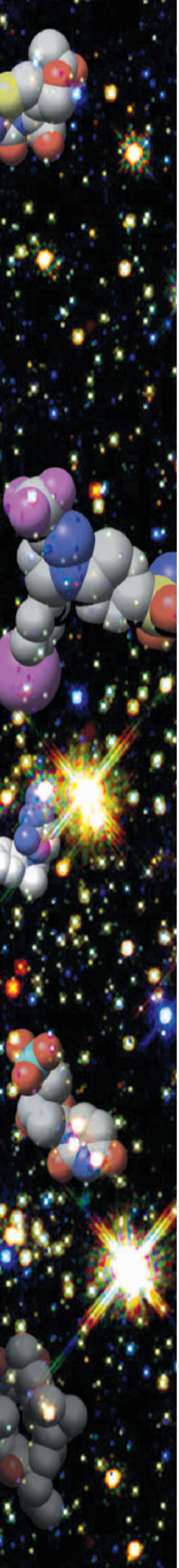

Natural and engineered nucleic acids as tools to explore biology

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RNA and DNA molecules can form complex, three-dimensional folded structures that have surprisingly sophisticated functions, including catalysing chemical reactions and controlling gene expression. Although natural nucleic acids make occasional use of these advanced functions, the true potential for sophisticated function by these biological polymers is far greater. An important challenge for biochemists is to take RNA and DNA beyond their proven use as polymers that form double-helical structures. Molecular engineers are beginning to harness the power of nucleic acids that form more complex three-dimensional structures, and apply them as tools for exploring biological systems and as therapeutics.

Exploring the full complexity of cells at the molecular level will require the fashioning of new tools that allow researchers to manipulate complex biological processes in unique ways. Small organic molecules that block or otherwise perturb the normal functions of the cellular machinery have long served as powerful tools for exploring biochemical processes. Similarly, new tools that take advantage of the natural functions of proteins and nucleic acids are proving to be enormously useful as researchers continue to probe the details of complex biochemical systems.

Living systems have been expanding and diversifying their natural collection of biochemical tools for billions of years. For example, enzymes build RNA, DNA and proteins with high fidelity and with impressive speed; in some cases more than 100 monomeric units are added to the polymer per second. Many other enzymes are known to selectively cut or join nucleic acids or proteins, and still others catalyse chemical reactions with great speed and accuracy. This provides us with a large set of verified technologies which, if harnessed by researchers, can be applied to understand and manipulate biological processes at their most fundamental level. Indeed, there is a considerable history of scientists taking bits and pieces of proteins and nucleic acids from natural sources, tailoring them by purposefully mutating or splicing them in different ways, and using them as reagents for biological study or for therapeutic applications.

More recently, researchers have begun to harness darwinian evolution to optimize existing functions of proteins[1,2] and nucleic acids[3,4], and to create new ones. In combination with rational design methods, these techniques for directing the evolution of biopolymers allow researchers to become a creative force for molecular change and invention. In many instances, we no longer need to be limited to using a less-than-optimal protein or nucleic acid molecule from natural sources. Some natural proteins and nucleic acids can be enhanced by using directed evolution or entirely new functions can be derived using similar engineering strategies.

Simple, engineered nucleic acids already provide us with useful tools for detecting and manipulating other nucleic acids. For example, the selective amplification of genomic fragments by the polymerase chain reaction (PCR)[5] or by related techniques requires the use of designed synthetic DNA primers. Similarly, the targeted inactivation of gene expression by using short synthetic oligonucleotides or small interfering RNAs (siRNAs)[6,7] is becoming increasingly routine. These applications are greatly aided by efficient methods for the sequence-specific chemical[8,9] and enzymatic[10] synthesis of RNA and DNA. In addition, the design of nucleic acids that bind to other nucleic acids with high affinity and specificity follows the simple and long-established rules of Watson–Crick base pairing[11].

However, it is becoming increasingly clear that nucleic acids can have far greater use than that shown by simple base-paired structures. For example, the hammerhead ribozyme consists of just over 30 nucleotides and can catalyse RNA-strand scission at a rate that is millions of times faster than spontaneous RNA cleavage[12]. At the opposite end of the spectrum is the ribosome, which at its core carries a staggeringly complex ribozyme structure that catalyses peptide-bond formation[13,14] (see section 'Ribozymes and deoxyribozymes' below).

These natural RNAs are just a small representation of the considerable untapped potential that nucleic acids have for forming complex structures and carrying out sophisticated tasks; it is this potential that nucleic acid engineers seek to harness. Nucleic acid design and synthesis techniques, along with powerful directed evolution strategies, are empowering the drive to design ever more complex RNA and DNA molecules. Here, I will focus on some of the surprising functions of novel 'designer' nucleic acids, and assess the potential for these new tools in biotechnology and therapeutics.

Manipulating life's central processes

To gauge the potential use of engineered nucleic acids in manipulating biological systems, we need only consider the roles of DNA and RNA in fundamental biological processes. The basic roles for DNA and RNA in information storage and transfer are well established, and numerous ways in which this process can be manipulated by using nucleic acids are being explored. Many existing technologies and several emerging ones can be used to selectively target gene expression and protein function at the DNA, RNA and protein levels (Fig. 1).

The instructions for protein synthesis, encoded by the nucleotide sequences of genomic DNA, are transferred to messenger RNAs that are subsequently 'read' by ribosomal RNAs and transfer RNAs. Therefore, the information stored in DNA or RNA can be manipulated by designing short complementary DNA or RNA oligonucleotides that bind the nucleic acids. For example, antisense oligonucleotides (whose sequences are complementary to their target genes) have been developed to selectively inhibit a variety of

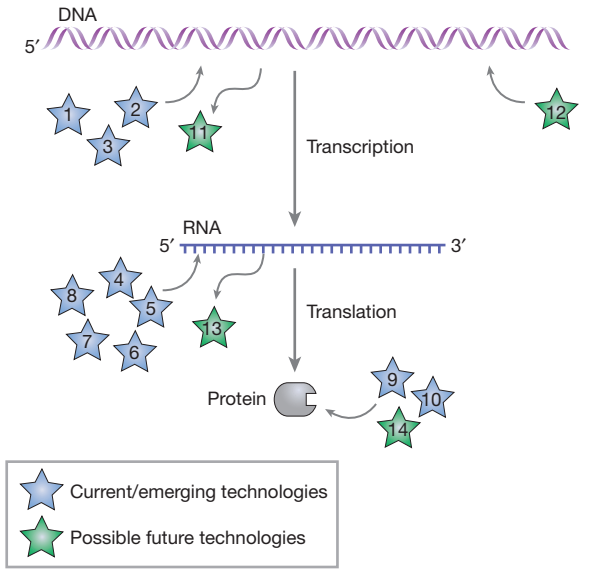

Figure 1 Manipulating the expression and function of proteins with nucleic acids. Depicted is a simplified schematic of the flow of biological information from DNA to proteins and the points of intervention in this process by nucleic acid tools. Current or emerging technologies include: oligonucleotide- or vector-mediated genetic engineering[109,110] (1); triplex-forming oligonucleotides (TFOs)[111] (2); ribozyme alteration of DNA sequence[112] (3); antisense oligonucleotides[15,16] (4); siRNAs[7] (5); mRNA cleavage by ribozymes and deoxyribozymes[35] (6); ribozyme repair of mRNAs[112,113] (7); engineered riboswitches[98–103] (8); aptamers[22–24,26] (9); nucleoside-analogue drugs (10). Potential future technologies include: replacement of protein with functional DNA (11); repair of DNA by functional RNA or DNA (12); replacement of protein with functional RNA (13); modification of protein by functional RNA or DNA (14).

genes[15,16]; one such molecule (Vitravene)[17] has been commercialized as an antiviral agent[18]. So, as with siRNAs, designing new oligonucleotides that downregulate gene expression can be as simple as creating a complementary sequence for the target mRNA.

However, antisense molecules typically function by different mechanisms[15] from those used by siRNAs[19], and thus do not take advantage of the natural siRNA processing enzymes. In general, more research and development is needed to ensure that each antisense oligonucleotide effectively targets its intended mRNA. Moreover, these and other approaches that use oligonucleotides *in vivo* must ensure that the RNA or DNA molecules being delivered are sufficiently resistant to chemical and enzymatic degradation in a cellular environment. Already, there have been numerous advances in the chemical synthesis of nucleic acid analogues[20]. These allow the oligonucleotide polymers to persist in the bloodstream for many hours, where otherwise they would have a half-life of seconds.

Another major role for nucleic acids in fundamental biological processes is not as polymers but as nucleotide-like fragments of essential metabolites and coenzymes. Small nucleotides like ATP and GTP, the cyclic nucleotides cAMP and cGMP, and numerous coenzymes and metabolic intermediates, including nucleotide fragments, are involved in many metabolic and signalling pathways. The proteins in these pathways are exploited by many drug compounds that mimic the basic structures of nucleotides and nucleotide-like coenzymes. Similarly, various nucleoside analogues (such as human immunodeficiency virus (HIV) reverse transcriptase chain terminators)[21] show antiviral activity because they interfere directly with the synthesis of new pathogen DNAs.

The purpose of this review, however, is to discuss the tremendous potential for more complex, folded nucleic acids which carry out functions that, until recently, had only been observed in proteins. These functions result when RNAs and DNAs form more globular structures, which usually include both helical structures and long-distance tertiary contacts, such as atypical molecular contacts between nucleotides and metal-ion binding to bases and phosphates. This means that researchers are not limited to exploiting the rules of Watson–Crick base pairing or designing nucleotide-like compounds that fortuitously occupy binding sites on proteins.

The diversity of sophisticated functions undertaken by structured nucleic acids opens many opportunities to create new tools that can be used to explore biological systems. Hints of this potential have been emerging in recent years with the development of aptamers (ligand-binding polynucleotides), ribozymes, deoxyribozymes and riboswitches (metabolite-sensing gene control elements). As with proteins, functional nucleic acids can be isolated from natural sources. But to tap into the full potential for structured nucleic acids, researchers will need to use engineering strategies, such as directed evolution[3,4], which can be used to generate RNAs and DNAs with entirely new tertiary structures. Specifically, this process works by selectively reproducing copies of RNAs or DNAs that have performed some task, such as ligand binding or self-cleavage. Some researchers believe that if an experimental protocol can be devised to identify functional molecules from trillions of inactive variants, then it is likely that a nucleic acid will be found that performs the desired task (as long as the task is compatible with the principles of chemistry). Some of the key advances in using directed evolution to acquire nucleic acid tools are described in the next section.

RNA and DNA aptamers

Dramatic examples of nucleic acids performing more complex functions have been provided by researchers who create and study aptamers (Fig. 2). Engineered aptamers are structured RNA or DNA molecules that form binding pockets for specific ligands[22,23]. They can be created by using directed evolution techniques: trillions of RNA or DNA molecules are prepared simultaneously and subjected to a process of selective amplification to enrich the population with variants that bind to a particular protein target. Directed or *in vitro* evolution of aptamers requires that some distinction be made between RNAs or DNAs that bind to a target ligand and those that do not. This is usually achieved by using some form of affinity chromatography to physically separate ligand-binding polynucleotides from the vast population of inactive variants. Isolation is then followed by amplification of the rare molecules.

Alternatively, domains of random sequences can be integrated with catalytic RNAs or DNAs such that ligand binding to specific variants triggers catalytic function. This arrangement, wherein ligand binding at one site controls the activity of a distant catalytic site, is defined as allosteric enzyme activity (see section on 'Allosteric ribozymes' below). For example, self-cleaving ribozymes that are triggered to cleave only when incubated in the presence of cyclic nucleotide monophosphates (for example, cAMP) have been created by using 'allosteric selection'[24]. Cleaved RNAs are then physically separated from those that are not cleaved, permitting the experimenter to isolate ligand-binding RNAs from large pools of random sequences. These composite aptamer–ribozyme constructs can subsequently be deconstructed to yield separate aptamer domains that retain their ligand-binding function[25].

Many aptamers have functional characteristics that are similar to antibodies[26]. Just like protein antibodies, aptamers can selectively recognize specific protein or small-molecule ligands, even in complex chemical or biological mixtures. Furthermore, they can bind to their cognate targets at target concentrations of the nanomolar or picomolar range, matching or even superseding the affinities of antibodies. Aptamers retain their function when they are immobilized for use in the test tube; they can also be delivered to organisms or expressed inside cells. Aptamers generated in the test tube can be made to bind a diverse array of targets, including highly toxic agents[27,28], and may perform their receptor functions under defined conditions that are far

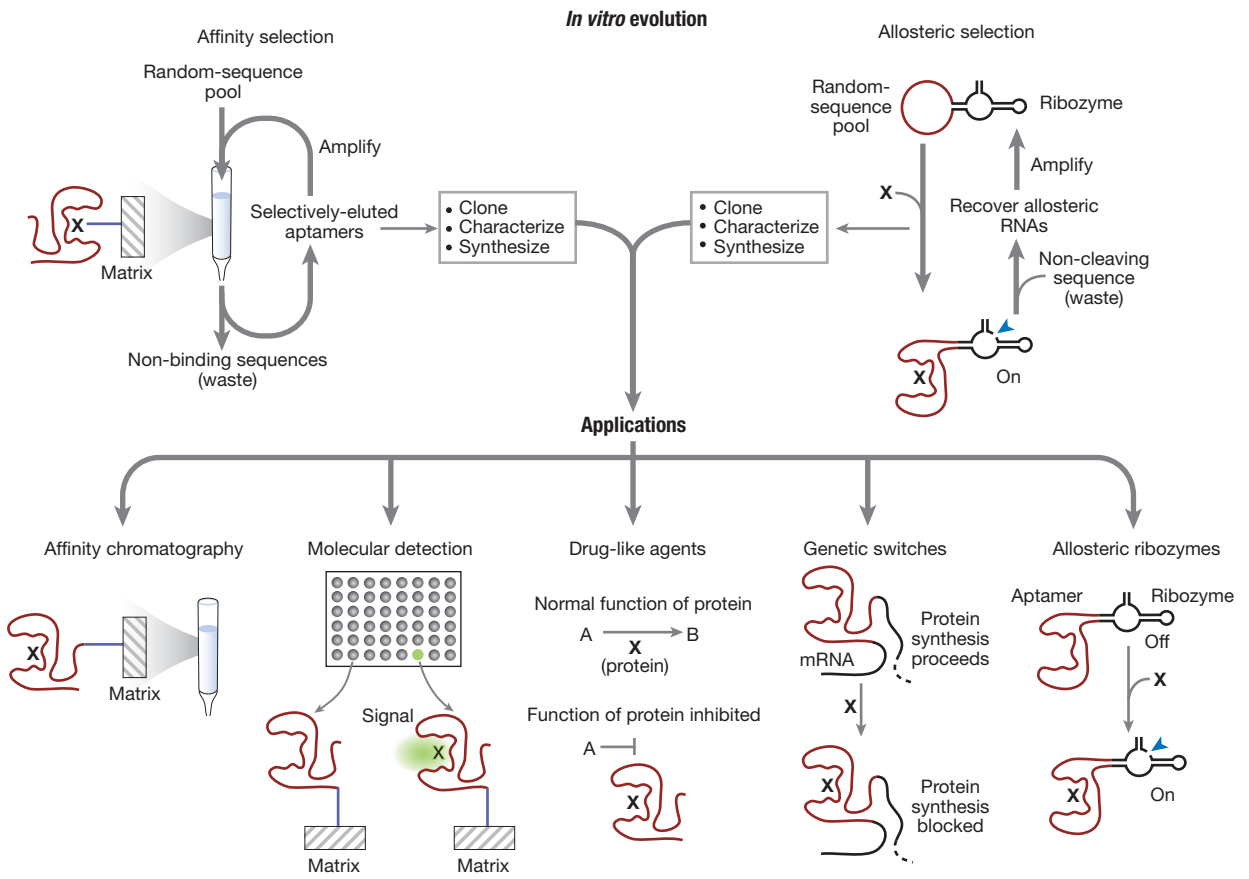

Figure 2 The generation and application of aptamers. Methods for the *in vitro* evolution of aptamers include the use of an affinity matrix to separate RNA variants that selectively bind an immobilized target (X; X represents any target molecule unless otherwise noted)[22,23,26], or the use of allosteric ribozymes that permit separation of ligand-binding RNAs by means of self-cleavage[81–83]. Once they have been engineered, aptamers can be used (among other applications) as chromatographic agents, biosensor elements, anti-protein drugs, gene-control elements and as components of allosteric ribozymes.

from the physiological norm. This last feature might be useful for biosensing applications, for which solvent conditions or desired temperatures might be disruptive to protein structures.

As with antibodies, the potential use of aptamers is considerable (Fig. 2). Aptamers can be immobilized on solid supports to yield designer matrices for affinity chromatography[29,30]. In most instances, aptamers undergo a change in shape on ligand binding[31]. This can be exploited to create biosensors (devices that use biological materials to monitor the presence of various chemicals in a substance) by the judicious integration of fluorescent tags with aptamers in solution or immobilized on surfaces. Such aptamer beacons have been used to detect specific proteins[32,33] or small molecules[34] through the change in fluorescence that occurs upon ligand binding.

Perhaps most profound is the possible application of protein-binding aptamers as therapeutic agents. A tremendous body of literature exists regarding the prospects for using nucleic acids for *in vivo* applications[35]. But, as is the case for other oligonucleotides, the delivery of aptamers into cells is problematic[36]. Also, the molecules must be resistant to nuclease degradation or they risk being rapidly destroyed by nucleases in the blood. Various approaches are being developed to improve the capabilities of aptamers *in vivo*. For example, the isolation of new aptamers (and ribozymes) can be conducted with chemical modifications already in place to facilitate nuclease resistance of the winning molecules, such as phosphorothioate linkages or modifications at the 2′ carbon of ribose[37,38]. A number of examples now exist in which aptamers expressed inside cells retain their function and bring about their intended inhibitory effect. These aptamers, sometimes called 'intramers'[39,40], can be stabilized by integrating them into a larger RNA construct. This RNA construct is inherently more resistant to degradation or can help route the RNA to its desired cellular compartment[41].

Furthermore, mirror-image aptamers, called spiegelmers[42,43], can be created to serve as highly stable receptors for their corresponding ligands. Spiegelmers have a chiral configuration (L-RNA) which is the mirror image of that for 'normal' or D-RNA. Despite this apparently radical alteration, spiegelmers can be made by using *in vitro* evolution in much the same way as that used to generate normal aptamers. First, normal D-RNA aptamers are generated that bind to the mirror image of the target that one wishes to bind with a spiegelmer. For example, an unnatural peptide target that is the mirror image (D-polypeptide) of that normally encountered in the cell (L-polypeptide) is used during the selection and amplification process. So, the resulting normal D-RNA aptamer would be functionally useless against the natural target analogue. Once in hand, however, the sequence of the normal D-RNA aptamer serves as a guide to make the L-configured spiegelmer by using L-nucleotides during chemical synthesis. This simple production trick creates an L-aptamer that can bind an L-polypeptide, starting from the D-aptamer–D-polypeptide complex that was originally created by *in vitro* evolution. Although spontaneous degradation by inherent

chemical instability of RNA should remain unchanged, spiegelmers are completely resistant to degradation by typical nucleases[44].

One of the challenging aspects of this technology is that new aptamers are not always easy to generate. The basic protocols for *in vitro* evolution are rather straightforward, but trivial problems with any of the selection or amplification steps can cause bottlenecks that restrict molecular variation, or can cause complete loss of the evolving population. Anything from losing trace amounts of nucleic acids because of non-specific binding to plastic tubes, to technical problems that create DNA amplification artifacts during PCR, can sap the efficiency of *in vitro* evolution experiments. Equally problematic is the emergence of 'selfish' RNA or DNA molecules. These 'molecular weeds' typically lack the desired ligand-binding function, but use alternative strategies to survive the selection process. For example, many aptamers have been isolated that bind to the chromatographic matrix (agarose, nitrocellulose) as opposed to the ligand that is immobilized on the matrix. Precautions can be taken to avoid or eliminate problems encountered during *in vitro* evolution, but these require additional steps or judgements to be made at each stage of the selection process. For example, matrix-binding aptamers can be disfavoured by using free ligand to selectively recover the desired aptamers from the chromatographic matrix. Free ligands compete for the aptamer binding sites, causing selective elution of aptamers that are bound to immobilized ligand versus those that simply bind the matrix.

Until recently, aptamer generation was a completely manual operation: it involved numerous pipetting and purification steps that had to be conducted with great care. However, there are several reports[45–48] of successful automated aptamer selections that require minimal hands-on effort. Aptamers produced by automated methods target diverse proteins, such as lysozyme[45] and the human U1A protein[48]. These automated methods and other manual protocols permit the pursuit of aptamers for many targets simultaneously. So far, promising drug-like aptamers, such as an anti-VEGF (vascular endothelial growth factor) aptamer[49] and two anti-clotting aptamers[50,51], have been created using manual selection methods. If hundreds or thousands of aptamers are demanded, technology appears to be advancing to the point where they could in principle be generated.

Ribozymes and deoxyribozymes

To date, there are nine known classes of natural ribozymes that catalyse phosphoester cleaving/forming or peptide-bond-forming reactions. The peptide-bond forming ribozyme is found at the core of the ribosome and is made up of the most highly conserved segments of rRNA[14,52]. Although this ribozyme has long been the target of many antibiotic drugs[53–55], there is considerable interest in using ribozymes themselves as therapeutic agents[56]. For example, RNase P, a phosphodiester-cleaving ribozyme that normally processes tRNA precursors, can be induced to cleave new RNA targets (such as mRNAs): an external 'guide' sequence that is delivered or expressed in cells directs the cleavage event[57–59] . A mix of conventional base pairing and tertiary structure formed by the guide sequence when docked to its target RNA is recognized as a substrate to be cleaved by RNase P. Two types of self-splicing ribozyme, called group I and group II because of their distinct structures and reaction mechanisms, have also been designed to catalyse *trans*-splicing of mRNAs[60,61] or to direct their own integration into genomic DNAs to yield genetic changes[62]. If these ribozymes were made to efficiently modify the mRNAs or the DNAs that serve as their templates, they could be used as new gene-repair systems.

Other ribozymes show promise as agents for the destruction of viral RNA or mRNAs. Indeed, most efforts to make therapeutic ribozymes have been directed towards developing the small self-cleaving ribozymes[35] into selective mRNA-cleaving agents. For example, the hammerhead ribozyme (Fig. 3) can be made to cleave different RNA substrates simply by tailoring the nucleotide sequences of its substrate-binding arms. RNA-cleaving ribozymes configured for therapeutic applications or for target validation[63] typically catalyse one reaction per minute. This is fast enough to have a biological impact, but not sufficiently fast to permit each ribozyme to process more than just a few substrate molecules before it is destroyed by cellular enzymes.

In vitro evolution can be used to create new ribozymes that catalyse RNA cleavage as well as many other chemical reactions[3,4]. Although there might not be an immediate use for self-alkylating RNAs[64] or for ribozymes that form the glycosidic linkage of nucleotides[65], other engineered ribozymes might find application much sooner. For example, ribozymes can be made to covalently attach to specific proteins[66], suggesting that designer ribozymes could be created that selectively couple to many different potential therapeutic or diagnostic protein targets.

A ribozyme-catalysed reaction that has more obvious use is that of RNA cleavage. The diversity of motifs that catalyse RNA cleavage by internal phosphoester transfer is substantial. Therefore, it is possible to design new sequences that have reaction characteristics tuned to the desired application. Some of these engineered ribozymes, such as the X-motif[67,68] (Fig. 3), have performance characteristics that are similar to that of the hammerhead ribozyme, indicating that new ribozymes could be created that destroy disease-causing RNAs with an efficiency equal to or greater than natural ribozymes.

Even DNA enzymes or 'deoxyribozymes' have been created that cleave RNA by using the same phosphoester transfer reaction[69]. The most studied catalytic DNA is the 10–23 deoxyribozyme[70] (Fig. 3). As with the hammerhead and X-motif ribozymes, 10–23 can be tailored to cleave almost any RNA molecule, and its catalytic efficiency allows it to affect gene expression[71]. For example, chemically synthesized 10–23 has been used to reduce the expression of a gene responsible for undesired tissue growth after artery damage[72,73]. As with other oligonucleotide therapeutics, there are concerns about deoxyribozyme delivery and pharmacokinetics, DNA stability, subcellular localization and biochemical access to target sites. However, results to date demonstrate that both ribozymes and deoxyribozymes can

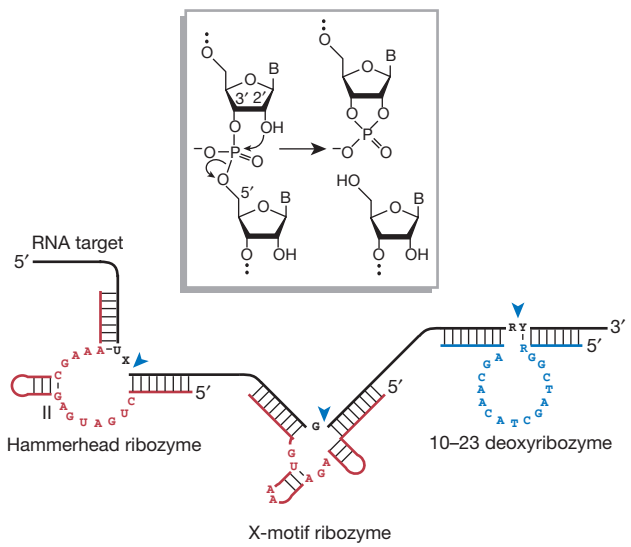

Figure 3 RNA-cleaving ribozymes and deoxyribozymes. The natural hammerhead ribozyme as well as the engineered X-motif ribozyme and 10–23 deoxyribozyme motifs catalyse RNA cleavage by promoting an internal phosphoester transfer reaction (inset). Base pairing between the RNA target and the substrate-binding arms of each catalyst can be tailored to target different RNA sequences. Nucleotides within the target RNA, the ribozymes and the deoxyribozyme that are not conserved are depicted with black, red and blue lines, respectively. B, base.

indeed function as agents for downregulating gene expression in a targeted fashion.

As noted above, engineered ribozymes and deoxyribozymes can catalyse reactions other than RNA cleavage. Some of these reactions would be ideal for manipulating the chemical structures of proteins and nucleic acids, either *in vitro* or *in vivo*. For example, ribozymes that selectively ligate RNA to protein have been created by *in vitro* evolution[66]. If these ribozymes were made to function inside cells, it might be possible to manipulate protein function by new mechanisms. In addition, numerous deoxyribozymes that use ATP to phosphorylate DNA[74,75] or to ligate DNA[76] have been generated. Although the efficiencies of most of these deoxyribozymes are far from sufficient to be of biological relevance, improvements to their action could be made. If so, unique tools for manipulating biological polymers would result (Fig. 1).

Allosteric ribozymes

The simplicity of the interactions that define the secondary structures of RNA and DNA molecules causes complications for those who study the structures and functions of nucleic acids. As the length of the molecule increases, so do the number of opportunities to form alternative base-pair or tertiary-structure interactions that prevent the desired fold from forming. Many different folding pathways that yield many alternately folded (and inactive) states are possible[77]. If conditions are right and the alternately folded structures are not very stable, these states can interchange on a timescale that is sufficiently short for the interchangeable state to be harnessed for useful functions. So, the conformational heterogeneity of nucleic acids can be turned into an important positive characteristic: this has been achieved by molecular engineers and in spectacular fashion by natural mRNAs.

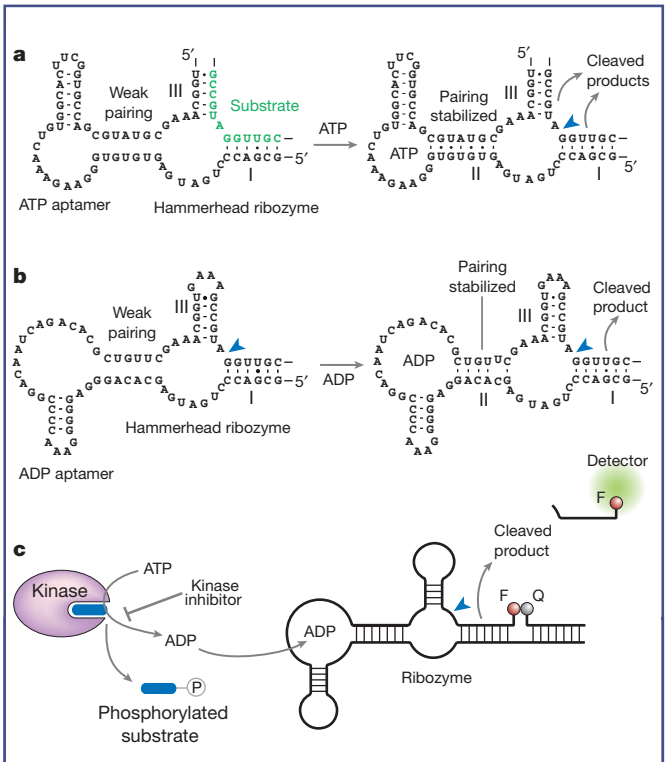

Figure 4 Allosteric ribozymes as precision biosensor elements. **a**, One of the first engineered allosteric ribozymes was created by fusing an ATP-binding aptamer to a hammerhead ribozyme by means of a disordered bridge element[80]. Ligand binding stabilizes the core of the aptamer and the weakly pairing stem (stem II of the ribozyme) to trigger increased ribozyme activity. **b**, A next-generation allosteric ribozyme or RiboReporter that senses ADP and disfavours binding of ATP by more than 100-fold. **c**, In the design shown here, a fluorescent readout is generated if ribozyme activity is triggered by ADP. As a result, fluorescence increase is prevented if an anti-protein-kinase drug, such as staurosoprine, is present[87]. RNA cleavage by the ribozyme occurs within the stem I/III junction between A and G (blue arrow). F and Q represent fluorophore and quencher moieties, respectively. The performance characteristics of this RNA switch are sufficient to permit its use in high-throughput screening assays.

For example, an ATP-binding aptamer created by *in vitro* evolution carries two base-paired elements that are pre-formed in the absence of ligand, whereas its ligand-binding core remains largely disordered[78,79] (Fig. 4a). However, the docking of ATP stabilizes the aptamer's core and lends additional stability to the adjoining base-paired stems. When it is appropriately fused to a weakened but essential stem of a hammerhead ribozyme, the aptamer acts as an allosteric binding site and permits the ribozyme to be activated by ATP binding[80].

This simple demonstration of allosteric activation of ribozymes has since been expanded upon; numerous RNA switches that are selectively triggered by many signals (including small organic compounds, proteins, nucleic acids, metal ions, pH and light) have been created[81,82]. Each engineered RNA switch can independently serve as a biosensor element for its corresponding ligand. Immobilized RNAs that are tagged with radioactive or fluorescent labels have been used to form biosensor arrays that report the presence and concentrations of targets, even in complex chemical or biological mixtures[83,84]. In other manifestations, ribozymes have been shown to function as diagnostics that sense the presence of pathogen-specific molecules, such as viral RNAs[85,86].

In these examples, the RNAs are not replacing the action of a small molecule but they could ultimately be used to identify small molecules that affect biological functions. For example, protein kinases typically convert ATP into ADP upon protein phosphorylation. A highly specific ADP-sensing RNA switch or RiboReporter[87,88] has been created and used to detect and report the amount of ADP by-product (Fig. 4b). This indirectly reflects the level of protein kinase activity in a given assay. A RiboReporter that yields a fluorescent report upon activation by ADP was used successfully to identify reaction mixtures containing the protein kinase inhibitor staurosporine (Fig. 4c)[87]. Similarly, this allosteric ribozyme could be used in large high-throughput screens to identify new protein kinase inhibitors or to find compounds that modulate the activity of any enzyme whose activity generates or destroys ADP. Allosteric ribozymes have also been created to respond to protein targets[89] such as lysozyme and the Rev peptide from HIV[84]. Furthermore, other protein-dependent allosteric ribozymes have recently been shown to be useful tools to screen for small molecules that disrupt protein–ligand interactions[90].

Riboswitches

Recent studies have begun to reveal that many bacteria already make extensive use of natural RNA aptamers for metabolite sensing and gene-control purposes[91,92]. These natural RNA switches, or riboswitches[93,94], show a wide range of target specificities and affinities. For example, they are known to be responsible for controlling the expression of about 2% of the genes in *Bacillus subtilis*[95]. Not only does the existence of riboswitches add validity to the notion that useful RNA switches can be engineered, but this mechanism for gene control also offers numerous opportunities to use natural or engineered aptamers *in vivo* for new applications.

The adenine-responsive riboswitch[95,96] from *B. subtilis* has characteristics that are typical of most other riboswitches. The riboswitch carries an aptamer domain that conforms to a consensus sequence and secondary structure (Fig. 5a). The aptamer resides immediately upstream of an 'expression platform' that modulates gene expression in response to metabolite binding. Its function is similar to that of the linker regions between aptamers and ribozymes in engineered RNA switches. Of the several mechanisms used by riboswitches in *B. subtilis*, the regulation of transcription through metabolite-mediated control of transcription termination is the most common. The adenine-specific

riboswitch shown in Fig. 5a uses this mechanism to activate expression of a gene encoding an adenine efflux pump when excess adenine is present[96,97]. Similar riboswitches repress gene expression upon introduction of the target metabolite. Given that these RNAs are highly modular and can be moved from one gene to the next, there is considerable potential to create transgenic organisms that express genes in response to several different metabolites.

Even more useful would be the creation of designer riboswitches that have entirely new ligand specificities. Already, several studies report the successful integration of aptamers with mRNAs to permit ligand-specific gene control[98–103]. For example, a reporter gene construct was made to express a theophylline-binding aptamer (itself previously created using *in vitro* evolution) located immediately upstream of its ribosome-binding site (RBS) for the coding region of the reporter-gene mRNA[103]. This aptamer–mRNA fusion allowed gene expression to be controlled by the addition of theophylline to a bacterial cell culture (Fig. 5b). Further iterations of these engineering efforts, perhaps augmented by reverse engineering of natural riboswitches, promise to provide designer gene-control switches for a variety of applications, such as *in vivo* metabolite sensing and/or the control of therapeutic genes delivered by retroviral vectors. It is already known that certain eukaryotic cells carry riboswitches[104]. Therefore, it seems reasonable to speculate that engineered riboswitches could function as designer gene-control elements in humans without provoking an undesired immune response as occurs with protein-based systems.

The ability to create new aptamers and riboswitches offers a way to create functions for non-natural compounds in gene control. It is also interesting to note that, because riboswitches have evolved to purposefully bind to metabolites, they should be able to serve as targets for drug compounds — much like their protein receptor counterparts. Indeed, it is now clear that riboswitches already serve as drug targets. The compound aminoethylcysteine (AEC), which for many years has been known to be toxic to bacterial cells, appears to work at least in part by binding to lysine-specific riboswitches and causing downregulation of lysine biosynthetic genes[105]. It is therefore likely that additional small compounds could be created to serve as anti-infective agents by targeting other crucial bacterial riboswitches.

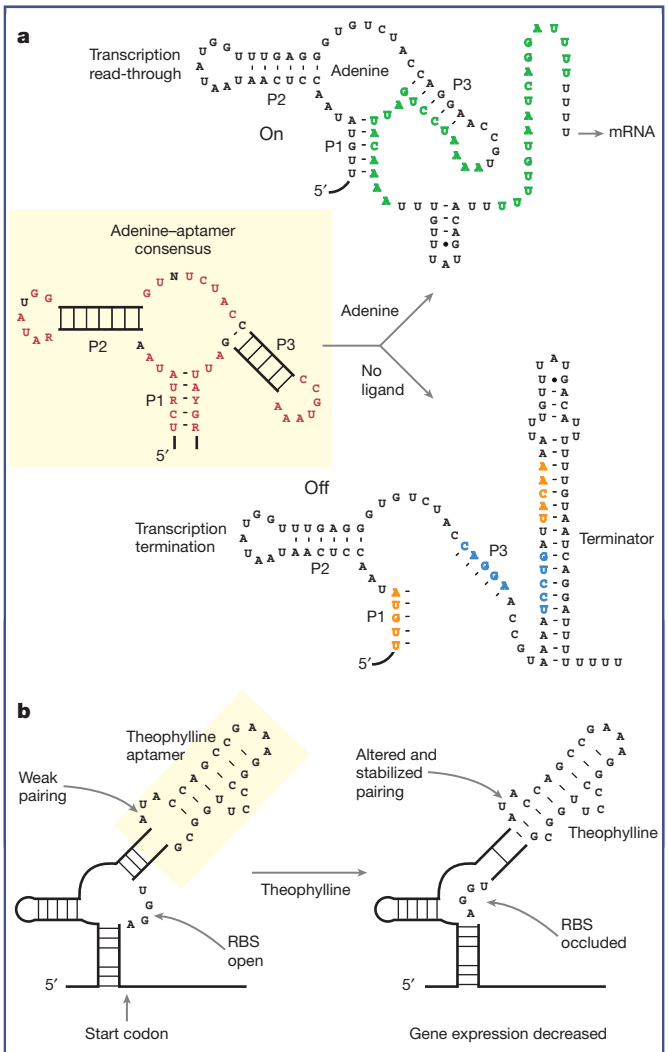

Figure 5 Natural and engineered riboswitches for controlling gene expression. **a**, A natural adenine-binding aptamer and its role in activating gene expression as part of an adenine riboswitch from the *ydhL* gene of *Bacillus subtilis*. The consensus sequence and secondary structure for the adenine aptamer domain is shaded. When sufficient adenine is present (top), the 5′ untranslated region (UTR) folds to form the full secondary structure for the aptamer bound to its ligand. This precludes nucleotides from forming an intrinsic terminator stem[114,115] (shown in green) and a complete mRNA is synthesized. In the absence of adenine (bottom), portions of the secondary structure required for the aptamer to bind its ligand (red and blue nucleotides) are not formed, which permits the intrinsic terminator to form and cause premature transcription termination. Gene expression is prevented because the complete mRNA is not synthesized. **b**, Proposed mechanism for an engineered genetic switch that uses a theophylline-specific aptamer[103]. The aptamer (shaded) and a short linker region is integrated with a 5′ UTR and the construct is fused upstream of an open reading frame. In the absence of theophylline (left), the protein is expressed because the RBS is available for interaction with ribosomes. In the presence of theophylline (right), the aptamer/linker structure becomes stabilized. This more stable structure presumably restricts ribosome access to the RBS, thus reducing gene expression.

Conclusions

The most mature nucleic acid technologies, such as those using antisense RNA and siRNAs, are widely used to selectively knock out the function of certain proteins by inhibiting gene expression. Engineered aptamers and designer enzymes can be used to modulate protein action once the polypeptide has been made; or these aptamers and enzymes can serve as biocatalysts in their own right. A high level of validation for these technologies exists in nature. For example, the most recently discovered class of ribozyme was found to be encoded upstream of the *glmS* gene in *B. subtilis*[106]. The *glmS* ribozyme is a small self-cleaving RNA that surprisingly also functions as a metabolite-sensing molecular switch. The ribozyme is selectively activated by a natural sugar compound, glucosamine-6-phosphate; its activity downregulates the expression of the protein that produces the sugar metabolite. This highlights the potential use of aptamers and ribozymes as agents for genetic control.

Other technologies, such as ribozyme-mediated modification of proteins or the complete replacement of a protein with an engineered nucleic acid, will require a substantial amount of research and development to make practical. However, the functional diversity of nucleic acids is enormous and future engineering efforts will certainly be made to expand the function and use of RNA and DNA tools. Currently, most research and development in nucleic acid engineering is being carried out as part of the basic research programmes of individual laboratories in academia. These efforts continue to provide proof of principle for an ever-widening array of nucleic acid tools.

Unfortunately, however, university-based research laboratories are not always the most appropriate settings for the maturation of emerging biotechnologies. Thus, it is encouraging to note that several small biotechnology companies are pursuing some of the more immediate applications of engineered nucleic acids, such as therapeutic aptamers and engineered RNA genetic switches. Undoubtedly, the continued exploration of the functional potential of nucleic acids will create new opportunities for those who seek to harness engineered RNAs and DNAs for practical applications. This process could be accelerated by providing support for research and development projects aimed at creating simple tools that could become useful for

basic research. Striking examples of this include the development of RNA-cleaving ribozyme constructs which, when expressed in cells, can be used to identify genes that are critical for certain cellular pathways[107,108]. Some of these tools for basic research will probably develop into treatments and become new classes of drugs to complement traditional small molecules. □

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