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One, Two, Infinity: Genomes Filled with Aptamers

Matters Arising

One, Two

Individual cells limit production of unnecessary metabolic capacities by monitoring their immediate environment and regulating gene expression appropriately. This presumably results in greater efficiency and energy utilization while allowing rapid response to environmental changes. Cells respond to changes in individual metabolites such as carbon and nitrogen sources or vitamins by regulating expression of genes necessary for synthesis or utilization of the specific metabolite. Curiously, although the ultimate chemical signal to which cells respond is the metabolite itself, until now the regulatory factotum usually has been a protein.

Ron Breaker and his colleagues [1, 2] have published two papers that demonstrate regulation of translation through direct interaction of a small molecule metabolite with mRNAs encoding proteins necessary for their uptake or utilization. In the first paper, the btuB leader sequence, 5′ to the translational initiation domain for btuB, interacts directly with coenzyme B_{12} in such a way as to cause structural changes to the RNA that sequester the translational initiation domain from ribosome inspection. The btuB gene encodes the receptor by which B_{12} is transported into the cell. Hence, the impact of this regulatory loop is to reduce synthesis of the transporter when intracellular B_{12} levels are sufficient. In the second paper, the 5′ leaders of thiM and thiC mRNAs are shown to bind directly to thiamine (vitamin B_1), again leading to an mRNA structural alteration such that the biosynthetic enzymes are depressed (translationally) when the intracellular thiamine levels are sufficient. The mechanisms for the translational repression are not known with certainty, but one can deduce the most likely scenarios. In *E. coli*, translation is initiated by a ribosomal interaction with the Shine and Dalgarno region/initiation codon of an mRNA as well as initiator tRNA and initiation factors [3, 4]. The easiest way to currently understand the two B-vitamin regulatory mechanisms described by Breaker's group is to imagine an mRNA with two alternative structures, open and closed, in equilibrium. The open structure allows translational initiation, while the closed hides the Shine and Dalgarno region from the ribosome (using different intramolecular base pairing). If the metabolite is preferentially recognized by the closed structure, thus altering the equilibrium, translation initiation will be lowered (Figure 1). Exactly the same principles can alter other mRNA (and pre-mRNA) functions (to be discussed later; Figures 1 and 4). These two systems described by Breaker represent a novel appreciation of the power of mRNA functional capacities, leading us to wonder (once again [5, 6]) how far such RNA magic may go.

The authors interpret their data in the glow of work by Harold B. White III [7]. White observed more than 20 years ago that many of the present nucleotide cofactors might have been among the nucleotides present in ancient RNA or DNA, thus providing oligonucleotides with both coding and catalytic capacities. He proposed, explicitly, that as the protein world emerged and became the modern basis of catalysis, a contemporaneous partitioning of function could have left cofactors free of oligonucleotides (to function as aids to the proteins that needed them for catalysis) and left oligonucleotides free to encode proteins. White predicted an earlier world in which "shapes and tapes" were found in the same ancient molecules, and that division of labor led to a simpler code (only four simple nucleotides) and better catalysts (proteins with cofactors, which were free to evolve or not, but were no longer bound by the rules for oligonucleotides). White had a substantial vision into what we now call the "RNA world," although he did not claim the idea as conclusively as did Gilbert [8]. Because these two examples in *E. coli* of aptamer activity within an mRNA are in the context of expression systems that participate in the synthesis of cofactors, Breaker interprets his findings as a reflection of the world that was. We love Harold B. White's and Ron Breaker's work, yet we wonder if remnants are less important to this discussion than continuous selection for coordinated regulation over the last several billion years, well after Harold B. White's cofactors had left their previous oligonucleotide homes.

Infinity

A more dramatic extrapolation of the findings would be to wonder if these two examples in *E. coli* reflect a rich world of regulatory phenomena involving RNAs expressing their "shape" coincidentally with their "tape"—that is, can we wonder if genomes encode large numbers of aptamers to alter metabolic pathways? Explicitly, we wish to consider the evolutionary pressures for a variety of aptamers that do exactly what Breaker has found, and to consider as well the organisms in which we are likely to find the phenomenon most frequently. We will conclude that these regulatory phenomena are even more important in the eukaryotes.

Aptamer Review 101

Let us first spend a few moments reviewing the SELEX protocol [9, 10] through which nonnatural aptamers are identified (Figure 2). Double-stranded DNA with a randomized sequence region is prepared chemically. One strand is synthesized with flanking fixed regions (for PCR or other amplification), and the complementary stand is prepared enzymatically. If the synthetic DNA is prepared on a 10 μmol scale, and 1 μmol of final product is obtained, the starting library may contain about 6×10^{17} unique sequences (when the random region is longer than about 30 nucleotides). The double-stranded *sequence* library (tape) is converted enzymatically to a single-stranded (DNA, RNA, or modified oligonucleotide) *shape* library from which shapes with desired properties are selected. Because the flanking sequences allow amplification of culled "winners," one can perform multiple rounds of enrichment, and eventually most targets yield an aptamer. In many ways, the SELEX protocol is merely the single-stranded oligonucleotide version of phage, ribosome, or mRNA display for peptides and larger proteins [11–14]. The primary difference is that

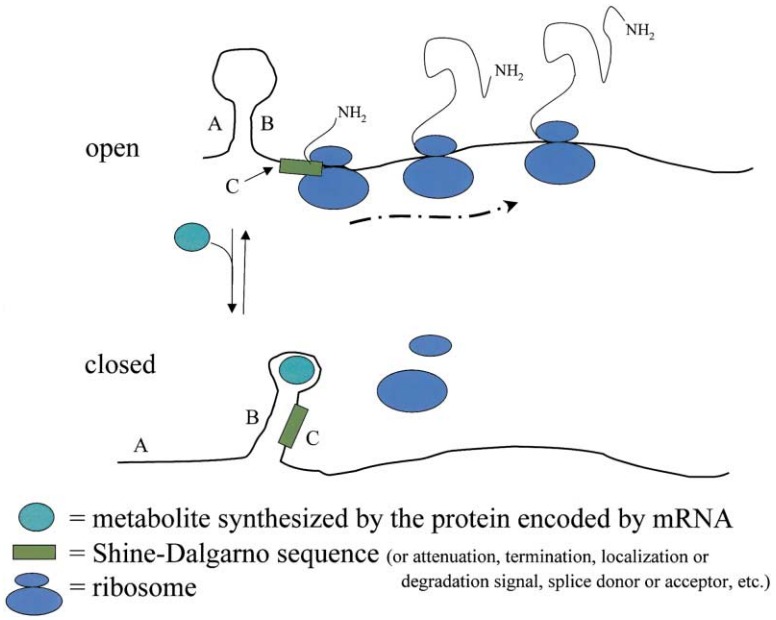

Figure 1. Breaker's Metabolic Feedback Regulation of Translation and a Generalization to Other mRNA Regulatory Pathways

Sequences A and C are complementary to sequence B. When the structure stabilized by A-B pairing is formed, the translation initiation sequences are available to the ribosome and translation proceeds. In the presence of metabolite, the structure stabilized by B-C pairing is favored, and the initiation region is unavailable to the ribosome.

Substituting other regulator sequences for the Shine-Dalgarno translation initiation sequence in this example allows the identical mechanism to moderate accessibility of other complexes necessary to effect processes such as splicing, termination, etc.

the identified aptamer is an oligonucleotide rather than a protein. Bruce Eaton has said repeatedly in his seminars that RNA/aptamer scientists must suffer from "side group envy" (B. Eaton, personal communication).

The length of the random regions in classical SELEX experiments is small [15–18]. At NeXstar, we focused on finding aptamers for therapeutics that could be synthesized at reasonable cost, and tended to use 30 to 40 random positions in a starting SELEX library. This was a compromise: longer single-stranded oligonucleotides must provide more shape opportunities than shorter oligonucleotides, but we knew that the cost of aptamer drugs would be high and a function of their length. Some SELEX experiments were performed with libraries containing up to 200 randomized positions, even though no aptamer derived from a long randomized region ever requires all or even most of the nucleotides of that aptamer to do the binding. In fact, no strong published data show that smaller randomized regions would have failed to yield an equivalent aptamer. The best short aptamer ever identified for a protein target may be the vascular endothelial growth factor (VEGF) aptamer that is presently in its pivotal clinical trial for macular degeneration; that molecule, now called Macugen by EyeTech Pharmaceuticals (http://www.eyetk.com), is but 28 nucleotides long, has an approximately 50 pM Kd, binds to VEGF with high specificity, and prevents VEGF from binding to its receptors [19].

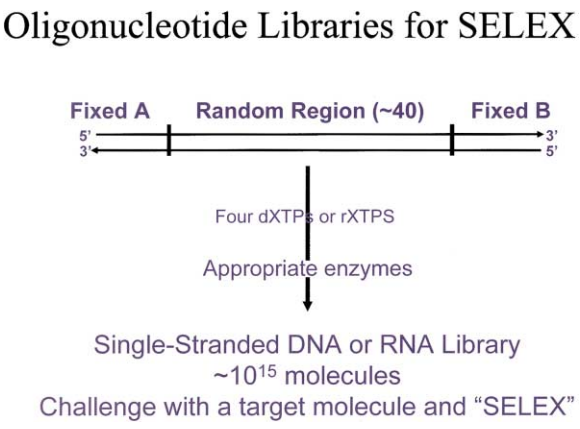

Figure 2. The Classic SELEX Protocol, Leading to Selection of Aptamers

It is true that most aptamers have been selected from very large starting libraries of oligonucleotide sequences, substantially larger than the sequence space available in modern genomes (SELEX starting libraries can be as large as 10^{15} unique molecules, about 10^5 times more than the human genome.). However, SELEX experiments are done in a week with 5 to 15 rounds of selection, while evolution and selection in biology had a billion years with perhaps a "round" of selection every day; the point is that 10^{11} divisions during unicellular growth can more than make up, via mutation, for a smaller starting library (the genome).

We know a great deal today about aptamers that have been selected against protein targets and also against small molecule (metabolite) targets [20, 21]. RNA aptamers have Kds between 10 nM and 10 pM for proteins (which are present usually in cells at concentrations between μM and nM), while aptamer Kds are between 10 μM and 10 nM for small molecules (which are present usually in cells at concentrations far higher than protein concentrations, perhaps mM to μM). Aptamers that have been selected in vitro seem ideally suited for interacting with proteins or small molecules at their physiological concentrations. This leads to wondering whether aptamer selection occurs frequently in vivo, leading to flagrant use of aptamers in all creatures.

Genomes Filled with Aptamers: Background

The first *natural* aptamer discovered in biology probably was the lac operator, although that "aptamer" consisted of double-stranded DNA. However, the large number of DNA sites to which specific proteins bind does reflect

our first principle: evolution can demand and get tight and specific binding between genomic nucleic acid sequences and proteins.

The second class of *natural* single-stranded RNA aptamers (and the first sites that really were aptamers) were discovered in the small *E. coli* RNA phages MS2, R17, and Qβ [22–24]. These phage face serious regulatory problems (such as clearing the input RNA genome of ribosomes before replication of the plus strand [to make a minus strand] results in a lethal collision between two immovable objects), and the phage all evolved wonderful binding sites comprising folded RNAs that allowed tight and specific binding of proteins; these sites are natural aptamers. Oddly, these fascinating viral RNA structures often were seen as the best these poor creatures could do because they had no chance to use transcriptional regulation. (People actually said in the 1970s and 1980s [in print] that translational regulation would not occur in an organism that had a chance to regulate gene expression transcriptionally.) Translational regulation was discovered in the bacteriophage T4 (which has a real DNA genome): T4 gene 32 protein regulates its own translation, T4 gene 43 protein regulates its own translation, and T4 regA protein regulates the translation of twenty or so mRNAs [25–28]. The sites responsible for translational regulation were called "translational operators" (a phrase chosen from transcription envy), *but these sites are natural aptamers*. A similar set of natural aptamers has been identified in *E. coli*: several ribosomal proteins were found to regulate their own translation by binding to the ribosome binding sites on their cognate mRNAs. Usually the proteins that regulated their own synthesis were proteins with binding sites for specific sites in ribosomal RNA, and thus the mRNA aptamers were thought to be mere mimics of that functional binding site [29, 30]. Nevertheless, one saw creeping into the literature mRNA sequences that (usually) were secondary sites that helped prevent excessive synthesis of specific proteins [31]. This type of autogenous translational repression is now quite common.

We think of transcriptional attenuation in bacteria as an example of regulatory events caused by a *natural* aptamer; the aptamer comprises specific codons within an mRNA [32]. When a ribosome is translating the 5′ end of a polycistronic mRNA that encodes the enzymes needed for the biosynthesis of an amino acid, codons in the leader peptide are recognized by the cognate aminoacylated tRNA (during translation) if the amino acid is present in sufficient quantities; those codons are not recognized if that amino acid concentration is too low. Translation of the leader peptide causes the remaining polycistronic mRNA to not be transcribed, thus signaling to the operon that additional biosynthetic enzymes are not needed. This example of feedback repression operates through an mRNA-amino acid recognition (thus an aptamer for the amino acid, sort of), but the actual mechanics of feedback involve the tRNA, the aminoacyl-tRNA synthetase, and the entire ribosome. There exist many other (far simpler) ways to have high amino acid concentration direct lower synthesis of the requisite enzymes for amino acid synthesis, but evolution takes what it can get (We will return to this point repeatedly, yet never make this point more exquisitely than Jacob made it more than 25 years ago [33].).

These observations are not restricted to bacteria. Years ago, Klausner and his colleagues described natural aptamers at the 5′ or the 3′ ends of mRNAs that could impact translation or mRNA stability [34–36], in either case altering the protein output by a feedback mechanism dependent on iron regulatory protein (IRP), which binds IREs (iron-responsive elements) in mRNA only in iron-depleted cells. Similarly, the number of proteins thought to enhance or repress splicing is growing; aptamer sequences near exon-intron or intron-exon junctions give splicing a remarkable flexibility and coordination with metabolism. In *Saccharomyces cerevisiae*, ribosomal protein L30 binds to a 36 nucleotide bulged stem-loop in rRNA. Excess L30 binds to a similar stem-loop structure containing the 5′ splice site in L30 pre-mRNA and inhibits utilization of this splice site. A further twist on aptamer use in this system is the finding that the translation initiation AUG of L30 mRNA is also wrapped up in a similar structure, so that excess L30 protein autogenously regulates both splicing and translation during gene expression [37, 38]. Splicing control in metazoans is even richer in aptamer like modulating sequences. Intronic enhancers [39] and exonic enhancers [40] participate in a cascade of sex-dependent splicing events in the development of *Drosophila melanogaster*. In mammals, splicing regulation seems to be the norm rather than the exception. It is now clear that SR proteins, a group that had been previously lumped together as one class because of their serine-arginine repeats, actually bind to individual aptamer sequences in exons to give exquisitely fine-tuned splicing patterns [41].

We note that when aptamers are selected against proteins that bind to natural aptamers, the resulting artificial aptamer (which is always short compared to the genomic window of opportunity) may not (and usually does not) resemble the natural aptamer. This is really not more than a statement that complex structures can utilize long-range RNA interactions that cannot happen in a randomized sequence only 30 or 40 nucleotides in length, although it must also be remembered that nature selects for optimal sequences (with respect to physiological needs), whereas artificial SELEX chooses maximal binding affinity.

The presence of many natural aptamers that serve to control protein expression is the intellectual space within which the Breaker papers reside. The startling element in the Breaker papers derives from the fact that the feedback inhibition occurs in response to the concentration of a small molecule rather than a protein, even though the specificity and affinity data for synthetic aptamers aimed at small molecules is so robust (see [21] for a wonderful review). RNA binding activities (as aptamers) can be generalized to include "open" and "closed" forms of attenuator sequences [32], splicing elements [37, 38], transcriptional termination sites [42], frame-shifting sequences [43], nuclear exit determinants [44, 45], RNA decay elements [46], etc.; that is, any RNA might fold into two states in equilibrium, such that the perturbation of that equilibrium by an aptamer-specific ligand can have a regulatory impact (Figure 1) which is manifested through either more or less RNA, more or

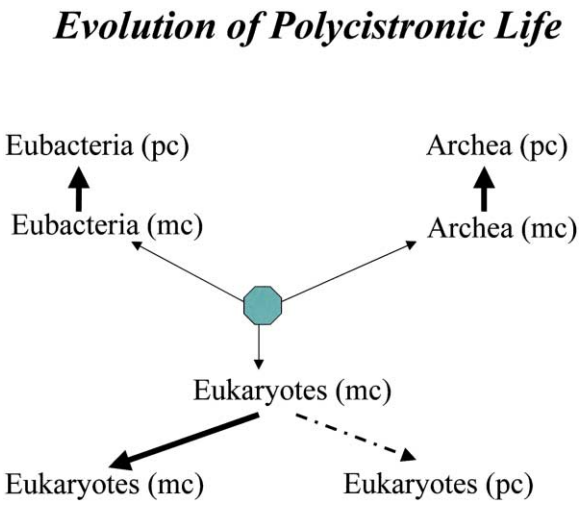

Figure 3. The Three Kingdoms of Life, Evolving from an All Monocistronic (mc) World into Polycistronic (pc) and Monocistronic Organisms

less activity, more or less transport to an appropriate intracellular location, and so on.

Speculation and More Speculation

Francois Jacob said it best in "Evolution and Tinkering" [33]. Evolution has a path, but that path is hard to predict and derives from historical accidents. While there is a perhaps illogical history to evolution, time (at least abundant time) can be the driver of metabolic coordination. Michael Savageau has spent a lifetime wondering about the likelihood that evolution selects, over abundant time, optimal solutions to serious problems [47]. At some important level, time allows playfulness (okay, call it randomness), and playfulness, when continued over abundant time, leads to optimal solutions. Jacob's analysis deals with the mechanism of evolution but does not deny the value of abundant time. Savageau argues, persuasively, that time is so abundant that we can forget sub-optimal solutions if they are not robust. The Savageau work suggests that evolution cannot hold a "position" unless it is sufficiently robust to survive playfulness. Jacob and Savageau represent the extremes of thought, even though for sure they would agree about almost everything in detail; each has made points that live comfortably within the other's worlds, once time is allowed into the dialog [48].

Why are we speaking about Jacob and Savageau? The regulation of gene expression is an area in which many quasi-equivalent/quasi-different solutions appear quasi-robust. This explains the extraordinarily different mechanisms by which bacterial phages control transcription during their developmental programs [49]; that is, evolutionary pressures cannot drive bacteriophages to all use the same mechanism because several biochemical mechanisms are equivalently robust (at least approximately equivalent). Thus, we need to understand the pressures that might allow Breaker's observations to be commonplace: once again can we extrapolate from two to infinity.

Coordination of Metabolic Capacity

Organisms struggle to utilize their biochemistry efficiently by coordinating vast numbers of different molecules and reactions within a cell or within an organism. What a struggle this must be. The world of biology has but a few robust mechanisms that ought to be selected and utilized whenever possible—one robust mechanism for coordination must be the use of polycistronic transcription. Clearly, nothing is more directly aimed at biochemical coordination than polycistronic transcription. If a cell requires enzymes 1 through 10 to accomplish some biochemical pathway, what could be more robust than to place those enzymes into an operon, express them coordinately from a single promoter, and allow the *product* (of the translational yield of each gene times the activity of each enzyme) to provide biochemical coordination?

Nothing is more straightforward than operons to effect coordinated biochemistry, and both eubacteria and archea use operons to accomplish coordinate biochemistry. This leads to a hypothesis (really based on the presumption of robustness in the Savageau sense, without recourse to mathematical modeling—Michael would hate this) that the evolution of polycistronic transcription is an enormous event that vastly simplifies intracellular biochemical coordination.

The recent literature gives us reason to rejoice. In some important papers by Tom Blumenthal and his col-

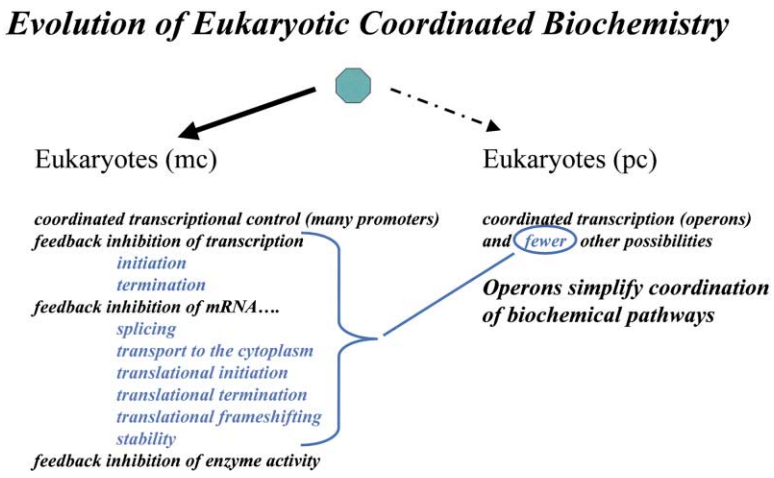

Figure 4. Monocistronic (mc) Organisms Coordinate Biochemistry with Less Dependence on Long Transcription Units and More Dependence on Regulation of Short Transcription Units and Regulation of Posttranscriptional mRNA Metabolism

leagues [50–52], we learn that *C. elegans* uses operons. Furthermore, we learn that *C. elegans* uses operons less frequently than *E. coli* but far more frequently than almost any other eukaryote. This leads to a tiny extension of the "tree" of life (Figure 3) and an idea that extends Breaker's work from remnant to novel coordination mechanisms. We believe that the division of life into the three kingdoms occurred prior to selection of polycistronic life, and that further (isolated within kingdoms) evolution had the opportunity to independently evolve polycistronic life as a solution to a big biochemical problem: *coordination*. Because both the eubacteria and the archea have had many more organismic replication events than have had eukaryotes, only those kingdoms have had the opportunity to optimize coordination through polycistronic life. *C. elegans* has had the good fortune, stochastically, to discover and use robustly that polycistronic life, but even *C. elegans* uses polycistronic life infrequently (about 15% of the genes are transcribed polycistronically). Interestingly, the coordination achieved in *C. elegans* is not followed by polycistronic translation; rather, *trans*-splicing events create individual mRNAs for translation.

Genomes Filled with Aptamers: Conclusion

Polycistronic life is a robust solution to the coordination problem. From within a world of polycistronic life, relatively minor "tinkering" probably allows pathways of all kinds to provide buffered responses to the external world those organisms face. But the world we care most about, mammalian life, has not evolved polycistronic expression. Life without polycistronic regulation must include robust alternatives. Mammals with large genomes and slow replication cycles require strategies that work at this moment, even though future mammals may well "discover" polycistronic transcription. Perhaps splicing flexibility (introduced into evolutionary mechanisms a la Jacob and refined a la Savageau) is sufficiently optimized to preclude polycistronic expression. Nevertheless, we are finally back to Breaker and his discovery.

An amazing number of regulatory events at the mRNA level (Figure 4) present themselves as alternatives to polycistronic life. Is mutagenesis, one nucleotide at a time, capable of elaborating aptamers in the genome that accomplish coordination of biochemistry? Again, we return to abundant time, the great problem solver.

In 1997, we wrote extensively about two evolutionary ideas visible through an experimental metric called "genomic SELEX" [5, 6]. We postulated that mutations within an RNA molecule could allow aptamers to form and be selected, and that one should search experimentally for RNA sequences that interact with proteins or metabolites in an organism. We proposed that SELEX libraries could be built for specific organisms using genome fragmentation, ligation of fixed sequences (as in Figure 3), and then the genomic SELEX protocol (which is identical to classic SELEX except that the starting library is natural). If organisms are testing RNA sequences/structures to see what kinds of feedback control might evolve, genomic SELEX would yield those molecules. Using the language of Stan Fields aimed at protein:protein interactions [53, 54], we called the protein-specific natural aptamers part of the nucleic acid-protein linkage map, and we called metabolite-specific natural aptamers part of the nucleic acid-metabolite linkage map.

Although the two examples described by Breaker and his colleagues are in *E. coli*, we expect that similar natural aptamers provide feedback regulation in monocistronic organisms. Computer search algorithms that seek structures in RNA [55–57] are one interesting means by which to see aptamers lurking within or outside of protein coding exons. Similarly, natural aptamers conferring end-product inhibition might be sought by scanning for conserved, structured RNA homologs among the mRNAs encoding sets of proteins involved in common biosynthetic pathways. The old notions of feedback regulation are likely to have a renaissance in the next few years as RNA investigations become even more sophisticated.

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