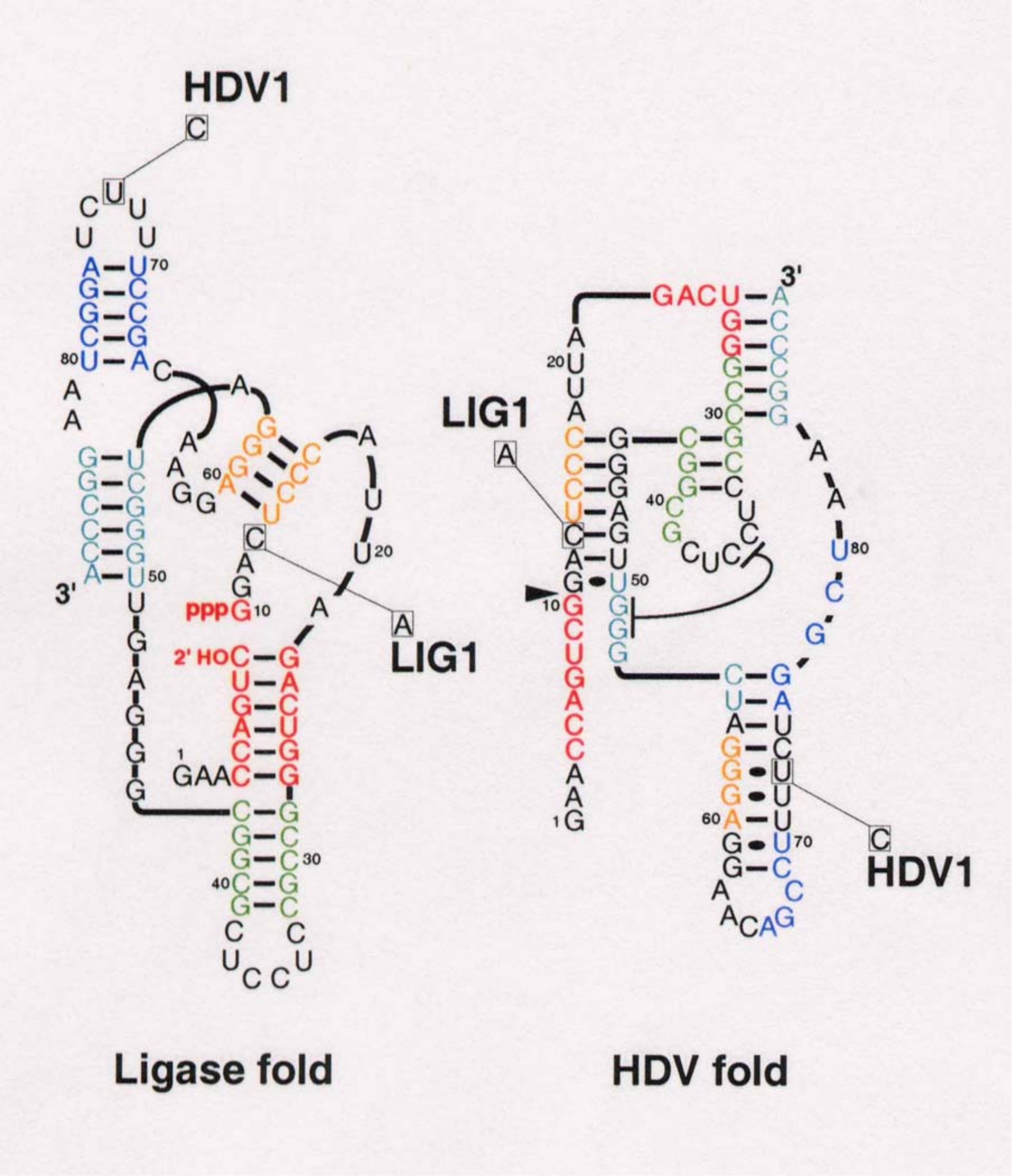
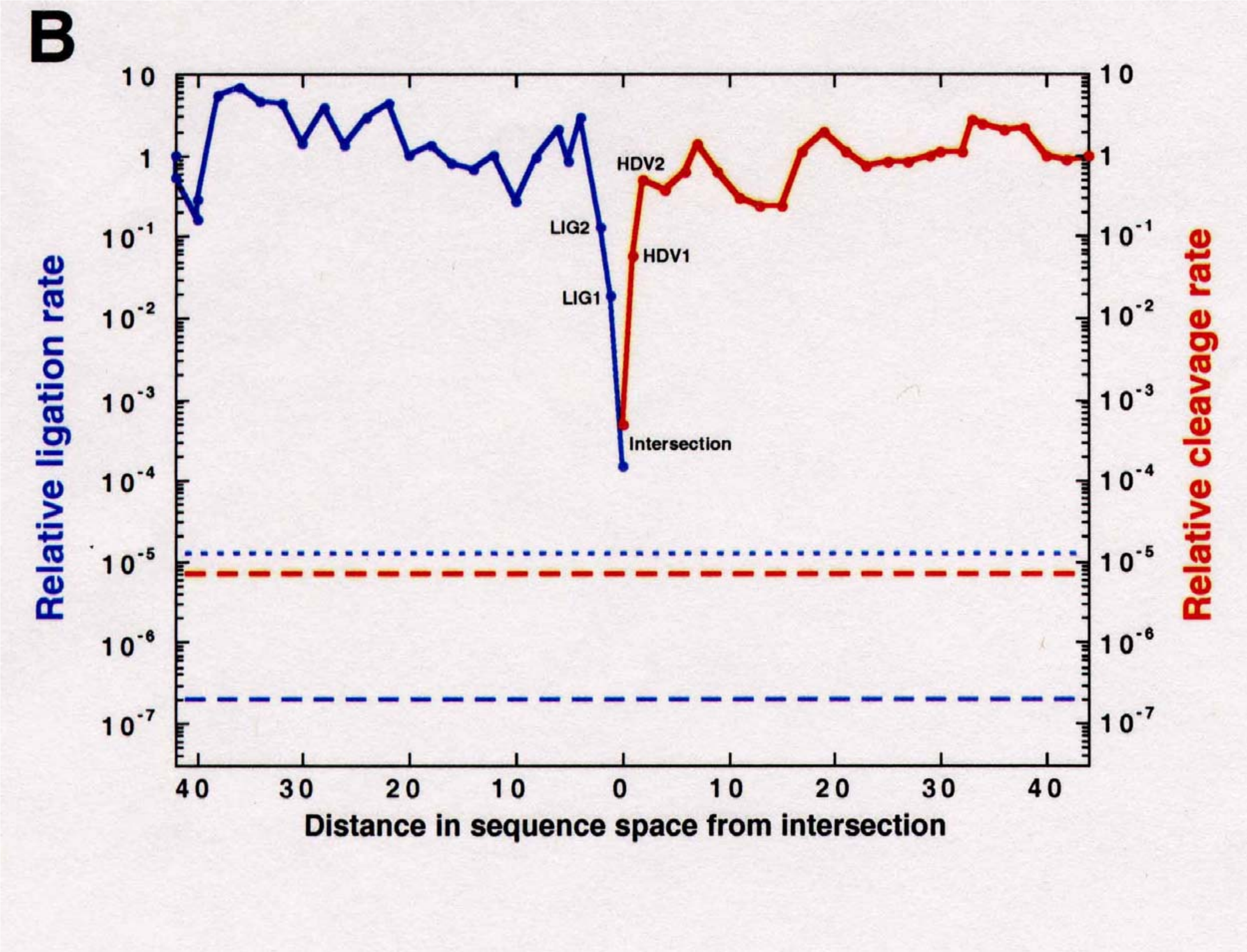


Two ribozymes of chain lengths n = 88 nucleotides: An artificial ligase (**A**) and a natural cleavage ribozyme of hepatitis-δ-virus (**B**)

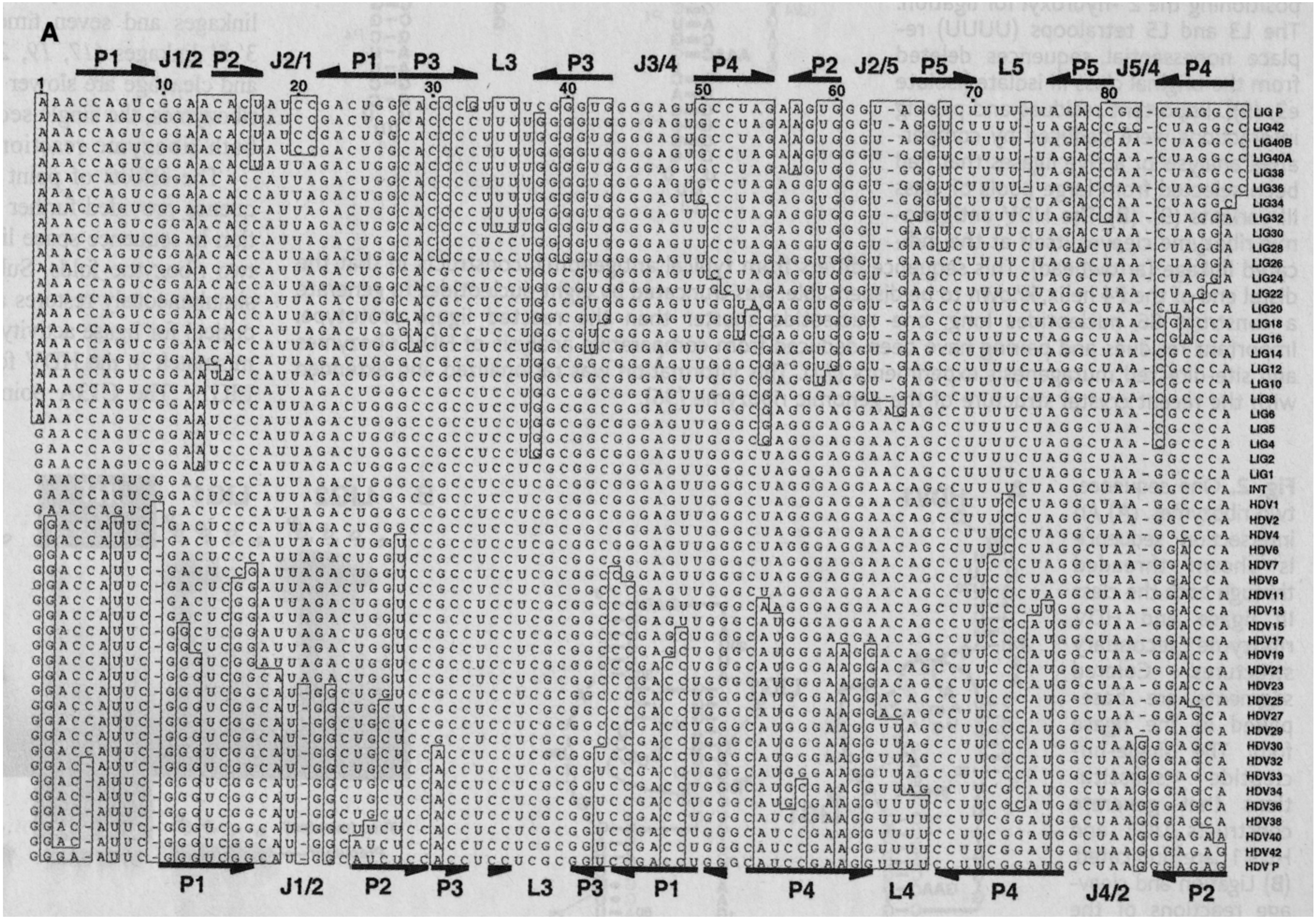
The sequence at the ***intersection***:

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





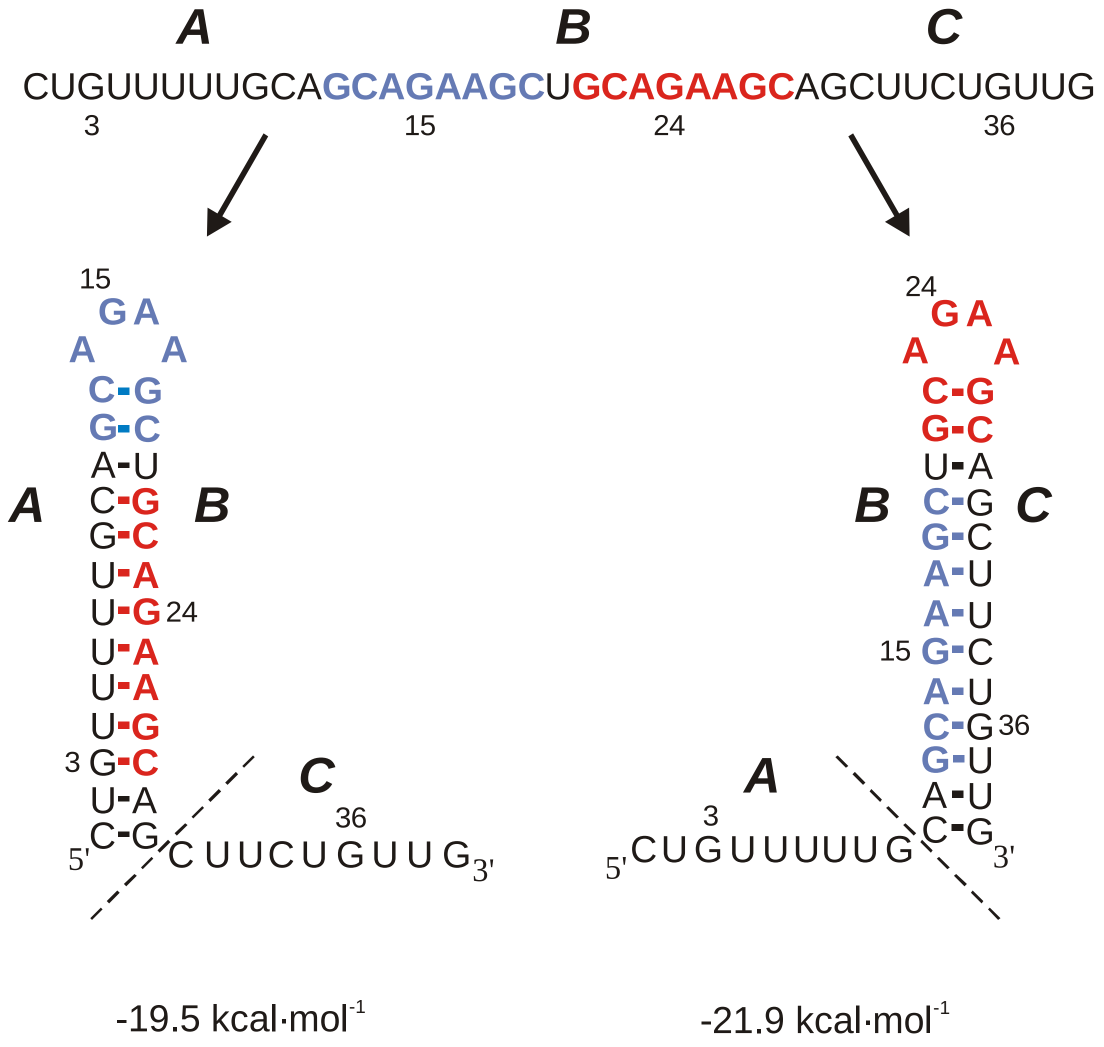
Two neutral walks through sequence space with conservation of structure and catalytic activity



Sequence of mutants from the intersection to both reference ribozymes

J. H. A. Nagel, C. Flamm, I. L. Hofacker, K. Franke, M. H. de Smit, P. Schuster, and C. W. A. Pleij. ***Structural parameters affecting the kinetic competition of RNA hairpin formation***, *Nucleic Acids Research*, submitted 2004.

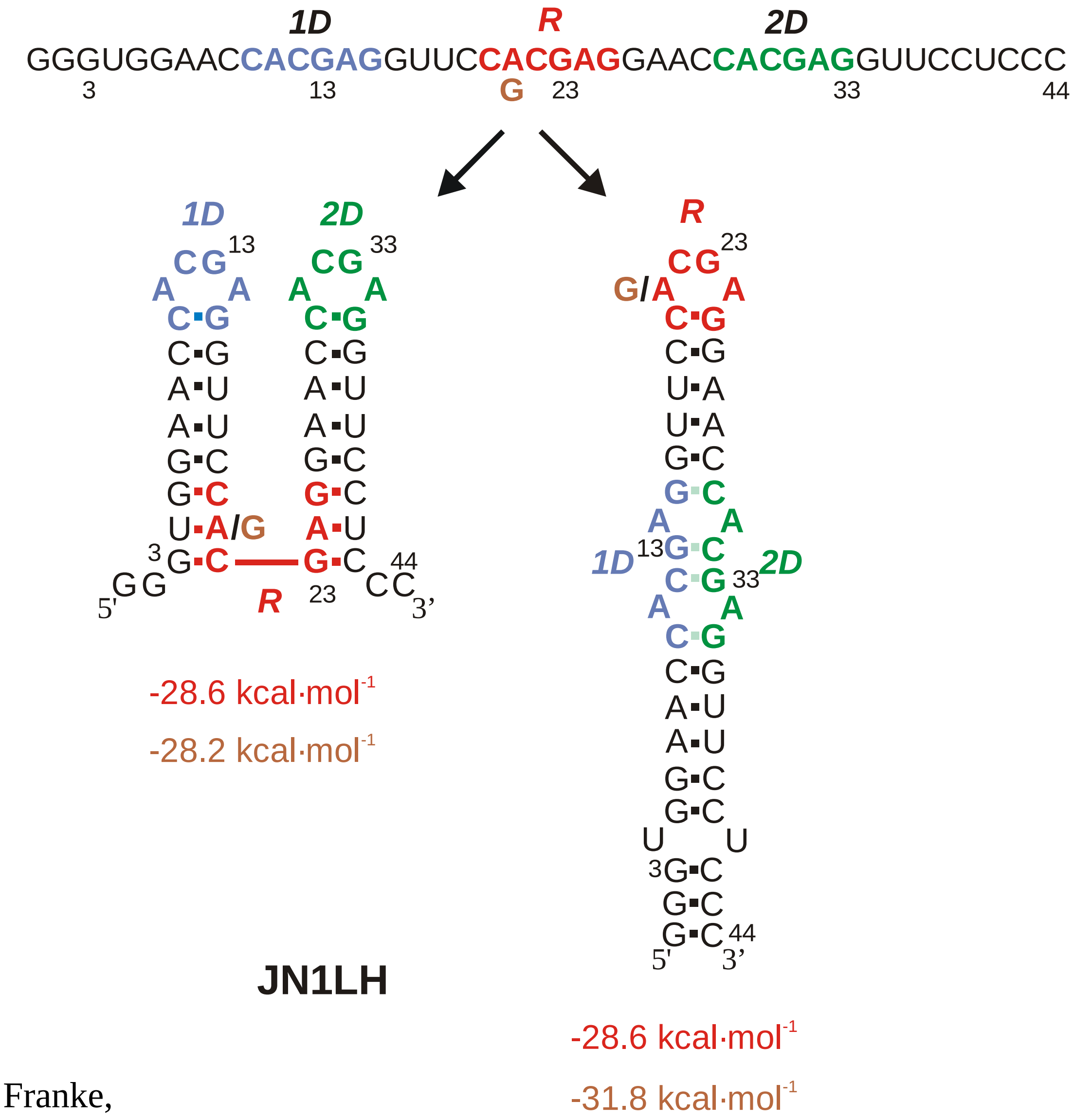
J. H. A. Nagel, J. Møller-Jensen, C. Flamm, K. J. Öistämö, J. Besnard, I. L. Hofacker, A. P. Gultyaev, M. H. de Smit, P. Schuster, K. Gerdes and C. W. A. Pleij. ***The refolding mechanism of the metastable structure in the 5'-end of the*** hok ***mRNA of plasmid*** R1, submitted 2004.



JN2C

J.H.A. Nagel, C. Flamm, I.L. Hofacker, K. Franke,
M.H. de Smit, P. Schuster, and C.W.A. Pleij.

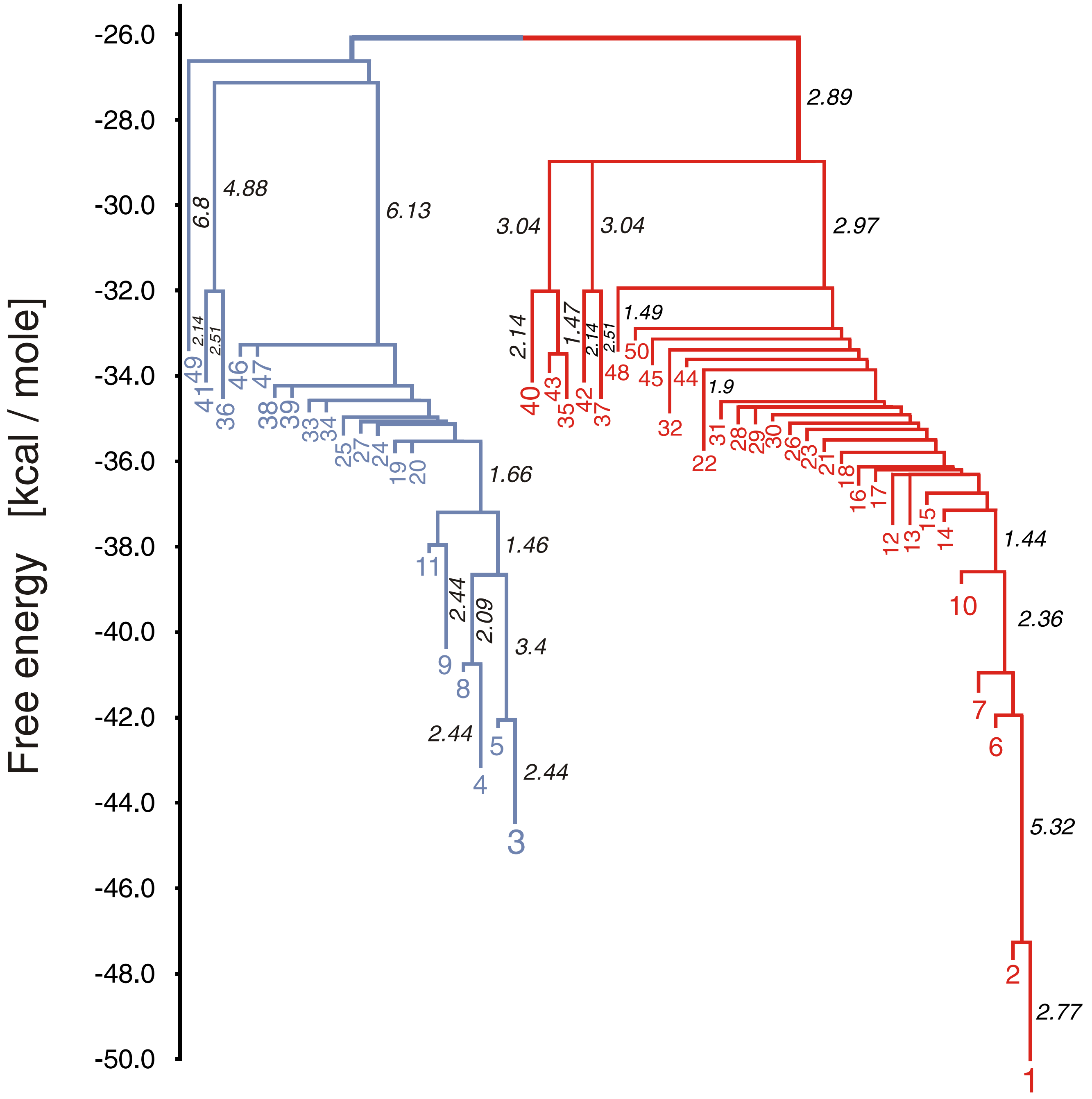
Structural parameters affecting the kinetic competition of RNA hairpin formation, in press 2004.



JN1LH

J.H.A. Nagel, C. Flamm, I.L. Hofacker, K. Franke,
M.H. de Smit, P. Schuster, and C.W.A. Pleij.

Structural parameters affecting the kinetic competition of RNA hairpin formation, Nucleic Acids Research, submitted 2004.



J1LH barrier tree

1. What are neutral networks ?
2. Mutations and structural stability
3. Structures from defective alphabets
4. Suboptimal conformations and structural stability
5. Metastable structures and RNA switches
6. **How to handle multiple constraints**

Multiple constraints on RNA structures

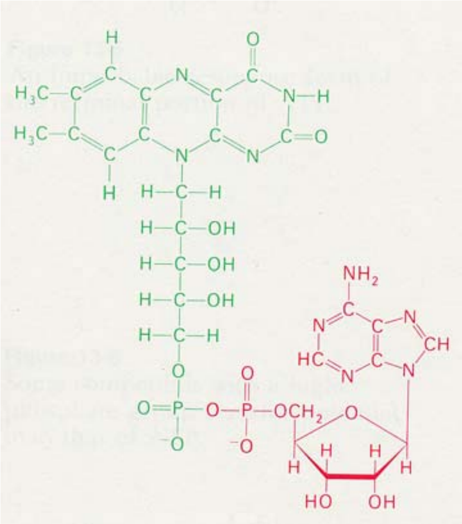
1. Two or more binding sites on one RNA molecule
2. Cofolding (hybridization) of two or more RNAs
3. Secondary structure and tertiary interactions
4. Switching RNAs with two functions

Examples: tRNAs, ribozyme with two functions, ...

Allosteric effectors:

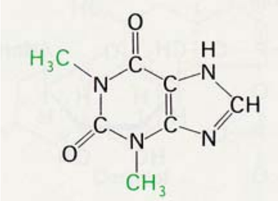
FMN = flavine mononucleotide

H10 – H12



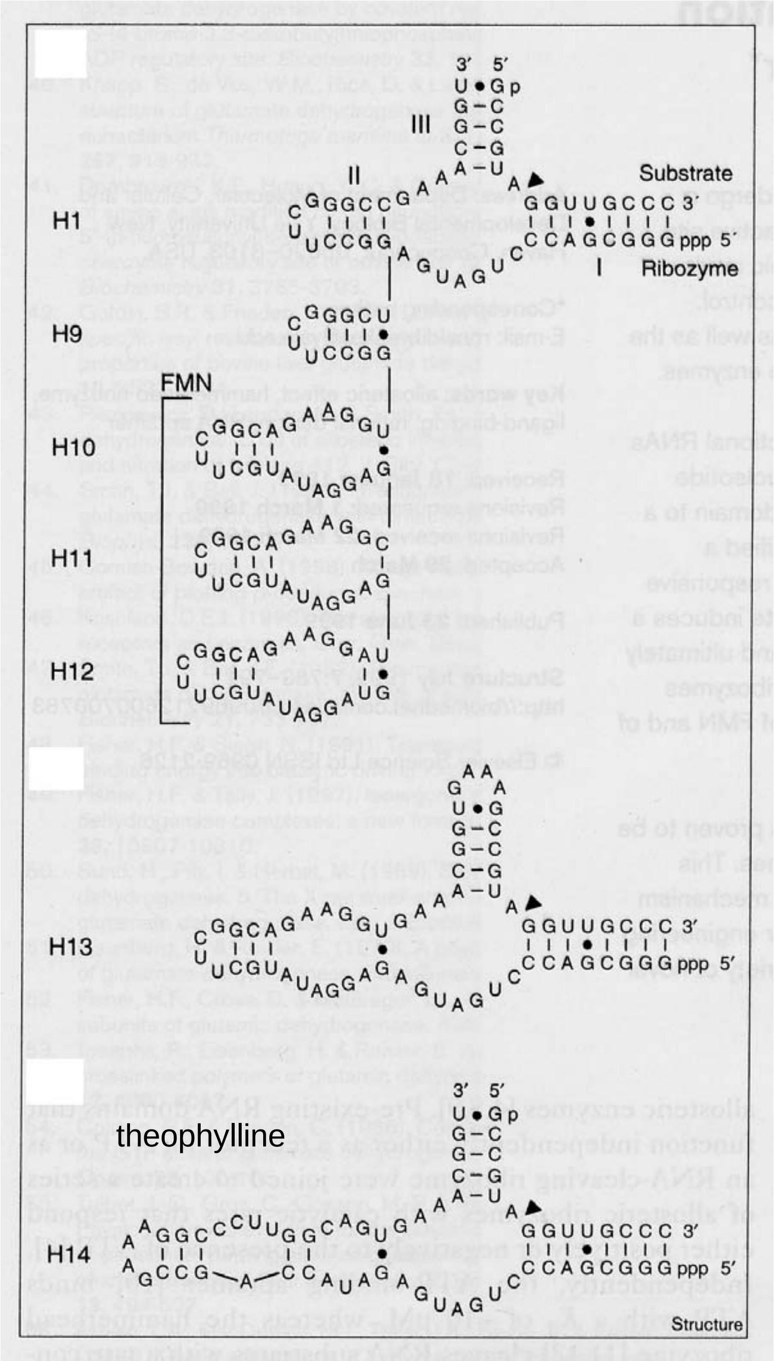
theophylline

H14

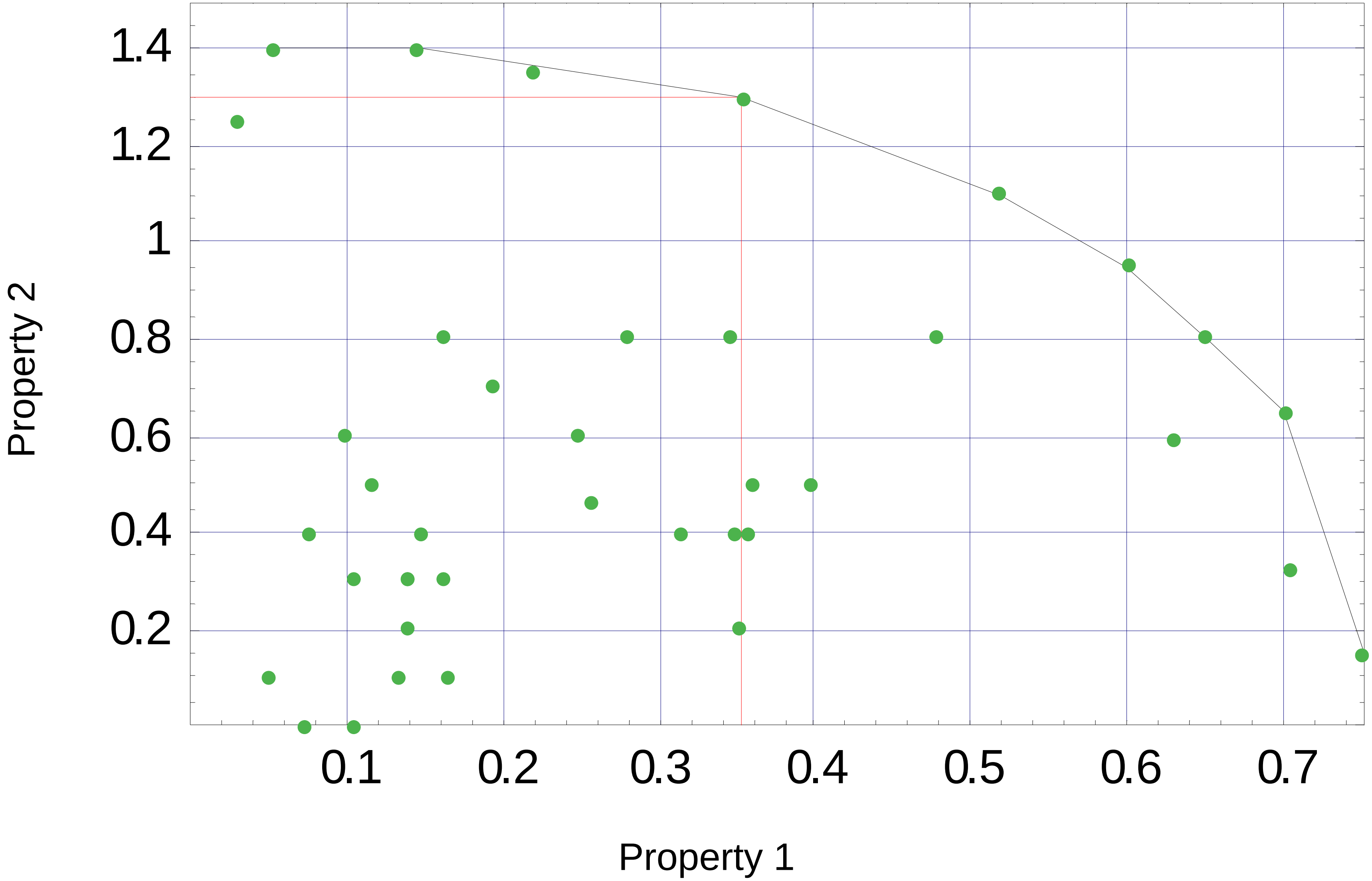


Self-splicing allosteric ribozyme

H13



Hammerhead ribozymes with allosteric effectors



Pareto set and Pareto front in optimization of two and more properties

Acknowledgement of support

Universität Wien

Fonds zur Förderung der wissenschaftlichen Forschung (FWF)
Projects No. 09942, 10578, 11065, 13093
13887, and 14898

Wiener Wissenschafts-, Forschungs- und Technologiefonds (WWTF)
Project No. Mat05

Jubiläumsfonds der Österreichischen Nationalbank
Project No. Nat-7813

European Commission: Contracts No. 98-0189, 12835 (NEST)

Austrian Genome Research Program – GEN-AU

Siemens AG, Austria

Universität Wien and the Santa Fe Institute

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Walter Fontana, Harvard Medical School, MA

Christian Forst, **Christian Reidys**, Los Alamos National Laboratory, NM

Peter Stadler, **Bärbel Stadler**, Universität Leipzig, GE

Jord Nagel, **Kees Pleij**, Universiteit Leiden, NL

Peter Roosen, „roko“ Aachen, GE

Christoph Flamm, **Ivo L.Hofacker**, **Andreas Svrček-Seiler**,
Universität Wien, AT

Kurt Grünberger, **Michael Kospach**, **Andreas Wernitznig**,
Stefanie Widder, Michael Wolfinger, Stefan Wuchty,Universität Wien, AT

Stefan Bernhart, **Jan Cupal**, **Lukas Endler, Ulrike Langhammer**,
Rainer Machne, Ulrike Mückstein, **Hakim Tafer,** Universität Wien, AT

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