Evolution von Molekülen

Von der Theorie zur Herstellung maßgeschneiderter Moleküle

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Institut für Theoretische Chemie, Universität Wien, Austria
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Biokatalyse und Graz, heute ein Begriff

Graz, 06.10.2010

Web-Page für weitere Informationen:

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

Charles Darwin, 1809-1882

Drei notwendige Bedingungen für Darwinsche Evolution:

1. **Vermehrung,**
2. **Variation**, und
3. **Selektion**.

Alle drei Bedingungen werden nicht nur von zellulären Organismen erfüllt sondern auch von **Nukleinsäuremolekülen** – **DNA** or **RNA** – **in** geeigneten **zellfreien Experimentalanordnungen**:

Darwinsche Evolution im Reagenzglas

1. Evolutionsexperimente im Reagenzglas
2. Kinetik der Evolution von Molekülen
3. Gezielte Evolution der „Molekülzüchter"
4. Strukturen und Fitnesslandschaften
5. Evolution *in silico*
6. RNA-Schalter

1. **Evolutionsexperimente im Reagenzglas**
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Reviews

G. F. Joyce

DOI: 10.1002/anie.200701369

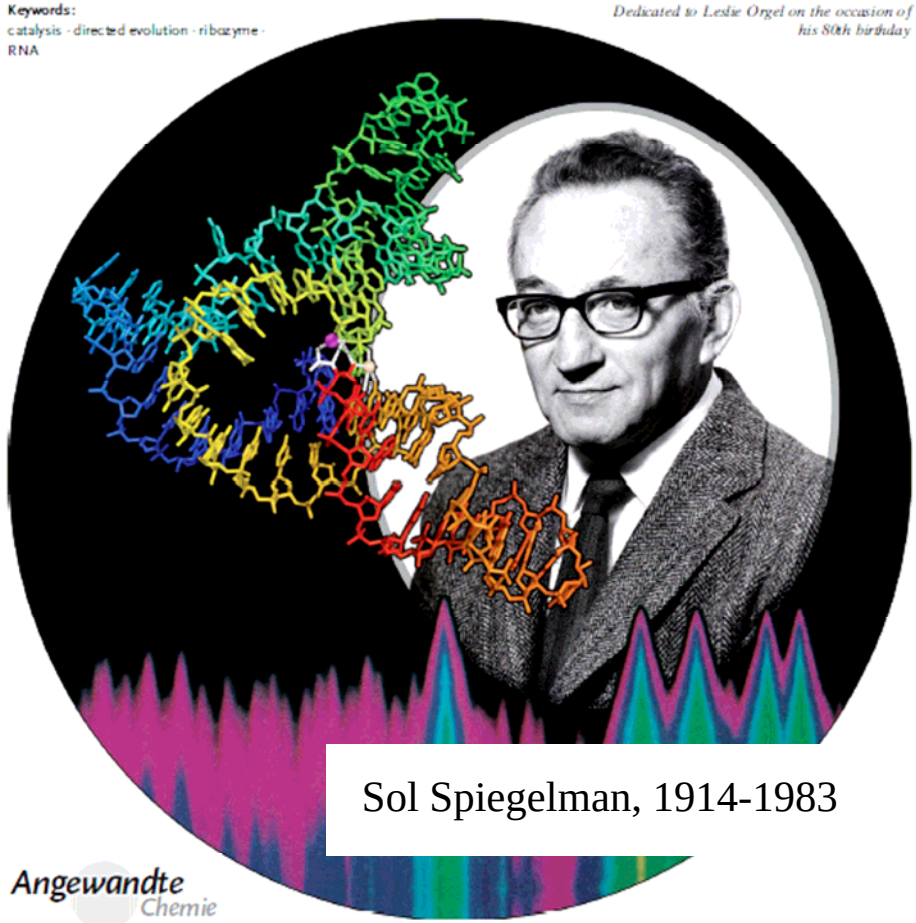
Molecular Evolution

Forty Years of In Vitro Evolution**

*Gerald F. Joyce**

Keywords:
catalysis · directed evolution · ribozyme · RNA

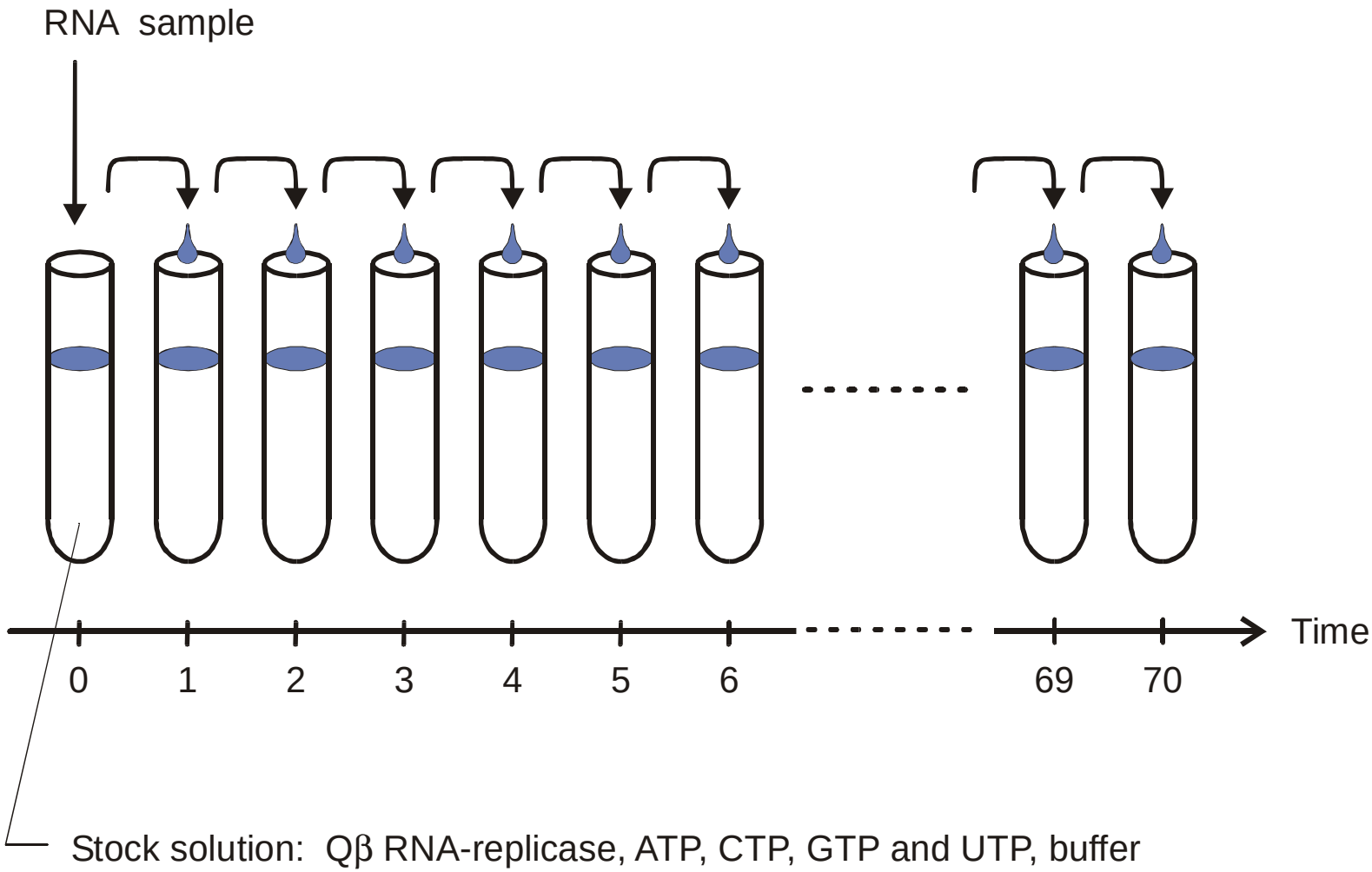
Dedicated to Leslie Orgel on the occasion of his 80th birthday



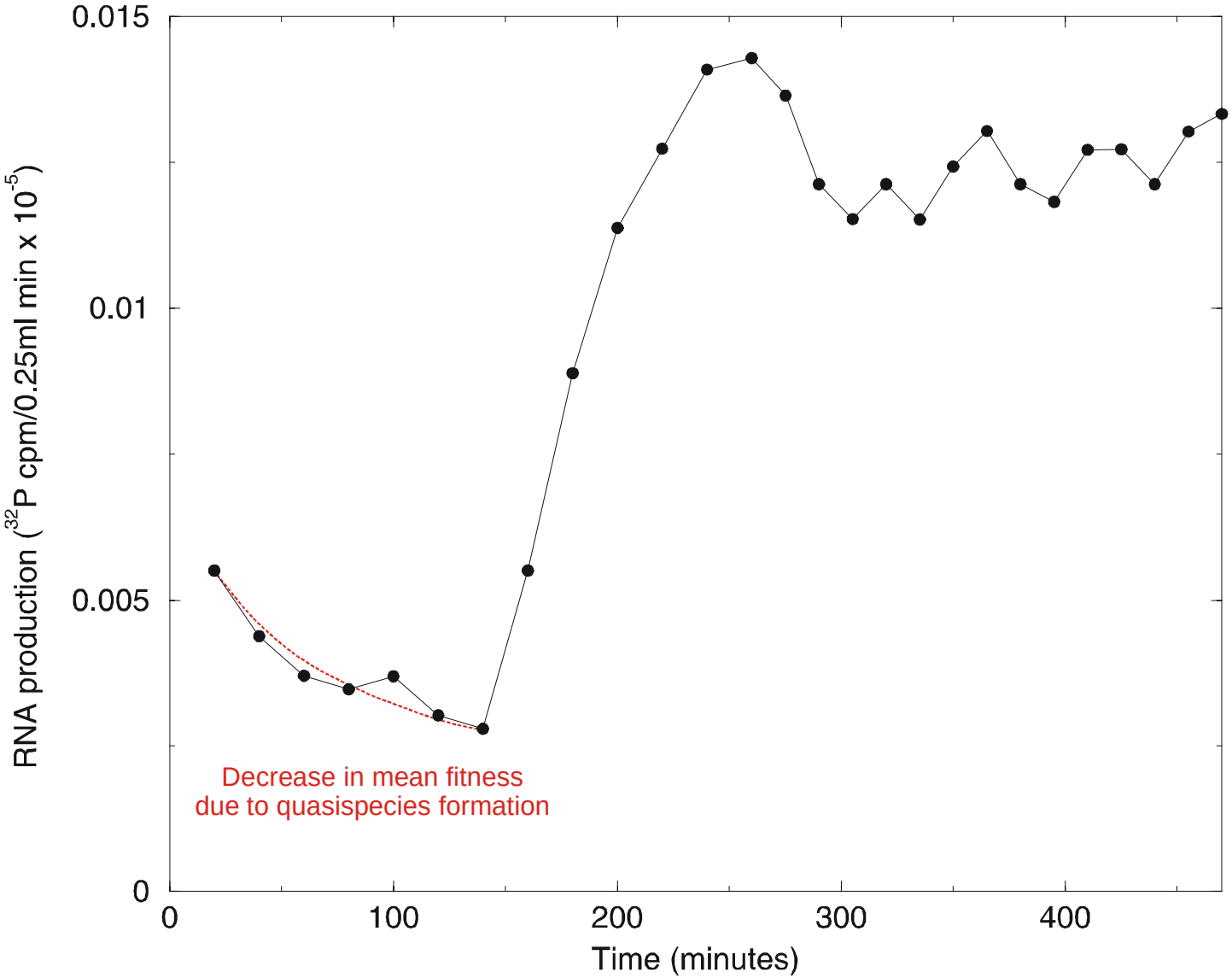
Sol Spiegelman, 1914-1983

Evolution im Reagenzglas

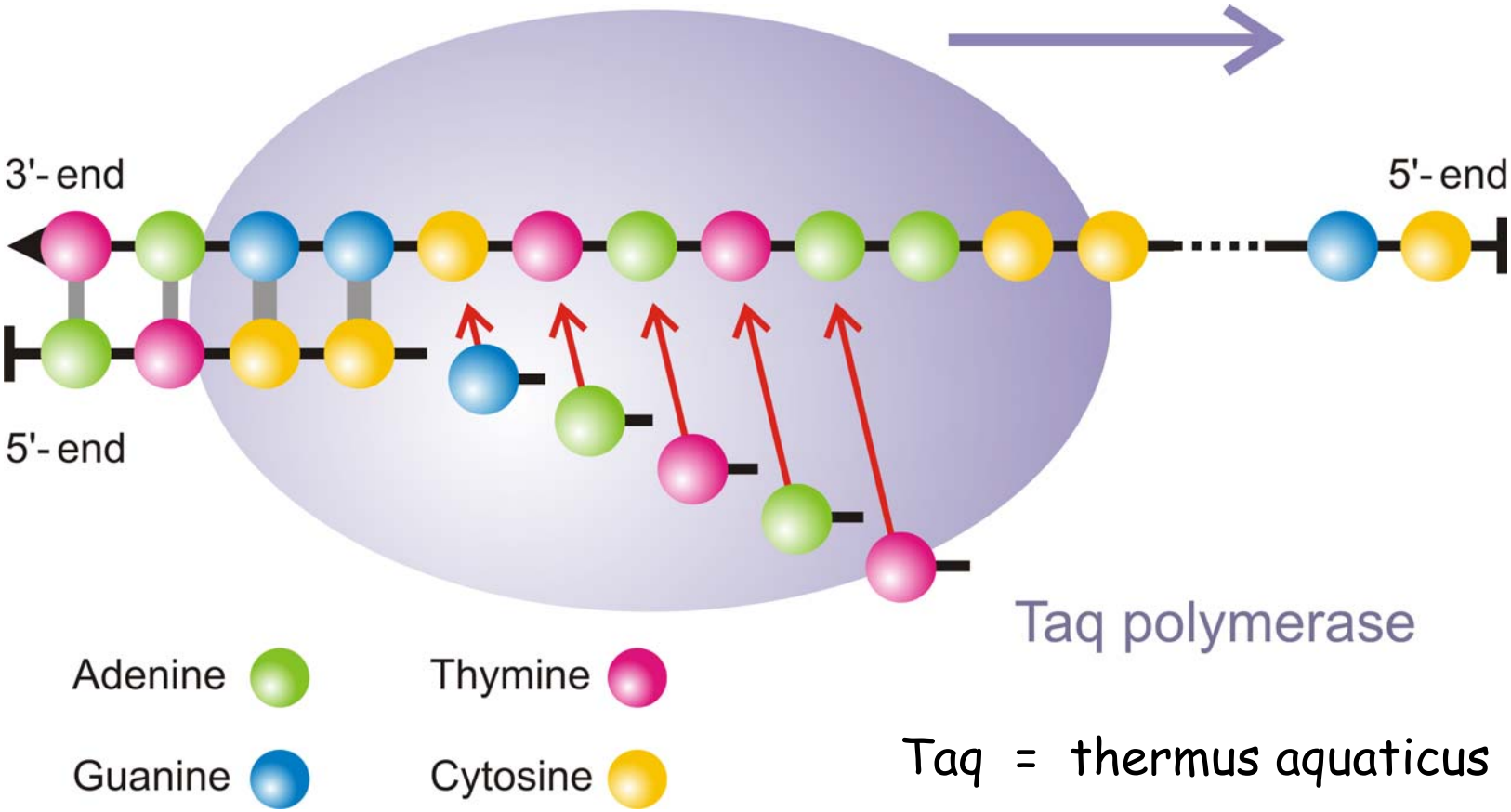
G.F. Joyce, *Angew.Chem.Int.Ed.* **46** (2007), 6420-6436



Anwendung der seriellen Überimpfungstechnik auf RNA-Evolution in Reagenzglas



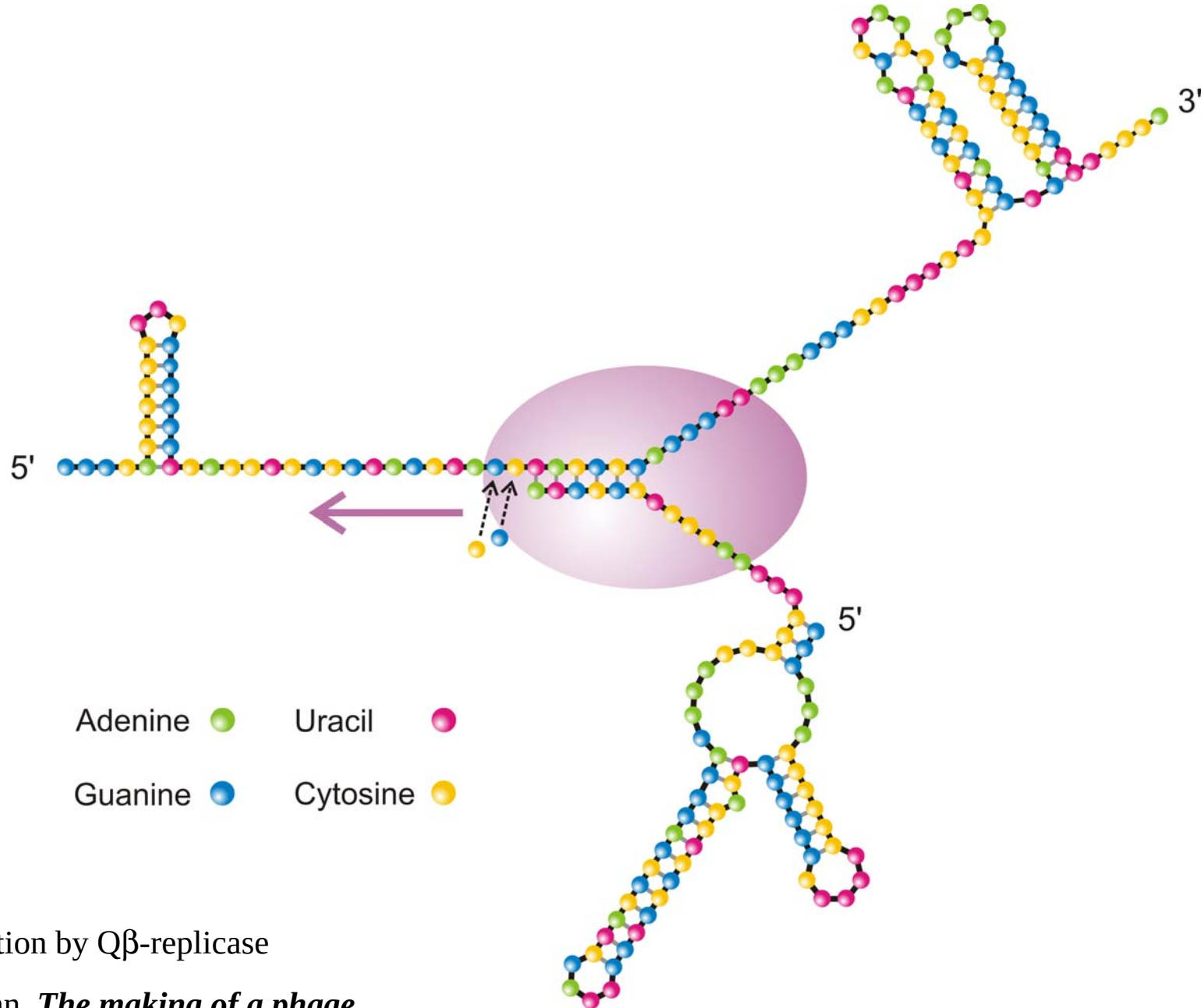
The increase in RNA production rate during a serial transfer experiment



Taq = thermus aquaticus

Accuracy of replication: $Q = q_1 \cdot q_2 \cdot q_3 \cdot \ldots \cdot q_n$

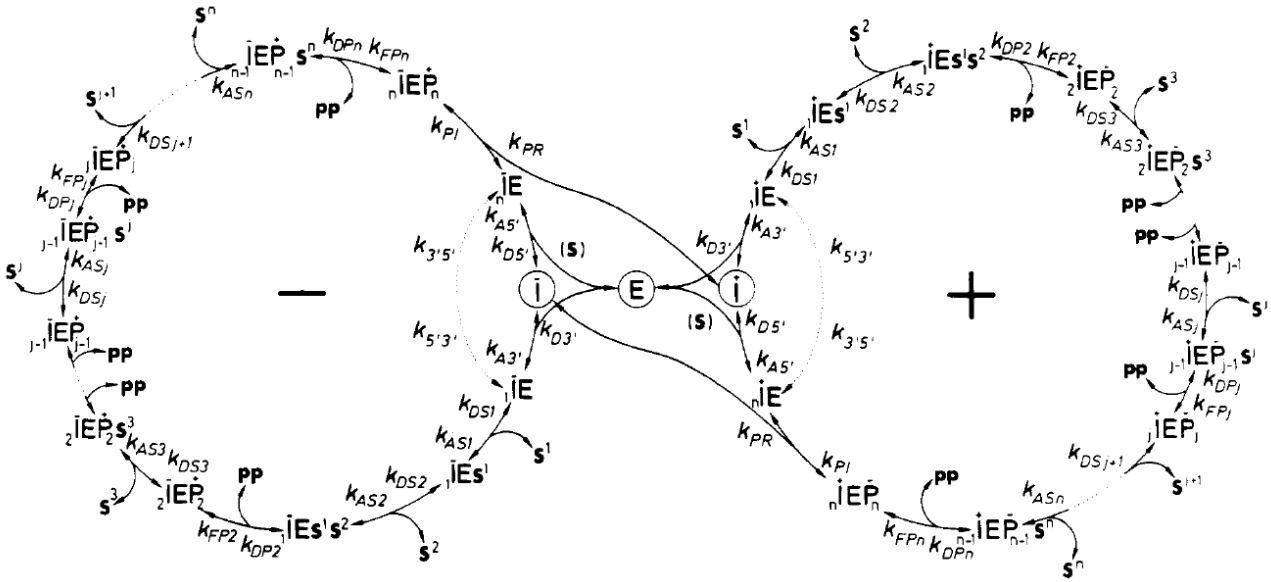
The logics of DNA replication

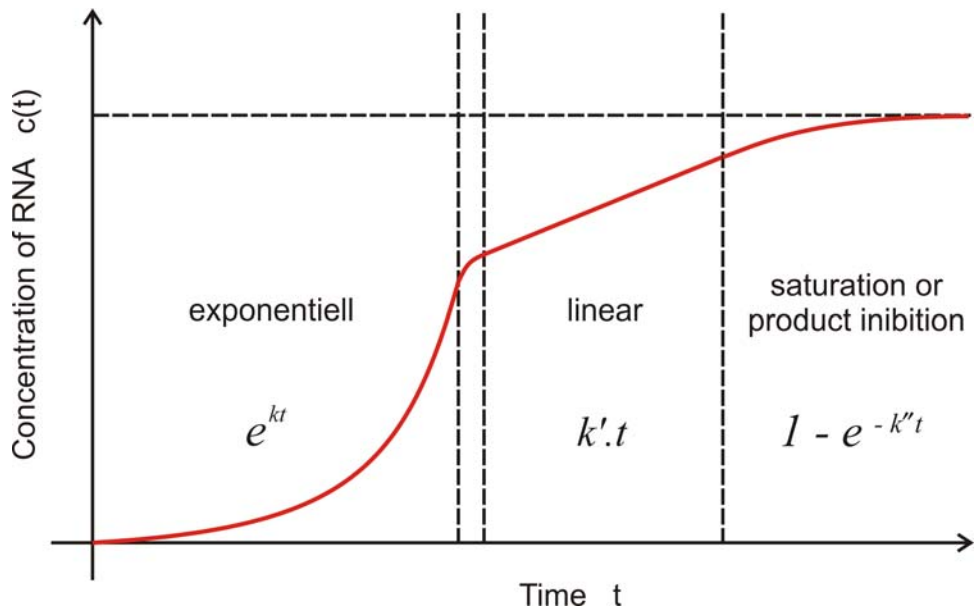


RNA replication by Qβ-replicase

C. Weissmann, ***The making of a phage***.
FEBS Letters **40** (1974), S10-S18

Christof K. Biebricher,
1941-2009

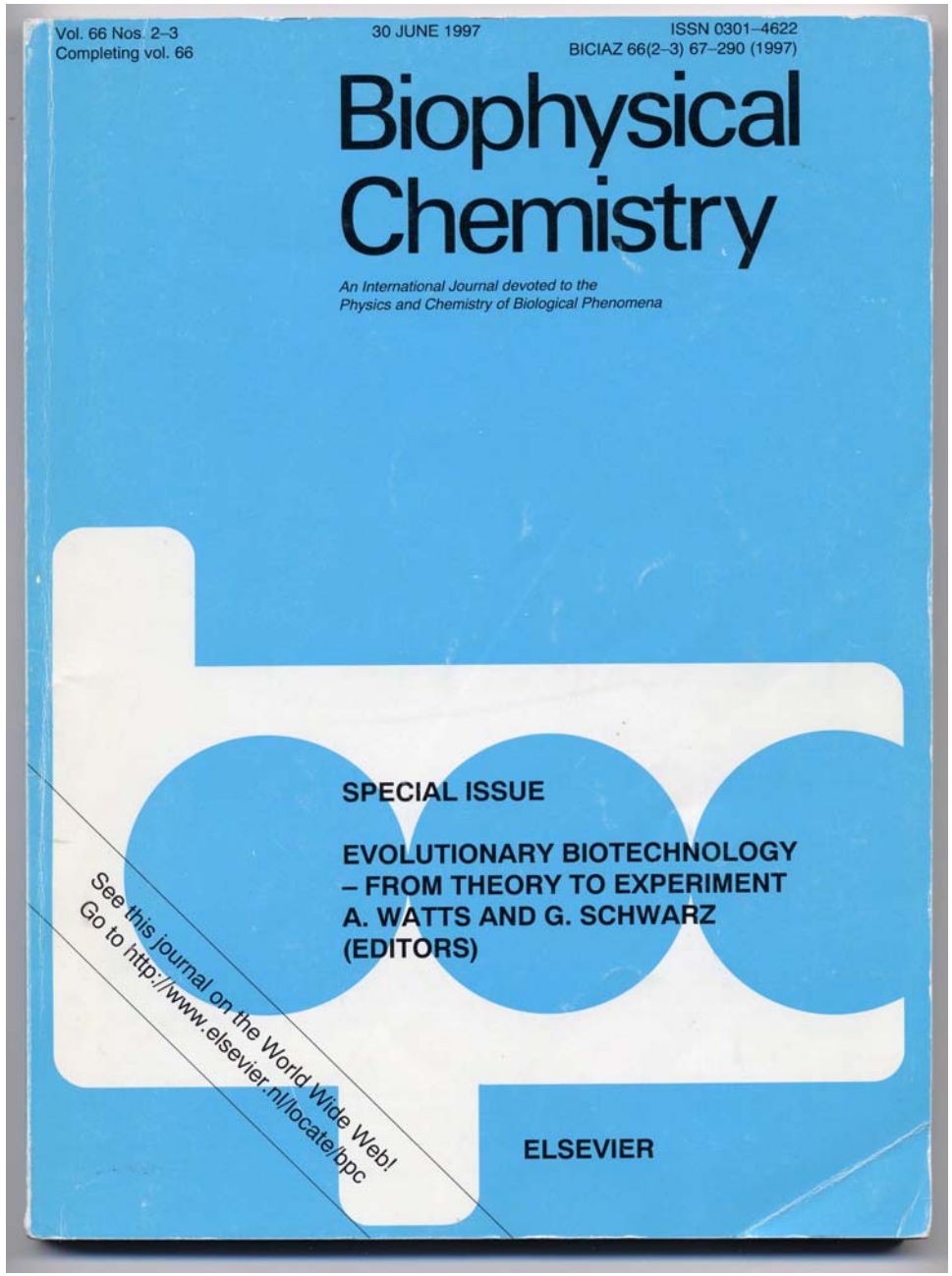




Kinetics of RNA replication

C.K. Biebricher, M. Eigen, W.C. Gardiner, Jr.
Biochemistry **22**:2544-2559, 1983

A collection of reviews on evolution *in vitro* and *in silico*



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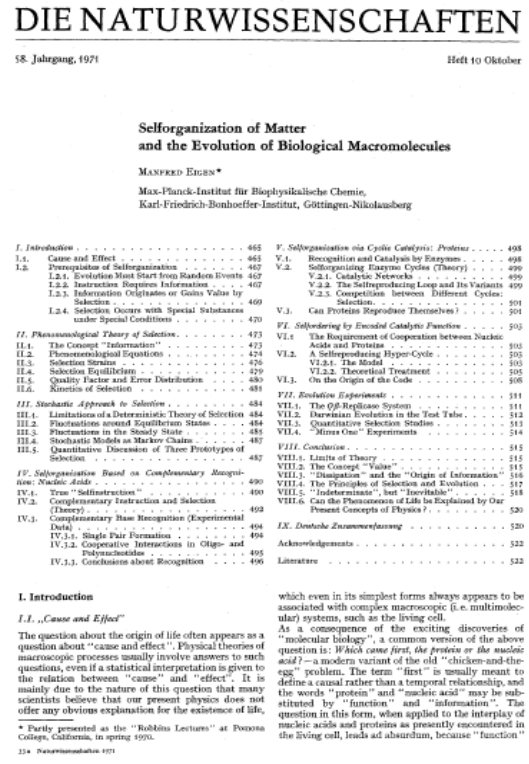

DIE NATURWISSENSCHAFTEN

58. Jahrgang, 1971 — Heft 10 Oktober

Selforganization of Matter and the Evolution of Biological Macromolecules

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* Partly presented as the "Robbins Lectures" at Pomona College, California, in spring 1970.

1. Introduction

1.1. „Cause and Effect"

The question about the origin of life often appears as a question about "cause and effect". Physical theories of macroscopic processes usually involve answers to such questions, even if a statistical interpretation is given to the relation between "cause" and "effect". It is mainly due to the nature of this question that many scientists believe that our present physics does not offer any obvious explanation for the existence of life, which even in its simplest forms always appears to be associated with complex macroscopic (i.e. multimolecular) systems, such as the living cell.

As a consequence of the exciting discoveries of "molecular biology", a common version of the above question is: *Which came first, the protein or the nucleic acid?*—a modern variant of the old "chicken-and-the-egg" problem. The term "first" is usually meant to define a causal rather than a temporal relationship, and the words "protein" and "nucleic acid" may be substituted by "function" and "information". The question in this form, when applied to the interplay of nucleic acids and proteins as presently encountered in the living cell, leads ad absurdum, because "function"

1971

Die Naturwissenschaften 64. Jahrgang Heft 11 November 1977

The Hypercycle

A Principle of Natural Self-Organization

Part A: Emergence of the Hypercycle

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Peter Schuster
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This paper is the first part of a trilogy, which comprises a detailed study of a special type of functional organization and demonstrates its relevance with respect to the origin and evolution of life. Self-replicative macromolecules, such as RNA or DNA in a suitable environment exhibit a behavior, which we may call Darwinian and which can be formally represented by the concept of the quasi-species. A quasi-species is defined as a given distribution of macromolecular species with closely interrelated sequences, dominated by one or several (degenerate) master copies. External constraints enforce the selection of the best adapted distribution, commonly referred to as the wild-type. Most important for Darwinian behavior are the criteria for internal stability of the quasi-species. If these criteria are violated, the information stored in the nucleotide sequence of the master copy will deteriorate irreversibly leading to an error catastrophy. As a consequence, selection and evolution of RNA or DNA molecules is limited with respect to the amount of information that can be stored in a single replicative unit. An analysis of experimental data regarding RNA and DNA replication at various levels of organization reveals, that a sufficient amount of information for the build up of a translation machinery can be gained only via integration of several different replicative units (or reproductive cycles) through *functional* linkages. A stable functional integration then will raise the system to a new level of organization and thereby enlarge its information capacity considerably. The hypercycle appears to be such a form of organization.

Preview on Part B: The Abstract Hypercycle

The mathematical analysis of dynamical systems using methods of differential topology, yields the result that there is only one type of mechanisms which fulfills the following requirements:
The information stored in each single replicative unit (or reproductive cycle) must be maintained, i.e., the respective master copies must compete favorably with their error distributions. Despite their competitive behavior these units must establish a cooperation which includes all functionally integrated species. On the other hand, the cycle as a whole must continue to compete favorably with any other single entity or linked ensemble which does not contribute to its integrated function.
These requirements are crucial for a selection of the best adapted functionally linked ensemble and its evolutive optimization. Only hypercyclic organizations are able to fulfil these requirements. Non-cyclic linkages among the autonomous reproduction cycles, such as chains or branched, tree-like networks are devoid of such properties.
The mathematical methods used for proving these assertions are fixed-point, Lyapunov- and trajectorial analysis in higher-dimensional phase spaces, spanned by the concentration coordinates of the cooperating partners. The self-organizing properties of hypercycles are elucidated, using analytical as well as numerical techniques.

Preview on Part C: The Realistic Hypercycle

A realistic model of a hypercycle relevant with respect to the origin of the genetic code and the translation machinery is presented. It includes the following features referring to natural systems:
1) The hypercycle has a sufficiently simple structure to admit an origination with finite probability under prebiotic conditions.
2) It permits a continuous emergence from closely interrelated (t-RNA-like) precursors, originally being members of a stable RNA quasi-species and having been amplified to a level of higher abundance.
3) The organizational structure and the properties of single functional units of this hypercycle are still reflected in the present genetic code in the translation apparatus of the prokaryotic cell, as well as in certain bacterial viruses.

I. The Paradigm of Unity and Diversity in Evolution

Why do millions of species, plants and animals, exist, while there is only one basic molecular machinery of the cell: one universal genetic code and unique chiralities of the macromolecules?
The geneticists of our day would not hesitate to give an immediate answere to the first part of this question. Diversity of species is the outcome of the tremendous branching process of evolution with its myriads of single steps of reproduction and mutation. It in-

1977

Molecular Quasi-Species†

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Max Planck Institut für biophysikalische Chemie, Am Fassberg, D 3400 Göttingen-Nikolausberg, BRD
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Institut für theoretische Chemie und Strahlenchemie, der Universität Wien, Währinger Strasse 17, A-1090 Wien, Austria (Received: June 9, 1988)

The molecular quasi-species model describes the physicochemical organization of monomers into an ensemble of heteropolymers with combinatorial complexity by ongoing template polymerization. Polynucleotides belong to the simplest class of such molecules. The quasi-species itself represents the stationary distribution of macromolecular sequences maintained by chemical reactions effecting error-prone replication and by transport processes. It is obtained deterministically, by mass-action kinetics, as the dominant eigenvalue of a *value* matrix, **W**, which is derived directly from chemical rate coefficients, but it also exhibits stochastic features, being composed to a significant fraction of unique individual macromolecular sequences. The quasi-species model demonstrates how macromolecular information originates through specific nonequilibrium autocatalytic reactions and thus forms a bridge between reaction kinetics and molecular evolution. Selection and evolutionary optimization appear as new features in physical chemistry. Concentration bias in the production of mutants is a new concept in population genetics, relevant to frequently mutating populations, which is shown to greatly enhance the optimization properties. The present theory relates to asexually replicating ensembles, but this restriction is not essential. A sharp transition is exhibited between a drifting population of essentially random macromolecular sequences and a localized population of close relatives. This transition at a threshold error rate was found to depend on sequence lengths, distributions of selective values, and population sizes. It has been determined generically for complex landscapes and for special cases, and, it was shown to persist generically in the presence of nearly neutral mutants. Replication dynamics has much in common with the equilibrium statistics of complex spin systems: the error threshold is equivalent to a magnetic order–disorder transition. A rational function of the replication accuracy plays the role of temperature. Experimental data obtained from test-tube evolution of polynucleotides and from studies of natural virus populations support the quasi-species model. The error threshold seems to set a limit to the genome lengths of several classes of RNA viruses. In addition, the results are relevant even in eucaryotes where they contribute to the exon–intron debate.

1. Molecular Selection

Our knowledge of physical and chemical systems is, in a final analysis, based on models derived from repeatable experiments. While none of the classic and rather besieged list of properties rounded up to support the intuition of a distinction between the living and nonliving—metabolism, self-reproduction, irritability, and adaptability, for example—intrinsically limit the application of the scientific method, a determining role by unique or *individual* entities comes into conflict with the requirement of repeatability. Combinatorial variety, such as that in heteropolymers based on even very small numbers of different bases, even just two, readily provides numbers of different entities so enormous that neither consecutive nor parallel physical realization is possible. The physical chemistry of finite systems of such macromolecules must deal with both known regularities and the advent of unique copolymeric sequences. Normally this would present no difficulty in a statistical mechanical analysis of typical behavior, where rare events play no significant role, but with autocatalytic polymerization processes even unique single molecules may be amplified to determine the fate of the entire system. Potentially creative, self-organizing around unique events, the dynamics of this simplest living chemical system is invested with regularities that both allow and limit efficient adaptation. The quasi-species model is a study of these regularities.

The fundamental regularity in living organisms that has invited explanation is adaptation. Why are organisms so well fitted to their environments? At a more chemical level, why are enzymes optimal catalysts? Darwin's theory of natural selection has provided biologists with a framework for the answer to this question. The present model is constructed along Darwinian lines but in terms of specific macromolecules, chemical reactions, and physical processes that make the notion of survival of the fittest precise. Not only does the model give an understanding of the physical limitations of adaptation, but also it provides new insight into the role of chance in the process. For an understanding of the structure of this minimal chemical model it is first necessary to recall the conceptual basis of Darwin's theory.

Darwin recognized that new inheritable adaptive properties were not induced by the environment but arose independently in the production of offspring. Lasting adaptive changes in a population could only come about by natural selection of the heritable traits or *genotype* based on the full characteristics or *phenotype* relevant for producing offspring. A process of chance, i.e., uncorrelated with the developed phenotype, controls changes in the genotype from one generation to the next and generates the diversity necessary for selection. Three factors have probably prevented chemists from gaining a clear insight into these phenomena in the past, despite the discovery of the polymeric nature of the genotype (DNA): the complexity of a minimum replication phenotype, the problem of dealing with a huge number of variants, and the nonequilibrium nature of these ongoing processes.

The formulation of a tractable chemical model based on Darwin's principle may be understood in several steps:

1. The major constituents of the system have to be inherently self-reproductive. Only two classes of molecules are presently

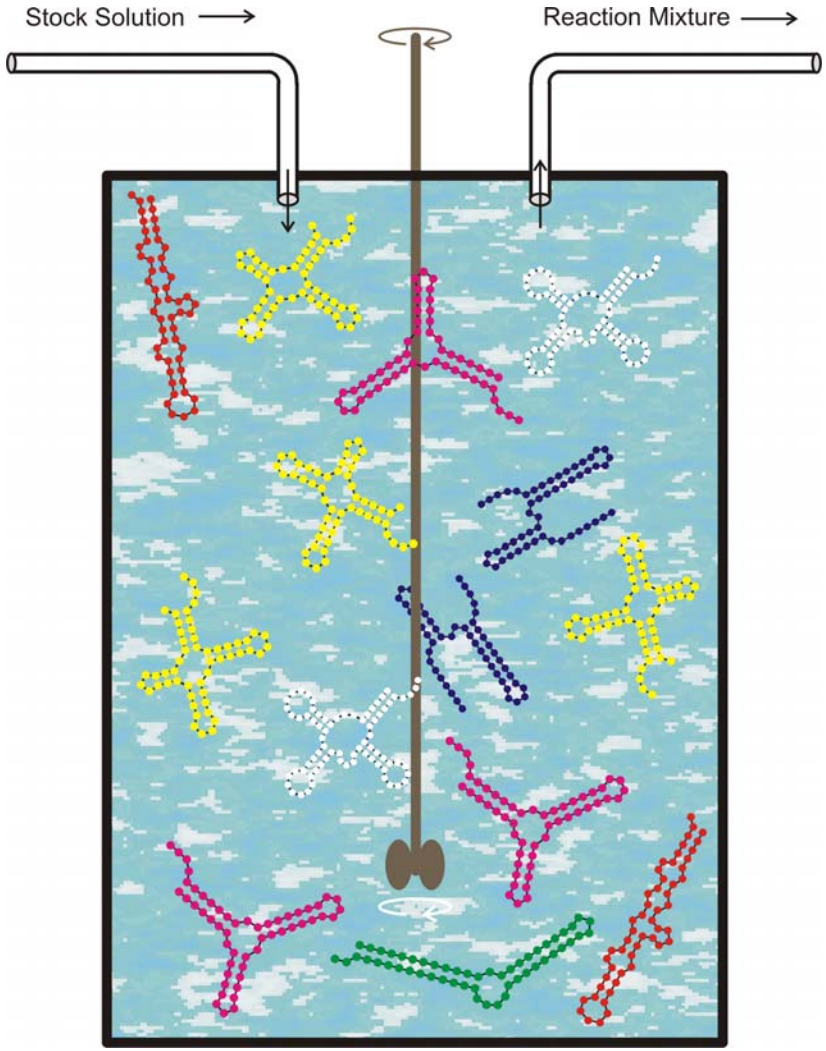
† This is an abridged account of the quasi-species theory that has been submitted in comprehensive form to *Advances in Chemical Physics*.[1]

(1) Eigen, M.; McCaskill, J. S.; Schuster, P. *Adv. Chem. Phys.*, in press.

0022-3654/88/2092-6881$01.50/0

1988

Chemische Kinetik der molekularen Evolution

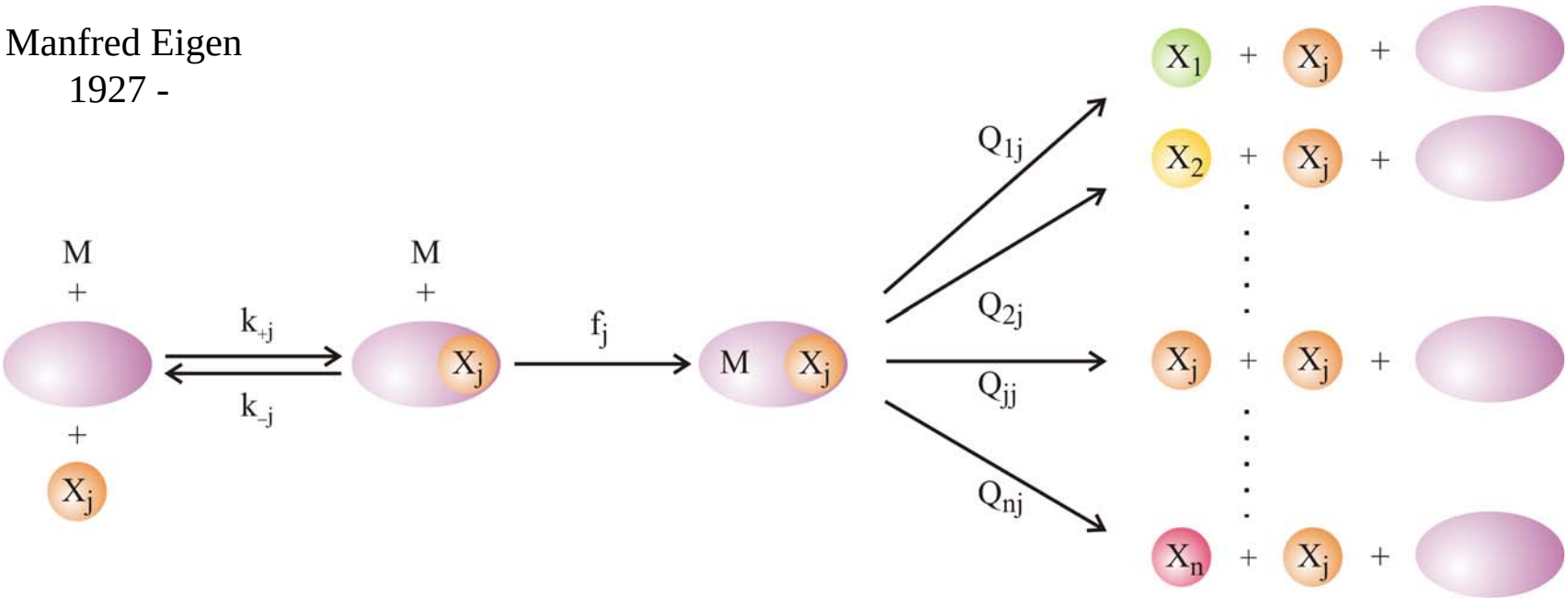


Chemische Kinetik von Replikation und Mutation als Differentialgleichungen

Manfred Eigen
1927 -

$$\frac{dx_j}{dt} = \sum_{i=1}^{n} Q_{ji} f_i x_i - x_j \phi(t); \quad j = 1, 2, \ldots, n$$

$$\phi(t) = \sum_{i=1}^{n} f_i x_i \Big/ \sum_{i=1}^{n} x_i$$



Mutation and (correct) replication as parallel chemical reactions

M. Eigen. 1971. *Naturwissenschaften* 58:465,
M. Eigen & P. Schuster.1977. *Naturwissenschaften* 64:541, 65:7 und 65:341

$$\frac{\mathrm{d}x_j}{\mathrm{dt}} = \sum_{i=1}^{N} Q_{ji}\, f_i\, x_i - \phi(t)\, x_j\,, \;\; j = 1,2,\ldots,N;\; \sum_{i=1}^{N} x_i = 1$$

$$\phi(\mathrm{t}) = \sum_{i=1}^{N} f_i\; x_i$$

$$\lim_{t\to\infty} \frac{\mathrm{d}x_i}{\mathrm{dt}} = 0;\;\; \lim_{t\to\infty} x_i(\mathrm{t}) = \bar{x}_i$$

$$\left\{\bar{x}_1, \bar{x}_2, \ldots, \bar{x}_n\right\} \ldots \text{Quasispezies}$$

Chemische Kinetik von Replikation und Mutation als Differentialgleichungen

$$\frac{dx_m^{(0)}}{dt} = \left(Q_{mm}\, f_m - \phi(t)\right) x_m^{(0)} =$$

$$= x_m^{(0)} \left(Q_{mm}\, f_m - \bar{f}_{-m} - x_m^{(0)} \left(f_m - \bar{f}_{-m}\right)\right)$$

$$\bar{x}_m^{(0)} = \frac{Q_{mm} - \sigma^{-1}}{1 - \sigma^{-1}} = \frac{1}{\sigma - 1}\left(\sigma (1-p)^n - 1\right) \quad \text{with}$$

$$\sigma = \frac{f_m}{\bar{f}_{-m}} \quad \text{and} \quad \bar{f}_{-m} = \frac{1}{1 - x_m} \sum_{i \neq m} x_i\, f_i$$

$$\bar{x}_m^{(0)} > 0 \quad \Rightarrow \quad Q \cdot \sigma = (1-p)^n \cdot \sigma > 1$$

Replication and mutation without mutational backflow

$$Q \cdot \sigma \quad = \quad (1-p)^n \cdot \sigma \quad \geq \quad 1 \quad \Rightarrow \quad n \cdot \ln(1-p) \geq -\ln\sigma$$

$$\left(n \cdot p\right)_{\text{cr}} \cong \ln\sigma$$

p … constant : $n_{\max} \approx \dfrac{\ln\sigma}{p}$

n … constant : $p_{\max} \approx \dfrac{\ln\sigma}{n}$

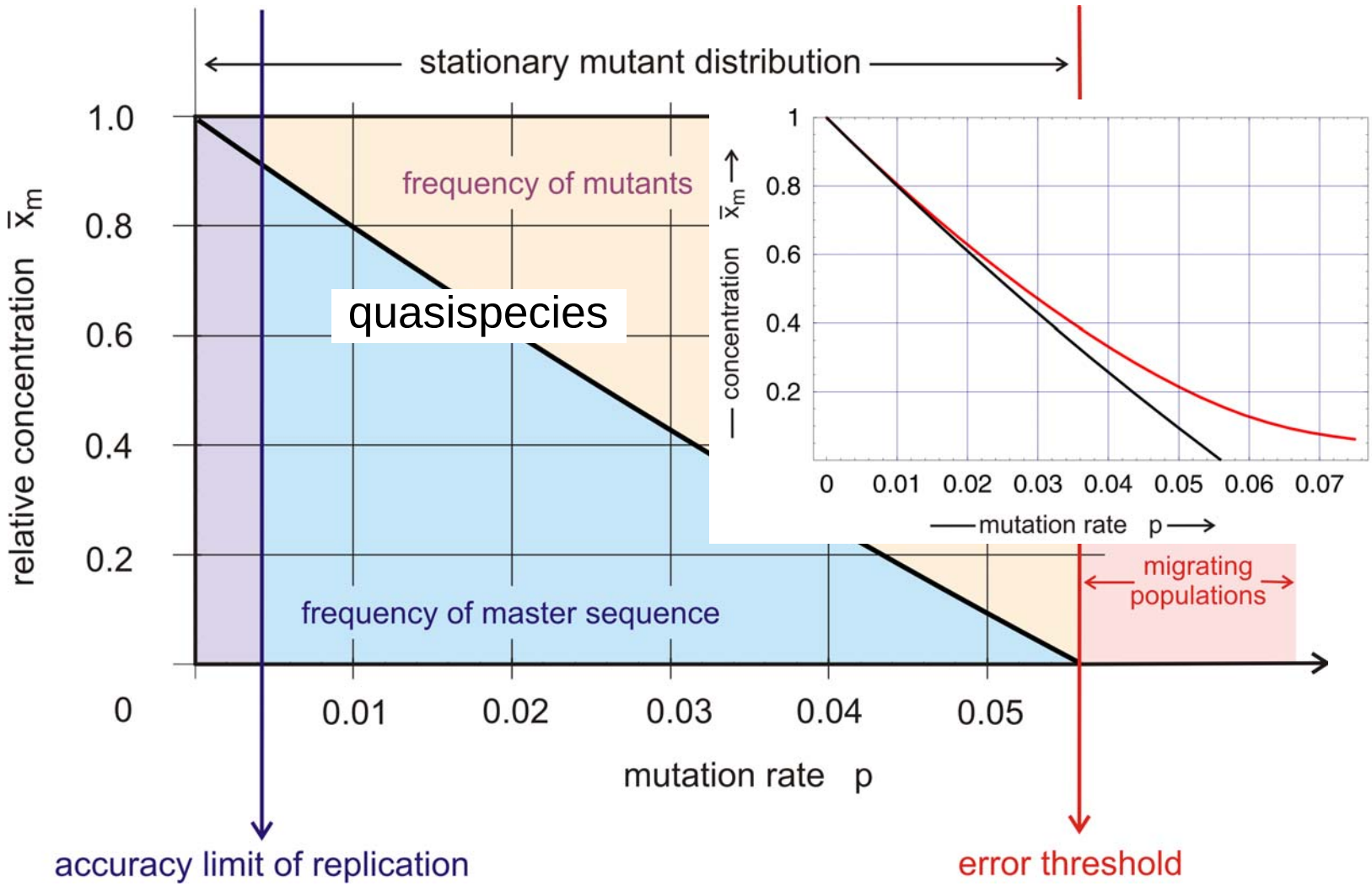
$Q = (1-p)^n$ … replication accuracy

p … error rate

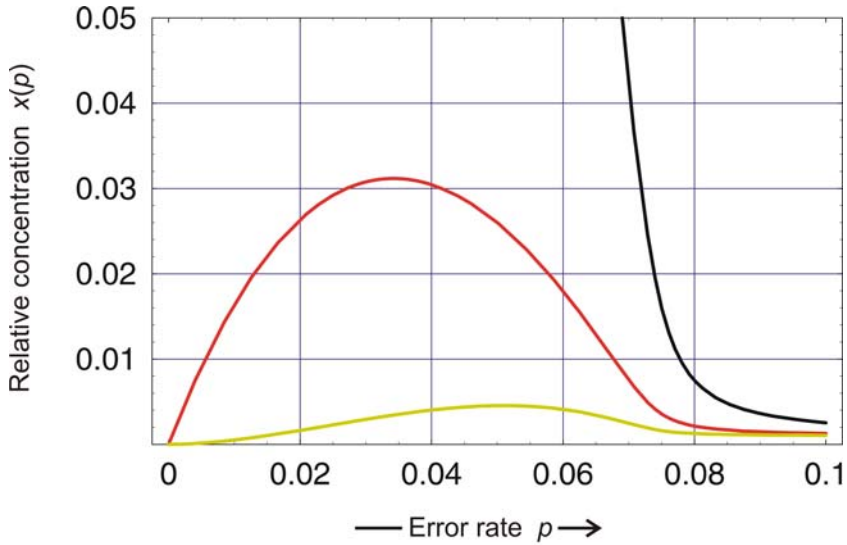
n … chain length

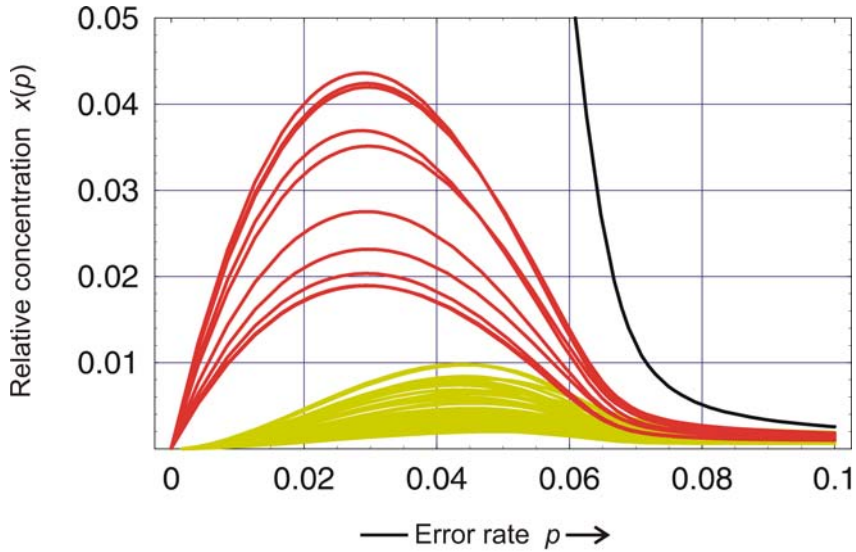
$\sigma = \dfrac{(1-x_m)\, f_m}{\sum_{i \neq m} x_i f_i}$ … superiority

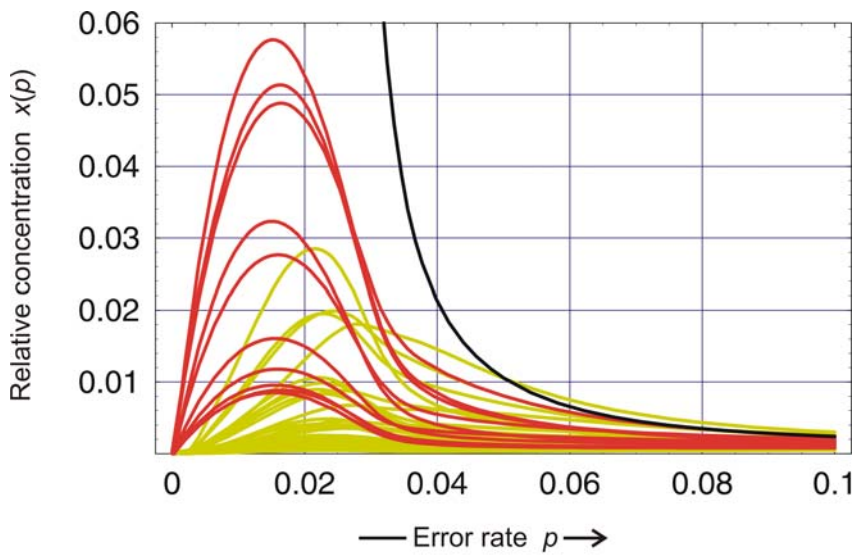
Chain lengths and error threshold



The error threshold in replication and mutation







Error threshold: Individual sequences

n = 10, σ = 2, s = 491 and d = 0, 1.0, 1.875

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Virus Research
www.elsevier.com/locate/virusres

Preface

Antiviral strategy on the horizon

Error catastrophe had its conceptual origins in the middle of the XXth century, when the consequences of mutations on enzymes involved in protein synthesis, as a theory of aging. In those times biological processes were generally perceived differently from today. Infectious diseases were regarded as a fleeting nuisance which would be eliminated through the use of antibiotics and antiviral agents. Microbial variation, although known in some cases, was not thought to be a significant problem for disease control. Variation in differentiated organisms was seen as resulting essentially from exchanges of genetic material associated with sexual reproduction. The problem was to unveil the mechanisms of inheritance, expression of genetic information and metabolism. Few saw that genetic change is occurring at present in all organisms, and still fewer recognized Darwinian principles as essential to the biology of pathogenic viruses and cells. Population geneticists rarely used bacteria or viruses as experimental systems to define concepts in biological evolution. The extent of genetic polymorphism among individuals of the same biological species came as a surprise when the first results on comparison of electrophoretic mobility of enzymes were obtained. With the advent of in vitro DNA recombination, and rapid nucleic acid sequencing techniques, molecular analyses of genomes reinforced the conclusion of extreme inter-individual genetic variation within the same species. Now, due largely to spectacular progress in comparative genomics, we see cellular DNAs, both prokaryotic and eukaryotic, as highly dynamic. Most cellular processes, including such essential information-bearing and transferring events as genome replication, transcription and translation, are increasingly perceived as inherently inaccurate. Viruses, and in particular RNA viruses, are among the most extreme examples of exploitation of replication inaccuracy for survival.

Error catastrophe, or the loss of meaningful genetic information through excess genetic variation, was formulated in quantitative terms as a consequence of quasispecies theory, which was first developed to explain self-organization and adaptability of primitive replicons in early stages of life. Recently, a conceptual extension of error catastrophe that could be defined as "induced genetic deterioration" has emerged as a possible antiviral strategy. This is the topic of the current special issue of ***Virus Research***.

Few would nowadays doubt that one of the major obstacles for the control of viral disease is short-term adaptability of viral pathogens. Adaptability of viruses follows the same Darwinian principles that have shaped biological evolution over eons, that is, repeated rounds of reproduction with genetic variation, competition and selection, often perturbed by random events such as statistical fluctuations in population size. However, with viruses the consequences of the operation of these very same Darwinian principles are felt within very short times. Short-term evolution (within hours and days) can be also observed with some cellular pathogens, with subsets of normal cells, and cancer cells. The nature of RNA viral pathogens begs for alternative antiviral strategies, and forcing the virus to cross the critical error threshold for maintenance of genetic information is one of them.

The contributions to this volume have been chosen to reflect different lines of evidence (both theoretical and experimental) on which antiviral designs based on genetic deterioration inflicted upon viruses are being constructed. Theoretical studies have explored the copying fidelity conditions that must be fulfilled by any information-bearing replication system for the essential genetic information to be transmitted to progeny. Closely related to the theoretical developments have been numerous experimental studies on quasispecies dynamics and their multiple biological manifestations. The latter can be summarized by saying that RNA viruses, by virtue of existing as mutant spectra rather than defined genetic entities, remarkably expand their potential to overcome selective pressures intended to limit their replication. Indeed, the use of antiviral inhibitors in clinical practice and the design of vaccines for a number of major RNA virus-associated diseases, are currently presided by a sense of uncertainty. Another line of growing research is the enzymology of copying fidelity by viral replicases, aimed at understanding the molecular basis of mutagenic activities. Error catastrophe as a potential new antiviral strategy received an important impulse by the observation that ribavirin (a licensed antiviral nucleoside analogue) may be exerting, in some systems, its antiviral activity through enhanced mutagenesis. This has encouraged investigations on new mutagenic base analogues, some of them used in anticancer chemotherapy. Some chapters summarize these important biochemical studies on cell entry pathways and metabolism of mutagenic agents, that may find new applications as antiviral agents.

This volume intends to be basically a progress report, an introduction to a new avenue of research, and a realistic appraisal of the many issues that remain to be investigated. In this respect, I can envisage (not without many uncertainties) at least three lines of needed research: (i) One on further understanding of quasispecies dynamics in infected individuals to learn more on how to apply combinations of virus-specific mutagens and inhibitors in an effective way, finding synergistic combinations and avoiding antagonistic ones as well as severe clinical side effects. (ii) Another on a deeper understanding of the metabolism of mutagenic agents, in particular base and nucleoside analogues. This includes identification of the transporters that carry them into cells, an understanding of their metabolic processing, intracellular stability and alterations of nucleotide pools, among other issues. (iii) Still another line of needed research is the development of new mutagenic agents specific for viruses, showing no (or limited) toxicity for cells. Some advances may come from links with anticancer research, but others should result from the designs of new molecules, based on the structures of viral polymerases. I really hope that the reader finds this issue not only to be an interesting and useful review of the current situation in the field, but also a stimulating exposure to the major problems to be faced.

The idea to prepare this special issue came as a kind invitation of Ulrich Desselberger, former Editor of ***Virus Research***, and then taken enthusiastically by Luis Enjuanes, recently appointed as Editor of ***Virus Research***. I take this opportunity to thank Ulrich, Luis and the Editor-in-Chief of ***Virus Research***, Brian Mahy, for their continued interest and support to the research on virus evolution over the years.

My thanks go also to the 19 authors who despite their busy schedules have taken time to prepare excellent manuscripts, to Elsevier staff for their prompt responses to my requests, and, last but not least, to Ms. Lucía Horrillo from Centro de Biología Molecular "Severo Ochoa" for her patient dealing with the correspondence with authors and the final organization of the issue.

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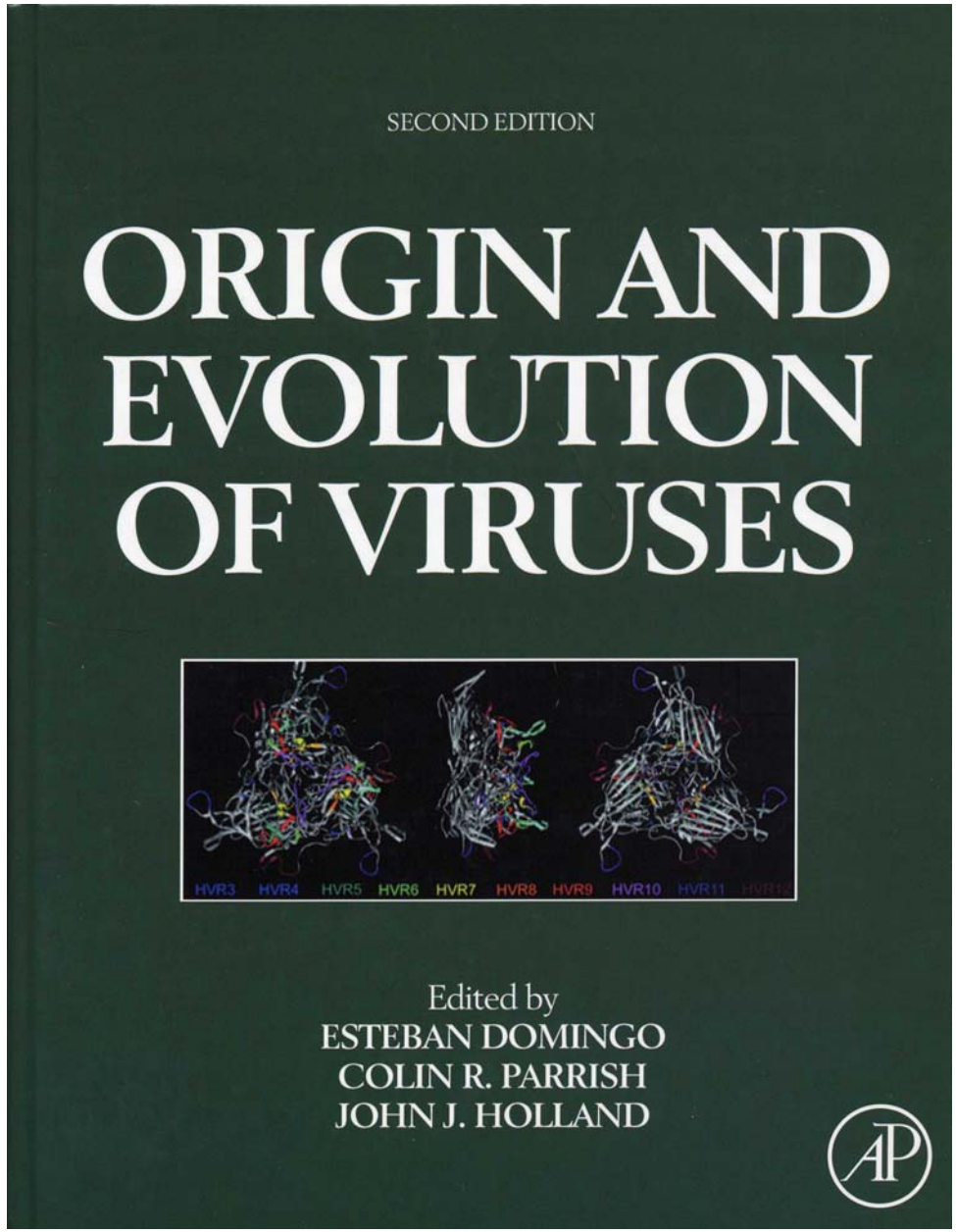

Molekulare Evolution von Viren

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Theory of Lethal Mutagenesis for Viruses▽†

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Mutation is the basis of adaptation. Yet, most mutations are detrimental, and elevating mutation rates will impair a population's fitness in the short term. The latter realization has led to the concept of lethal mutagenesis for curing viral infections, and work with drugs such as ribavirin has supported this perspective. As yet, there is no formal theory of lethal mutagenesis, although reference is commonly made to Eigen's error catastrophe theory. Here, we propose a theory of lethal mutagenesis. With an obvious parallel to the epidemiological threshold for eradication of a disease, a sufficient condition for lethal mutagenesis is that each viral genotype produces, on average, less than one progeny virus that goes on to infect a new cell. The extinction threshold involves an evolutionary component based on the mutation rate, but it also includes an ecological component, so the threshold cannot be calculated from the mutation rate alone. The genetic evolution of a large population undergoing mutagenesis is independent of whether the population is declining or stable, so there is no runaway accumulation of mutations or genetic signature for lethal mutagenesis that distinguishes it from a level of mutagenesis under which the population is maintained. To detect lethal mutagenesis, accurate measurements of the genome-wide mutation rate and the number of progeny per infected cell that go on to infect new cells are needed. We discuss three methods for estimating the former. Estimating the latter is more challenging, but broad limits to this estimate may be feasible.

Error threshold and lethal mutagenesis

DOI: 10.1534/genetics.108.091413

Lethal Mutagenesis of Bacteria

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ABSTRACT

Lethal mutagenesis, the killing of a microbial pathogen with a chemical mutagen, is a potential broad-spectrum antiviral treatment. It operates by raising the genomic mutation rate to the point that the deleterious load causes the population to decline. Its use has been limited to RNA viruses because of their high intrinsic mutation rates. Microbes with DNA genomes, which include many viruses and bacteria, have not been considered for this type of treatment because their low intrinsic mutation rates seem difficult to elevate enough to cause extinction. Surprisingly, models of lethal mutagenesis indicate that bacteria may be candidates for lethal mutagenesis. In contrast to viruses, bacteria reproduce by binary fission, and this property ensures their extinction if subjected to a mutation rate >0.69 deleterious mutations per generation. The extinction threshold is further lowered when bacteria die from environmental causes, such as washout or host clearance. In practice, mutagenesis can require many generations before extinction is achieved, allowing the bacterial population to grow to large absolute numbers before the load of deleterious mutations causes the decline. Therefore, if effective treatment requires rapid population decline, mutation rates $\gg 0.69$ may be necessary to achieve treatment success. Implications for the treatment of bacteria with mutagens, for the evolution of mutator strains in bacterial populations, and also for the evolution of mutation rate in cancer are discussed.

Error threshold and lethal mutagenesis

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B

Phil. Trans. R. Soc. B (2010) **365**, 1943–1952
doi:10.1098/rstb.2010.0076

Review

Pathways to extinction: beyond the error threshold

Susanna C. Manrubia[1,*], Esteban Domingo[1,2,3] and Ester Lázaro[1]

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[2]*Centro de Biología Molecular 'Severo Ochoa', CSIC-UAM, Cantoblanco 28049, Madrid, Spain*
[3]*Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas, 08036 Barcelona, Spain*

Since the introduction of the quasispecies and the error catastrophe concepts for molecular evolution by Eigen and their subsequent application to viral populations, increased mutagenesis has become a common strategy to cause the extinction of viral infectivity. Nevertheless, the high complexity of virus populations has shown that viral extinction can occur through several other pathways apart from crossing an error threshold. Increases in the mutation rate enhance the appearance of defective forms and promote the selection of mechanisms that are able to counteract the accelerated appearance of mutations. Current models of viral evolution take into account more realistic scenarios that consider compensatory and lethal mutations, a highly redundant genotype-to-phenotype map, rough fitness landscapes relating phenotype and fitness, and where phenotype is described as a set of interdependent traits. Further, viral populations cannot be understood without specifying the characteristics of the environment where they evolve and adapt. Altogether, it turns out that the pathways through which viral quasispecies go extinct are multiple and diverse.

Keywords: quasispecies; viral extinction; mutagen; antiviral therapy; genotype–phenotype map; complex phenotype

Error threshold and lethal mutagenesis

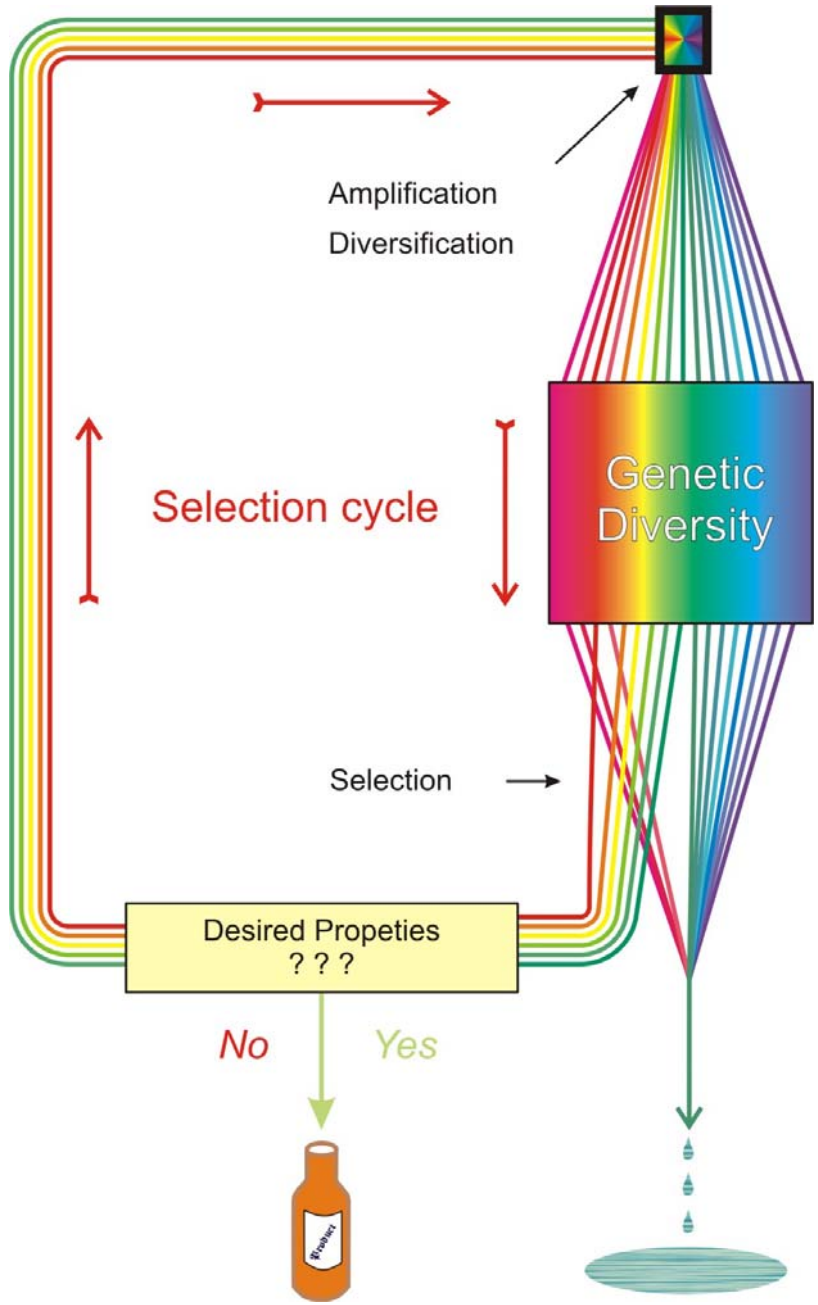
1. Evolutionsexperimente im Reagenzglas
2. Kinetik der Evolution von Molekülen
3. **Gezielte Evolution der „Molekülzüchter"**
4. Strukturen und Fitnesslandschaften
5. Evolution *in silico*
6. RNA-Schalter

How would you explain synthetic biology to Charles Darwin?

"You had it just right! And now I can build new components of life -- for example, the proteins of which we are made -- by artificial selection. When I decide who gets to reproduce, these components evolve just like the organisms that make them do, because they are encoded by the same stuff and follow the same principles of evolution that you showed us for all forms of life."

Frances H. Arnold, 1956 -

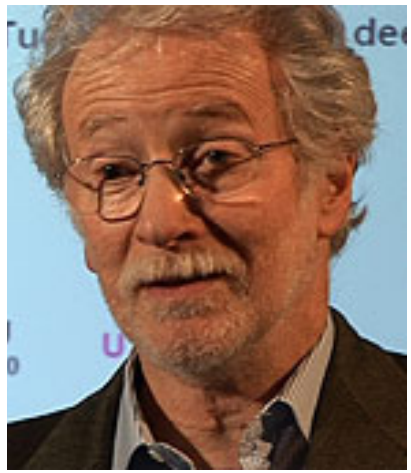
Ein Beispiel für Selektion von Molekülen mit vorbestimmbaren Eigenschaften im Laborexpriment



Andrew D. Ellington,
1959 -

Jack W. Szostak,
1952 -

Craig Tuerk,

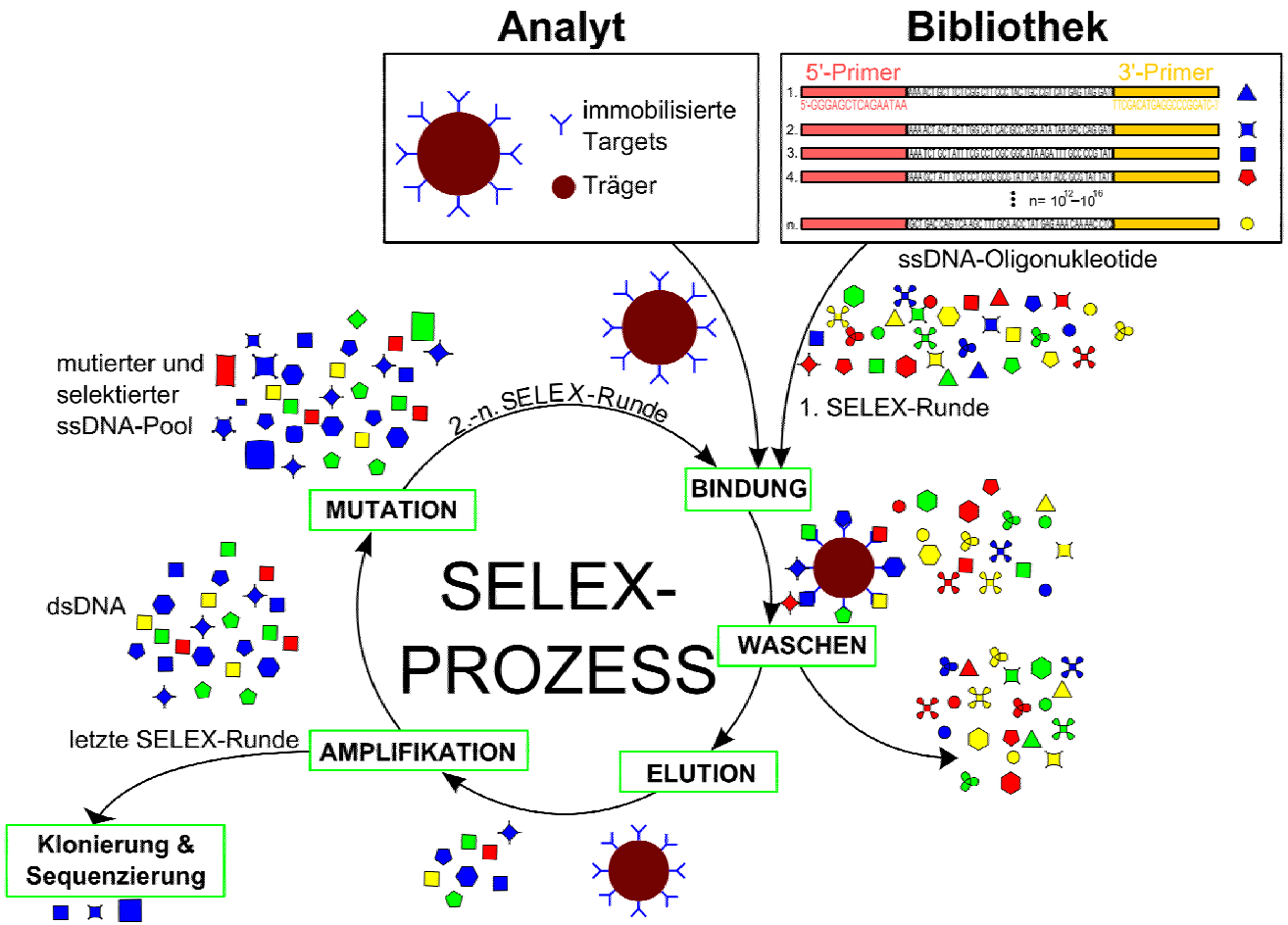


Larry Gold,

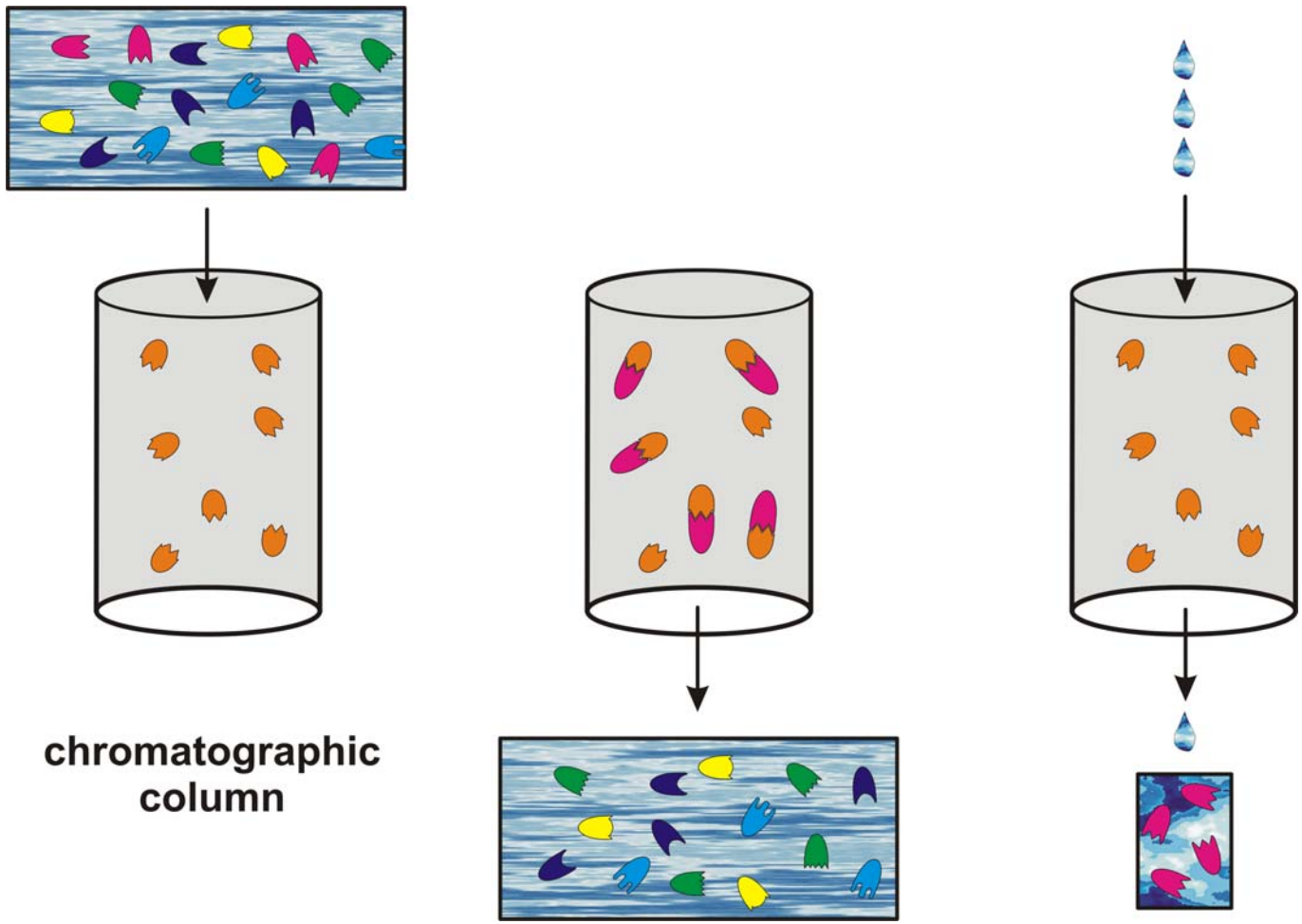
A. D. Ellington, J. W. Szostak. 1990. *In vitro* selection of RNA molecules that bind specific ligands. Nature 346:818-822.

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

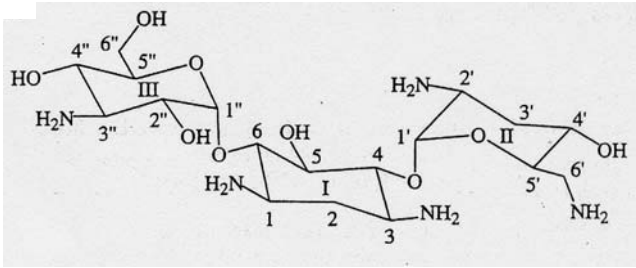
Die SELEX Methode



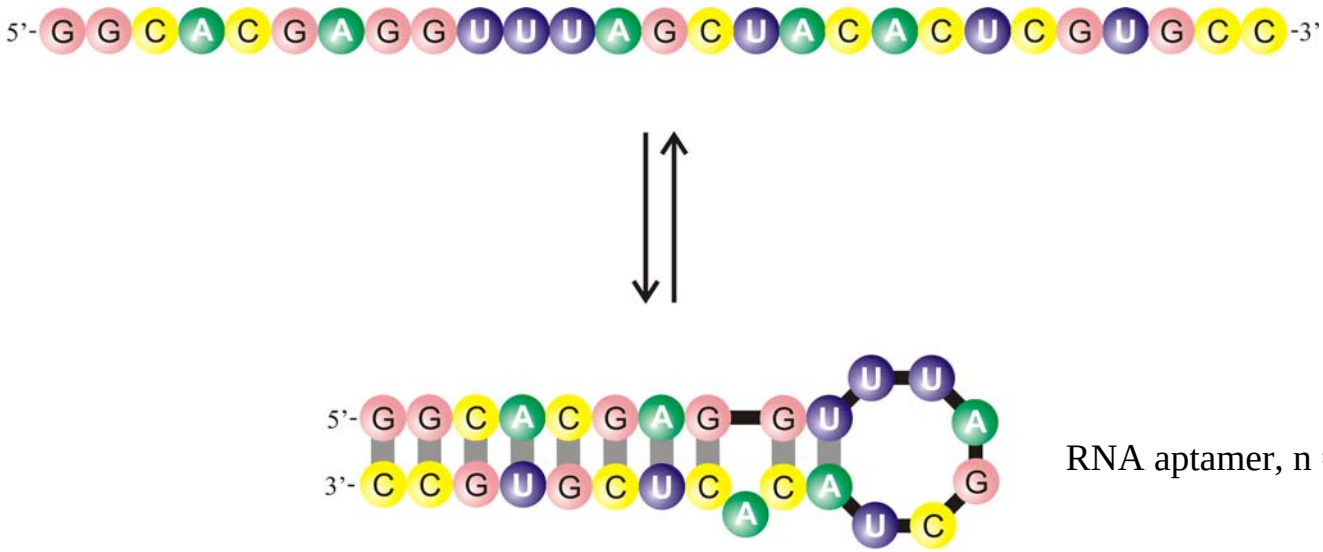
Die SELEX Methode



Die SELEX-Technik zur evolutionären Erzeugung von stark bindenden Molekülen



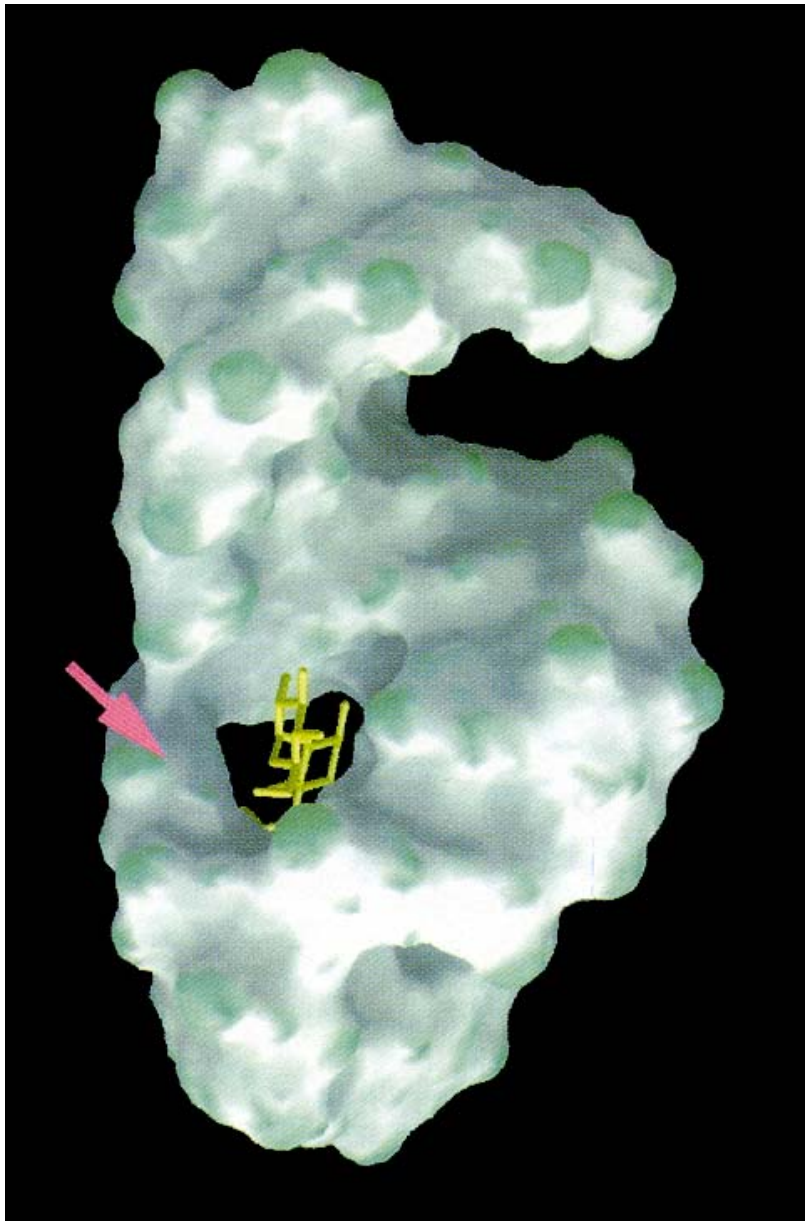
tobramycin



RNA aptamer, n = 27

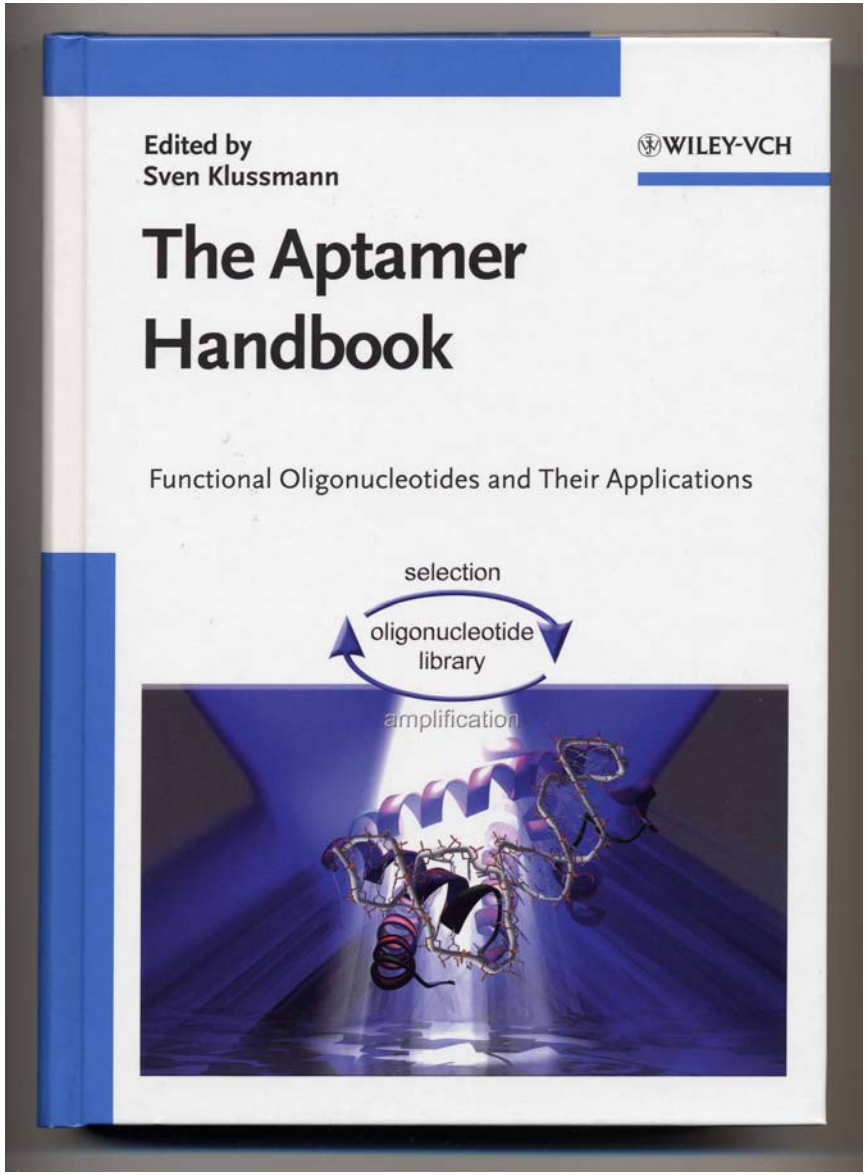
Formation of secondary structure of the tobramycin binding RNA aptamer with K_D = 9 nM

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

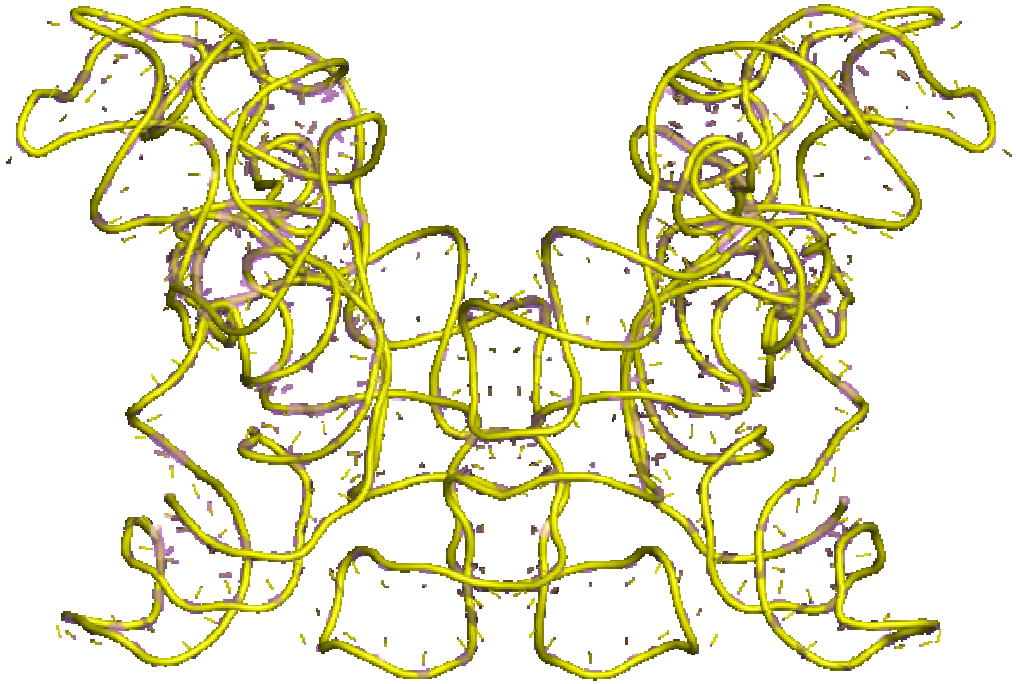


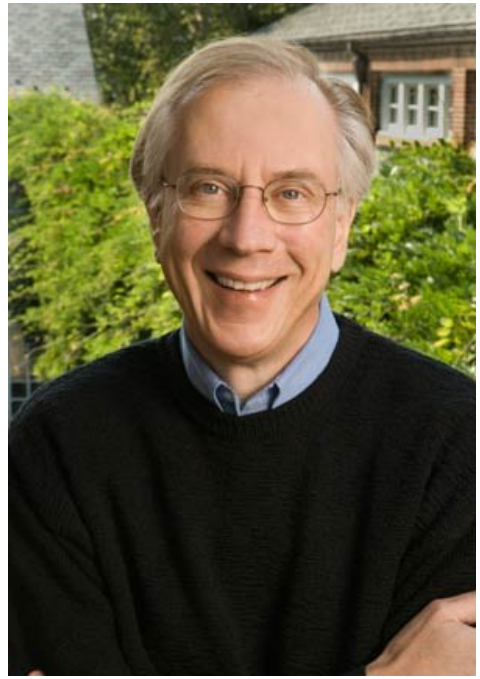
The three-dimensional structure of the tobramycin aptamer complex

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



Application of molecular evolution to problems in RNA biotechnology





Thomas R. Cech, 1947 -

T. R. Cech. 1990. Self-splicing group I introns.
Annu. Rev. Biochem. 59:543-568.

Entdeckung der RNA-Katalyse

Annu. Rev. Biochem. 2004. 73:791–836
doi: 10.1146/annurev.biochem.73.011303.073717

First published online as a Review in Advance on March 18, 2004

DIRECTED EVOLUTION OF NUCLEIC ACID ENZYMES

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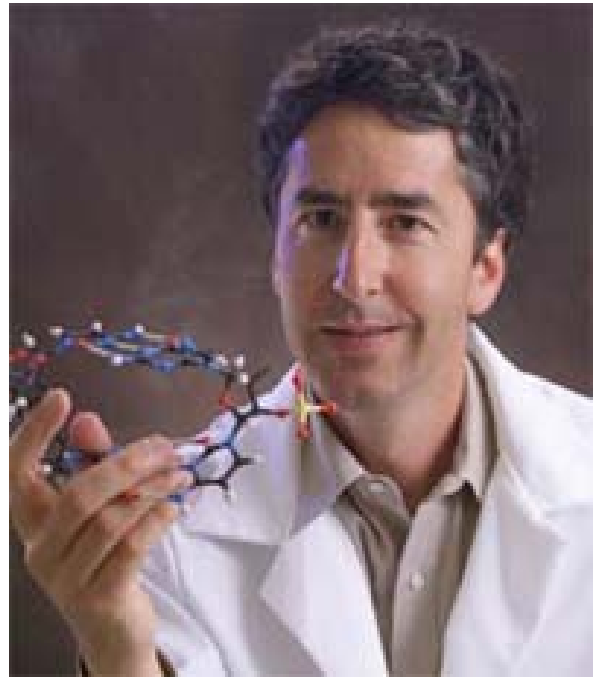
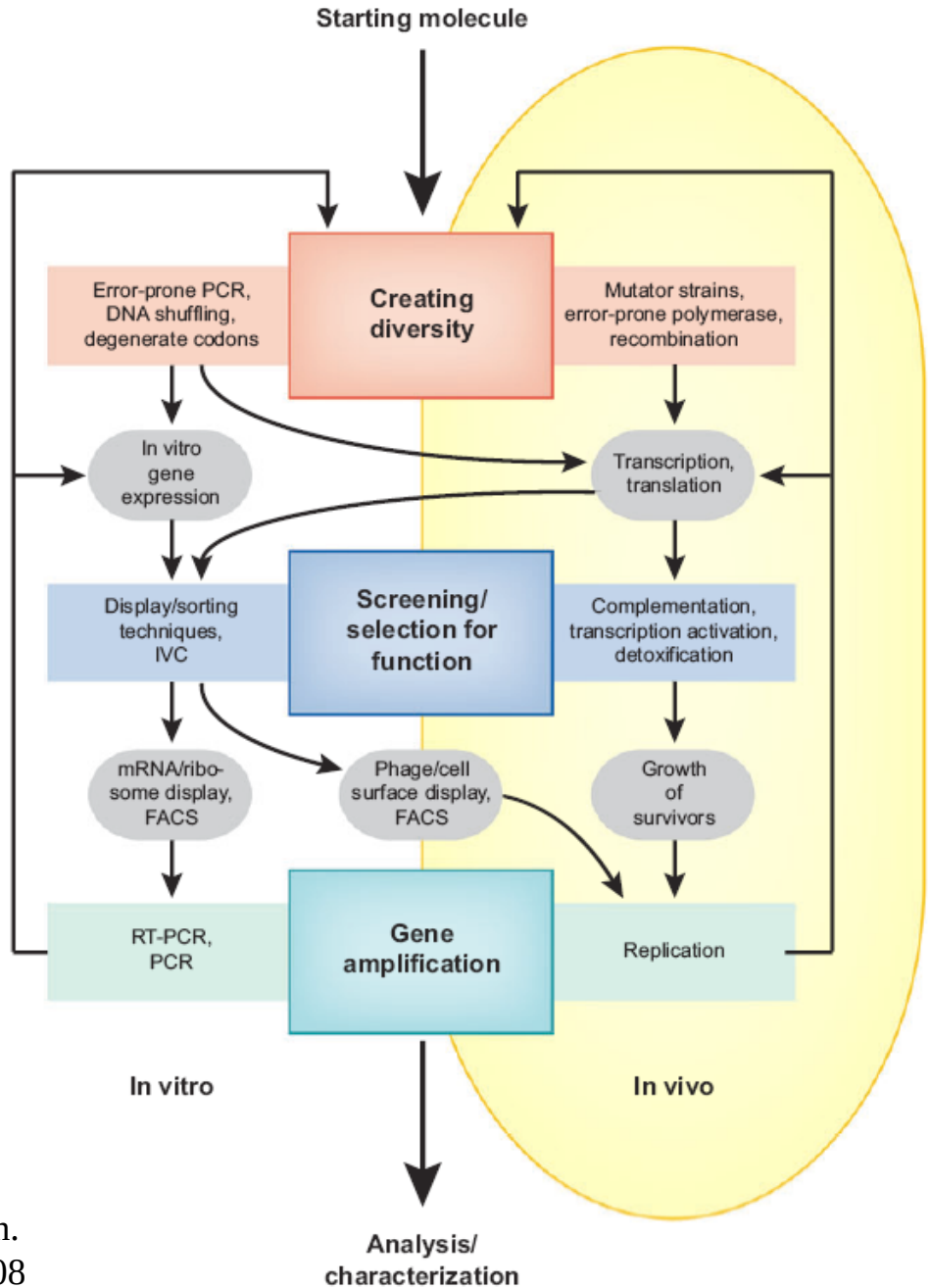
Key Words combinatorial library, DNA enzyme, in vitro evolution, in vitro selection, ribozyme

■ **Abstract** Just as Darwinian evolution in nature has led to the development of many sophisticated enzymes, Darwinian evolution in vitro has proven to be a powerful approach for obtaining similar results in the laboratory. This review focuses on the development of nucleic acid enzymes starting from a population of random-sequence RNA or DNA molecules. In order to illustrate the principles and practice of in vitro evolution, two especially well-studied categories of catalytic nucleic acid are considered: RNA enzymes that catalyze the template-directed ligation of RNA and DNA enzymes that catalyze the cleavage of RNA. The former reaction, which involves attack of a 2′- or 3′-hydroxyl on the α-phosphate of a 5′-triphosphate, is more difficult. It requires a comparatively larger catalytic motif, containing more nucleotides than can be sampled exhaustively within a starting population of random-sequence RNAs. The latter reaction involves deprotonation of the 2′-hydroxyl adjacent to the cleavage site, resulting in cleaved products that bear a 2′,3′-cyclic phosphate and 5′-hydroxyl. The difficulty of this reaction, and therefore the complexity of the corresponding DNA enzyme, depends on whether a catalytic cofactor, such as a divalent metal cation or small molecule, is present in the reaction mixture.



Gerald F. Joyce, 1956 -



Schematic overview of the principal processes, strategies, and techniques of directed evolution. Today, numerous experimental methods are available to perform the fundamental processes of true Darwinian evolution (*central boxes*) in the laboratory, either in vivo within microorganisms or entirely in vitro in the test tube. Arrows indicate possible routes for connecting individual evolutionary steps. Abbreviations: PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR; IVC, in vitro compartmentalization; FACS, fluorescence-activated cell sorting.

Christian Jäckel, Peter Kast, and Donald Hilvert.
Protein design by directed evolution.
Annu.Rev.Biophys. **37**:153-173, 2008

Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 12809–12813, October 1998
Biochemistry

Directed evolution of a thermostable esterase

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Division of Chemistry and Chemical Engineering, Mail Code 210-41, California Institute of Technology, Pasadena, CA 91125

Edited by Norman R. Pace, University of California, Berkeley, CA, and approved September 5, 1998 (received for review April 30, 1998)

ABSTRACT We have used *in vitro* evolution to probe the relationship between stability and activity in a mesophilic esterase. Previous studies of these properties in homologous enzymes evolved for function at different temperatures have suggested that stability at high temperatures is incompatible with high catalytic activity at low temperatures through mutually exclusive demands on enzyme flexibility. Six generations of random mutagenesis, recombination, and screening stabilized *Bacillus subtilis p*-nitrobenzyl esterase significantly (>14°C increase in T_m) without compromising its catalytic activity at lower temperatures. Furthermore, analysis of the stabilities and activities of large numbers of random mutants indicates that these properties are not inversely correlated. Although enhanced thermostability does not necessarily come at the cost of activity, the process by which the molecule adapts is important. Mutations that increase thermostability while maintaining low-temperature activity are very rare. Unless both properties are constrained (by natural selection or screening) the evolution of one by the accumulation of single amino acid substitutions typically comes at the cost of the other, regardless of whether the two properties are inversely correlated or not correlated at all.

Gezielte Evolution von Proteinen

RESEARCH ARTICLE

Directed evolution of a *para*-nitrobenzyl esterase for aqueous-organic solvents

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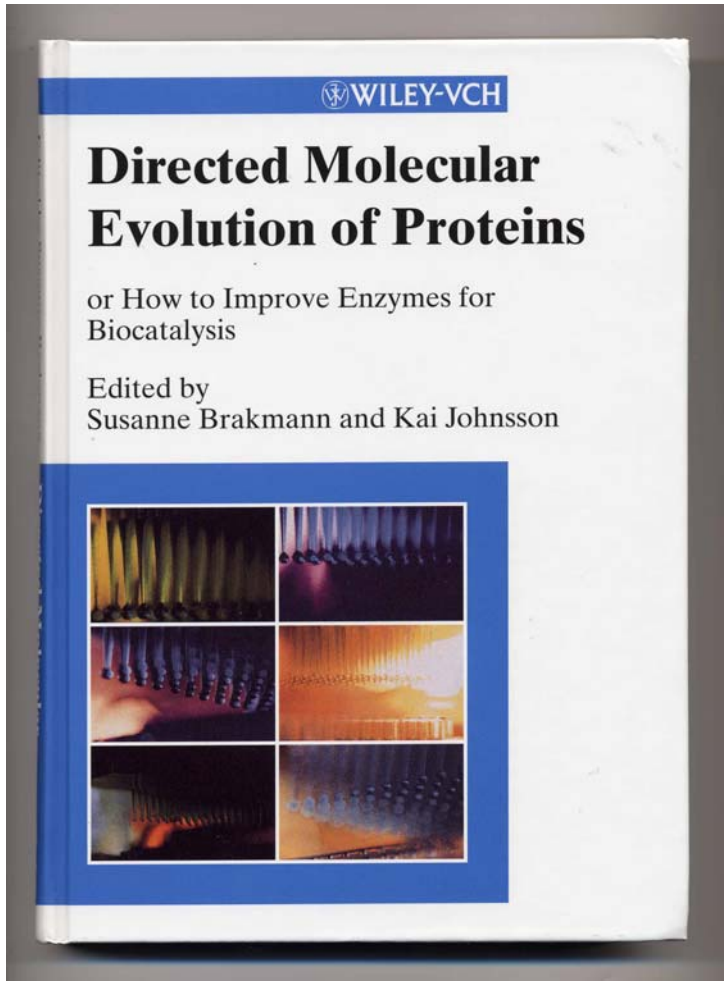
**Corresponding author (e-mail: frances@cheme.caltech.edu).*

Received 13 December 1995; accepted 24 January 1996.

Through sequential generations of random mutagenesis and screening, we have directed the evolution of an esterase for deprotection of an antibiotic *p*-nitrobenzyl ester in aqueous-organic solvents. Because rapid screening directly on the desired antibiotic (loracarbef) nucleus *p*-nitrobenzyl ester was not feasible, the *p*-nitrophenyl ester was employed. Catalytic performance on the screening substrate was shown to reasonably mimic enzyme activity toward the desired ester. One *p*-nitrobenzyl esterase variant performs as well in 30% dimethylformamide as the wildtype enzyme in water, reflecting a 16-fold increase in esterase activity. Random pairwise gene recombination of two positive variants led to a further two-fold improvement in activity. Considering also the increased expression level achieved during these experiments, the net result of four sequential generations of random mutagenesis and the one recombination step is a 50–60-fold increase in total activity. Although the contributions of individual effective amino acid substitutions to enhanced activity are small (<2-fold increases), the accumulation of multiple mutations by directed evolution allows significant improvement of the biocatalyst for reactions on substrates and under conditions not already optimized in nature. The positions of the effective amino acid substitutions have been identified in a pNB esterase structural model developed based on its homology to acetylcholinesterase and triacylglycerol lipase. None appear to interact directly with the antibiotic substrate, further underscoring the difficulty of predicting their effects in a 'rational' design effort.

Keywords: random mutagenesis, antibiotic synthesis, enzymatic deprotection

Gezielte Evolution von Proteinen



Application of molecular evolution to problems in protein biotechnology

Artificial evolution in biotechnology and pharmacology

G.F. Joyce. 2004. Directed evolution of nucleic acid enzymes. *Annu.Rev.Biochem*. **73**:791-836.

C. Jäckel, P. Kast, and D. Hilvert. 2008. Protein design by directed evolution. *Annu.Rev.Biophys*. **37**:153-173.

S.J. Wrenn and P.B. Harbury. 2007. Chemical evolution as a tool for molecular discovery. *Annu.Rev.Biochem*. **76**:331-349.

Exploring protein fitness landscapes by directed evolution

Philip A. Romero and Frances H. Arnold

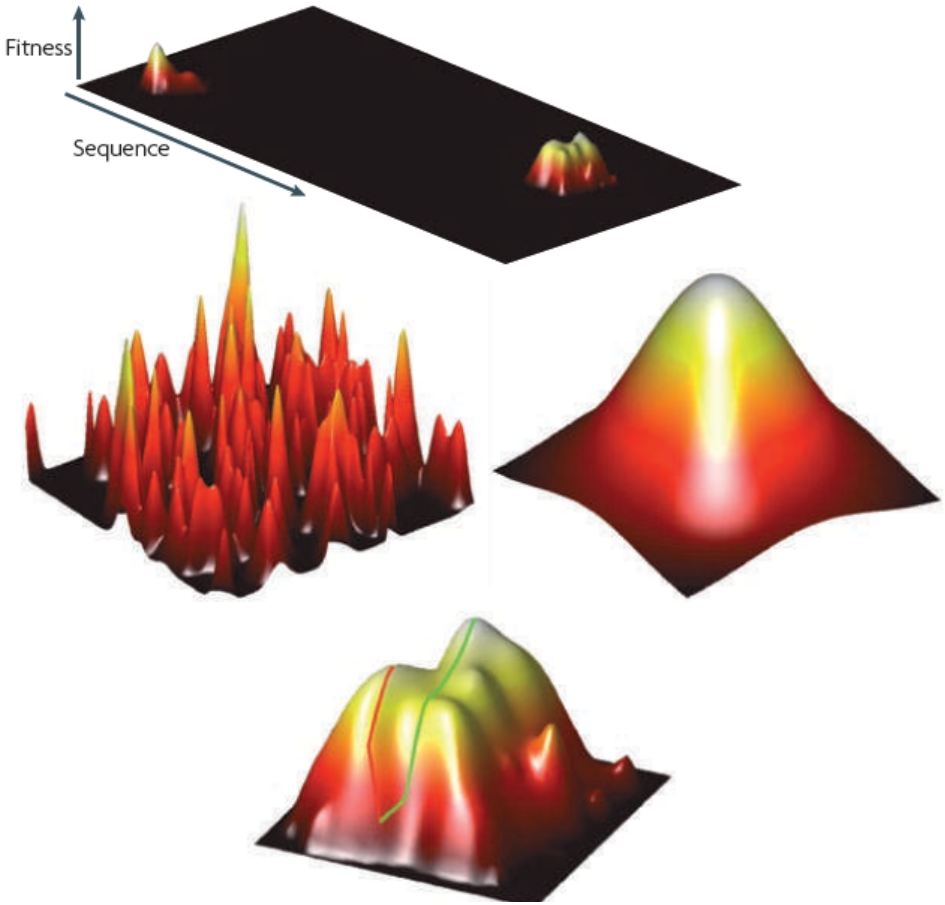
Darwin200

Abstract | Directed evolution circumvents our profound ignorance of how a protein's sequence encodes its function by using iterative rounds of random mutation and artificial selection to discover new and useful proteins. Proteins can be tuned to adapt to new functions or environments by simple adaptive walks involving small numbers of mutations. Directed evolution studies have shown how rapidly some proteins can evolve under strong selection pressures and, because the entire 'fossil record' of evolutionary intermediates is available for detailed study, they have provided new insight into the relationship between sequence and function. Directed evolution has also shown how mutations that are functionally neutral can set the stage for further adaptation.

Frances H. Arnold, 1956 -

Nature Reviews Molecular Cell Biology 10:866-876 (2009)

Fitnesslandschaften von Proteinen



Epistasis occurs when the effect of one mutation depends on the presence of another, which can create landscape ruggedness and local optima. Landscapes could range from the rugged 'Badlands' landscape (left panel), which is nearly impossible to climb by mutational steps, to the 'Fujiyama' landscape (right panel), in which any beneficial mutation brings the search closer to the optimum[2]

Fitnesslandschaften von Proteinen

1. Evolutionsexperimente im Reagenzglas
2. Kinetik der Evolution von Molekülen
3. Gezielte Evolution der „Molekülzüchter“
4. **Strukturen und Fitnesslandschaften**
5. Evolution *in silico*
6. RNA-Schalter

Peter Schuster. 2006.

Prediction of RNA secondary structures: From theory to models and real molecules.

Rep.Prog.Phys. 69:1419-1477

INSTITUTE OF PHYSICS PUBLISHING REPORTS ON PROGRESS IN PHYSICS

Rep. Prog. Phys. **69** (2006) 1419–1477 doi:10.1088/0034-4885/69/5/R04

Prediction of RNA secondary structures: from theory to models and real molecules

Peter Schuster[1,2]

[1]Institut für Theoretische Chemie der Universität Wien, Währingerstraße 17, A-1090 Vienna, Austria
[2]The Santa Fe Institute, 1399 Hyde Park Road, Santa Fe, NM 87501, USA

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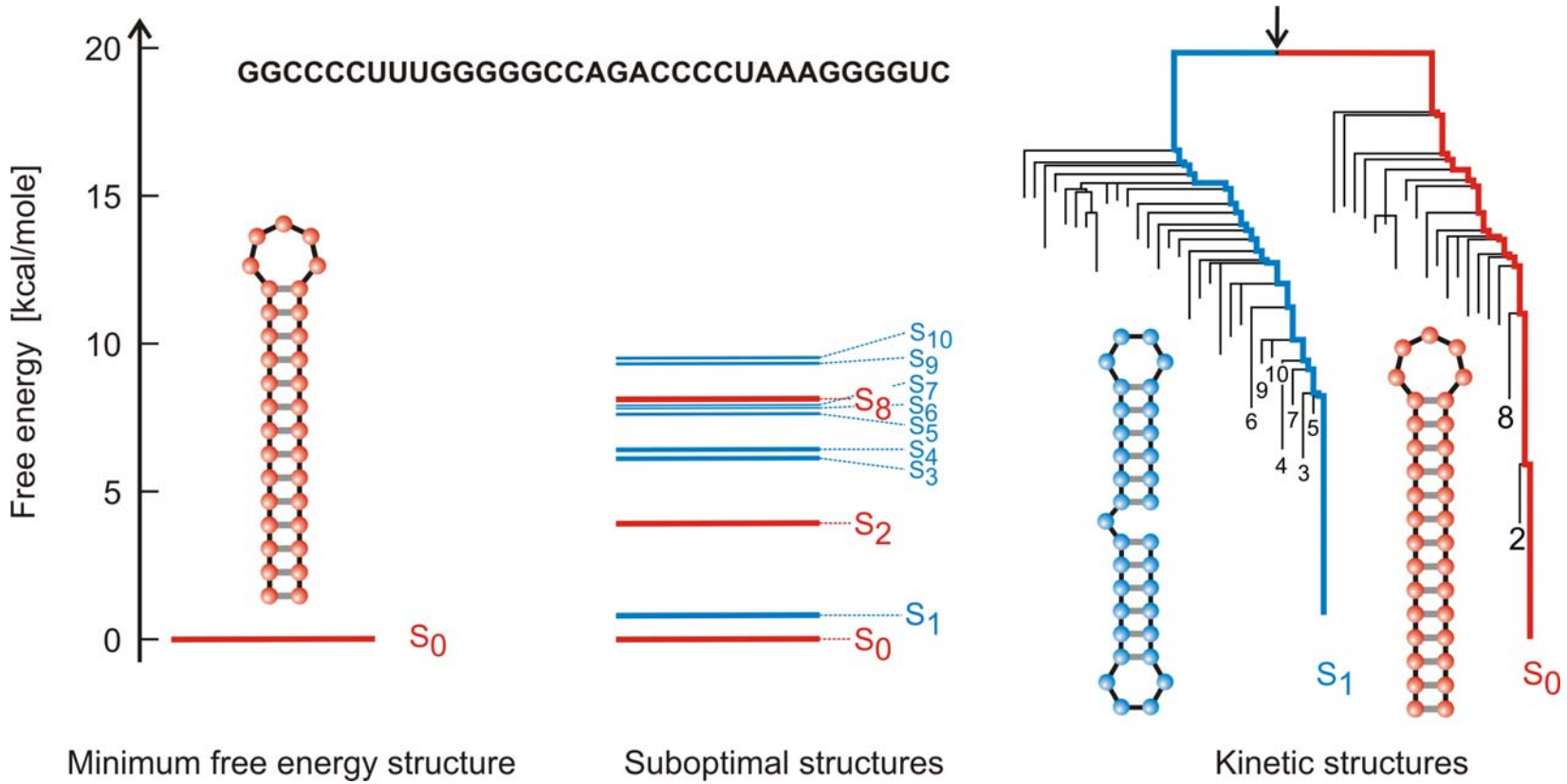
Published 18 April 2006
Online at stacks.iop.org/RoPP/69/1419

Abstract

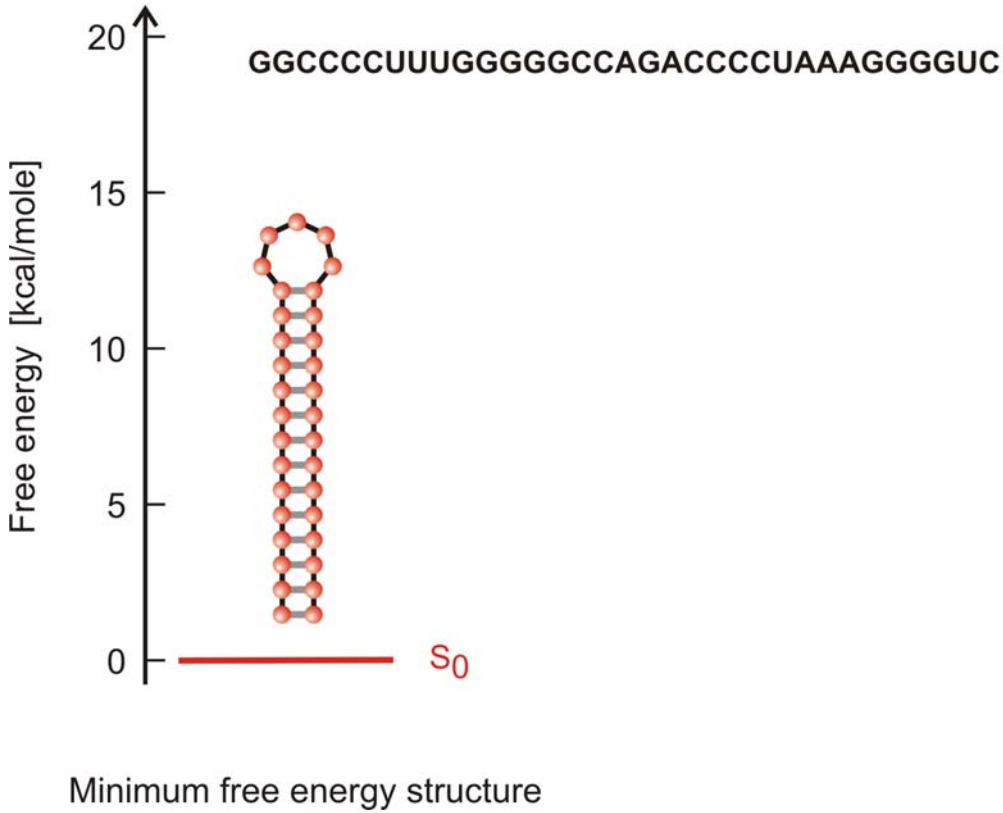
RNA secondary structures are derived from RNA sequences, which are strings built form the natural four letter nucleotide alphabet, {**AUGC**}. These coarse-grained structures, in turn, are tantamount to constrained strings over a three letter alphabet. Hence, the secondary structures are discrete objects and the number of sequences always exceeds the number of structures. The sequences built from two letter alphabets form perfect structures when the nucleotides can form a base pair, as is the case with {**GC**} or {**AU**}, but the relation between the sequences and structures differs strongly from the four letter alphabet. A comprehensive theory of RNA structure is presented, which is based on the concepts of *sequence space* and *shape space*, being a space of structures. It sets the stage for modelling processes in ensembles of RNA molecules like evolutionary optimization or kinetic folding as dynamical phenomena guided by mappings between the two spaces.

The number of minimum free energy (mfe) structures is always smaller than the number of sequences, even for two letter alphabets. Folding of RNA molecules into mfe energy structures constitutes a non-invertible mapping from sequence space onto shape space. The preimage of a structure in sequence space is defined as its *neutral network*. Similarly the set of *suboptimal structures* is the preimage of a sequence in shape space. This set represents the *conformation space* of a given sequence. The evolutionary optimization of structures in populations is a process taking place in sequence space, whereas kinetic folding occurs in molecular ensembles that optimize free energy in conformation space. Efficient folding algorithms based on dynamic programming are available for the prediction of secondary structures for given sequences. The inverse problem, the computation of sequences for predefined structures, is an important tool for the design of RNA molecules with tailored properties. Simultaneous folding or *cofolding* of two or more RNA molecules can be modelled readily at the secondary structure level

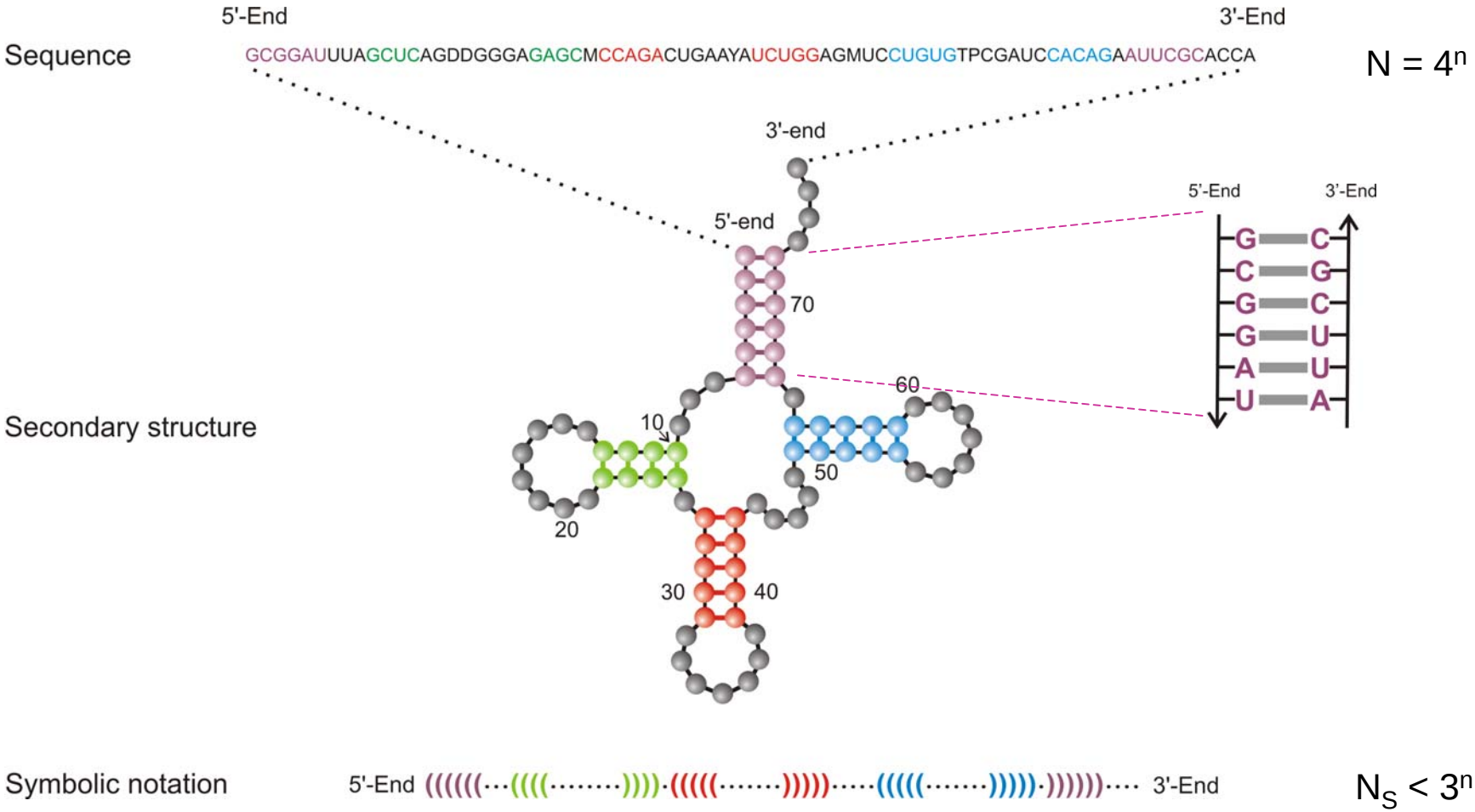
This article was invited by Professor T J Newman.



Strukturen von RNA-Molekülen



Strukturen von RNA-Molekülen



Criterion: Minimum free energy (mfe)

Rules: _ (_) _ ∈ {**AU**,**CG**,**GC**,**GU**,**UA**,**UG**}

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

Monatshefte für Chemie 125, 167–188 (1994)

Monatshefte für Chemie
Chemical Monthly

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]

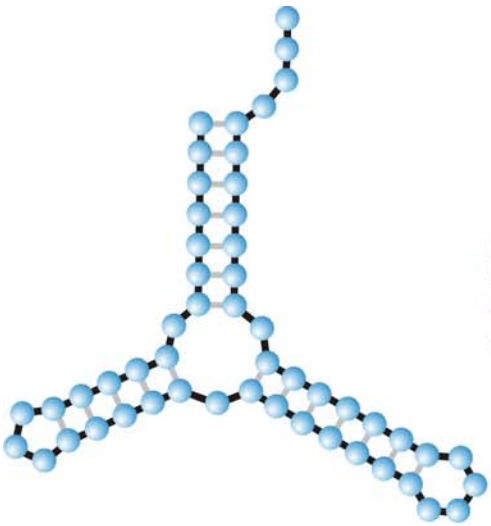
[1] Institut für Theoretische Chemie, Universität Wien, A-1090 Wien, Austria
[2] Institut für Molekulare Biotechnologie, D-07745 Jena, Federal Republic of Germany
[3] Santa Fe Institute, Santa Fe, NM 87501, U.S.A.
[4] Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, U.K.

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.



Minimum free energy criterion

1st
2nd
3rd trial
4th
5th

Inverse folding

UUUAGCCAGCGCGAGUCGUGCGGACGGGGUUAUCUCUGUCGGGCUAGGGCGC

GUGAGCGCGGGGCACAGUUUCUCAAGGAUGUAAGUUUUUGCCGUUUAUCUGG

UUAGCGAGAGAGGAGGCUUCUAGACCCAGCUCUCUGGGUCGUUGCUGAUGCG

CAUUGGUGCUAAUGAUAUUAGGGCUGUAUUCCUGUAUAGCGAUCAGUGUCCG

GUAGGCCCUCUUGACAUAAGAUUUUUCCAAUGGUGGGAGAUGGCCAUUGCAG

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

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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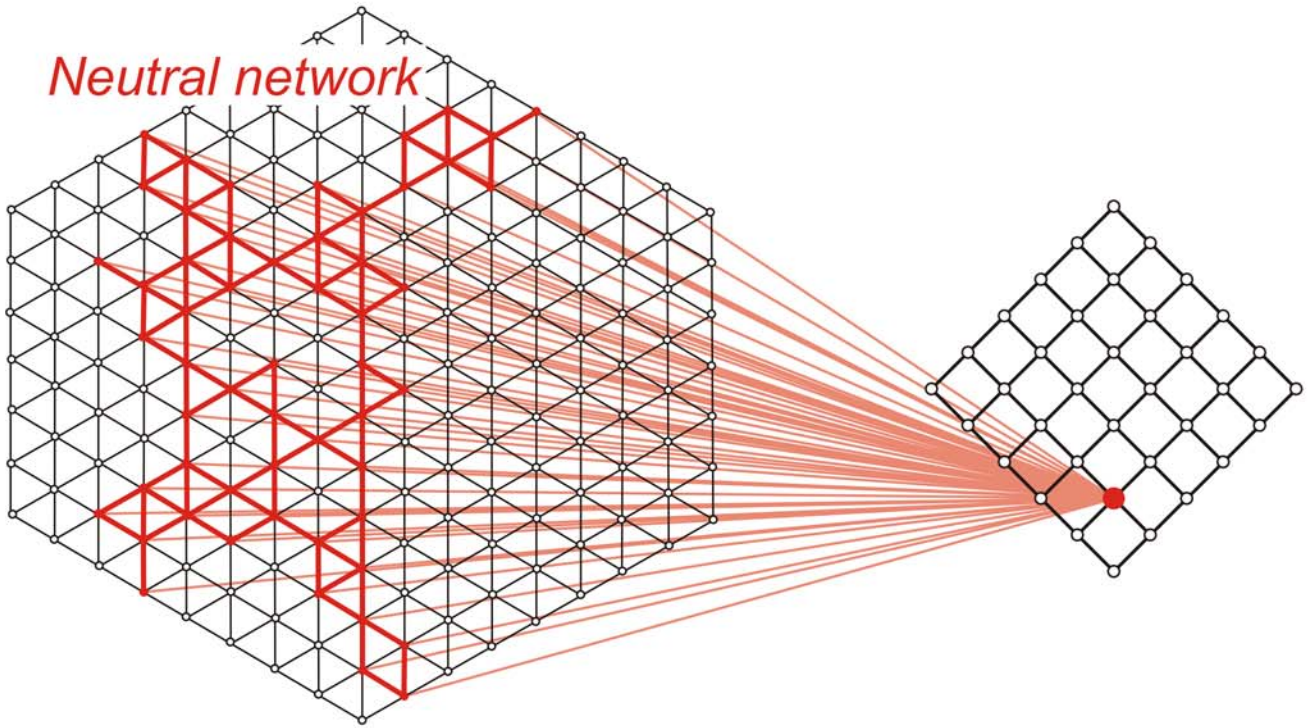
[3] *Santa Fe Institute, Santa Fe, U.S.A.*

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.

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



many genotypes ⇒ one phenotype

Bulletin of Mathematical Biology, Vol. 59, No. 2, pp. 339–397, 1997
Elsevier Science Inc.

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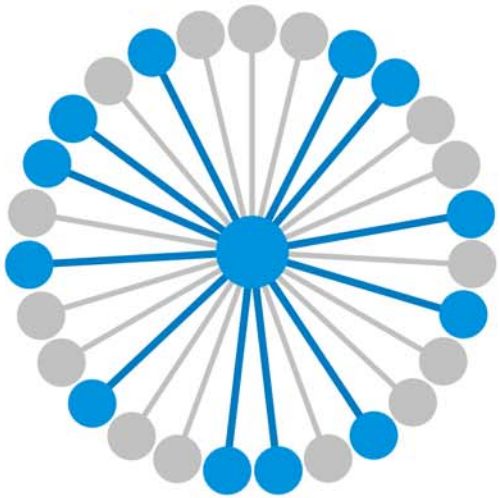
Random graph theory is used to model and analyse the relationships between sequences and secondary structures of RNA molecules, which are understood as mappings from sequence space into shape space. These maps are non-invertible since there are always many orders of magnitude more sequences than structures.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



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

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

$\lambda_j = 12 / 27 = 0.444$

Alphabet size κ :

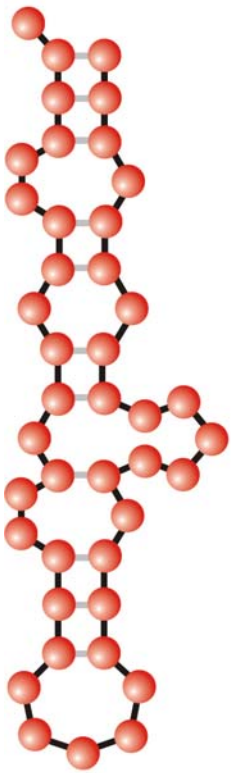
κ	λ_{cr}	
2	0.5	**AU,GC,DU**
3	0.423	**AUG , UGC**
4	0.370	**AUGC**

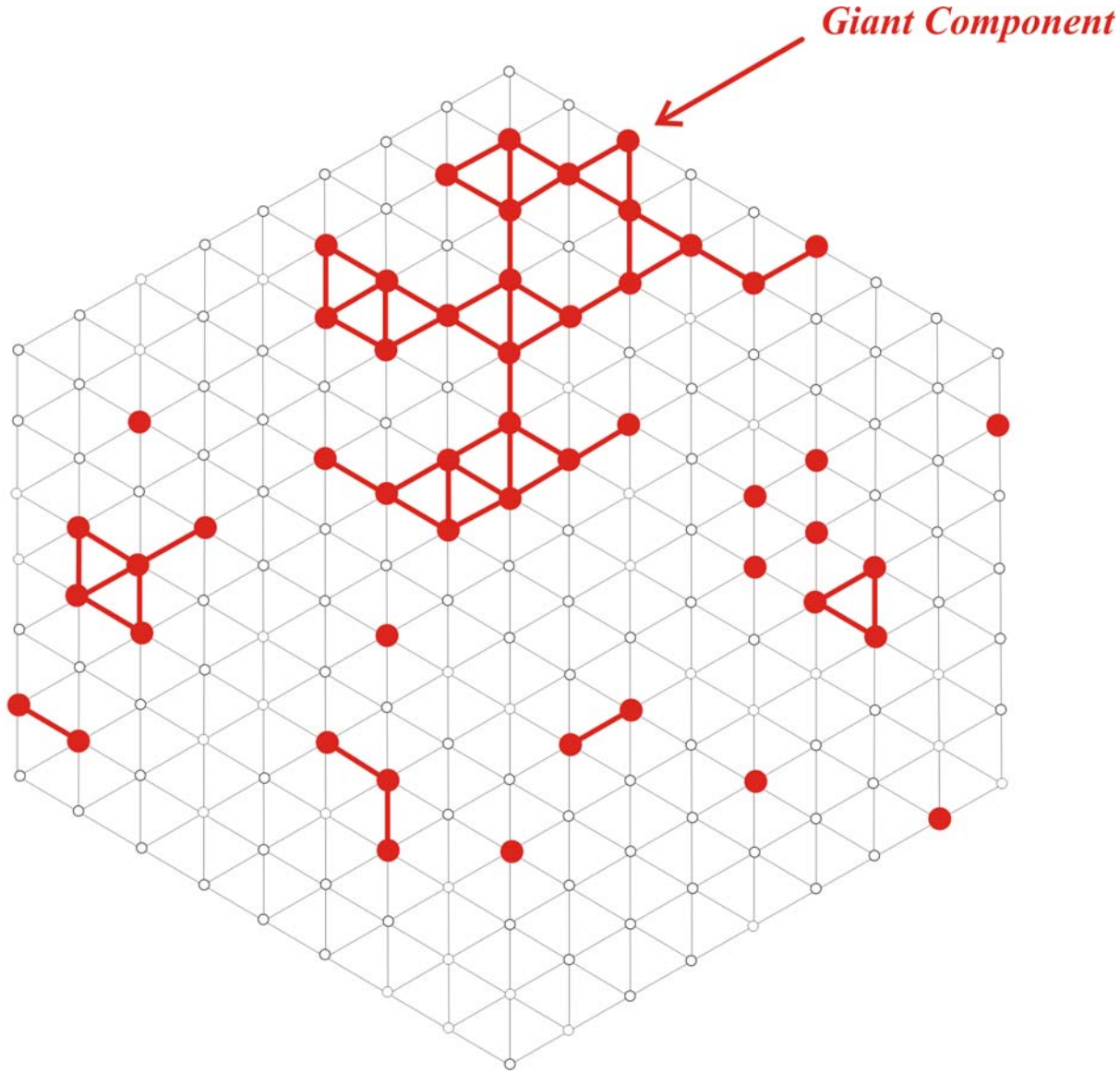
$\bar{\lambda}_k > \lambda_{cr}$ .... network G_k is connected

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

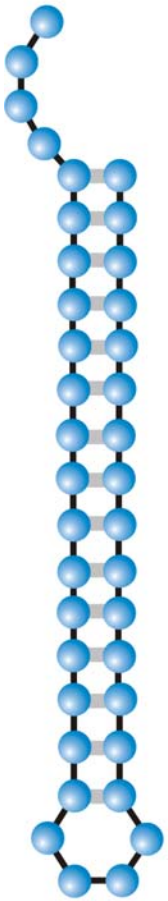
Connectivity threshold: $\lambda_{cr} = 1 - \kappa^{-1/(\kappa-1)}$

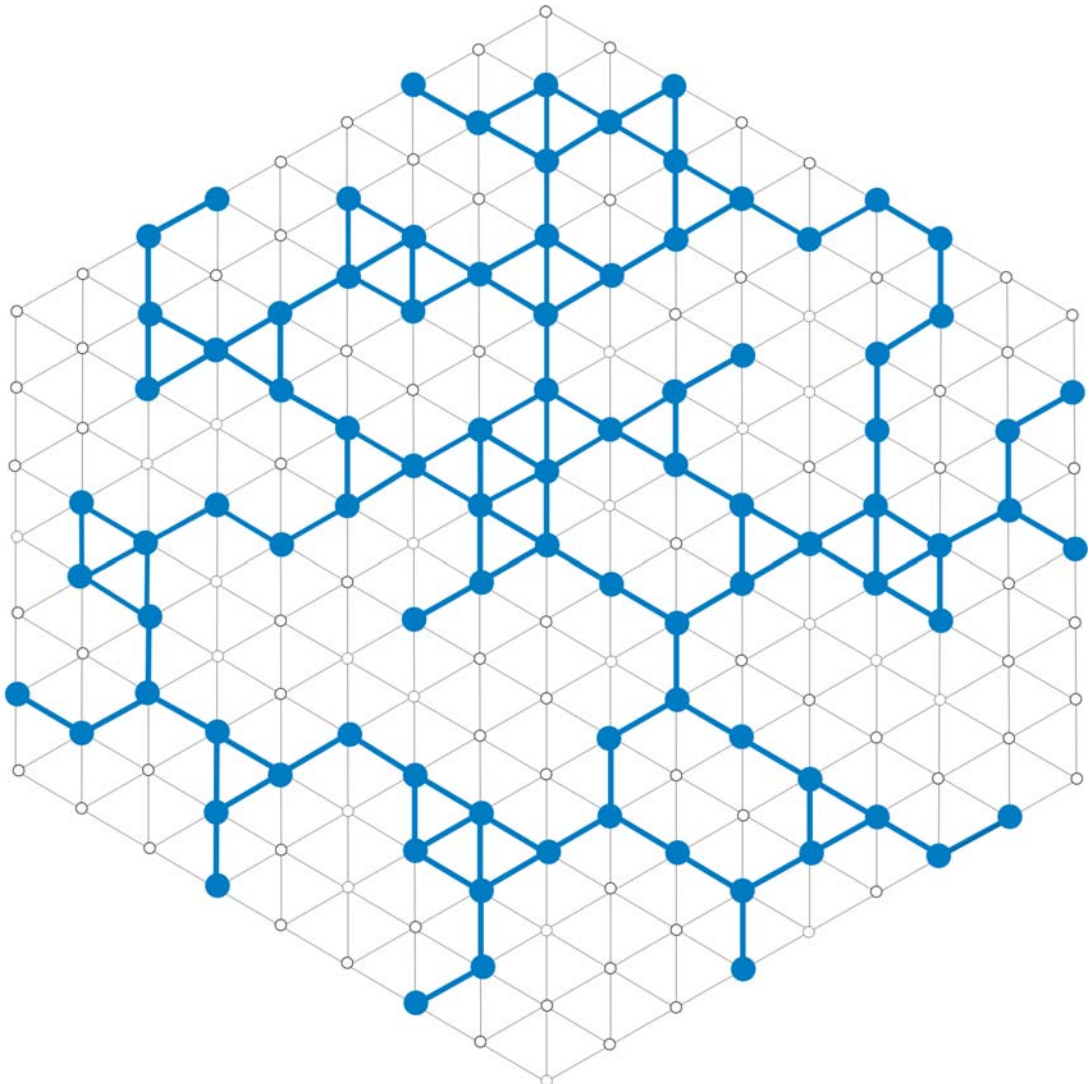
Degree of neutrality of neutral networks and the connectivity threshold





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





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

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

Evidence for neutral networks and intersection of apatamer functions

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

JOURNAL OF MOLECULAR EVOLUTION

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

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

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

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Introduction

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

1. Evolutionsexperimente im Reagenzglas
2. Kinetik der Evolution von Molekülen
3. Gezielte Evolution der „Molekülzüchter"
4. Strukturen und Fitnesslandschaften
5. **Evolution *in silico***
6. RNA-Schalter

Evolution *in silico*

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

random individuals. The primer pair used for genomic DNA amplification is 5′-TCTCCCTGGATTCTCATTTA-3′ (forward) and 5′-TCTTTGTCTTCTGTTCCACC-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′-GCATGACCTGCCGGCTAATGGG-3′; reverse, 5′-CTCACGGCTTCTGCATGGTGCTCGGCTGGC-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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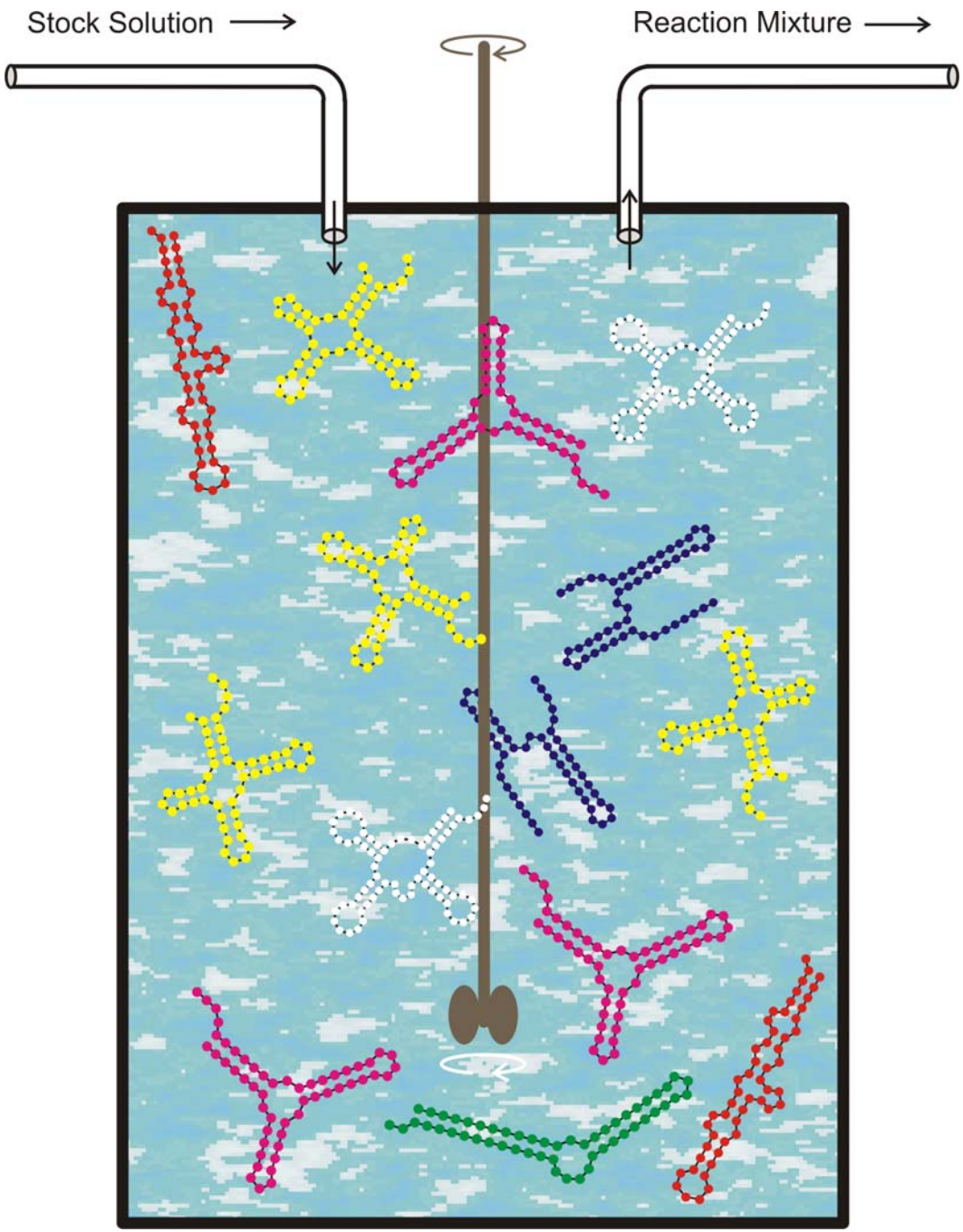


Replikationsparameter:

$$f_k = \gamma / [\alpha + \Delta d_S^{(k)}]$$

$$\Delta d_S^{(k)} = d_H(S_k, S_\tau)$$

Selektionsbedingung:

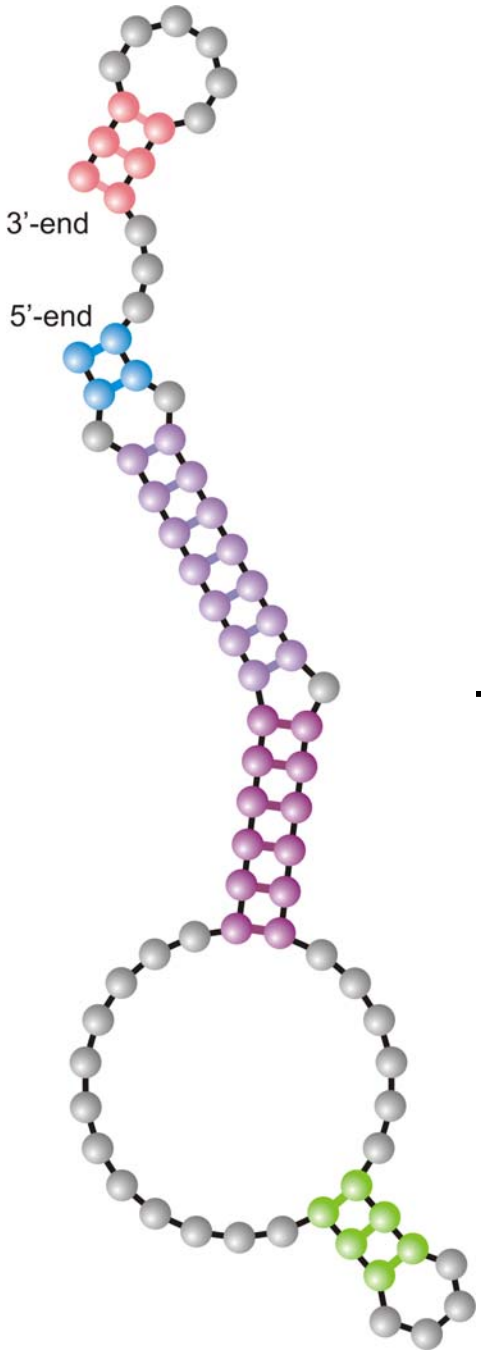
Die Populationsgröße, N = # RNA-Moleküle, wird durch den Fluss kontrolliert:

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

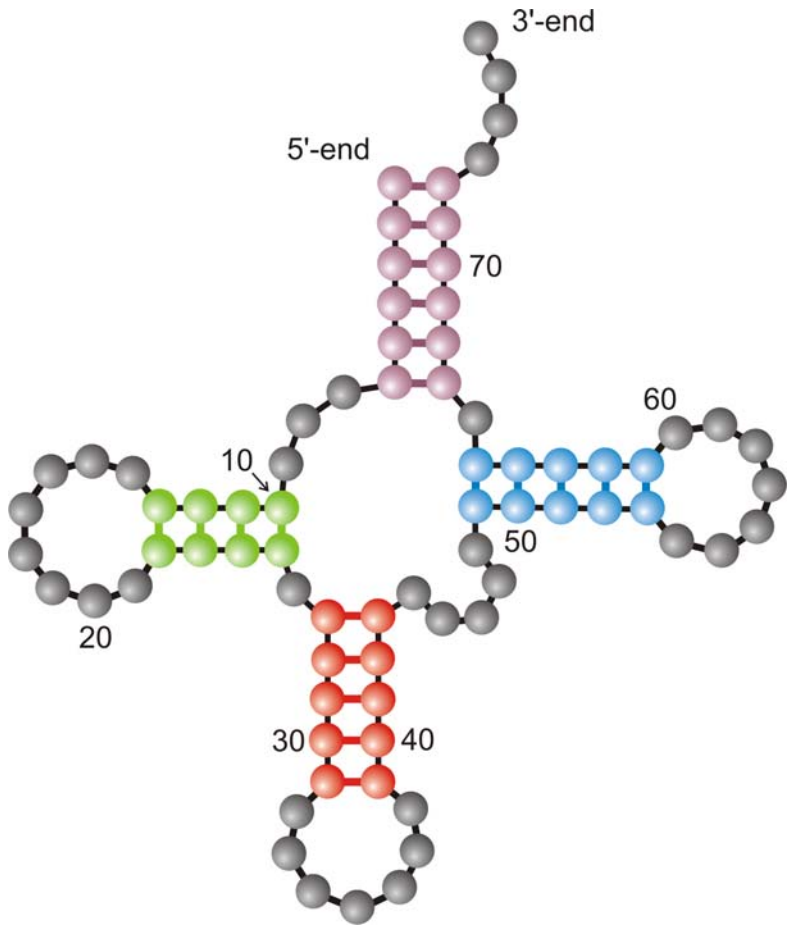
Mutationsrate:

p = 0.001 / Nukleotid × Replikation

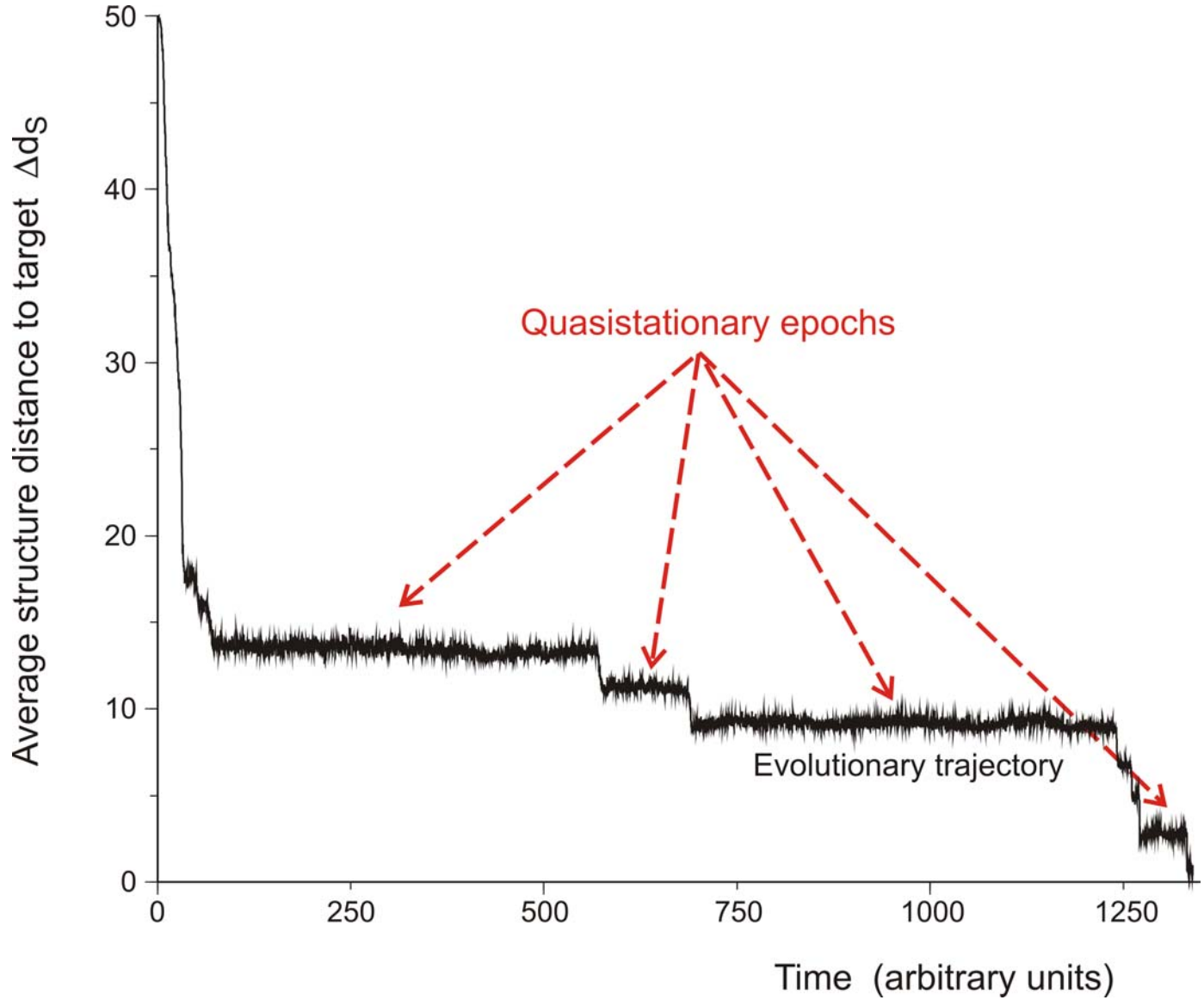
Der Flussreaktor zum Studium von Evolution ***in vitro*** and ***in silico***



Struktur einer
zufällig gewählten
Anfangssequenz



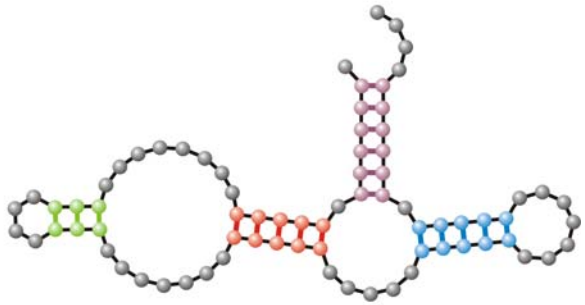
Phenylalanyl-tRNA
als Zielstruktur



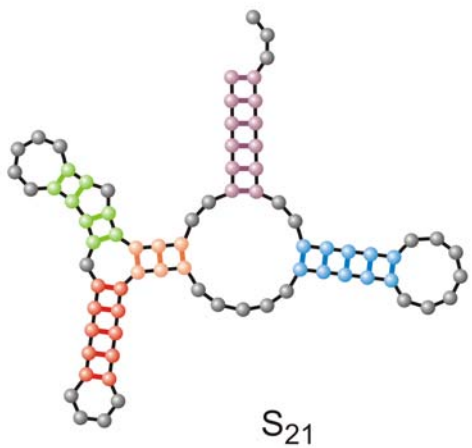
In silico Strukturoptimierung im Flussreaktor: eine Trajektorie des Evolutionsprozesses

Zufällig gewählte
Anfangsstruktur

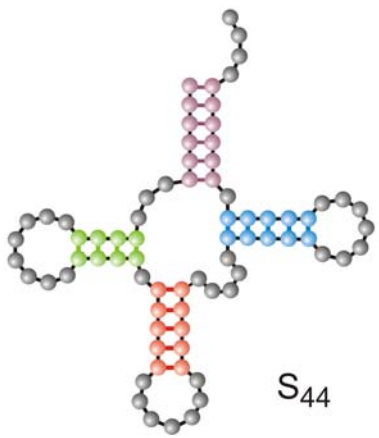
S_0



S_9



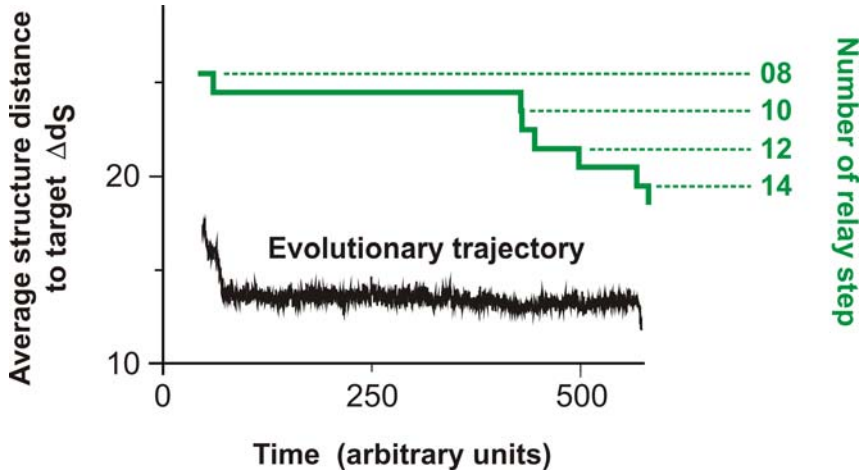
S_{21}



S_{44}

Phenylalanyl-tRNA
als Zielstruktur

28 neutrale Punktmutationen
während einer langen quasi-stationären Epoche

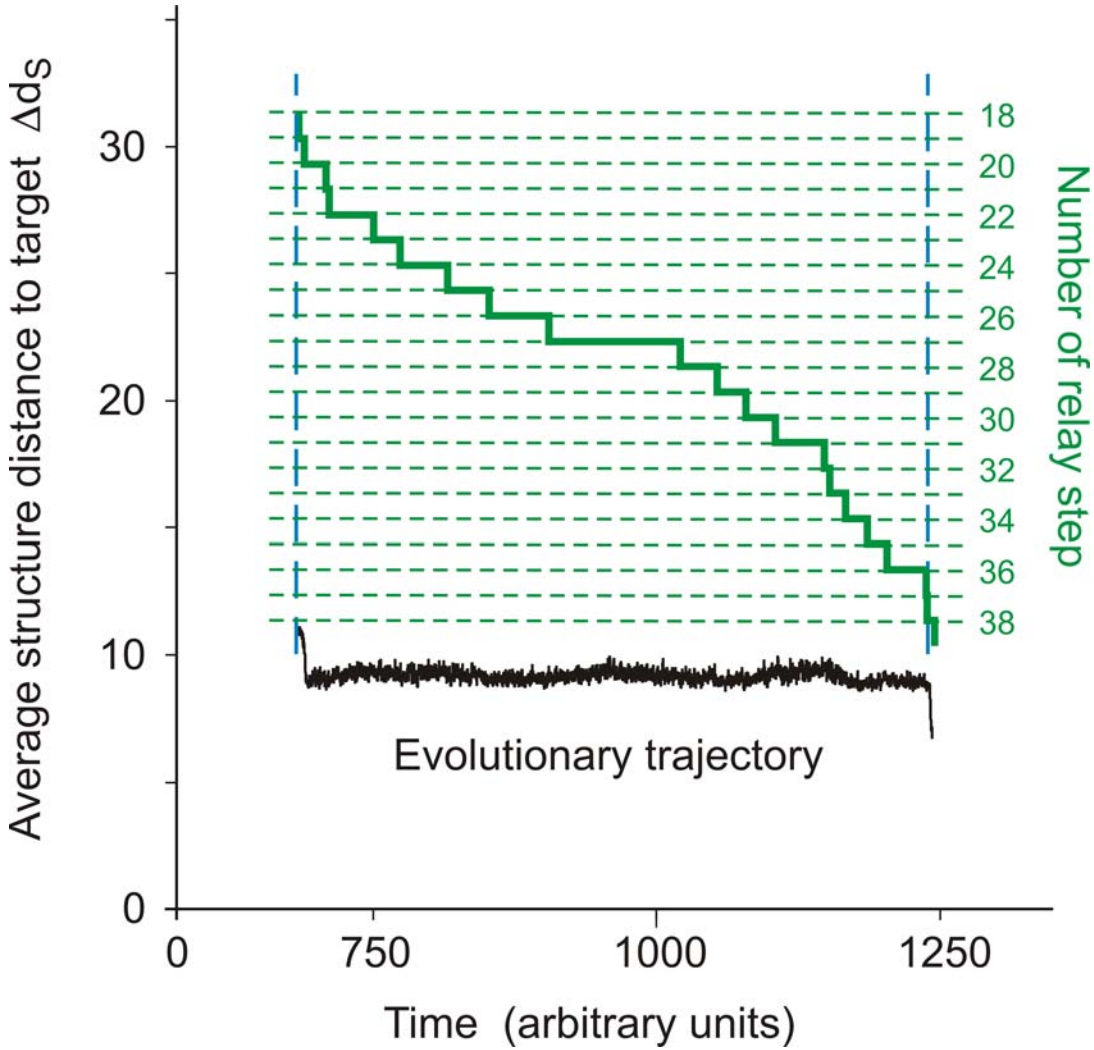



```
entry   GGUAUGGGCGUUGAAUAGUAGGGUUUAAACCAAUCGGCCAACGAUCUCGUGUGCGCAUUUCAUAUCCCGUACAGAA
8       .((((((((((((.........(((....)))).......)))))).....((((.......))))))))))....
exit    GGUAUGGGCGUUGAAUAAUAGGGUUUAAACCAAUCGGCCAACGAUCUCGUGUGCGCAUUUCAUAUCCCAUACAGAA
entry   GGUAUGGGCGUUGAAUAAUAGGGUUUAAACCAAUCGGCCAACGAUCUCGUGUGCGCAUUUCAUAUACCAUACAGAA
9       .((((((.(((((.........(((....)))).......)))))).....((((.......)))))..))))))....
exit    UGGAUGGACGUUGAAUAACAAGGUAUCGACCAAACAACCAACGAGUAAGUGUGUACGCCCCACACACCGUCCCAAG
entry   UGGAUGGACGUUGAAUAACAAGGUAUCGACCAAACAACCAACGAGUAAGUGUGUACGCCCCACACAGCGUCCCAAG
10      .(((((..(((((.........(((....)))).......)))))).....((((.......)))))..)))))....
exit    UGGAUGGACGUUGAAUAACAAGGUAUCGACCAAACAACCAACGAGUAAGUGUGUACGCCCCACACAGCGUCCCAAG
```

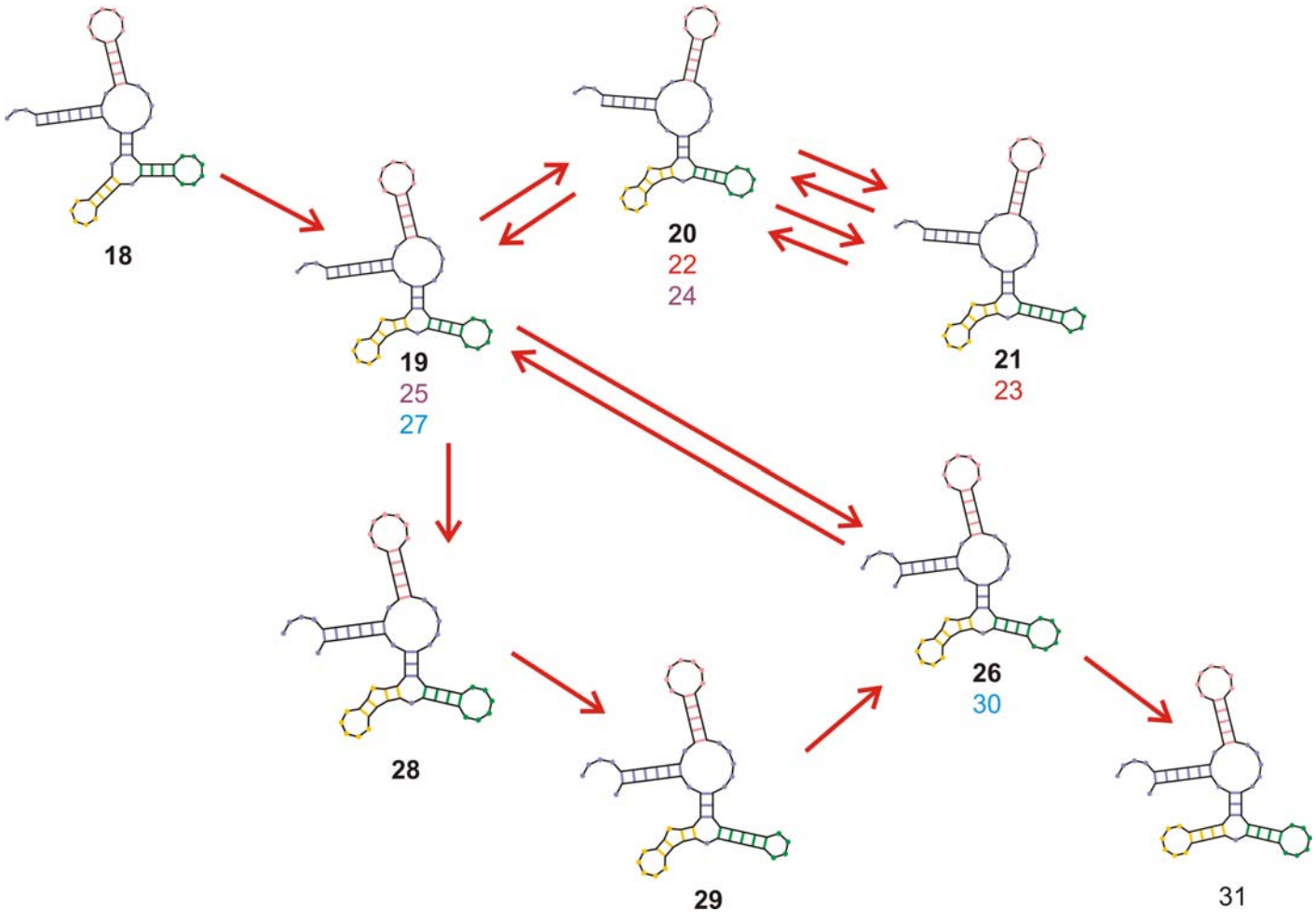
Übergänge induzierende Punktmutationen
ändern die molekulare Struktur

Neutrale Punktmutationen lassen die molekulare Struktur unverändert

Neutrale Evolution von Sequenzen bei konstanter Struktur



Eine quasistationäre Epoche mit wechselnden Strukturen

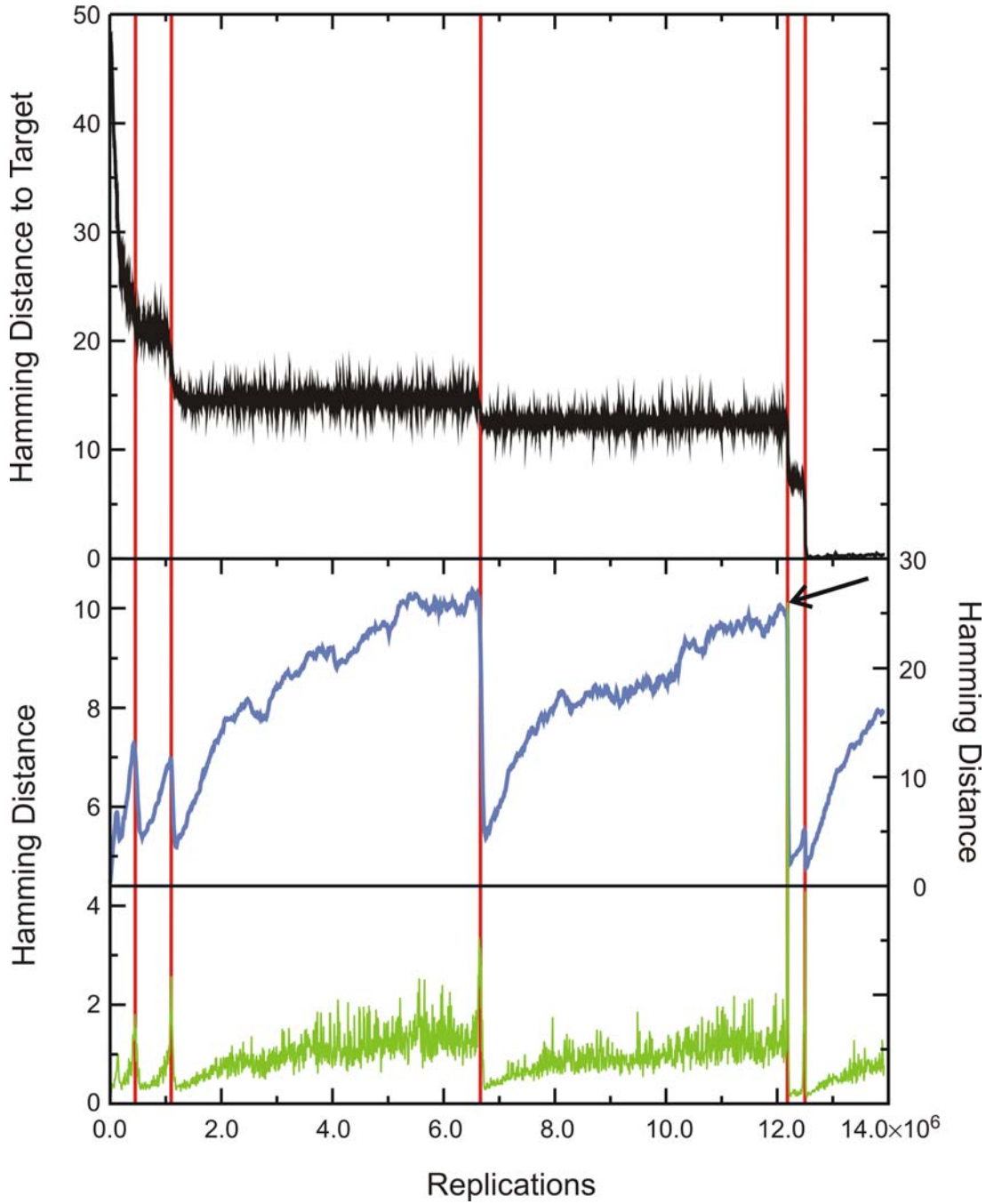


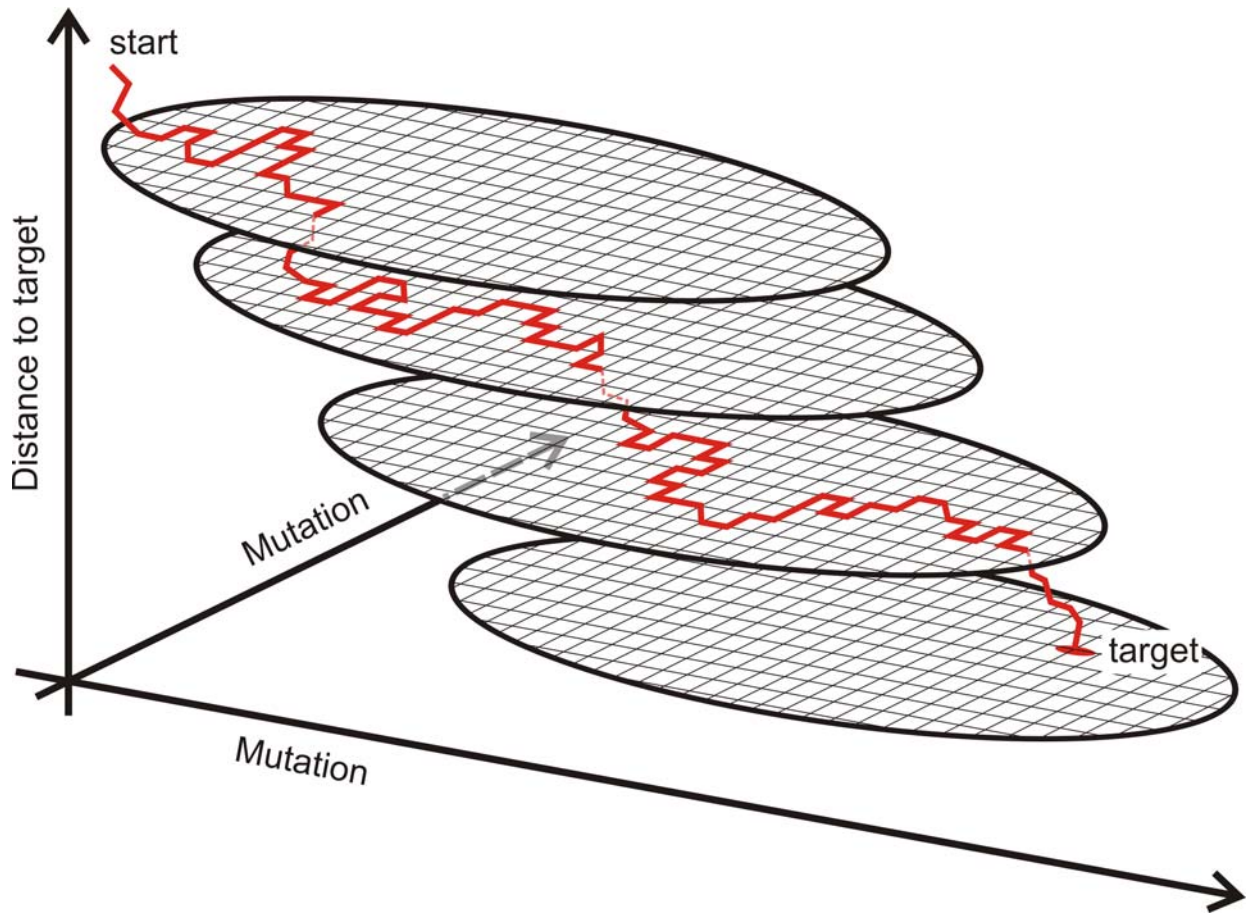
Ein ‚Irrflug‘ im Raum gleichwertiger Strukturen

Trajektorie eines
Evolutionsprozesses

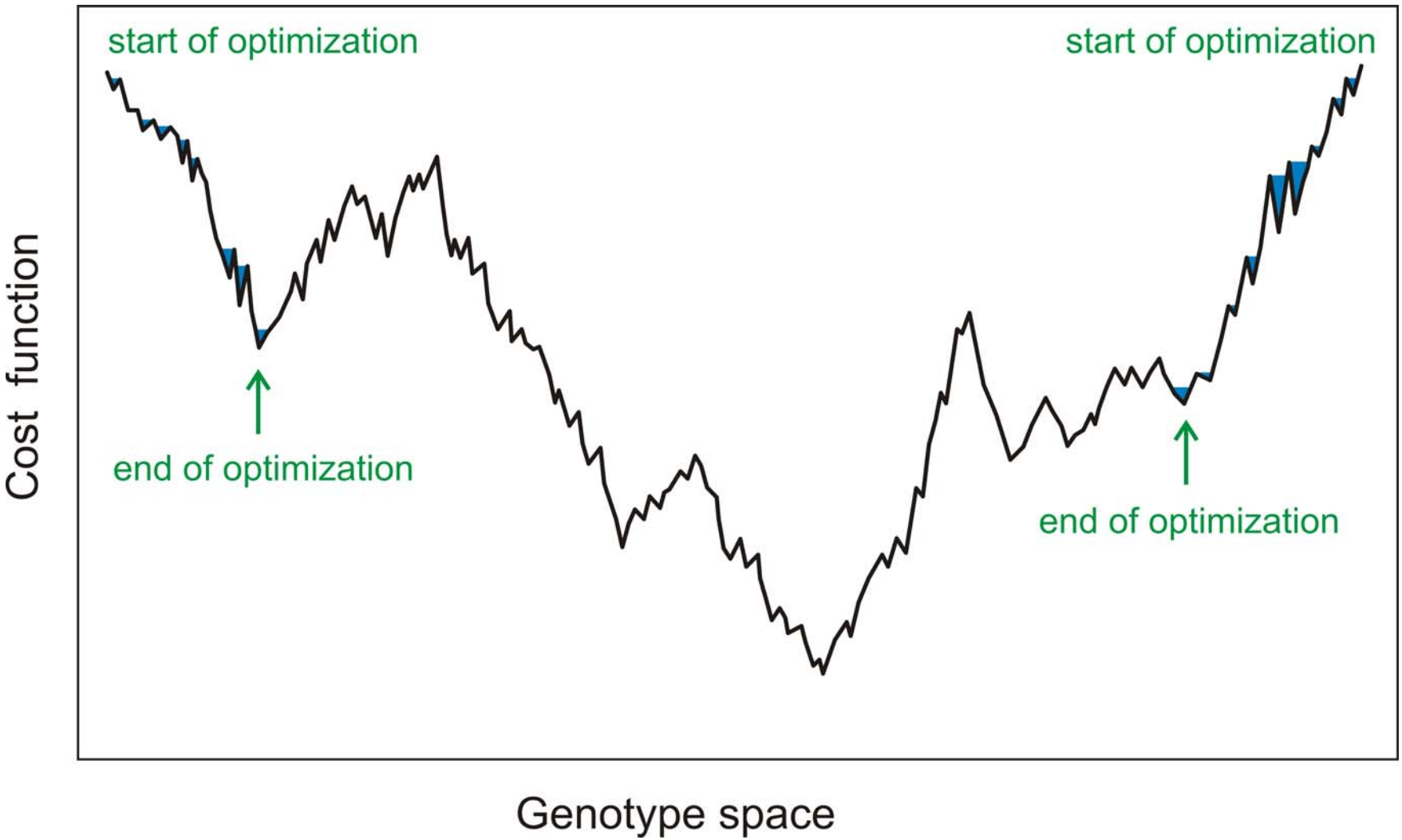
Ausbreitung der Population
auf neutralen Netzwerken

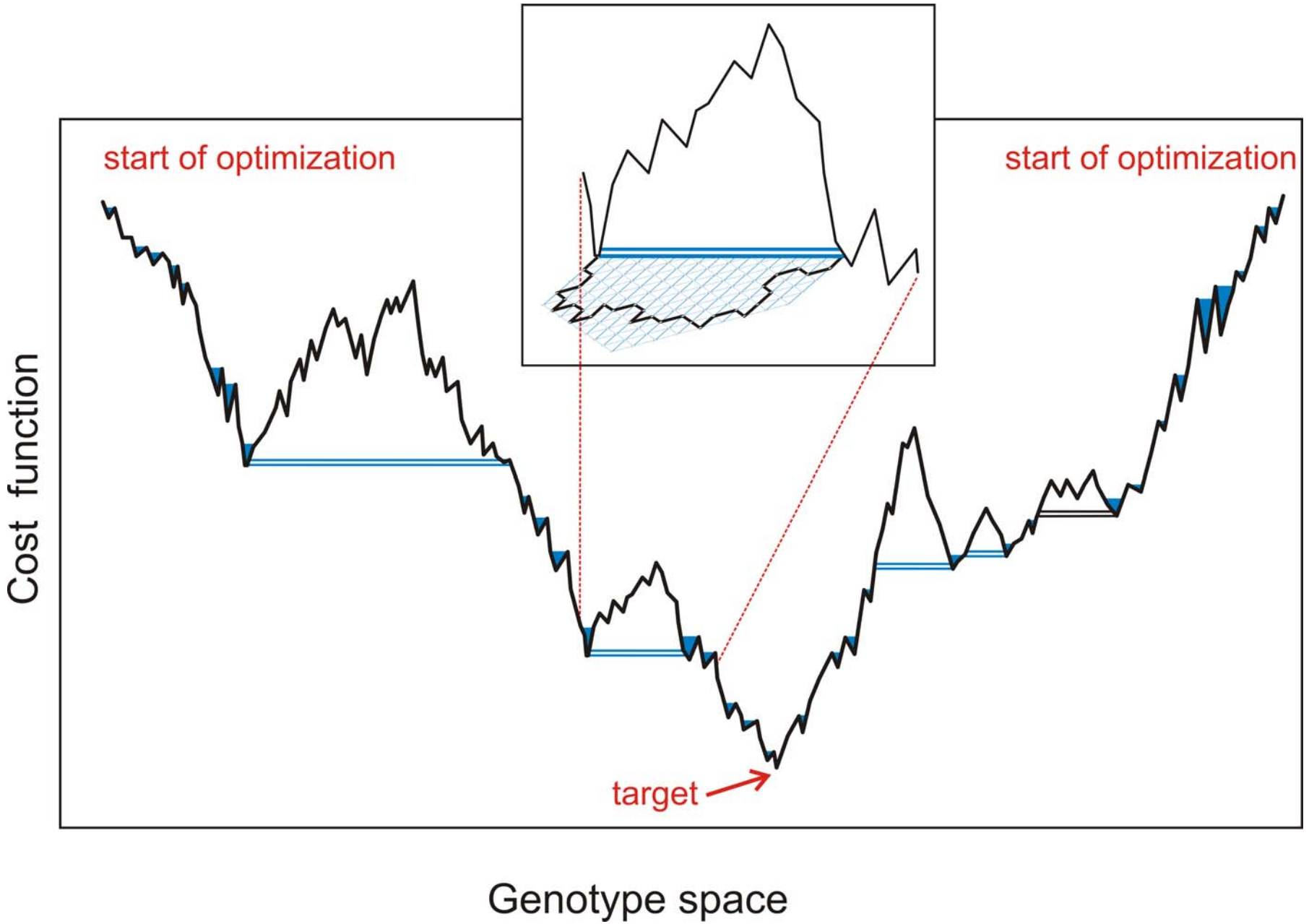
Drift des Populations-
schwerpunktes im
Sequenzraum





Skizze der Optimierung auf neutralen Netzwerken


start of optimization
start of optimization
end of optimization
end of optimization
Cost function
Genotype space


start of optimization
start of optimization
target
Cost function
Genotype space

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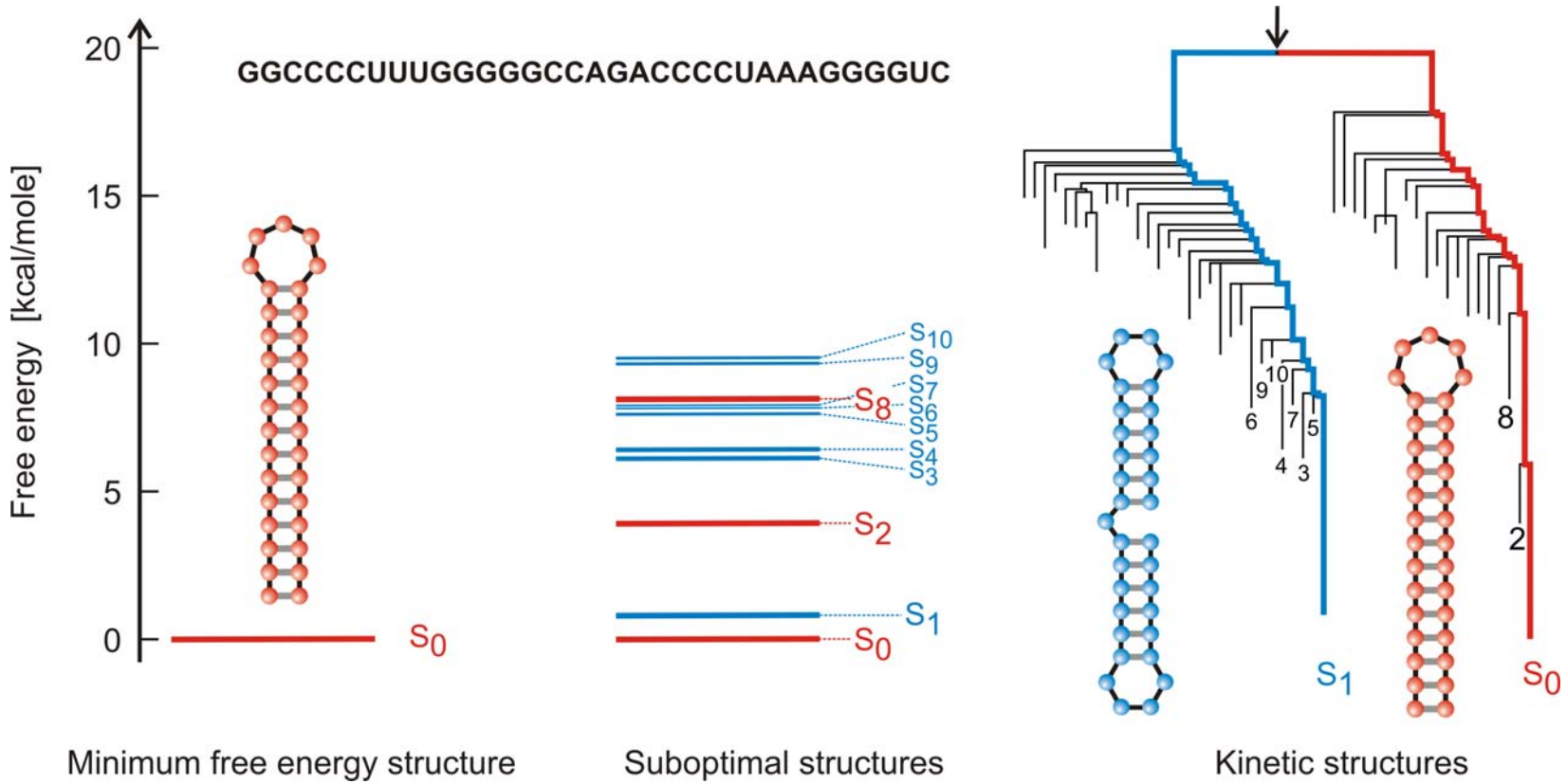
4. Strukturen und Fitnesslandschaften
5. Evolution *in silico*
6. **RNA-Schalter**



Strukturen von RNA-Molekülen

3568–3576 Nucleic Acids Research, 2006, Vol. 34, No. 12
doi:10.1093/nar/gkl445

Structural parameters affecting the kinetics of RNA hairpin formation

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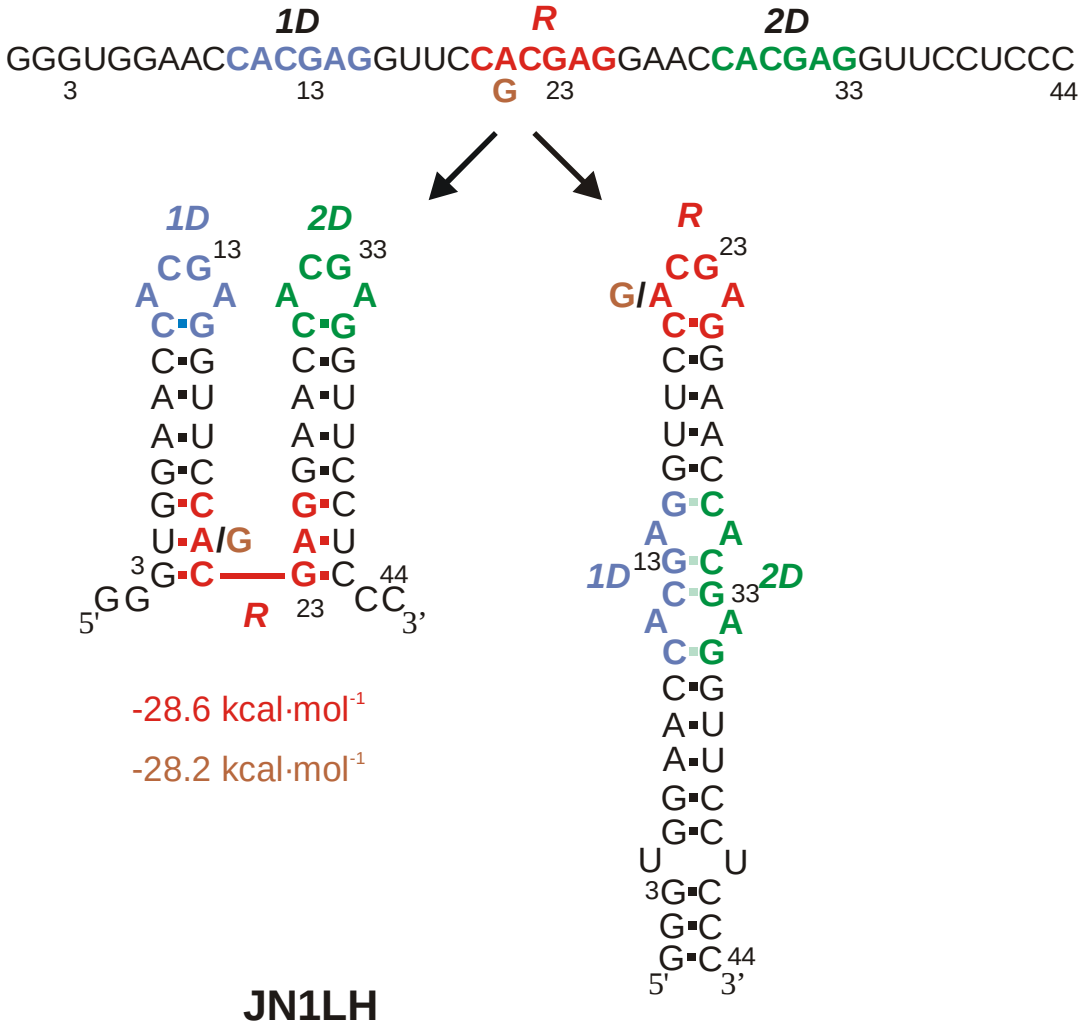
ABSTRACT

There is little experimental knowledge on the sequence dependent rate of hairpin formation in RNA. We have therefore designed RNA sequences that can fold into either of two mutually exclusive hairpins and have determined the ratio of folding of the two conformations, using structure probing. This folding ratio reflects their respective folding rates. Changing one of the two loop sequences from a purine- to a pyrimidine-rich loop did increase its folding rate, which corresponds well with similar observations in DNA hairpins. However, neither changing one of the loops from a regular non-GNRA tetra-loop into a stable GNRA tetra-loop, nor increasing the loop size from 4 to 6 nt did affect the folding rate. The folding kinetics of these RNAs have also been simulated with the program 'Kinfold'. These simulations were in agreement with the experimental results if the additional stabilization energies for stable tetra-loops were not taken into account. Despite the high stability of the stable tetra-loops, they apparently do not affect folding kinetics of these RNA hairpins. These results show that it is possible to experimentally determine relative folding rates of hairpins and to use these data to improve the computer-assisted simulation of the folding kinetics of stem–loop structures.

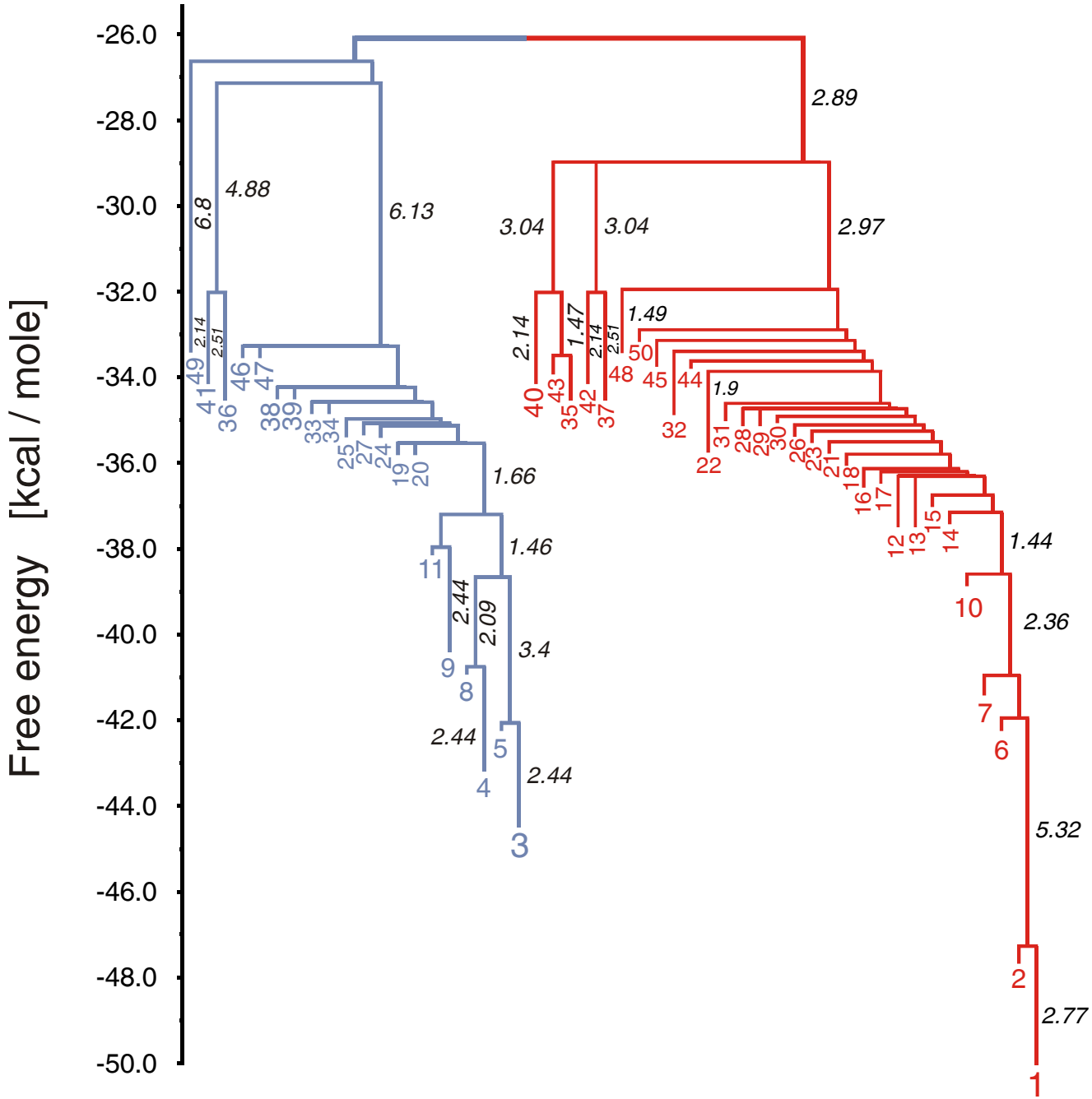


An experimental RNA switch

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 Res.*** **34**:3568-3576 (2006)



J1LH barrier tree

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.).

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One Sequence, Two Ribozymes: Implications for the Emergence of New Ribozyme Folds

Erik A. Schultes and David P. Bartel*

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

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

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

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

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

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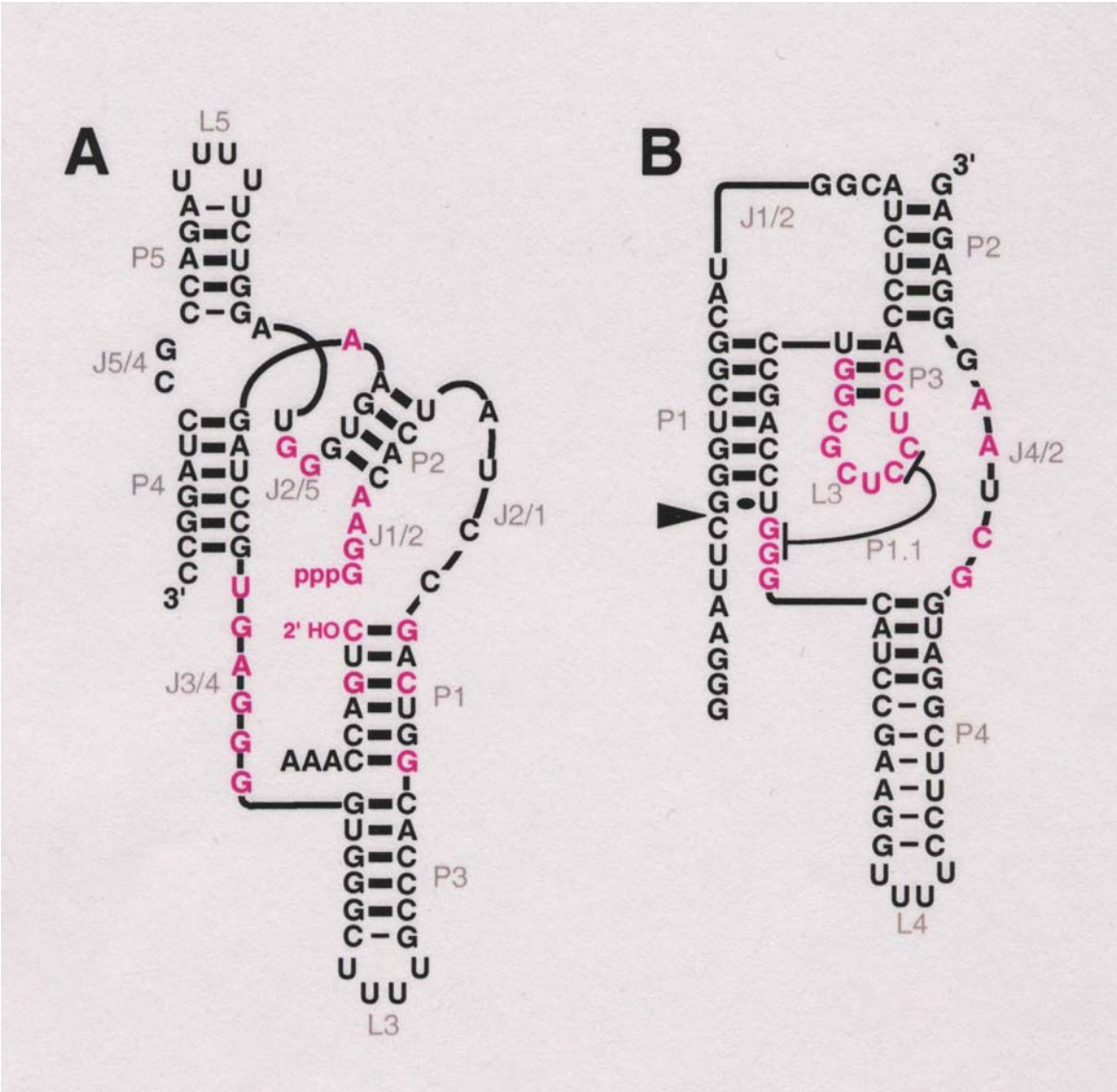
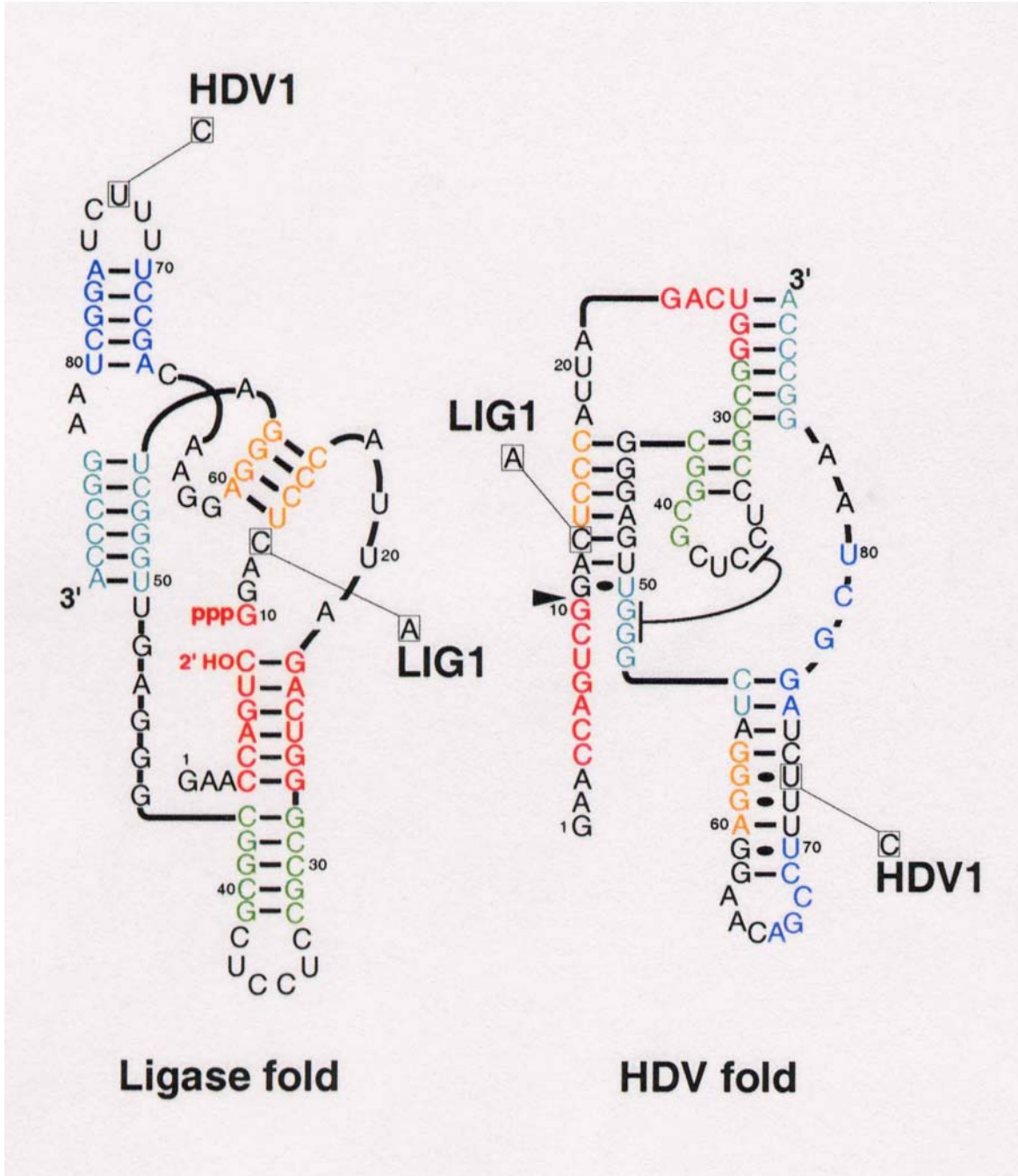
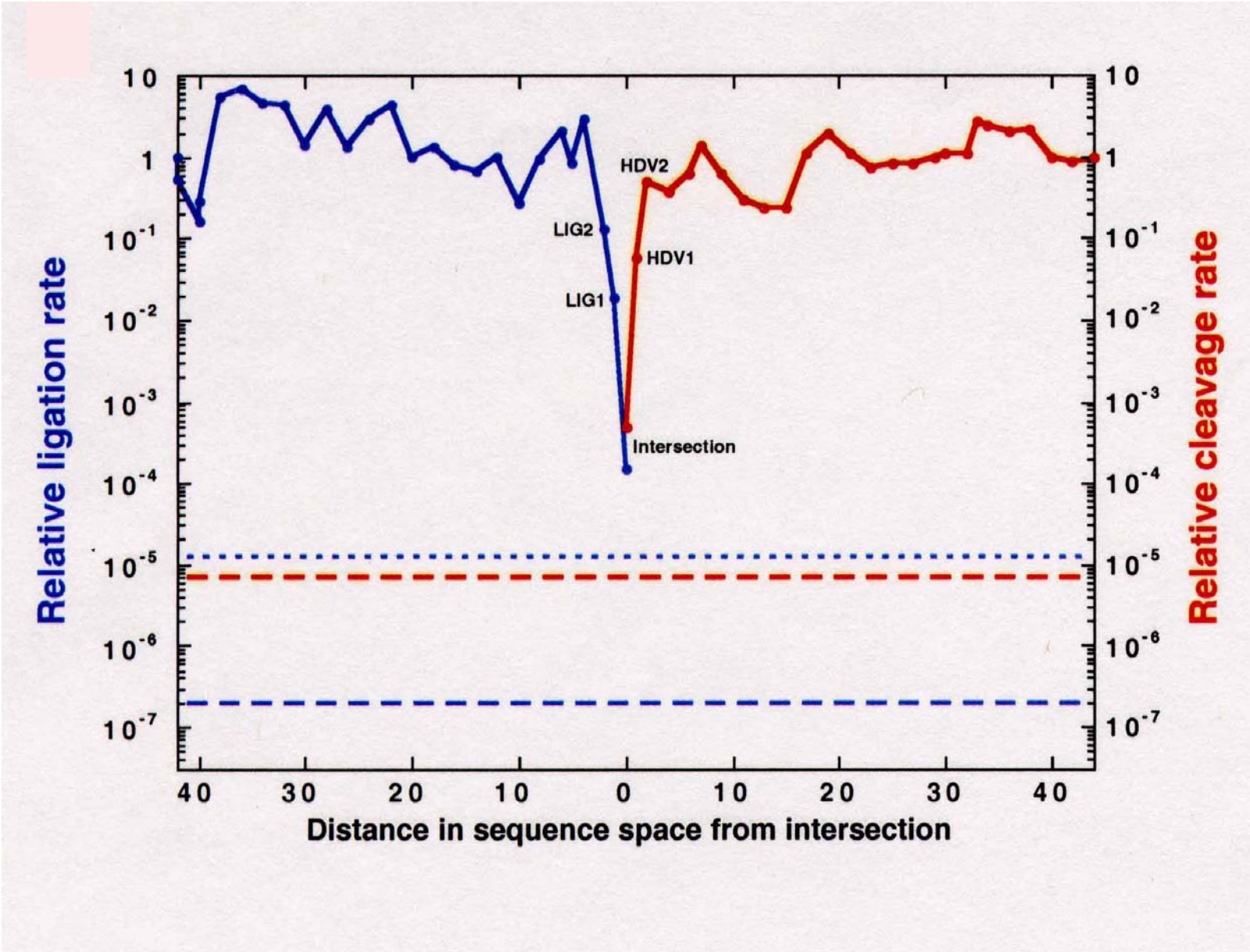


Two ribozymes of chain lengths n = 88 nucleotides: An artificial ligase (**A**) and a natural cleavage ribozyme of hepatitis-δ-virus (**B**)

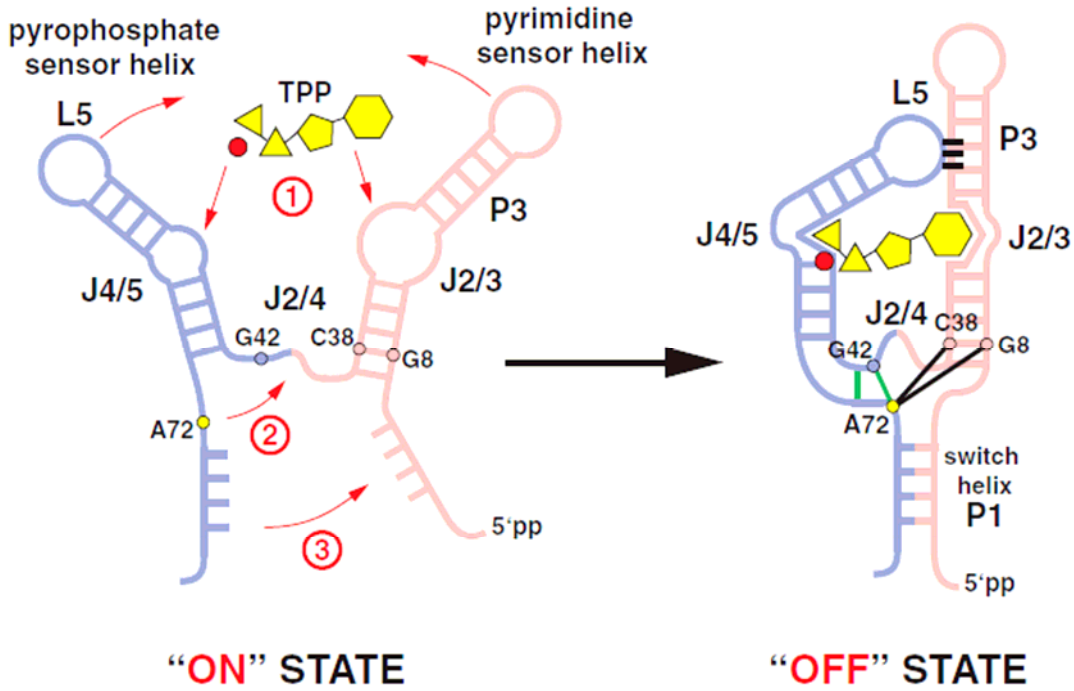
The sequence at the *intersection*:

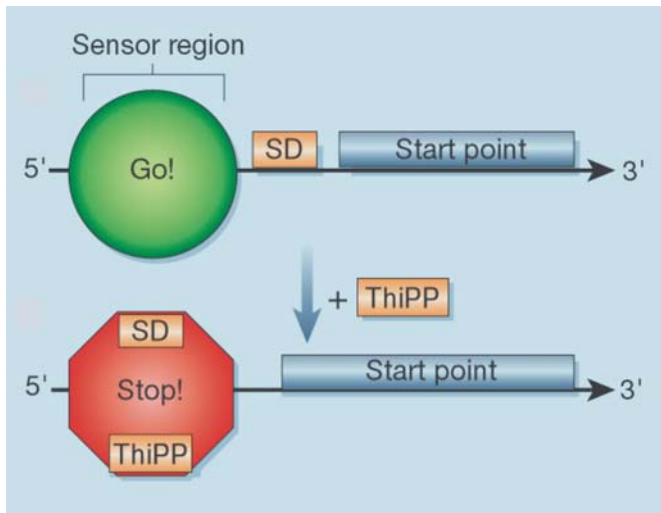
An RNA molecule which is 88 nucleotides long and can form both structures.





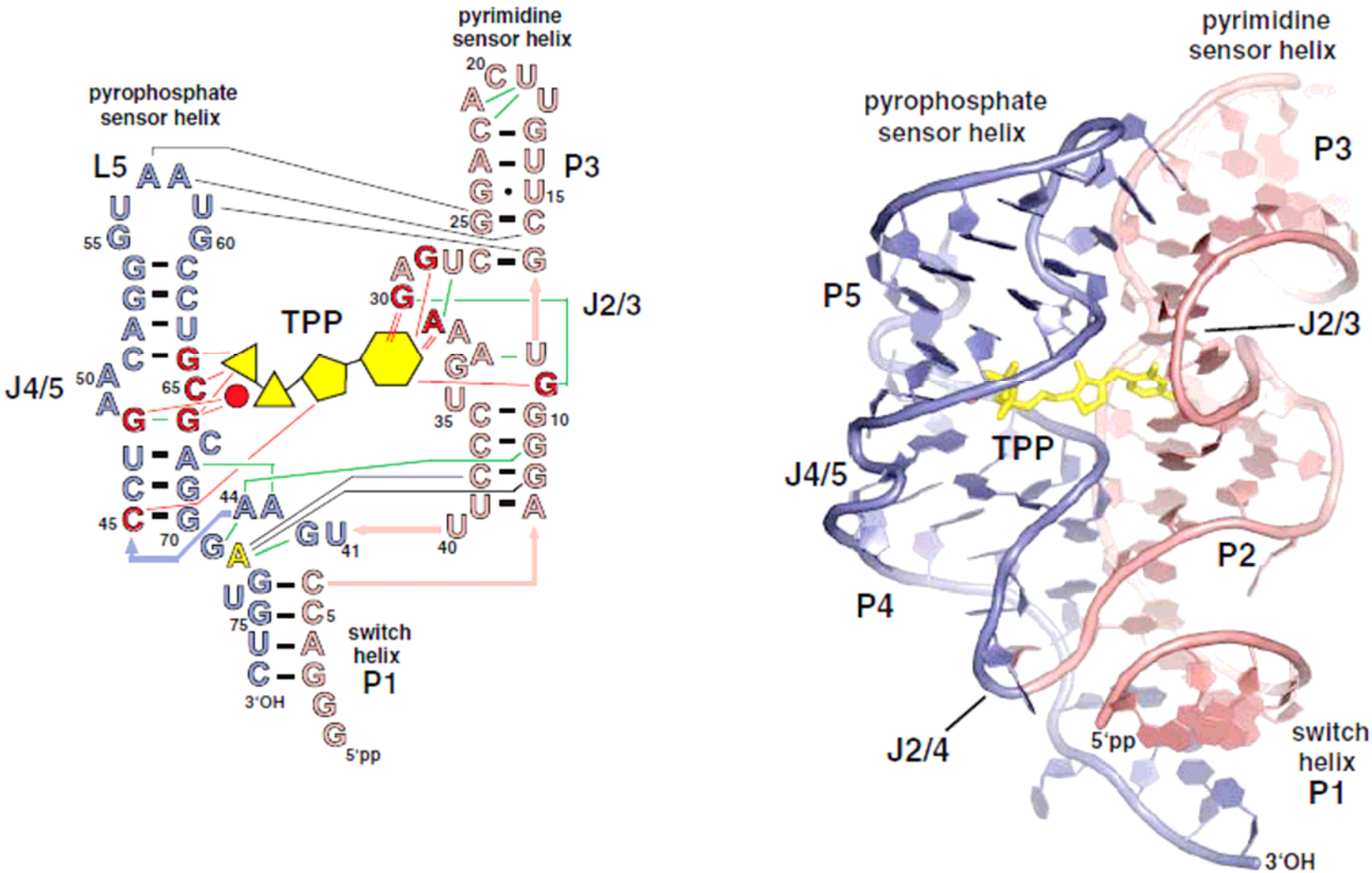
Two neutral walks through sequence space with conservation of structure and catalytic activity





The thiamine-pyrophosphate riboswitch

S. Thore, M. Leibundgut, N. Ban.
Science **312**:1208-1211, 2006.



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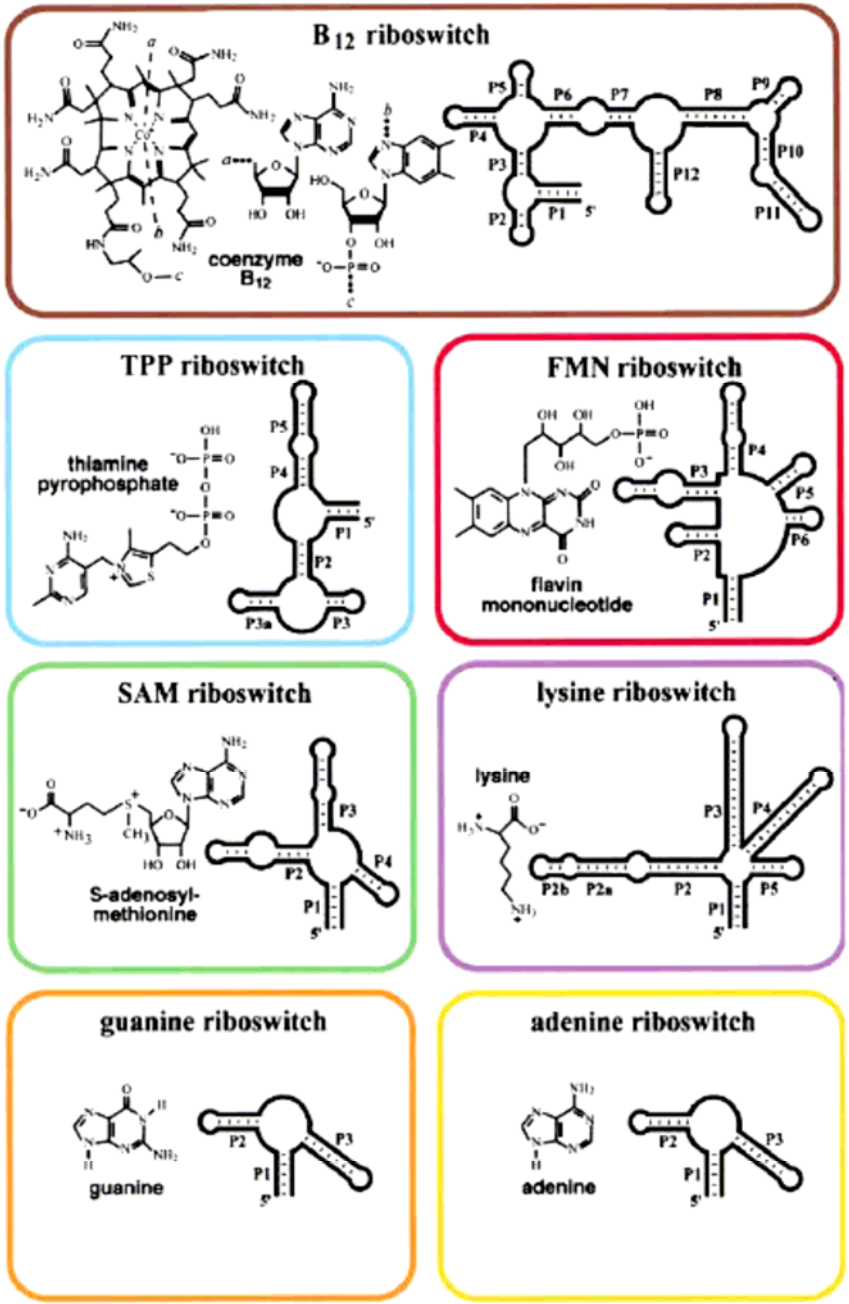

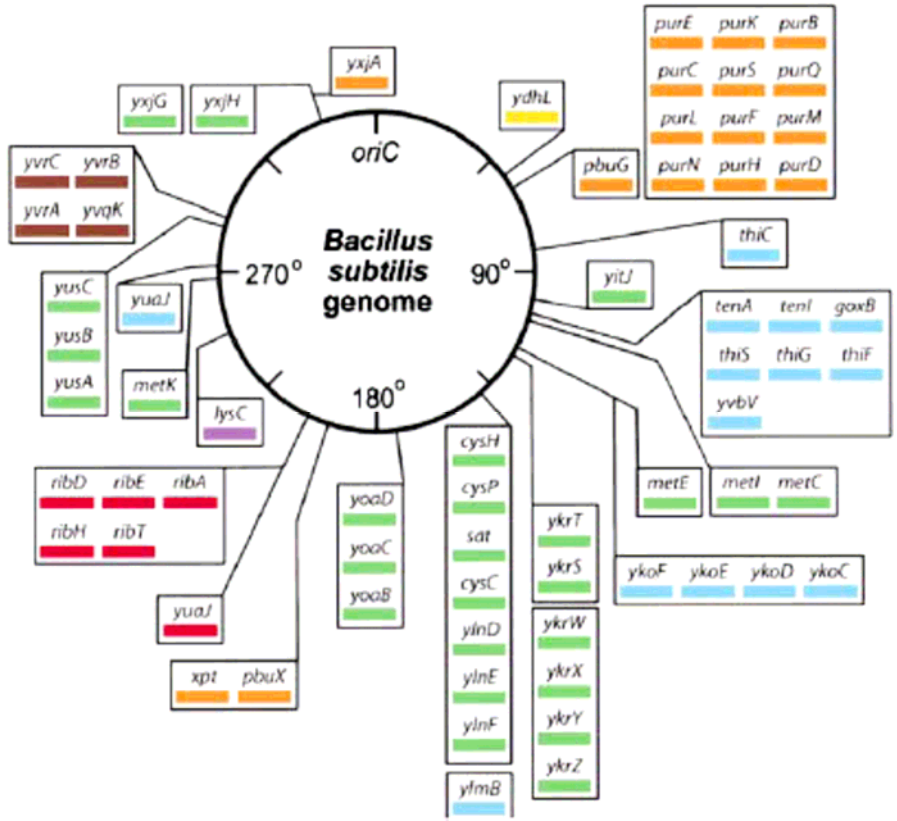

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