

Hypothesis

A positive selection function for microRNA: an adaptor hypothesis revisited



Dr Pieczenik is a notable graduate of Andover Academy, and studied at Harvard University. He worked with Fred Sanger, Francis Crick, Sydney Brenner, Bruce Merrifield, Norton Zinder and other early founders of molecular biology at the MRC Laboratory of Molecular Biology in Cambridge, UK and the Rockefeller Institute in New York, USA. He is considered the father of combinatorial chemistry and made the first combinatorial libraries at the MRC Laboratory of Molecular Biology. For the past several years, Dr Pieczenik has been involved, with his co-authors and others, in studies related to whole-cell screening of gametes and early embryos. This work has led to several intriguing findings some of which are presented in the current manuscript.

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Abstract

A hypothesis for coding different combinations of mRNA segments by a novel postulated function of microRNAs (miRNA) is presented. In this hypothesis, miRNA act as both coding adaptors and ligating enzymes. This hypothesis postulates an enzyme–substrate intermediate comprising a RNA triplex structure consisting of a miRNA adaptor and two mRNA configured as two turns of an A-form RNA helix. Postulating the existence of differentially expressed sets of adaptors (codes) may explain biological functions such as imprinting, differentiation, immunological escape and immunological tolerance, *inter alia*.

Keywords: adaptor, combinatorial, hypothesis, ligase, miRNA, tRNA

Introduction

In this paper, a model of mRNA ligation by microRNA (miRNA) acting as coding adaptor and enzyme is postulated. This hypothesis requires a triplex RNA structure for the enzyme–substrate intermediate complex. The concept of a triplex RNA structure has an antecedent analogous structural basis in a protein translation model originally proposed by Pieczenik (1980) and Pieczenik and Crick (Crick *et al.*, 1976).

Hypothesis

Pieczenik (1980) proposed a mechanism for genotypic selection and the survival of ‘fitness’ of transfer RNA (tRNA)–mRNA interactions, and Pieczenik and Crick (Crick *et al.*, 1976) proposed a model for the origin of the primordial protein synthesis machinery that allowed for two tRNA anticodon loops to bind to a mRNA, forming an A-form double-stranded RNA helix with three interacting RNA molecules – a triplex interaction.

stranded RNA helix. In this structure, the mRNA is bound by two anticodon loops (tRNA1, tRNA2). Each anticodon loop has five bases interacting with the mRNA. The anticodon loops are in one of two possible configurations, the FH (Fuller–Hodgson) and hf configuration, each with five bases stacked (**Figure 1A1**). The mechanism of translation requires that hf configuration tRNA2 loop flips into the FH configuration by bringing up two nucleotides on the 3' end and bringing down two nucleotides on the 5' end (**Figure 1B**) This flip mechanism, thereby displaces the adjacent FH tRNA1 anticodon loop. After this flip, the tRNA2 is now in a similar configuration to the tRNA1's previous configuration, but now the translational structure has been moved by three nucleotides. This mRNA decoding machinery requires sequence constraints on both the mRNA sequence and the tRNA sequences. The constraint on mRNA sequences is a purine, N, pyrimidine bias, where N is any of the four possible nucleotides. The sequence constraint on tRNA anticodon loops is a uracil 5' to the anticodon and a purine 3' to the anticodon. These constraints are preserved and exist in known tRNA and mRNA, suggesting the co-evolution of mRNA with their respective tRNAs and continuous genotypic selection for this postulated interaction (Pieczenik, 1980). These sequence constraints on tRNA and mRNA create

Figure 1A2 shows a triplex RNA interaction in a stable double-

a structural comma-free decoding. Even though there are five bases interacting at any time, it is a triplet code. The tRNA translation moves along three nucleotides at a time because the flip moves two nucleotides down and two nucleotides up. ($7 - 2 = 5 - 2 = 3$). These constraints also create an interacting triplex RNA complex. In this model, the messenger RNA holds together two separate tRNA anticodon loops in a stable RNA helix. This triplex interaction has two half turns of two tRNA anticodon loops forming a full turn of a double stranded RNA helix (**Figure 1A2**).

Previously, Crick *et al.* (1957) had proposed a comma-free code that required an adaptor hypothesis to explain certain observations that there were no dipeptide amino acid constraints on known protein sequences. Direct chemical interactions between amino acids and nucleic acids would create an overlapping coding constraint and consequently a strong dipeptide constraint on protein sequences. This comma-free code, which Crick and colleagues proposed, turned out to be completely wrong.

However, the requirement of an adaptor for such a code turned out to be correct, in that adaptors do exist as tRNA.

Without the concept of an adaptor function for tRNA, tRNA function could not have been identified from its sequence or its structure (Crick *et al.*, 1957). The crystal structure shows the anticodon has five bases stacked in the anticodon (Hingerty *et al.*, 1978; Pieczenik, 1980), and not three bases which would be expected from a molecule that decodes a triplet code or structurally follows a 'wobble' decoding (Crick, 1966). The crystal structure of tRNA appeared to be an artefact of crystallization because it showed five bases stacked in the FH configuration in the anticodon loop. It did not have three bases stacked and exposed as was expected for a structural explanation of a triplet code.

MicroRNA (miRNA) are a class of RNA that are 21 to 22 nucleotides long, believed to be involved as their primary function in inhibiting translation of mRNA and also involved in cleavage reactions involving mRNA. Over 3000 miRNA have been sequenced (<http://microrna.sanger.ac.uk/sequences/index.shtml>). Unique miRNA have been identified in human embryonic stem cells (Suh *et al.*, 2004), suggesting the differential expression of classes of miRNA creating different coding combinations. While miRNA are involved in cleavage reactions, more commonly in plants, they do not have such function in animals. This differential suggests an alternative function for miRNA not directly related to cleavage.

However, the conceptual understanding of miRNA function is in a very similar situation to the lack of understanding of tRNA function prior to the introduction of Crick's adaptor hypothesis. Decoding mRNA required the introduction of a concept of an intermediate adaptor where one side of the molecule interacted with the mRNA and the other side was involved in bringing amino acids to a location that allowed them to be ligated together in a protein-synthesizing mechanism.

In this paper, a coding adaptor function for miRNA, with a mechanism that requires RNA-mediated ligation is proposed. RNA self-ligation has been previously observed (Buzayun *et al.*, 1986; Sharmeen *et al.*, 1989).

Biochemical functional constraints

As biochemical reactions are reversible, and as RNAs are known to self-cleave and to act as catalysts, i.e. ribozymes, it is formally possible that RNA can self-ligate and also act as a template-enzyme to ligate other RNAs.

Two characteristics of miRNA lend themselves to a ligation mechanism. One is their unique size and, the second is the additional stacking energy of binding that a turn of an A-form double-stranded RNA helix contributes.

Almost all miRNA are 22 nucleotides in length. This is exactly two turns of an A-form RNA double-stranded helix. RNA and A-form DNA both have 11 base pairs per turn of their helix, with a pitch of 30 Å. A complete turn of the helix of 11 base pairs of RNA is a stable structure in that stacking forces are combined with hydrogen-binding forces.

The model proposed suggests that for the miRNA to act as a catalyst it can bring together two mRNA in sufficiently close proximity to allow two water molecules both to cleave mRNA and to allow one of these water molecules to be removed to ligate the cleaved pieces. One can presume that cyclic 2' to 3' intermediates will occur in this reaction (Renz *et al.*, 1971).

Two turns of a helix, where each turn binds to a different RNA, will naturally bring these two different RNAs together in close proximity, forming an RNA triplex interaction with an A-form double-stranded helical structure.

Figure 2 shows how this reactive proximity can be accomplished. The first 11 bases in the 3' to 5' direction of the miRNA base

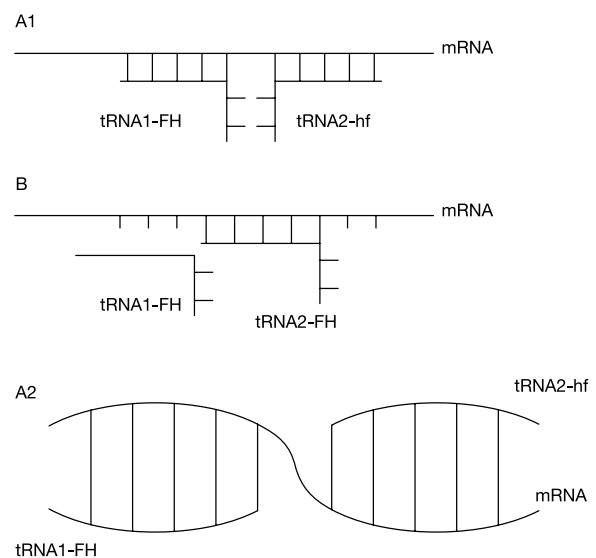


Figure 1. (A1) Transfer RNA (tRNA) anticodons in alternate configurations (FH and hf) binding to messenger RNA (mRNA) in a triplex configuration. (B) tRNA2 flipping from hf configuration to FH configuration and displacing tRNA1. (C) Two tRNA anticodons base pairing to mRNA with five bases each forming a single turn of the helix.

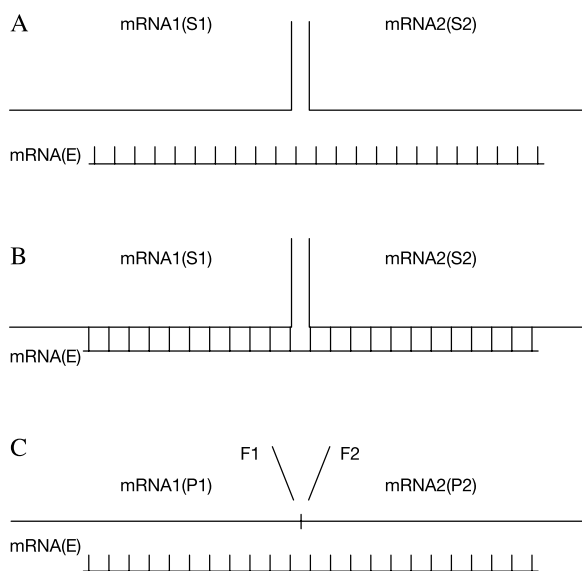


Figure 2. (A) mRNA1 and mRNA2 and miRNA [miRNA + (mRNA1 + mRNA2)]; E = enzyme. (B) mRNA1 and mRNA2 base pairing to miRNA with 11 bases each forming the enzyme–substrate intermediate [miRNA(mRNA1+mRNA2)]. (C) miRNA + (P1 + P2) [mRNA1 (cleavage product) ligated to mRNA2 (cleavage product)] and F1 fragment from mRNA1 and F2 fragment form mRNA2.

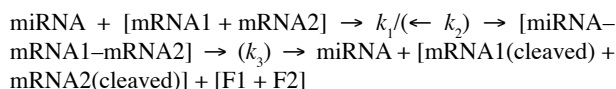
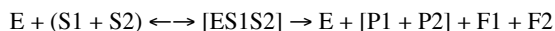
pairs with 11 antiparallel complementary bases of mRNA1 in the 5' to 3' direction and the second 11 bases of the miRNA in the 3' to 5' base pairs with 11 antiparallel complementary bases of mRNA2 in the 5' to 3' direction (**Figure 2A**). This creates the transition state intermediate [(miRNA)(mRNA1–mRNA2)] (**Figure 2B**). This transition state will allow the two mRNA to ligate at the junction enzymatically determined by the miRNA sequence, thereby creating a novel RNA that is a new combination of segments of the two mRNA. This reaction then goes to release the enzyme, i.e. miRNA, and a new longer RNA composed of P1 and P2 (P1 + P2), the cleavage products which are now ligated together (**Figure 2C**).

The numbers of combinations allowable are determined by the number of miRNA adaptors in each cell. This addresses the issue of how so few coding sequences i.e. 19,000–30,000 can code for over a million proteins. Combinatorial solutions determined by adaptors, such as the class of miRNA in each cell, will allow for novel combinatorial ligations between mRNA and mRNA precursors that will allow for novel protein combinations using similar coding sequences. This can also explain species and cellular differences of expression using the same initial limited number of coding sequences.

While this hypothesis is speculative, it formally suggests that deconvoluting the coding structure will require analysis of the relationship between miRNA coding sequences and the constraints they can impose on creating and limiting combinatorial possibilities of the genome's transcribed

sequences. While a present positive selection mechanism imposing constraints is postulated, these constraints can also be viewed as historical constraints from an evolutionary history for a similar primordial mechanism.

Figure 2 also shows several steps in the proposed model for miRNA-directed RNA ligation of two separate mRNA sequences. The enzymatic equation describing this reaction can for a first order approximation follow Michaelis–Menton enzyme kinetics:



where E is the enzyme, in this case miRNA; S is the substrate, in this case two separate mRNA sequences with regions complementary to miRNA; (\leftrightarrow) represents the forward and back reaction rate constants, k_1 , k_2 for the triplex complex and (\rightarrow) represents the forward rate constant, k_3 , for the ligation reaction, and P is the ligated RNA composed of sections of mRNA1 and mRNA2. P is composed of P1 the cleaved product of mRNA1 which is ligated to P2 the cleaved product of mRNA2. (E + S1 + S2) is [(miRNA) + [mRNA1 + mRNA2]] as in **Figure 2A**. The transition state [ES1S2] is [(miRNA)(mRNA1–mRNA2)] as in **Figure 2B**. The reaction product and enzyme ([E] + [P1 + P2] + F1 + F2) is [(miRNA) + [P1 + P2] + [F1] + [F2]] as in **Figure 2C**. In **Figure 2C**, [P1 + P2] is the new ligated mRNA composed of [mRNA1 (cleaved) + mRNA2 (cleaved)] and [F1] + [F2] are the fragments remaining after cleavage from mRNA1 and mRNA2, respectively. The approximate first-order Michaelis–Menton miRNA enzyme kinetic equation can then be written as:

$$V_o = (V_{\max} [\text{mRNA1} + \text{mRNA2}] / (K_{\text{mi}} + [\text{mRNA1} + \text{mRNA2}]))$$

where V_o is the initial velocity of this ligation reaction, V_{\max} is the maximum velocity of the ligation reaction, with a K_{mi} , the miRNA constant for this ligation, equal to $(k_2 + k_3) / k_1$, and [mRNA1 + mRNA2] are the concentrations of the two mRNA to be ligated by miRNA.

Testing this model: biochemical tests

This model can be tested by creating miRNA-dependent mRNA ligation and consequent protein-synthesizing systems dependent on this ligated mRNA. This is biochemically difficult.

An initial simpler chemical experiment would be to synthesize two small artificial mRNA sequences exactly complementary to each of the two 11-base sequences known in a miRNA sequence. 5'-end-labelling with [γ - ^{32}P] ATP with polynucleotide kinase of either of the small mRNA will allow identifying a labelled final larger product dependent on miRNA's ability to ligate.

Engineered genetic tests

Designing organisms with two reporter molecules, using for example, green and red fluorescent protein coding, inserted at the 5' and 3' ends of mRNAs which have sequences complementary to miRNA can be used to identify whether these ligations actually occur *in vivo*. Novel synthetic miRNA sequences creating new combinations that are inserted in the genome might also define if these reactions occur *in vivo* and suggest novel mechanisms to change, 'cure' improper combinations and/or create new coding combinations.

Sequence constraints of this model

This model suggests sequence constraints in the genome. Therefore, firstly, one can identify regions in the genome that are antiparallel complements of half of each miRNA. Secondly, one can identify known protein sequences which correspond to the predicted coding from antiparallel complementary sequences to miRNA. These sequences would be in the range of seven amino acids.

Sequences of amino acids that are approximately seven amino acids long coded by the antiparallel complement of miRNA should appear in higher frequency than expected from equally frequent random amino acid distributions, if this mechanism proposed is commonly used. The frequency of this class of seven-amino-acid sequences should occur in a higher frequency than $1/20^7$ or $1/1.28$ billion of all possible seven-amino-acid-long sequences. If one considers the *gedunken* (or 'thought') experiment that all three billion nucleotides of the genome are coding, then there would be an expectation of a protein 1 billion amino acids long. The chance of finding any given seven-amino-acid sequence is 1 in 20^7 or 1 in 1.28 billion. This suggests that one should expect only one such antiparallel complementary miRNA coding sequence in the genome's coding. An additional constraint is that less than 4% of the genome is coding and, therefore, the random expectation is that any given seven-amino-acid sequence would be found should be much less than once in the proteome.

What is found is that sequences that are coded by antiparallel complements of miRNA, which is what would be created in the new ligated message, do appear much more frequently than once in the protein data base of known protein sequences.

For example, miRNA let-7 (6) in the 5' to 3' has one open reading frame and three open reading frames in its antiparallel complement in the 5' to 3' direction. The antiparallel complement would correspond to coding sequences which would appear in mRNA which are ligated with the mechanism postulated. As one example, the coding sequences for the three phases of the antiparallel complement of let-7, (i) Asn.Tyr.Thr.Thr.Tyr.Tyr.Leu; (ii) Thr. Ile. Gln. Pro.Thr.Thr.Ser; and (iii) Leu.Tyr.Asn. Leu.Leu.Pro.His/Gln are found in several proteins e.g. splicing factor U2Af, bromodomain-containing protein (stimulates transcription activity), testis-determining factor, coiled-coil domain-containing protein 3 precursor, DNA-directed RNA polymerase I subunit 2, *inter alia*. This study used the small protein-protein (blastp) sequence search rather than nucleotide-nucleotide (blastn) search which will not identify 11-nucleotide length matches. Expressed sequence tags (EST) are being

examined for their relationship to this model and whether they are 'valid' coding sequences under these constraints.

In addition, if this model is correct, the genome can be deconvoluted into regions bounded by regions of antiparallel complementarity to miRNA sequences, which would in turn correspond to combinatorial coding of proteins. For example, if any of the human 19,000–30,000 possible coding sequences in the human genome (Petsko, 2001) could be combined with any other of these coding sequences, then, there are a possible 361–900 million coding sequences i.e. $19,000^2$ – $30,000^2$.

If it is postulated that only those coding sequences using miRNA as template adaptors are functionally viable, then these 361–900 million possible coding sequences are limited. For example, in humans there are 321 miRNA which would allow only $1/321$ of the 361–900 million possible combinations. Therefore, approximately 1.1–2.8 million possible human proteins are coded with this additional miRNA adaptor coding constraint. This number is in the range of known protein sequences.

This is another example, of a genotypic selectionist constraint on possible mRNA sequences. The 'classical' Crick central dogma, where DNA is a tape machine instructing replication, transcription, processing and translation, has to be modified into a selectionist framework where there is direct positive and negative selection on DNA and RNA for their ability to be replicated, transcribed, processed and translated. This selection is called genotypic selection (Pieczenik, 1980). It is a consequence of combinatorial variability at the DNA, RNA and protein level. That variability is acted upon and selected by the replication machinery, the transcription machinery, the processing machinery (of which this model is now an addition), and the translation machinery (of which the tRNA–mRNA model presented by Crick *et al.* (1976) and Pieczenik (1980) is another). Evidence for the nature of this direct selection on nucleic acids can be seen in the relationship between this postulated positive selection ligating function of miRNA and the known negative selection function of 'silencing' unwanted mRNA.

Evolutionary relationship and functional relationship between ligation and cleavage

The evolutionary relationship between the adaptor ligation function and the selective 'silencing' cleavage function of miRNA is one where there is positive selection for sequence combinations of single turns of an RNA helix complementary to miRNA, and negative selection for improper sequence combinations that are complementary to miRNA, which form two turns of an RNA helix. The negative selection is effected by an RNase III-type enzyme function, either by RNase III proper or Dicer. The positive selection is effected by miRNA acting as a ligating-directing enzyme.

Once a mechanism evolves to ligate two RNA molecules using two turns of an A-form RNA helix in a triplex enzyme-substrate intermediate, then it is evolutionarily adaptive to eliminate any individual sequence that can compete by forming an A-form double helix with two turns

of the helix. These 22-nucleotide-long double-stranded RNA sequences would interfere with the triplex enzyme–substrate intermediate. They would necessarily need to be removed if combinatorial ligation is the preferred positive selection. Therefore, any double-stranded two-turn A-form helical RNA structure would be selectively cleaved, by any endonuclease with RNase III-like function. This suggests that RNase III can measure off a 60 Å pitch of an A-form double helix but not a 30 Å pitch of an A-form double helix. A crystal structure determination of RNase III complexed with double-stranded RNA would confirm this prediction.

Figure 3A, B and C shows what happens if, in the triplex enzyme–substrate interaction (**Figure 3A**), mRNA2 is complementary to two turns of the helix rather than just one turn of the helix. One turn would be required for ligation to mRNA1. If the sequence constraint creates a second turn of the helix, then mRNA1 is displaced (**Figure 3B**), and a duplex with two turns of an A-form double-stranded RNA is formed (**Figure 3C**). This structure is susceptible to cleavage by RNase III-type enzymes. Therefore, RNase III-type enzymes exist for a strong negative intracellular genotypic selection (Pieczenik, 1980) against this duplex structure. **Figure 3C** shows this ‘silencing’ of mRNA2 by cleavage elimination which has its own adaptive functions. Many sequences are called but few are chosen by miRNA adapter enzymes. The rest are eliminated by RNase III.

In a classic 1968 paper, Robertson and coworkers (Robertson *et al.*, 1968) emphasized the specificity of RNase III for the A-form double-stranded RNA. They said ‘RNA is more closely related to the A helix than to the B helix of DNA. The A lattice of DNA was first described by Franklin and Gosling as occurring at low relative humidities, in contrast to the B lattice normally found at high relative humidities. Since all the natural substrates for RNase III may be in such A-type helices, it is possible that part of the specificity of RNase III resides in its ability to recognize structures similar to the A helix’. This insight was very prophetic. While RNase III cuts down to smaller than two turns of the helix, Dicer (Zamore *et al.*, 2000) seems to preserve this length constraint.

Therefore, the functional relationship between the triplex enzyme–substrate intermediate structure required for ligation, and the duplex structure required for cleavage is simply a flip of one mRNA turn of the helix, displacing another mRNA (**Figure 3A, B and C**). This is analogous to the translation mechanism postulated and discussed previously where the flip of a tRNA displaces another tRNA by five nucleotides, a half turn of the helix (**Figure 1A, B and C**).

Sequence constraints on miRNA

In addition to structural constraints, there are sequence constraints on miRNA. At the junction between the two A-form helices, in many of the miRNA, there are the characteristic splicing recognition sequences (AG/GU). For example, in human let-7A, 7B, 7C, 7D, and 7E miRNAs, the characteristic splicing sequences AG/GU are at positions 11–12, exactly in the middle, (<http://microrna.sanger.ac.uk/sequences/index.shtml>), which would suggest a conserved sequence recognition constraint for cleavage exactly between the structural constraint

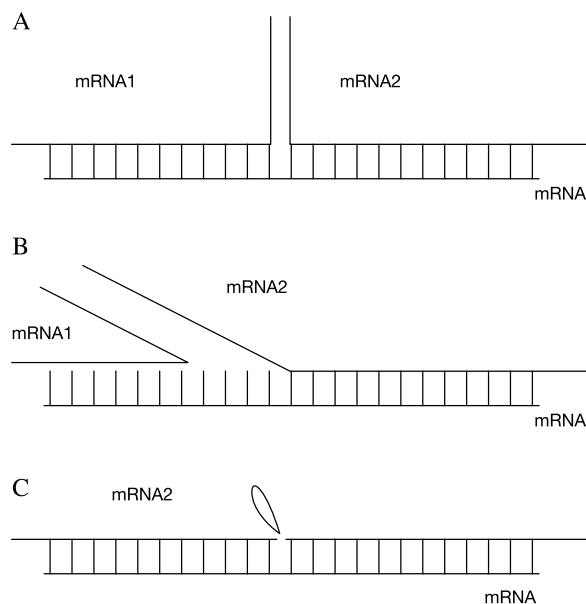


Figure 3. (A) Triplex enzyme substrate intermediate [miRNA + (mRNA1+mRNA2)]. (B) mRNA2 displacing mRNA1 with an additional 11-base pair complementary to miRNA. (C) Double-stranded A-form RNA of miRNA and mRNA2 susceptible to cleavage by RNase III.

of where two RNA turns form. In these cases, the sequence constraint and the structural constraint are synergistic.

These adaptors allow one to deconvolute the genome into its proper expression modalities as a ‘code’ prior to their translation by the genetic code. While the new novel combinations are large, they are discrete and not infinite in number. These miRNA adaptors thereby limit random combinations of coding sequences to a miRNA ‘coded’ set. This set can be variable, and more extensive, if one allows for G–U base pairs.

Pieczenik (2003) postulated an equivalence principle between the discrete-sized universes of antibodies and antigens. This hypothesis and miRNA adaptors can explain immunological escape by tumours and pathogens such as *Leishmania tarentolae* (which have their own class of analogous guide RNA (gRNA)) by the differential expression of different classes of positively and negatively functioning RNA adaptors at different times or locations. Immunological tolerance can be explained by a differential temporal and spatial expression of different classes of coding adaptors and mRNA sequence modifiers.

In conclusion, it is proposed that miRNAs act as template adaptors to determine novel combinatorial possibilities of sections of mRNA, and as enzymes to ligate these combinations. It is another example of genotypic selection (Pieczenik, 1980), where there is intracellular positive selection, using miRNA as adaptors and enzymes for combinatorial adaptive mRNA from a large combination of non-adaptive sequences, which are ‘silenced’ by negative selection, using RNase III-type enzymes.

Acknowledgements

This paper is dedicated to the memories of Hugh Robertson, the discoverer of RNase III, a friend and colleague who recently suddenly passed away and to my brilliant colleague, Francis HC Crick, who also passed away this year.

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