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Artificial functional nucleic acids: Aptamers, ribozymes, and deoxyribozymes identified by in vitro selection

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Abstract. The discovery of natural RNA catalysts (ribozymes) inspired the use of in vitro selection methodology to develop artificial functional nucleic acids. In vitro selection is the experimental process by which large random-sequence pools of RNA or DNA are used as the starting point to identify particular nucleic acid sequences that have desired functions. When this function is binding of a molecular target, the functional nucleic acid is an RNA or DNA "aptamer". When this function is catalysis of a chemical reaction, the functional nucleic acid is a "ribozyme" or "deoxyribozyme"; these are collectively termed "nucleic acid enzymes". Since the first in vitro selection experiments in 1990, a wide variety of aptamers and nucleic acid enzymes have been identified. This chapter describes how aptamers, ribozymes, and deoxyribozymes are obtained by in vitro selection methodologies. Also addressed are the scope of the molecular targets that are bound and the chemical reactions that are catalyzed. Biochemical and structural characterizations of aptamers and nucleic acid enzymes are discussed. A final section introduces aptazyme, which are allosterically regulated nucleic acid enzymes.

1 Introduction to Artificial Functional Nucleic Acids

As described in Chapter 1, naturally occurring functional nucleic acids (FNAs) are now widely recognized and investigated. In parallel, the development of artificial FNAs is well underway. Broadly speaking, artificial FNAs are either aptamers or enzymes. The term "aptamer" derives from the Latin *aptus*, the past participle of "to fit" (Ellington and Szostak 1990). Aptamers are RNA or DNA molecules that bind molecular targets. Nature developed RNA aptamers long before researchers reproduced the achievement. The discovery of self-splicing group I introns (Kruger et al. 1982) inherently showed that RNA can bind small molecules, because guanosine or one of its derivatives is an obligatory and specifically bound cofactor for the splicing reaction (Bass and Cech 1984). Other studies revealed that viruses encode small

RNAs which have biological activity. For example, adenovirus encodes the short virus-associated (VA) RNA that inhibits interferon-induced protein kinase R (PKR) activity (O'Malley et al. 1986), and HIV encodes functionally relevant small RNA oligomers such as the TAR (trans-activation response) RNA that binds to the viral Tat protein (Cullen and Greene 1989; Marciniak et al. 1990). In the same year that the first in vitro aptamer selection experiments were published (Ellington and Szostak 1990; Tuerk and Gold 1990), the naturally occurring TAR aptamer was reported to inhibit HIV replication in vivo (Sullenger et al. 1990). The first artificial nucleic acid enzyme was published in the same year (Robertson and Joyce 1990).

All of these FNAs were identified by in vitro selection. The procedure of in vitro selection was dubbed "Systematic Evolution of Ligands by EXponential Enrichment", or SELEX, by Tuerk & Gold (1990). Although this acronym is often applied to all selection experiments, strictly speaking it applies only to aptamer selections. Methodologies for in vitro selection are growing increasingly sophisticated, for example including the use of such techniques as microfluidics. Nevertheless, the simple principles and methods that were used in the initial experiments are still quite useful and commonly applied. Several general reviews on the in vitro selection of aptamers, ribozymes, and deoxyribozymes have been published (Gold et al. 1995; Breaker 1997; Osborne and Ellington 1997; Wilson and Szostak 1999; Joyce 2004).

2 Methodology for In Vitro Selection of RNA and DNA Aptamers

The most common methodology for in vitro selection of RNA and DNA aptamers depends on exposing a "pool" of random RNA or DNA sequences to a solid support that has been covalently derivatized with the binding target of interest. Sequences that can bind the target remain noncovalently associated with the solid support and can therefore be separated due to their affinity. This section discusses the affinity chromatography approach and also other methods for identifying nucleic acid sequences that bind to desired targets.

The molecular species bound by an aptamer is often called a "ligand", although the same term is also commonly used for the aptamer itself (note what the L of SELEX stands for). To avoid confusion, in this chapter the molecular species bound by an aptamer is designated as the "target" of that aptamer.

2.1 In Vitro Selection of Aptamers by Affinity Chromatography

Although many experimental approaches are used for identification of aptamers by in vitro selection (SELEX), the methodology typically relies upon immobilization of the target on a solid-phase support such as agarose beads (Ellington and Szostak 1990). Then, a pool of random RNA or DNA sequences is exposed to the target-derivatized support. Binding of the very small fraction of RNA or DNA sequences that have a high affinity for the target allows enrichment of those sequences, which

are then eluted by addition of underivatized target. Appropriate biochemical manipulations are used to prepare the enriched pool for initiating another round of the selection process. Selection rounds are iterated until an adequately high level of binding is observed. Typically 5-15 rounds are required until no further enrichment occurs, although occasionally more rounds are needed. Finally, individual aptamer sequences are identified, and their binding and biochemical properties are examined in more detail. The aptamers that emerge from such studies have been used in numerous applications (see Sections II and III of this book).

2.1.1 The Basic Procedures of In Vitro Selection (SELEX) of Aptamers

The fundamental choice between RNA and DNA aptamers is largely a matter of personal preference, because both types of nucleic acid are capable of binding a wide range of targets (Sections 3 and 4). Once the choice of nucleic acid has been made, a typical aptamer selection experiment (Fig. 1) begins with solid-phase synthesis of a long DNA oligonucleotide that contains a stretch of random nucleotides. Each random position is introduced by using an appropriate mixture of the four standard DNA phosphoramidites during the solid-phase synthesis coupling step. For RNA aptamers, the long DNA oligonucleotide is amplified by PCR, and the initially random RNA pool is prepared by transcription using the DNA as a template (Fig. 1, steps A and B). For DNA aptamers, the initially random DNA pool is directly prepared by either primer extension or PCR using the long DNA oligonucleotide as a template. These reactions are facilitated by constant sequence regions that are included adjacent to the random region within the long DNA oligonucleotide.

A key choice at the outset of selection is the length of the random region within the initial pool. The optimal size of the random region is often debated for both binding and catalysis (Sabeti et al. 1997). For a wide range of aptamer targets, random regions as small as 22 nucleotides (i.e., N_{22} regions) have been used successfully (Lozupone et al. 2003; Legiewicz et al. 2005). N_{25} regions have been used to select aptamers for small-molecule targets (Connell and Yarus 1994; Majerfeld and Yarus 1994), proteins (Missailidis et al. 2005; Ferreira et al. 2006), and a misactivated tRNA (Hale and Schimmel 1996). Random regions of N_{40} to N_{80} are more typical, although pools as large as N_{120} (Ellington and Szostak 1992; Famulok and Szostak 1992; Sassanfar and Szostak 1993; Conrad et al. 1994; Tian et al. 1995; Kumar et al. 1997; Yamamoto et al. 2000), N_{134} (Seiwert et al. 2000), or in an unusual case even N_{228} (!) have been utilized (Li et al. 1996). In one set of experiments, the optimal random-region length was found to reflect a balance between factors (Legiewicz et al. 2005), although the generality of the outcome for other targets is uncertain. For a typical N_{40} pool, the size of "sequence space" is 4^{40}, or 10^{24} possible sequences. In practice, technical considerations limit the number of random-pool sequences that can actually be used for initiating the selection to 0.01–10 nmol, which is only 10^{13}–10^{16} sequences Therefore, sequence space is substantially undersampled, with only

one out of every 10^{10} possible sequences examined experimentally (i.e., 10^{-10} coverage of sequence space). Larger random regions such as N_{80} have even less coverage of sequence space. Despite these statistical challenges, aptamer selections do succeed, which means that functional sequences (i.e., those that bind the target) must be quite common within sequence space. The weight of experimental evidence indicates that longer random regions are not always superior, for at least two reasons. First, longer stretches of nucleotides offer more possibilities for forming inhibitory interactions with sequences that would otherwise be good target-binders. Second, the "tyranny of the short motif" (Conrad et al. 1996; Osborne and Ellington 1997) must be avoided. Short motifs have a selective advantage due to their very high probability of being present within a random sequence (Knight and Yarus 2003b), even if a longer motif would be more highly functional (i.e., have a higher affinity for the target). Initiating the selection process with a longer stretch of random nucleotides increases the probability that a suboptimal short motif may be present and outcompete a more functional but less probable longer motif.

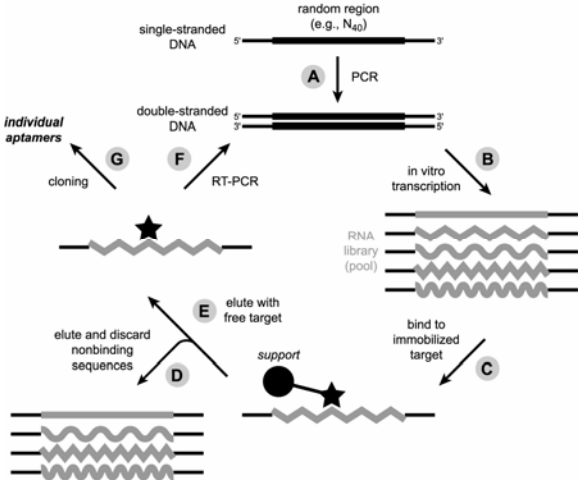

Fig. 1. Typical aptamer selection procedure, illustrated for RNA aptamers.

As the next part of the selection procedure, the random pool is exposed to the target-derivatized solid support (step C). The particular incubation conditions must be chosen carefully. Many experimental variables must be considered, including pH, temperature, buffer identity and concentration, organic cosolvent (if included), metal ion identities and concentrations, and incubation time. The choices made for all of these variables depend on the specific target being used, and there is no general rule for optimal incubation conditions.

The incubation time is usually arranged such that equilibration occurs during the binding time period. Typical incubation times range from 5 min to 1 h. Once equilibration has occurred, nonbinding sequences are eluted and discarded (step D), and the target-binding sequences are eluted with free target (step E). If the selection is for RNA aptamers, then RT-PCR (step F) is used to regenerate a DNA template, which is now enriched in those sequences that (when made as RNA) are capable of binding the target. The new RNA pool is then prepared by transcription from the DNA template. If the selection is for DNA aptamers, then PCR is used directly to regenerate the pool DNA (reverse transcription is of course unnecessary). For DNA aptamer selections, the PCR can be performed "asymmetrically" by including a skewed ratio of the two primers (Gyllensten and Erlich 1988; Ellington and Szostak 1992; Wilson and Szostak 1998; Yang et al. 1998), or "unidirectionally" by including only one primer and obtaining linear amplification (Missailidis et al. 2005). Alternatively, the PCR can be performed normally, but arranged such that the desired single-stranded DNA can be separated physically from its undesired complement. The most common approaches are by binding of the biotinylated form of the undesired DNA strand to immobilized avidin or streptavidin (Bock et al. 1992; Harada and Frankel 1995; Li et al. 1996; Kato et al. 2000a; Blank et al. 2001; Vianini et al. 2001) or by electrophoretic separation of the undesired biotinylated strand (Green et al. 1996; Hicke et al. 1996; Wiegand et al. 1996; Charlton et al. 1997; Daniels et al. 2003).

To achieve the desired affinities between the nucleic acid aptamer and the target, the stringency of the selection procedure can be increased as the selection rounds progress. This is often achieved by decreasing the concentration of the target that is used to displace the binding sequences from the target-derivatized support. A mathematical analysis has been developed for determining the best initial concentration of target to use for elution (Ciesiolka et al. 1996). For the later rounds of selection, empirical feedback from the results of previous rounds is usually used to guide the choices.

After the selection rounds have been completed, the final PCR product is cloned, leading to individual aptamer sequences (step G). By aligning the sequences of tens or even hundreds of individual clones, consensus aptamer sequences may often be identified. However, highly functional aptamers are sometimes "orphans" with little or no sequence homology to the other sequences, and it is unwise to assume that only repeatedly found sequences are functional. Aptamer candidates from the cloning process are screened to determine their target-binding properties. The "winning" sequences are then studied in greater detail, for example to determine their binding constants and secondary structures.

2.1.2 Additional Considerations for Aptamer Selection Procedures

Since the first RNA aptamer selection experiments (Ellington and Szostak 1990; Tuerk and Gold 1990), many refinements to the basic procedures have been made. As a basic component of the affinity chromatography procedure, different solid supports may be used. The original Tuerk & Gold (1990) experiment with the protein T4 DNA polymerase as the target used nitrocellulose filters, as have numerous other experiments with protein targets (Schneider et al. 1992; Tuerk et al. 1992; Giver et al. 1993a; Jellinek et al. 1994; Allen et al. 1995; Binkley et al. 1995; Jellinek et al. 1995; Nazarenko and Uhlenbeck 1995; Tian et al. 1995; Green et al. 1996; Wiegand et al. 1996; Klug et al. 1997; Kubik et al. 1997; Kumar et al. 1997; Bridonneau et al. 1998; Ruckman et al. 1998; Baskerville et al. 1999; Biesecker et al. 1999; Klug et al. 1999; Lebruska and Maher 1999; Fukuda et al. 2000; Hirao et al. 2000; Kawakami et al. 2000; Seiwert et al. 2000; White et al. 2001; Kimoto et al. 2002; Rusconi et al. 2002; Vuyisich and Beal 2002; Chen et al. 2003; Santulli-Marotto et al. 2003; White et al. 2003; Hirao et al. 2004; Gopinath et al. 2006). For most small-molecule aptamer selections, agarose (e.g., Sepharose) is used as the support material. Protein targets can sometimes be immobilized directly on agarose rather than nitrocellulose. Alternatively, the protein of interest can be expressed as a GST fusion and bound to glutathione-agarose (Weiss et al. 1997; Hirao et al. 2000); the protein can be bound to beads via biotin-streptavidin or antibody interactions (Davis et al. 1998); or the protein can be bound to colloidal gold (Moreno et al. 2003).

In all of these approaches, selectively bound nucleic acid sequences are eluted from the protein-derivatized support during each selection round either by changing the ionic strength (Ferreira et al. 2006), by adding SDS, Triton X-100, urea, or guanidinium as denaturant (Giver et al. 1993a; Conrad et al. 1994; Allen et al. 1995; Binkley et al. 1995; Doudna et al. 1995; Jellinek et al. 1995; Nazarenko and Uhlenbeck 1995; Hicke et al. 1996; Lee and Sullenger 1996; O'Connell et al. 1996; Wiegand et al. 1996; Xu and Ellington 1996; Klug et al. 1997; Kubik et al. 1997; Kumar et al. 1997; Lee and Sullenger 1997; Weiss et al. 1997; Bridonneau et al. 1998; Davis et al. 1998; Ruckman et al. 1998; Baskerville et al. 1999; Biesecker et al. 1999; Klug et al. 1999; Lebruska and Maher 1999; Fukuda et al. 2000; Hirao et al. 2000; Kawakami et al. 2000; Seiwert et al. 2000; Srisawat and Engelke 2001; Proske et al. 2002; Vuyisich and Beal 2002; Chen et al. 2003; Santulli-Marotto et al. 2003; White et al. 2003; Hirao et al. 2004; Gopinath et al. 2006), or by cleaving the protein-support linker (Nieuwlandt et al. 1995; White et al. 2001; Rusconi et al. 2002). Alternatively, the protein target can be immobilized on agarose that has been derivatized with a second protein which interacts tightly with the target; e.g., concanavalin A that binds strongly to thrombin (Bock et al. 1992). In such a case, an unrelated ligand that in-

teracts well with the second protein is used to displace and therefore elute selectively bound nucleic acid sequences during each selection round (e.g., α-methylmannoside as a concanavalin A ligand). Other solid supports that have been used to identify aptamers for protein targets include plastic plates or flasks (Hicke et al. 2001; Gopinath et al. 2006) or the surfaces of PCR tubes (Missailidis et al. 2005).

In some experiments with small-molecule targets, the functional RNA or DNA sequences are eluted from the affinity column not by adding free target but by changing the ionic strength of the elution buffer or by chelating divalent metal ions, which therefore denatures the nucleic acid. These approaches have been particularly effective in several selections of aptamers that bind to amino acids (Famulok and Szostak 1992; Harada and Frankel 1995; Tao and Frankel 1996).

Measures must be taken to ensure that the RNA or DNA sequences do not merely bind directly to the support itself, regardless of its chemical makeup. Binding directly to the support is usually avoided by exposing the pool to the underivatized support and discarding all sequences that are retained before the pool is exposed to the target-derivatized support. Of course, any sequences that would have been capable of binding to the target but also bind to the support itself are lost. This is an unavoidable drawback of the affinity chromatography procedures. Without discarding the support-binding sequences, in most cases they would overwhelm the selection process and prevent any true target-binding sequences from being discovered.

Some degree of selectivity for the target is usually desired, and several approaches to ensure high selectivity have been developed. Negative selection, also termed counter-selection, is commonly applied (Ellington and Szostak 1992; Jenison et al. 1994; Majerfeld and Yarus 1994; Harada and Frankel 1995; Lauhon and Szostak 1995; Geiger et al. 1996; Haller and Sarnow 1997; Burke and Hoffman 1998; Rink et al. 1998; Wallace and Schroeder 1998; Gebhardt et al. 2000; Seiwert et al. 2000; Brockstedt et al. 2004). In negative selection, the pool of nucleic acid sequences is exposed to a secondary target for which binding is *not* desired, and any sequences that bind are discarded. The remaining sequences are then allowed to bind with the desired support-bound target as usual, and the successfully binding sequences are retained to be input into the next round of selection. Exposure of the pool to the underivatized support (as described above) is an essentially universal form of negative selection against nonselective binding to the support material itself.

The first ATP aptamer (Sassanfar and Szostak 1993) was re-discovered independently two additional times (Burgstaller and Famulok 1994; Burke and Gold 1997), but a different aptamer was found when discrimination between AMP and ATP was enforced by negative selection (Sazani et al. 2004). Interestingly, the discriminating aptamer was not found in the other selection experiments, even though it binds to ATP only slightly less well than the earlier aptamers. Although negative selection is very useful to increase target selectivity, such selectivity does not necessarily *require* the use of negative selection; selectivity may instead emerge fortuitously. For example, the Huizenga & Szostak (1995) selection of a DNA ATP aptamer provided a sequence that selectively binds ATP over other NTPs, even though negative selection was never applied (DNA aptamers are discussed in more detail in Section 3 below). Similarly, an aptamer that binds both guanine and xanthine did not bind to any of adenine, cytosine, or uracil, even though negative selection against binding of these other nucleobases was never applied (Kiga et al. 1998; Meli et al. 2002). Other examples of discrimination against binding of closely related small-molecule targets without negative selection have been described (Wallis et al. 1995; Koizumi and Breaker 2000; Win et al. 2006). With or without negative selection, selectivity among closely related protein targets has also been achieved in several cases (Conrad et al. 1994; Doudna et al. 1995; Lee and Sullenger 1997; Seiwert et al. 2000; Rusconi et al. 2002; White et al. 2003).

Sometimes, target generality rather than selectivity is sought. For this purpose with protein targets, investigators applied “toggle SELEX”, in which the RNA pool is alternately exposed to human and animal versions of the same proteins. This is advantageous for preclinical studies to ensure cross-reactivity; i.e., to make certain that both forms of the proteins are bound by the aptamer (White et al. 2001). A similar approach could certainly be applied for other targets such as small molecules, to ensure that undesired selectivity is not inadvertently obtained.

Aptamer selections do not necessarily need to begin with an entirely random pool. Instead, in a process termed “reselection”, a known aptamer may be partially randomized (mutagenized) to provide the starting point for a new selection effort (Connell and Yarus 1994; Lorsch and Szostak 1994b; Huizenga and Szostak 1995; Lauhon and Szostak 1995; Burke et al. 1996; Geiger et al. 1996; Burke et al. 1997; Mannironi et al. 1997; Burke and Hoffman 1998; Holeman et al. 1998; Wilson et al. 1998; Biesecker et al. 1999; Koizumi and Breaker 2000; Held et al. 2003). The aptamer starting point for reselection may even be derived from a natural RNA sequence (Nazarenko and Uhlenbeck 1995; Klug et al. 1997). The reselection approach takes advantage both of known target-binding sequences and of the power of selection to improve the original binding activity. Reselection is also used in nucleic acid enzyme selections (see Section 6.1.2). As an alternative to reselection via solid-phase synthesis of a mutagenized pool, error-prone PCR (Cadwell and Joyce 1994) or nonstandard nucleotide triphosphates (Tahiri-Alaoui et al. 2002) may be used to introduce sequence variation (Nieuwlandt et al. 1995). In one case, appending an additional entirely random region to a known aptamer and repeating the selection process was used to improve the binding affinity of the aptamer (Hwang and Lee 2002).

One important consideration for aptamer selection experiments is whether or not the constant primer-binding sequences will become involved in the target-binding event. This is not necessarily detrimental, although a requirement for the primer-binding sequences may make it difficult to minimize (truncate) any aptamers as part of a practical application. Therefore, several approaches have been developed to ensure that the primer-binding sequences do not participate directly or indirectly in

target binding. These methods sequester the primer-binding sequences within duplex structures either intramolecularly (Jarosch et al. 2006) or intermolecularly (Nutiu and Li 2005), using the logic that duplex nucleic acids are less likely than single strands to interact with a target. Alternatively, ligation is used to append primer-binding sequences during each round of selection, but the primer-binding sequences are absent during the key selection step itself (Vater et al. 2003).

2.2 Aptamer Selection Methods Other than Affinity Chromatography

In addition to approaches based on binding of nucleic acids to agarose beads or nitrocellulose filters, many other aptamer selection methods have been developed. In one study, the protein target was attached to colloidal gold (Moreno et al. 2003). This effectively increased the mass of the protein and allowed a more efficient separation of successfully bound DNA sequences simply by centrifugation to pellet the gold. Another technical advance is the use of magnetic beads, which allow more convenient separations (faster and consuming less material). His_6-tagged proteins may be bound to Ni-NTA magnetic beads to identify aptamers for a protein target (Murphy et al. 2003). Magnetic beads may also be used with non-His_6-tagged proteins (Daniels et al. 2002; Lupold et al. 2002; Tahiri-Alaoui et al. 2002). Antibody-coated magnetic beads have been used for immunoprecipitation during selection (Doudna et al. 1995; Lee and Sullenger 1996, 1997). Immunoprecipitation using non-magnetic beads has also been reported (Tsai et al. 1992; Bridonneau et al. 1998).

A variant of the magnetic bead approach that additionally uses fluorescence has been termed “FluMag-SELEX” (Mann et al. 2005; Stoltenburg et al. 2005). In this technique, fluorescent labels replace radioactive (^{32}P) tags to quantify the bound and unbound oligonucleotides during each selection step. This could be particularly advantageous if a laboratory wished completely to avoid the use of radioactivity. In principle, a fluorescence change in the appropriately derivatized target (Babendure et al. 2003) could even be used as the basis for selecting aptamers via a compartmentalization approach (Tawfik and Griffiths 1998; Miller et al. 2006). However, this approach has not yet been reported.

As an alternative to relying on noncovalent affinity, photoinduced covalent bond formation has been used as the basis for selection (Golden et al. 2000). In one set of experiments, selection rounds with or without photocrosslinking were alternated, leading to aptamers that have both high affinity to the target (HIV-1 REV protein) and high photocrosslinking efficiency (Jensen et al. 1995). The photochemistry required incorporation of a nonstandard 5-iodouracil nucleotide into the RNA.

Nondenaturing gel electrophoresis has been used as the basis for separating RNA or DNA that binds to protein targets (Conrad et al. 1994; Smith et al. 1995; Green et al. 1996; Charlton et al. 1997; Weiss et al. 1997; Ruckman et al. 1998; Srisawat and Engelke 2001; Chen et al. 2003). Often this has been used as an alternative approach to filter binding within a single selection experiment. Alternation of two physical

approaches helps to suppress artifacts such as sequences that might opportunistically survive the selection procedure if only one approach were used.

Surface plasmon resonance (SPR; Biacore) has been used directly as part of the selection process in several instances (Khati et al. 2003; Pileur et al. 2003; Misono and Kumar 2005). Enrichment of binding sequences is based on the slow dissociation rate of aptamers from the surface-bound target.

Methodology that avoids the need to derivatize the target and then bind it to a bead is desirable for practical reasons. Capillary electrophoresis (CE) methods have been used to identify new DNA aptamers (Mendonsa and Bowser 2004; Berezovski et al. 2005; Berezovski et al. 2006; Drabovich et al. 2006; Tang et al. 2006), although such methods could presumably be applied to RNA aptamers as well. At present these methods appear promising, and it will be interesting to see the extent to which they supplant the original solid-support methods. CE and other analytical techniques that depend upon aptamers for the binding events are also being developed (Ravelet et al. 2006) (see Section III of this book).

Finally, several automated aptamer selection processes have been developed. This has been done by the laboratory of Ellington (Cox and Ellington 2001; Sooter et al. 2001; Cox et al. 2002a; Cox et al. 2002b; Bryant et al. 2005) and also by others (Drolet et al. 1999; Eulberg et al. 2005; Hybarger et al. 2006). The importance of automated selection will certainly increase as aptamer technologies improve and as aptamers are more widely used in practical applications.

3 Molecular Targets and Properties of RNA Aptamers

A particularly striking feature of RNA and DNA aptamers is the wide range of targets that may be bound. Not considering divalent metal ion targets (Ciesiolka et al. 1995; Ciesiolka and Yarus 1996; Hofmann et al. 1997), the smallest artificial target for which an aptamer has been developed is ethanolamine, which has only four heavy (non-hydrogen) atoms and a molecular weight of merely 61 (Mann et al. 2005). Despite this small size, the molecule is bound by its (DNA) aptamer with binding constant (K_d) of 6 nM. If nucleic acids can bind tightly to such a small and relatively featureless target, then larger targets should certainly be bindable as well. Indeed, RNA aptamers have been identified that bind well to many targets (Fig. 2). This section describes these molecular targets of RNA aptamers and discusses some features of the aptamers themselves. Several examples of aptamers for transition-state analogues are presented in the discussion on ribozymes (see Section 6.1.3).

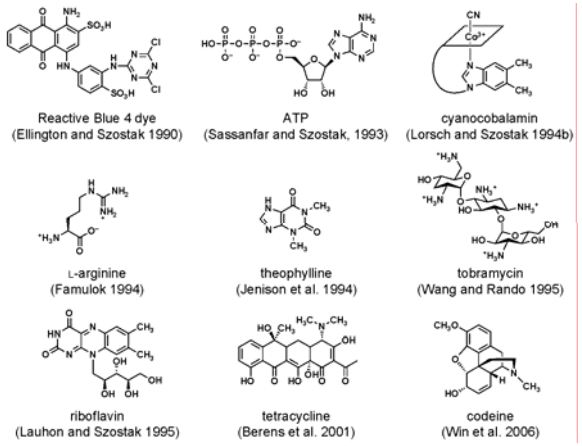

Fig. 2. Representative small-molecule targets of RNA aptamers.
The corrin ring of cyanocobalamin (vitamin B_{12}) is shown schematically.

3.1 Molecular Targets Bound by RNA Aptamers

3.1.1 The First RNA Aptamers

The first RNA aptamers were reported in 1990 (Ellington and Szostak 1990; Tuerk and Gold 1990). Ellington and Szostak identified RNAs that bind to small organic dyes, whereas Tuerk and Gold found RNAs that bind to T4 DNA polymerase. These pioneering experiments established that in vitro selection for target binding by nucleic acids is a viable experimental approach. The studies of Ellington and Szostak used organic dyes attached to agarose beads, and the experiments of Tuerk and Gold used T4 DNA polymerase bound to nitrocellulose filters.

One of the first RNA aptamers for a small biomolecule as the target was a sequence that binds ATP and its derivatives (Sassanfar and Szostak 1993). To enable the selection effort, ATP was bound to an agarose support via a tether connected to

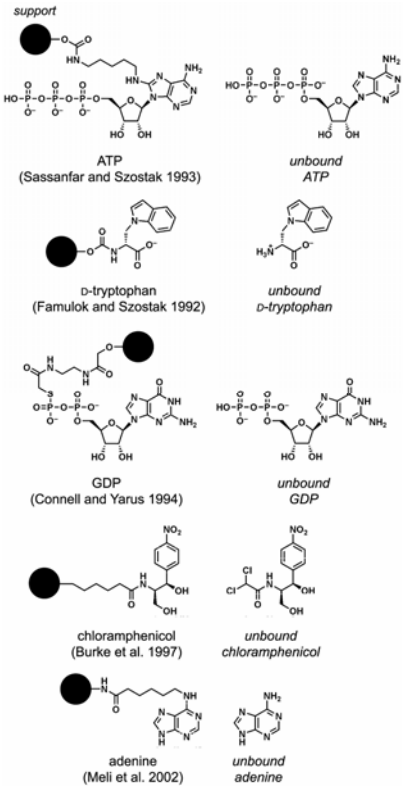

Fig. 3. Attachment of small-molecule RNA aptamer targets to solid support.
On the right are shown unbound versions of each target.

the C8 position of the adenine nucleobase (Fig. 3). Elution of binding sequences with free ATP ensured that the resulting RNA aptamers can bind to untethered ATP as well as to the support-tethered compound. After eight rounds of selection, an aptamer consensus sequence was identified by comparing the sequences of 39 clones. Based on the experimental results, a short 40-nucleotide RNA aptamer (Fig. 4) was designed and tested for binding to ATP and numerous derivatives. The data revealed that the adenine nucleobase and ribose sugar moieties are recognized by the RNA aptamer, whereas the 5'-triphosphate is dispensable for binding. At 5 mM Mg^{2+}, which was the concentration of Mg^{2+} present during the selection procedure, the K_d of the aptamer for ATP was on the order of 10 μM, which wasimproved to 0.7 μM by strategic nucleotide changes. The precise K_d values depended on which measurement method was used (see Section 3.2 for more on biochemical characterization of aptamers).

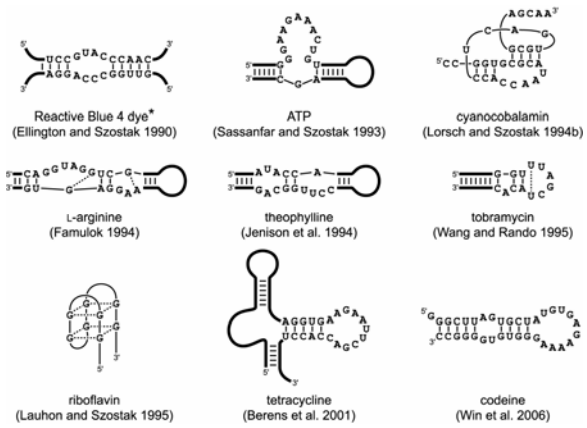

Fig. 4. Representative RNA aptamers, shown in proposed secondary structures.
* = Only the core motif of the dye binding site is illustrated.

3.1.2 Small-Molecule Targets of RNA Aptamers

Small-molecule antibiotic compounds have long been known to interact with ribosomal RNA (Moazed and Noller 1987; Hermann 2003) and to disrupt group I intron splicing by either competitive or noncompetitive inhibition (von Ahsen and Schroeder 1990; von Ahsen et al. 1991; von Ahsen and Schroeder 1991; von Ahsen et al. 1992). The more recent discovery of metabolite-binding "riboswitch" RNAs (see Chapter 1) has strikingly demonstrated that binding of small molecules by RNA can have important natural effects in vivo. Both to advance fundamental understanding and possibly to assist in therapeutic interventions, a large number of in vitro selection experiments have focused on identifying RNA aptamers that bind to small-molecule targets. Here, "small molecules" are defined as compounds of relatively low molecular weight (<1,000), which among other targets includes various antibiotics, cofactors, and metabolites. See Table 1 for a compilation of RNA aptamer binding constants for small-molecule targets.

Organic dyes were used not only in the initial aptamer selection experiments (Ellington and Szostak 1990) but in several subsequent studies (Holeman et al. 1998; Werstuck and Green 1998; Grate and Wilson 1999). As mentioned above, one of the first RNA aptamers was selected for binding to ATP (Sassanfar and Szostak 1993). Soon thereafter, an aptamer for theophylline was reported (Jenison et al. 1994). This aptamer was identified using negative selection against binding of caffeine, and excellent (10^4-fold) discrimination was observed. Aptamers were subsequently identified that bind to nucleobases (Kiga et al. 1998), to nucleosides and nucleotides (Connell and Yarus 1994; Koizumi and Breaker 2000; Meli et al. 2002; Sazani et al. 2004), or to their modified or damaged analogues (Haller and Sarnow 1997; Rink et al. 1998). Aptamers have also been found for the adenosine moiety of S-adenosylmethionine (SAM) (Burke and Gold 1997) and for S-adenosylhomocysteine (SAH) (Gebhardt et al. 2000).

Many aptamers have been developed for naturally occurring antibiotics such as tobramycin (Wang and Rando 1995; Wang et al. 1996), neomycin (Wallis et al. 1995), lividomycin (Lato et al. 1995; Lato and Ellington 1996), kanamycin A (Lato et al. 1995), kanamycin B (Kwon et al. 2001), chloramphenicol (Burke et al. 1997), streptomycin (Wallace and Schroeder 1998), tetracycline (Berens et al. 2001), and the peptide antibiotic viomycin (Wallis et al. 1997). The example of chloramphenicol illustrates that even key functional groups of the target molecule can be missing when tethered to the solid support (Fig. 3), yet the selection process still leads to highly functional aptamers.

Aptamers have been identified for biological cofactors such as cyanocobalamin (vitamin B_{12}) (Lorsch and Szostak 1994b), flavin derivatives (Burgstaller and Famulok 1994; Lauhon and Szostak 1995; Roychowdhury-Saha et al. 2002), biotin

Small-molecule target	K_d, μM	Reference
tobramycin	0.002	(Wang and Rando 1995; Wang et al. 1996)
sialyl Lewis X (sLe^X)	0.003	(Jeong et al. 2001)
cyanocobalamin (vitamin B_{12})	0.09	(Lorsch and Szostak 1994b)
theophylline	0.1	(Jenison et al. 1994)
S-adenosylhomocysteine	0.1	(Gebhardt et al. 2000)
neomycin	0.1	(Wallis et al. 1995)
kanamycin B	0.2	(Kwon et al. 2001)
L-arginine	0.3	(Geiger et al. 1996)
sulforhodamine B dye	0.3	(Holeman et al. 1998)
8-oxo-deoxyguanosine	0.3	(Rink et al. 1998)
flavin mononucleotide (FMN)	0.5	(Burgstaller and Famulok 1994)
7-methyl-guanosine	0.5	(Haller and Sarnow 1997)
coenzyme A (CoA)	0.5	(Burke and Hoffman 1998)
4,4′-methylenedianiline	0.5	(Brockstedt et al. 2004)
ATP	0.7	(Sassanfar and Szostak 1993)
riboflavin	1	(Lauhon and Szostak 1995)
guanine	1	(Kiga et al. 1998)
streptomycin	1	(Wallace and Schroeder 1998)
Malachite Green dye	1	(Grate and Wilson 1999)
tetracycline	1	(Berens et al. 2001)
chloramphenicol	2	(Burke et al. 1997)
L-dopamine	2	(Mannironi et al. 1997)
nicotinamide mononucleotide (NMN)	3	(Lauhon and Szostak 1995)
codeine	3	(Win et al. 2006)
ATP (recognizing triphosphate)	5	(Sazani et al. 2004)
biotin	6	(Wilson et al. 1998)
viomycin	10	(Wallis et al. 1997)
cAMP	10	(Koizumi and Breaker 2000)
adenine	10	(Meli et al. 2002)
D-tryptophan	20	(Famulok and Szostak 1992)
L-tyrosine	40	(Mannironi et al. 2000)
flavin adenine dinucleotide (FAD)	50	(Roychowdhury-Saha et al. 2002)
L-arginine	60	(Famulok 1994)
L-citrulline	60	(Famulok 1994)
Cibacron Blue 3G-A dye	100	(Ellington and Szostak 1990)
L-arginine	200	(Connell et al. 1993)
L-isoleucine	200	(Majerfeld and Yarus 1998)
Reactive Blue 4 dye	600	(Ellington and Szostak 1990)
L-isoleucine	1000	(Lozupone et al. 2003)
L-arginine	4000	(Connell and Yarus 1994)
L-valine	10000	(Majerfeld and Yarus 1994)

Table 1. Binding constants (K_d values) of RNA aptamers for small-molecule targets. Listed is the best K_d value for each target from the indicated reference.

(Wilson and Szostak 1995; Wilson et al. 1998), and coenzyme A (CoA) (Burke and Hoffman 1998; Saran et al. 2003). Aptamers have also been identified for free amino acids or their derivatives such as dopamine (Famulok and Szostak 1992; Connell et al. 1993; Famulok 1994; Geiger et al. 1996; Mannironi et al. 1997; Mannironi et al. 2000). One set of experiments found aptamers that bind to either arginine or guanosine, presumably due to the related structures of these targets (Connell and Yarus 1994). Another experiment found arginine-binding RNAs that resemble the natural TAR RNA of HIV (Tao and Frankel 1996). A series of experiments from the Yarus laboratory has identified aptamers for aliphatic amino acids and explored their properties (Majerfeld and Yarus 1994, 1998; Lozupone et al. 2003). These efforts have also provided empirical evidence regarding the optimal size of the random region for selection experiments (Legiewicz et al. 2005).

Other small-molecule targets that are bound by RNA include codeine (Win et al. 2006) and carcinogenic amines (Brockstedt et al. 2004). An RNA aptamer has been found that binds with nanomolar affinity to the sialyl Lewis X tetrasaccharide (Jeong et al. 2001). Finally, aptamers for carbohydrates such as sephadex may have utility for purification of RNA or RNA-protein complexes (Srisawat et al. 2001).

Because nucleic acids are inherently polyanionic, monovalent or divalent metal ions are always required for structure and therefore function. Mg^{2+} is commonly included in the binding buffer during the selection process. In some cases, the divalent metal ion requirements of the resulting aptamers have been investigated in detail (Wallis et al. 1995; Burke and Hoffman 1998). The Mg^{2+} may not actually be required for binding (Lorsch and Szostak 1994b), and indeed no divalent metal ions at all have been included in some selection procedures (Lato et al. 1995; Lato and Ellington 1996; Tao and Frankel 1996). In other experiments, more than one divalent metal ion has been included, and ions other than Mg^{2+} such as Mn^{2+} or Zn^{2+} can be required for target binding (Majerfeld and Yarus 1998; Gebhardt et al. 2000; Koizumi and Breaker 2000).

3.1.3 Peptide and Protein Targets of RNA Aptamers

Many manuscripts have been published on RNA aptamers for peptides and proteins. This reflects the immense practical interest in proteomics methods that use aptamer technologies (see Section II of this book) and also indicates the attention devoted to therapeutic applications of protein-aptamer interactions (Lee et al. 2006). Table 2 contains a compilation of RNA aptamer binding constants for protein targets.

Protein target	K_d, nM	Reference
keratinocyte growth factor	0.0003	(Pagratis et al. 1997)
vascular endothelial growth factor (VEGF)	0.05	(Ruckman et al. 1998)
thrombin	0.05	(White et al. 2001)
HIV-1 Tat protein	0.1	(Yamamoto et al. 2000)
P-selectin	0.2	(Jenison et al. 1998)
haemagglutinin	0.2	(Gopinath et al. 2006)
basic fibroblast growth factor	0.4	(Jellinek et al. 1995)
human CD4	0.4	(Davis et al. 1998)
rat GPCR for neurotensin	0.4	(Daniels et al. 2002)
coagulation factor IXa	0.6	(Rusconi et al. 2002)
nuclear factor κB (NF-κB)	1	(Lebruska and Maher 1999)
antibody against human insulin receptor	2	(Doudna et al. 1995)
interferon-γ	2	(Kubik et al. 1997)
human nonpancreatic secretory phospholipase A_2	2	(Bridonneau et al. 1998)
human complement C5 component	2	(Biesecker et al. 1999)
G6-9 anti-DNA autoantibody	2	(Kim et al. 2003)
colicin E3	2	(Hirao et al. 2004)
transforming growth factor-β type III receptor	2	(Ohuchi et al. 2006)
elongation factor Tu (EF-Tu)	3	(Nazarenko and Uhlenbeck 1995)
L-selectin	3	(O'Connell et al. 1996)
formamidopyrimidine glycosylase (Fpg)	3	(Vuyisich and Beal 2002)
angiopoietin-2	3	(White et al. 2003)
HIV-1 reverse transcriptase (RT)	5	(Tuerk et al. 1992)
bacteriophage R17 coat protein	5	(Schneider et al. 1992)
extracellular regulated kinase 2 (ERK2)	5	(Seiwert et al. 2000)
fibrinogen-like domain of tenascin-C	5	(Hicke et al. 2001)
HIV-1 surface glycoprotein gp120	5	(Khati et al. 2003)
hepatitis C virus nonstructural protein 3 protease	6	(Fukuda et al. 2000)
antibody against MIR of AChR	6	(Hwang and Lee 2002)
streptavidin	7	(Tahiri-Alaoui et al. 2002)
coagulation factor VIIa	10	(Rusconi et al. 2000)
cytotoxic T cell antigen-4 (CTLA-4)	10	(Santulli-Marotto et al. 2003)
elongation factor SelB	20	(Klug et al. 1997)
pepocin	20	(Hirao et al. 2000)
prion protein (PrP^{Sc})	20	(Rhie et al. 2003)
immunoglobulin IgE	30	(Wiegand et al. 1996)
HTLV-1 Rex ARM peptide	30	(Baskerville et al. 1999)
Alzheimer's βA4(1-40) amyloid peptide	30	(Ylera et al. 2002)
heparan sulfate (on cell surface)	40	(Ulrich et al. 2002)
human epidermal growth factor receptor-3	50	(Chen et al. 2003)
herpes simplex virus-1 US11 protein	70	(Bryant et al. 2005)
HIV-1 integrase	80	(Allen et al. 1995)
prion protein (PrP)	100	(Proske et al. 2002)
fibronectin (on cell surface)	100	(Ulrich et al. 2002)
substance P	200	(Nieuwlandt et al. 1995)
human nerve growth factor	200	(Binkley et al. 1995)
streptavidin	200	(Srisawat and Engelke 2001)
laminin (on cell surface)	200	(Ulrich et al. 2002)
Ras-binding domain of Raf-1	200	(Kimoto et al. 2002)
HIV-1 Tat protein	300	(Kawakami et al. 2000)
thrombospondin (on cell surface)	400	(Ulrich et al. 2002)

Table 2. Binding constants (K_d values) of RNA aptamers for protein targets. Listed is the best K_d value for each target from the indicated reference.

Peptide targets for RNA aptamers include techykinin substance P (Nieuwlandt et al. 1995), a peptide derived from the Rev protein (Xu and Ellington 1996), the HTLV-1 Rex ARM peptide (Baskerville et al. 1999), and an Alzheimer's disease amyloid peptide (Ylera et al. 2002). Protein targets for RNA aptamers include streptavidin (Tahiri-Alaoui et al. 2002), which may find use in affinity purifications (Srisawat and Engelke 2001), as well as many proteins of more therapeutic interest. These proteins include HIV-1 reverse transcriptase (Tuerk et al. 1992; Burke et al. 1996), HIV-1 REV protein (Giver et al. 1993a; Giver et al. 1993b), HIV-1 integrase (Allen et al. 1995), bacteriophage R17 coat protein (Schneider et al. 1992), protein kinase C (Conrad et al. 1994), vascular endothelial growth factor (VEGF; see next subsection) (Jellinek et al. 1994; Green et al. 1995b; Ruckman et al. 1998) and other growth factors (Jellinek et al. 1995; Pagratis et al. 1997), trans-activating protein Tax from human T-cell leukemia virus (Tian et al. 1995), antibodies (Tsai et al. 1992; Doudna et al. 1995; Lee and Sullenger 1996, 1997; Hwang and Lee 2002; Kim et al. 2003), immunoglobulins (Wiegand et al. 1996), selectins (O'Connell et al. 1996; Jenison et al. 1998), interferon-γ (Kubik et al. 1997), thrombin (Drolet et al. 1999; White et al. 2001) and other serine proteases (Kumar et al. 1997; Fukuda et al. 2000; Rusconi et al. 2000; Rusconi et al. 2002), prion proteins (Weiss et al. 1997; Proske et al. 2002; Rhie et al. 2003), nuclear factor κB (Lebruska and Maher 1999), angiopoietin-2 (White et al. 2003), T cell antigens (Santulli-Marotto et al. 2003), integrins (Blind et al. 1999; Mi et al. 2005), and many other proteins (Binkley et al. 1995; Nazarenko and Uhlenbeck 1995; Klug et al. 1997; Bridonneau et al. 1998; Davis et al. 1998; Biesecker et al. 1999; Klug et al. 1999; Hirao et al. 2000; Kawakami et al. 2000; Hicke et al. 2001; Daniels et al. 2002; Kimoto et al. 2002; Lupold et al. 2002; Ulrich et al. 2002; Vuyisich and Beal 2002; Khati et al. 2003; Hirao et al. 2004; Bryant et al. 2005; Romero-López et al. 2005; Gopinath et al. 2006; Homann et al. 2006; Ohuchi et al. 2006).

An RNA aptamer that binds two orders of magnitude more tightly to the Tat protein than the natural TAR RNA aptamer was identified (Yamamoto et al. 2000). This indicates that even when nature has already provided aptamers, improvements to binding can still be made. Several reports show that aptamers selected for a particular "epitope" can bind that target when it is present in a different context; e.g. within a full-length protein (Xu and Ellington 1996; Blind et al. 1999; Proske et al. 2002). An RNA aptamer for a serine protease was developed by conjugating a known small-molecule covalent inhibitor to a random RNA pool and then performing selection (Smith et al. 1995).

3.1.4 RNA Aptamers for In Vivo and Therapeutic Applications

Substantial efforts have focused upon applying RNA aptamers in vivo and increasingly for therapeutic purposes. For example, aptamers have been identified that act as transcriptional activators (Buskirk et al. 2003) and can be controlled in vivo by a

small molecule that binds to a fused aptamer domain (Buskirk et al. 2004). Target-regulated aptamers have been developed to control protein activity (Vuyisich and Beal 2002).

Several aptamers for extracellular targets have been pursued (Pestourie et al. 2005). The first aptamer approved for human medicinal use by the US Food and Drug Administration (FDA) was Macugen (Tucker et al. 1999; Kim et al. 2002; Eyetech Study Group 2003), which is effective against age-related macular degeneration and has been marketed since January 2005. This aptamer (which is heavily modified chemically; see Section 3.3 below for a general discussion) binds to the extracellular vascular endothelial growth factor VEGF and suppresses angiogenesis. At present, many other RNA aptamers are in various stages of the drug development process. Many of the aptamers mentioned in the previous subsection (e.g., those that bind to growth factors and coagulation factors) interact with extracellular targets.

Aptamers have been selected using live cells as the targets (Morris et al. 1998; Ulrich et al. 2002; Lorger et al. 2003; Cerchia et al. 2005; Chu et al. 2006; Ohuchi et al. 2006; Shangguan et al. 2006), in some cases with selectivity for certain cell types. These aptamers are thought to interact with particular molecules on the cell surface (Pestourie et al. 2005). By alternation of purified protein with live cells expressing that protein as the target in a "crossover" SELEX technique, aptamers for the tenascin-C protein were identified that bind selectively to tumor cells expressing this protein (Hicke et al. 2001). Viral particles (Pan et al. 1995; Wang et al. 2000; Gopinath et al. 2006) or even whole organisms (Homann and Göringer 1999; Lorger et al. 2003; Homann et al. 2006) have also been used successfully as targets.

Aptamers expressed inside of cells ("intramers") can function just as well as they do in vitro (Werstuck and Green 1998; Famulok et al. 2001; Famulok and Verma 2002; Kim and Jeong 2004; Theis et al. 2004; Toulmé et al. 2004; Choi et al. 2006). Indeed, intramers are increasingly being investigated for use in human medicine (Cerchia et al. 2002; Nimjee et al. 2005; Ireson and Kelland 2006).

3.1.5 Other Targets for RNA Aptamers

RNA aptamers have been identified for binding to nucleic acid structures (Pei et al. 1991; Mishra et al. 1996; Soukup et al. 1996; Boiziau et al. 1997; Ducongé and Toulmé 1999; Scarabino et al. 1999; Tok et al. 2000; Aldaz-Carroll et al. 2002; Kikuchi et al. 2003; Da Rocha Gomes et al. 2004; Fauzi et al. 2005; Kikuchi et al. 2005). Aptamers have also been selected for binding to host-guest complexes (Manimala et al. 2004). A bis-boronic acid host was used to bind guests such as citrate or tartrate, and aptamers were identified that bind to the complex. In this fashion, RNA aptamers for these very small and anionic targets were found.

3.2 Biochemical Characterization of RNA Aptamers

Due to their fundamental implications for understanding how nucleic acids interact with other molecules, and also for their practical utility, many aptamers have been characterized extensively by biochemical means. These characterizations are presented here. NMR and X-ray structural studies of both RNA and DNA aptamers are discussed together in Section 5 below.

3.2.1 Secondary Structure Analysis of RNA Aptamers

Prior to analysis of secondary structure, the minimal range of nucleotides that is required by an aptamer for target binding may be determined by boundary mapping (Connell et al. 1993; Wallis et al. 1995; Green et al. 1996; Wiegand et al. 1996; Burke and Gold 1997; Burke et al. 1997; Kubik et al. 1997; Mannironi et al. 1997; Bridonneau et al. 1998; Burke and Hoffman 1998; Wallace and Schroeder 1998; Wilson et al. 1998; Biesecker et al. 1999; Lebruska and Maher 1999; Fukuda et al. 2000; Koizumi and Breaker 2000; Mannironi et al. 2000; Meli et al. 2002; Roychowdhury-Saha et al. 2002). In this procedure, the aptamer is radiolabeled at either its 5'-terminus or 3'-terminus. After partial alkaline hydrolysis is used to generate a ladder of cleaved aptamers, the RNA sequences that can bind to (and be eluted from) target-derivatized solid support are revealed by PAGE. Alternatively, restriction endonucleases can be used to truncate the DNA that encodes an RNA aptamer, and the resulting RNAs can be screened to determine a cutoff point in the sequence beyond which target-binding function is lost (Lorsch and Szostak 1994b).

Aptamers can often tolerate nucleotide changes at particular positions, as long as Watson-Crick base-pairing interactions are maintained (Carothers et al. 2004). Via analysis of such base-pair covariations, which may be revealed by sequence alignments of many initial clones (Davis et al. 1996), individual secondary structures may be proposed (Fig. 4). Such secondary structures may also be proposed even for orphan sequences via secondary structure prediction programs such as mfold (Zuker 2003). Alternatively, an "artificial phylogeny" may be generated by randomization and reselection (Hirao et al. 2004; Ohuchi et al. 2006). By whatever means they are generated, the secondary structure predictions may then be validated by enzymatic and chemical probing experiments (Sassanfar and Szostak 1993; Burgstaller et al. 1995; Burgstaller and Famulok 1996; Haller and Sarnow 1997; Kiga et al. 1998; Majerfeld and Yarus 1998; Wilson et al. 1998; McGregor et al. 1999; Fukuda et al. 2000; Hirao et al. 2000; Kawakami et al. 2000; Seiwert et al. 2000; Kimoto et al. 2002; Roychowdhury-Saha et al. 2002; Sayer et al. 2004; Dey et al. 2005) or by mutagenesis in which Watson-Crick covariations are tested empirically. If enzymatic or chemical probing experiments are performed in both the absence and presence of the target, then the data may be used to provide a "footprint" of the target upon the aptamer (Connell and Yarus 1994; Lorsch and Szostak 1994b; Allen et al. 1995; Binkley et al. 1995; Lauhon and Szostak 1995; Wallis et al. 1995; Geiger et al. 1996;

Tao and Frankel 1996; Burke et al. 1997; Klug et al. 1997; Mannironi et al. 1997; Wallis et al. 1997; Wallace and Schroeder 1998; Klug et al. 1999; Hirao et al. 2000; Mannironi et al. 2000; Yamamoto et al. 2000; Berens et al. 2001; Kwon et al. 2001; Meli et al. 2002; Tahiri-Alaoui et al. 2002; Vuyisich and Beal 2002; Kim et al. 2003; Hirao et al. 2004; Bryant et al. 2005; Gopinath et al. 2006; Win et al. 2006).

Some studies have suggested the value of embedding a non-random structured RNA fragment such as a stem-loop within an otherwise random region, which enables more highly functional aptamers to be identified (Davis and Szostak 2002; Carothers et al. 2004). Although it may be useful, this approach has not been widely adopted.

3.2.2 Binding Constants of RNA Aptamers: Methodology

Aptamer binding constants are determined by one of several experimental methods, or sometimes by more than one approach. One particularly common method to determine K_d for a small-molecule target is isocratic elution (Dunn and Chaiken 1974; Arnold and Blanch 1986; Arnold et al. 1986), which has been applied many times (Ellington and Szostak 1990; Famulok and Szostak 1992; Connell et al. 1993; Sassanfar and Szostak 1993; Burgstaller and Famulok 1994; Connell and Yarus 1994; Famulok 1994; Lorsch and Szostak 1994b; Majerfeld and Yarus 1994; Tao and Frankel 1996; Mannironi et al. 1997; Mannironi et al. 2000; Roychowdhury-Saha et al. 2002; Ylera et al. 2002; Lozupone et al. 2003; Brockstedt et al. 2004; Win et al. 2006). Gradient elution may instead be used if a standard curve is first constructed on the basis of isocratic elution data (Tao and Frankel 1996).

A second common method for K_d determination is equilibrium filtration (Sassanfar and Szostak 1993; Famulok 1994; Jenison et al. 1994; Lauhon and Szostak 1995; Burke et al. 1997; Mannironi et al. 1997; Wallis et al. 1997; Burke and Hoffman 1998; Kiga et al. 1998; Koizumi and Breaker 2000; Meli et al. 2002; Roychowdhury-Saha et al. 2002; Sazani et al. 2004). Binding constants may be determined from the fraction of RNA bound as a function of immobilized target concentration (Wilson et al. 1998). Competition binding analysis has been performed using a DNA oligonucleotide that contains the target, which was a damaged DNA nucleotide (Rink et al. 1998). Other approaches include titration of chemical modification (Wallis et al. 1995; Berens et al. 2001), equilibrium dialysis (Lorsch and Szostak 1994b; Geiger et al. 1996), and electrophoretic mobility shift assay (Rink et al. 1998; Tahiri-Alaoui et al. 2002; Chen et al. 2003).

If the target is itself a fluorophore, then observables such as fluorescence emission intensity (Lauhon and Szostak 1995), fluorescence anisotropy (Holeman et al. 1998), or maximum emission wavelength (Grate and Wilson 1999) may be used to determine K_d. Alternatively, if a fluorophore can be conjugated to the target and the properties of this attached fluorophore change upon aptamer-target assocation, then

fluorescence-based assay methods can be used (Wang and Rando 1995; Wang et al. 1996; Kwon et al. 2001).

Determination of binding constants can be achieved by Biacore (Gebhardt et al. 2000; Jeong et al. 2001; Kwon et al. 2001; Davis and Szostak 2002; Tahiri-Alaoui et al. 2002; Khati et al. 2003; Ferreira et al. 2006; Ohuchi et al. 2006; Win et al. 2006). Related to measurements of binding constants are analysis of aptamer-target interactions by atomic force microscopy (Jiang et al. 2003; Jiang et al. 2004; Basnar et al. 2006).

For peptide and protein targets, binding constants have been determined by equilibrium dialysis (Nieuwlandt et al. 1995), electrophoretic mobility shift assay (Lee and Sullenger 1996; Xu and Ellington 1996; Klug et al. 1997; Baskerville et al. 1999; Klug et al. 1999; Yamamoto et al. 2000; Srisawat and Engelke 2001; Hwang and Lee 2002; Bryant et al. 2005), filter-binding assay (Schneider et al. 1992; Jellinek et al. 1994; Allen et al. 1995; Binkley et al. 1995; Jellinek et al. 1995; Tian et al. 1995; Green et al. 1996; Hale and Schimmel 1996; Hicke et al. 1996; O'Connell et al. 1996; Wiegand et al. 1996; Klug et al. 1997; Kubik et al. 1997; Kumar et al. 1997; Pagratis et al. 1997; Bridonneau et al. 1998; Ruckman et al. 1998; Biesecker et al. 1999; Klug et al. 1999; Lebruska and Maher 1999; Fukuda et al. 2000; Hirao et al. 2000; Seiwert et al. 2000; White et al. 2001; Daniels et al. 2002; Proske et al. 2002; Vuyisich and Beal 2002; Santulli-Marotto et al. 2003; White et al. 2003; Hirao et al. 2004; Tang et al. 2006), immunoprecipitation (Doudna et al. 1995), flow cytometry (Davis et al. 1998), binding to protein immobilized on plates (Hicke et al. 2001), Biacore (Kawakami et al. 2000; Hicke et al. 2001), and saturation analysis of binding to the surface of live cells (Ulrich et al. 2002). In some cases, binding is assessed indirectly, via determining the effect of the aptamer on an appropriate biochemical activity of the protein target (Smith et al. 1995; Burke et al. 1996; Kumar et al. 1997; Lupold et al. 2002).

3.2.3 Binding Constants of RNA Aptamers: Quantitative Data

Binding constants (K_d values) for small-molecule targets of RNA aptamers are collected in Table 1. The tobramycin aptamer (Wang and Rando 1995; Wang et al. 1996) currently has the highest reported affinity for its small-molecule target (~2 nM), although much weaker K_d values can be determined experimentally. It is not surprising that an aminoglycoside antibiotic such as tobramycin binds particularly well to RNA, because the protonated amino groups of an aminoglycoside can interact electrostatically with the polyanioninc nucleic acid. However, the binding is not solely electrostatic, because substantial selectivity in target binding is observed.

For protein targets, aptamers are frequently compared favorably with antibodies (Jayasena 1999). Aptamers have several advantages compared with antibodies, such as selectability against toxic or nonimmunogenic targets; selectability under non-physiological conditions; uniformity of synthetic batches; longer shelf-life; relatively

small size; and ease of chemical modification. In general, the binding constants of aptamers for their protein targets are competitive with antibody binding constants. Several K_d values for aptamers to protein targets are collected in Table 2. Many of these K_d values are low nanomolar or better; currently the best reported is 0.3 pM for keratinocyte growth factor (Pagratis et al. 1997). The origin of this high affinity is not entirely clear, but the 2'-fluoropyrimidine nucleotides used in this selection effort (see Section 3.3) are required to achieve the affinity.

To place the nanomolar or stronger K_d values for protein targets in context, it is important to note that even a random RNA pool can bind a protein target with sub-micromolar affinity. For example, the K_d of the random pool was 200 nM for the VEGF aptamer selection (Jellinek et al. 1994) and 30 nM for the keratinocyte growth factor aptamer selection (Pagratis et al. 1997). A typical observation is that two to three orders of magnitude increase in pool affinity (relative to the initial random pool) is obtained during the selection process.

3.2.4 Evolutionary Considerations for RNA Aptamers

The relationship between binding ability and "informational complexity" has been explored experimentally with GTP aptamers (Carothers et al. 2004). One important conclusion was that increasing the binding strength by a factor of 10 requires an RNA structure that is approximately 1000-fold less frequent within a pool of random sequences. Importantly, the authors concluded on the basis of additional data that selectivity for the target does not automatically increase when the affinity is raised (Carothers et al. 2006). In addition, the authors noted that improving aptamer binding (i.e., lowering K_d) is easier to achieve via increasing RNA tertiary stability rather than via improving RNA-target interactions, at least in their particular system.

Evolution of one aptamer sequence into another has been explored several times (Famulok 1994; Mannironi et al. 2000; Held et al. 2003; Huang and Szostak 2003). Stringent minimization of RNA aptamers has been achieved by a combination of computational and empirical approaches (Anderson and Mecozzi 2005a, 2005b). Some RNA aptamers for amino acids have a coding triplet in the binding site, which may have implications for the "RNA World" hypothesis and the origin of the genetic code (Yarus 2000).

3.3 RNA Aptamers with Chemical Modifications

Due to the interest in using aptamers in vivo (see Section 3.1.4), chemical approaches to stabilize RNA in vivo have been investigated. One acute challenge for in vivo application of aptamers is their rapid degradation by cellular nucleases. Chemical modifications to RNA that prevent nuclease degradation allow aptamers to be used in vivo with greater efficacy.

Common chemical modifications are substitutions of the 2'-hydroxyl group of pyrimidine nucleotides with 2'-fluoro or 2'-amino (Lin et al. 1994; Green et al. 1995b; Jellinek et al. 1995; Pan et al. 1995; Smith et al. 1995; Hicke et al. 1996; Lee and Sullenger 1996; Lin et al. 1996; O'Connell et al. 1996; Wiegand et al. 1996; Kubik et al. 1997; Lee and Sullenger 1997; Pagratis et al. 1997; Bridonneau et al. 1998; Davis et al. 1998; Ruckman et al. 1998; Biesecker et al. 1999; Beaudry et al. 2000; Wang et al. 2000; Hicke et al. 2001; White et al. 2001; Daniels et al. 2002; Hwang and Lee 2002; Proske et al. 2002; Rusconi et al. 2002; Tahiri-Alaoui et al. 2002; Ulrich et al. 2002; Khati et al. 2003; Lorger et al. 2003; Rhie et al. 2003; Santulli-Marotto et al. 2003; White et al. 2003; Cerchia et al. 2005). Such modifications lead to two to four orders of magnitude increase in the serum half-life (Lin et al. 1994; Jellinek et al. 1995; Lin et al. 1996), although 2'-fluoro-RNAs appear superior to 2'-amino-RNAs in terms of target affinities, at least in one study (Pagratis et al. 1997). Nucleotides with 2'-O-methyl and other modifications are also often used (Beigelman et al. 1995; Kato et al. 2005), and these alterations can improve the half-life considerably (Green et al. 1995a). To reduce renal clearance of aptamers in vivo, addition of polyethylene glycol (PEG) chains (Bell et al. 1999; Tucker et al. 1999), covalent appendage of cholesterol (de Smidt et al. 1991; Rusconi et al. 2004) or biotin-streptavidin (Dougan et al. 2000), or anchoring to a liposome (Willis et al. 1998) can be employed. Locked nucleic acids (LNAs) can improve secondary structure stability and in vivo stability of an aptamer (Schmidt et al. 2004). Chemically modified nucleotides may be introduced to enhance target binding rather than for stability reasons (Battersby et al. 1999; Teramoto et al. 2001; Vaish et al. 2003), or to allow variations of a selection procedure (see Section 2.2).

3.4 Mirror-Image RNA Aptamers (Spiegelmers)

Natural RNA and DNA nucleotides are built using D-ribose monomers. Symmetry demands that an D-ribose-based aptamer which binds the mirror image of the desired target (such as the opposite enantiomer of a desired peptide target) can be used to subsequently synthesize an L-ribose-based aptamer that binds the correct enantiomer of the target. Cellular nucleases do not degrade RNA made from L-ribose monomers, and therefore L-ribose aptamers should be much more stable than their conventional D-ribose analogues. Based on this logic, a selection strategy has been applied to identify biologically stable L-ribose aptamers for many targets (Vater and Klussmann 2003). The mirror-image L-ribose RNA aptamers are termed "Spiegelmers", deriving from the German *Spiegel* meaning "mirror". It is appropriate that this term also honors the name of Sol Spiegelman, whose work in the 1960s pre-dated almost all of the current studies on functional nucleic acids (see introduction to Section 6 below). Spiegelmers were first reported for binding to D-adenosine (Klussmann et al. 1996) and L-arginine (Nolte et al. 1996), and others have been identified (Leva et al. 2002).

Spiegelmers can have excellent binding activity both in vitro and in vivo (Faulhammer et al. 2004; Helmling et al. 2004).

4 Molecular Targets and Properties of DNA Aptamers

Many parallels may be drawn between RNA and DNA aptamers. The selection methodologies are very similar. As described in Section 2, when selecting DNA aptamers the functional DNA sequences are simply converted directly into DNA for a new selection round by PCR rather than RT-PCR. In general, RNA and DNA aptamers are similar in terms of size and apparent structural complexity, and no obvious distinction has been reported in the range of targets that may be bound. This section describes the molecular targets and biochemical characterization of DNA aptamers.

4.1 Molecular Targets Bound by DNA Aptamers

The first DNA apatamers were obtained by selection for binding of small organic dyes (Ellington and Szostak 1992) or the protein thrombin (Bock et al. 1992). Since then, many DNA aptamers have been developed for a variety of targets, both small molecules (Table 3) and proteins (Table 4).

One of the first DNA aptamers binds adenosine and related compounds (Huizenga and Szostak 1995). The DNA aptamer for adenosine and its 5'-phosphate derivatives (e.g., AMP, ATP) binds two molecules of target, whereas the RNA aptamer for ATP (Sassanfar and Szostak 1993) binds just one molecule (Lin and Patel 1997). The RNA aptamer does not bind ATP when made as DNA and vice versa. This lack of RNA-DNA cross-reactivity appears to be a general rule, although a riboflavin RNA aptamer did retain some binding ability when made as the corresponding DNA sequence (Lauhon and Szostak 1995).

DNA aptamers have been identified for assorted small molecules such as organic dyes (Ellington and Szostak 1992; Wilson and Szostak 1998), cocaine (Stojanovic et al. 2001; Stojanovic and Landry 2002), cholic acid and other steroids (Kato et al. 2000a; Kato et al. 2000b), porphyrins (Li et al. 1996; Chinnapen and Sen 2002), amino acids (Harada and Frankel 1995) and derivatives (Vianini et al. 2001), cellobiose and other carbohydrates (Yang et al. 1998), thalidomide (Shoji et al. 2007), and ethanolamine (Mann et al. 2005). As mentioned in Section 3, ethanolamine—with its molecular weight of merely 61—represents the smallest organic target for an aptamer known to date. DNA aptamers have also been identified for nucleic acid structures (Boiziau et al. 1999; Sekkai et al. 2002).

In the realm of peptides and proteins, DNA aptamers have been identified for tumor marker peptides (Ferreira et al. 2006), growth factors (Green et al. 1996), immunoglobulins (Wiegand et al. 1996), selectins (Hicke et al. 1996), and other

Small-molecule target	K_d, μM	Reference
ethanolamine	0.006 *	(Mann et al. 2005)
N-methylmesoporphyrin IX (NMM)	0.4	(Li et al. 1996)
cocaine	0.4	(Stojanovic et al. 2001)
cellobiose	0.6	(Yang et al. 1998)
sulforhodamine B dye	0.7	(Wilson and Szostak 1998)
(*R*)-thalidomide	1	(Shoji et al. 2007)
cholic acid	5	(Kato et al. 2000a)
adenosine / ATP	6	(Huizenga and Szostak 1995)
reactive green 19 dye	30	(Ellington and Szostak 1992)
L-tyrosinamide	50	(Vianini et al. 2001)
L-arginine	3000	(Harada and Frankel 1995)

Table 3. Binding constants (K_d values) of DNA aptamers for small-molecule targets. Listed is the best K_d value for each target from the indicated reference. * = The target was immobilized on beads for the K_d measurement.

Protein target	K_d, nM	Reference
platelet-derived growth factor B-chain	0.1	(Green et al. 1996)
MUC1 tumor marker	0.1	(Ferreira et al. 2006)
L-selectin	0.3	(Hicke et al. 1996)
immunoglobulin IgE	10	(Wiegand et al. 1996)
thrombin	30	(Bock et al. 1992)
anti-MUC1 IgG3 monoclonal antibody	50	(Missailidis et al. 2005)
ricin	60	(Tang et al. 2006)
tenascin-C	150	(Daniels et al. 2003)
misactivated tRNA synthetase	15000	(Hale and Schimmel 1996)

Table 4. Binding constants (K_d values) of DNA aptamers for protein targets. Listed is the best K_d value for each target from the indicated reference.

proteins (Moreno et al. 2003; Missailidis et al. 2005; Tang et al. 2006). This includes proteins expressed on surface of live cells (Blank et al. 2001; Daniels et al. 2003) or even anthrax spores (Bruno and Kiel 1999), although in the latter case the molecular target of the aptamer was not investigated. As was achieved for an RNA aptamer (Smith et al. 1995), a DNA aptamer for a serine protease was developed by conjugating a known small-molecule covalent inhibitor to a random DNA pool and then selecting for binding (Charlton et al. 1997). Finally, a DNA aptamer has been developed that causes editing of protein synthesis by inducing hydrolysis of a misacylated tRNA synthetase (Hale and Schimmel 1996).

4.2 Biochemical Characterization of DNA Aptamers

The methods to characterize DNA aptamers are essentially the same as those used to characterize RNA aptamers (Section 3.2). An approach apparently reported only for DNA is UV differential absorption spectroscopy (Wilson and Szostak 1998; Yang et al. 1998). Representative binding constants are listed in Tables 3 and 4. Structural

characterization of DNA aptamers is somewhat more difficult than for RNA aptamers, in part because enzymatic probing with ribonucleases cannot be applied. Procedures functionally analogous to the "boundary mapping" approach applicable to RNA aptamers may be used with DNA (Green et al. 1996).

4.3 Other Considerations for DNA Aptamers

DNA aptamers have been identified using chemically modified nucleotides to increase biological stability (Latham et al. 1994; Masud et al. 2004) and also to increase binding affinity (Shoji et al. 2007). DNA Spiegelmers have been developed, in analogy to RNA Spiegelmers (Section 3.4). The first DNA Spiegelmer was developed for the peptide hormone vasopressin (Williams et al. 1997). Others have since been identified (Leva et al. 2002; Wlotzka et al. 2002; Purschke et al. 2003).

5 Direct Structural Analysis of RNA and DNA Aptamers

5.1 NMR and X-Ray Crystallography Analysis of Aptamers

Direct structural studies of aptamer-target complexes by NMR spectroscopy and X-ray crystallography have revealed several basic principles behind the binding events. Aptamers are thought—and in some cases known—to be largely unstructured in solution in the absence of their target. Upon binding, aptamers adopt highly organized conformations that allow selective target recognition ("adaptive folding"). The target itself can also undergo conformational changes upon being bound by the aptamer (induced fit) (Nguyen et al. 2002). Common features of aptamer-target contacts include stacking interactions between π systems, hydrogen bonding, hydrophobic contacts, and electrostatic interactions. (Hermann and Patel 2000). Shape complementarity also plays an important role in many cases. Selectivity for a target generally arises from some combination of steric hindrance, hydrogen bonding, and electrostatic complementarity.

Below are three case studies of aptamers for theophylline, ATP, and thrombin. These experiments used either NMR spectroscopy, X-ray crystallography, or a combination of both methods. In addition, NMR spectroscopy has been used to study aptamers that bind argininamide (Lin and Patel 1996; Lin et al. 1998; Kawakami et al. 2000), arginine and citrulline (Yang et al. 1996), flavin mononucleotide (Fan et al. 1996), tobramycin (Jiang et al. 1997; Jiang and Patel 1998), neomycin B (Jiang et al. 1999), HIV-1 Rev peptide (Ye et al. 1996), HIV-1 TAR RNA (Collin et al. 2000), and an organic dye (Flinders et al. 2004). X-ray crystallography has provided structures of aptamers that bind to an organic dye (Baugh et al. 2000), biotin (Nix et al. 2000), vitamin B_{12} (Sussman et al. 2000; Sussman and Wilson 2000), streptomycin (Tereshko et al. 2003) and various proteins (Convery et al. 1998; Rowsell et al. 1998; Horn et al. 2004). Additional structural information on RNA-target interactions is available from structures of several natural riboswitches (see Chapter 1).

5.2 Case Study #1: Theophylline RNA Aptamer

NMR structures of the theophylline RNA aptamer (Jenison et al. 1994) have revealed fundamental principles underlying both aptamer-target interactions and binding selectivity (Zimmermann et al. 1997; Zimmermann et al. 2000). In the free aptamer, the binding site is not stably formed. In contrast, the 15-nucleotide binding pocket within the aptamer-target complex is well-ordered, with stacked base triples and numerous hydrogen bonds holding theophylline in place. Selectivity for theophylline versus caffeine (which differs solely by having an additional methyl group) is enforced at least in part by steric clashes involving the methyl group. In addition, two hydrogen bonds would be disrupted by introduction of caffeine's extra methyl group, and stacking interactions would likely be affected as well. Biochemical studies showed that the high affinity of the aptamer for theophylline is due primarily to a slow dissociation rate (Jucker et al. 2003). This dissociation rate increases several orders of magnitude in the absence of Mg^{2+}, which explains the requirement for this divalent metal ion.

One particular nucleotide of the theophylline aptamer, C27, can be replaced with an abasic residue while retaining high binding affinity. Nevertheless, this nucleotide may only be A or C; either G or U leads to a much lower affinity. The origin of this requirement was traced using NMR spectroscopy to stable interactions involving the particular nucleobase in the free state of the RNA (Zimmermann et al. 1998). No unfavorable contacts are formed in the target-bound state; indeed, C27 is highly dynamic in the aptamer-target complex. These results serve as a reminder that nucleotide requirements do not always originate in interactions involve the bound state of the RNA. In this case, the unbound state of the RNA is clearly the source of the nucleotide requirement.

5.3 Case Study #2: ATP RNA and DNA Aptamers

The ATP aptamer identified by Sassanfar and Szostak (1993) also binds ADP, AMP, and adenosine but discriminates against other nucleotide triphosphates. The solution structure of the aptamer-AMP complex was solved by NMR spectroscopy in two research groups (Dieckmann et al. 1996; Jiang et al. 1996). The structures agree in both the topology and the key hydrogen bonding and stacking interactions that lead to target recognition (Patel et al. 1997). The nucleobase (A) moiety is recognized through a G-A mismatch involving the minor groove edge of G and Watson-Crick edge of A, and not via a Watson-Crick base pair. Follow-up studies further identified the hydrogen-bonding patterns that assist in both target recognition and binding-site organization (Dieckmann et al. 1997; Nonin et al. 1997).

An all-DNA version of the RNA aptamer does not bind ATP (Dieckmann et al. 1997). Indeed, NMR evidence was obtained for specific 2'-hydroxyls of the RNA aptamer that are important for binding, in addition to the 2'-hydroxyl of the adenosine nucleotide itself. Of course, a DNA aptamer for ATP has been identified (Huizenga and Szostak 1995), and this aptamer was also studied by NMR spectroscopy (Lin and Patel 1997; Nonin-Lecomte et al. 2001). The RNA-aptamer and DNA-aptamer complexes have different tertiary structures and even different binding stoichiometries; the RNA aptamer binds one molecule of ATP but the DNA aptamer binds two molecules of ATP. In the DNA aptamer complex, the nucleobase moieties of the two bound target molecules are recognized by noncanonical interactions of the same type as found in the RNA aptamer complex, despite the differences in overall architecture and stoichiometry.

5.4 Case Study #3: Thrombin DNA Aptamer

The thrombin-binding DNA aptamer (Bock et al. 1992) has been examined by both NMR spectroscopy (Macaya et al. 1993; Wang et al. 1993a; Wang et al. 1993b; Schultze et al. 1994) and X-ray crystallography (Padmanabhan et al. 1993). The structural data agree in revealing two stacked guanine quadruplexes (G-quartets). However, the data disagree with regard to the connectivities among the nucleotides that compose these units. A comparative re-evaluation of the two types of data suggested that the original X-ray structure was incorrect with regard to the connectivities (Kelly et al. 1996). This conclusion was confirmed by the authors of the original X-ray structure (Padmanabhan and Tulinsky 1996). The local but not global symmetry of the aptamer was the source of the structural ambiguity.

6 In Vitro Selection of Ribozymes

From one viewpoint, aptamers are inherently simpler than nucleic acid enzymes. Both types of FNA must bind to something else, but only nucleic acid enzymes then proceed to catalyze a chemical reaction. However, the majority of known nucleic acid enzymes have nucleic acids as their substrates, and these substrates are bound by straightforward Watson-Crick base-pairing interactions. The segregation of many nucleic acid enzymes into "binding" and "catalysis" regions greatly simplifies the binding process and essentially frees a nucleic acid enzyme to focus its attention on catalysis.

Experiments in the 1960s with Qβ replicase by Sol Spiegelman and coworkers indicated that "test tube evolution" can provide fundamental insight into nucleic acid catalysis (Mills et al. 1967; Spiegelman 1971). Nevertheless, artificial ribozymes did not become a truly realistic objective until researchers were motivated by the discovery of natural ribozymes in the early 1980s (Kruger et al. 1982; Guerrier-Takada et al. 1983). Within a decade after those discoveries, selection experiments had demonstrated that RNA aptamers could readily be identified, and soon thereafter reports on artificial ribozymes identified by in vitro selection began to be published. The first in vitro selection procedures to identify artificial ribozymes were initiated with the natural *Tetrahymena* group I intron ribozyme and evolved activities that were related to RNA splicing (Green et al. 1990; Robertson and Joyce 1990). Since those experiments, many efforts have focused on the selection of new catalytic activities starting from completely random-sequence RNA. One of the earliest selections sought ribozymes that ligate two RNA substrates (Bartel and Szostak 1993). This selection experiment will be used here to illustrate several facets of ribozyme selection methodology.

6.1 Methodology to Identify Ribozymes

Most ribozyme selections have employed a common general approach, although the details necessarily differ for each study depending on the details of the reaction that is catalyzed. As is the case for aptamers, a ribozyme selection typically begins by solid-phase synthesis of a long DNA oligonucleotide that has a random region embedded between two constant regions (see Section 2). In vitro transcription is used to provide the initially random RNA pool for the first round of selection. The key design element of any ribozyme selection is to arrange for any catalytically active RNA sequences to become chemically modified during catalysis. In particular, this chemical modification must occur in such a way that the modified RNA sequences may be readily separated from sequences that are catalytically inactive and therefore chemically unmodified.

6.1.1 The Basic Procedures of In Vitro Selection of Ribozymes

For the first-reported RNA ligase ribozymes (Bartel and Szostak 1993), selection was achieved by using one substrate oligonucleotide with a 5'-terminal "sequence tag" (Fig. 5). The other substrate oligonucleotide was covalently joined with the random region, such that ligation of the two substrates inherently connects the sequence tag to the random region. After reverse transcription, the sequence tag then served as one of the two required PCR primer binding sites for amplification of the catalytically active sequences. Because inactive RNA sequences did not acquire the sequence tag, they were not PCR-amplified and did not survive into the next selection round. Other efforts have instead depended upon addition or removal of a biotin moiety (Wilson and Szostak 1995; Lohse and Szostak 1996; Zhang and Cech 1997; Jenne and Famulok 1998; Suga et al. 1998b; Tuschl et al. 1998; Seelig and Jäschke 1999; Lee et al. 2000; Seelig et al. 2000; Teramoto et al. 2000; Saito et al. 2001; Sengle et al. 2001; Baskerville and Bartel 2002; Tsukiji et al. 2003), addition of a thiol group (Lorsch and Szostak 1994a; Kumar and Yarus 2001), or shift of a band on polyacrylamide gel electrophoresis (PAGE) (Pan and Uhlenbeck 1992a, 1992b;

Williams et al. 1995; Jayasena and Gold 1997). Occasionally more than one method has been used during the same selection experiment (Illangasekare et al. 1995; Wecker et al. 1996; Tarasow et al. 1997; Wiegand et al. 1997; Unrau and Bartel 1998; Johnston et al. 2001; Chapple et al. 2003; Nieuwlandt et al. 2003; Lau et al. 2004; Tarasow et al. 2004; Curtis and Bartel 2005). This has been done either by using more than one method in the same round or in more serial fashion, with some rounds using one method and other rounds using a different method.

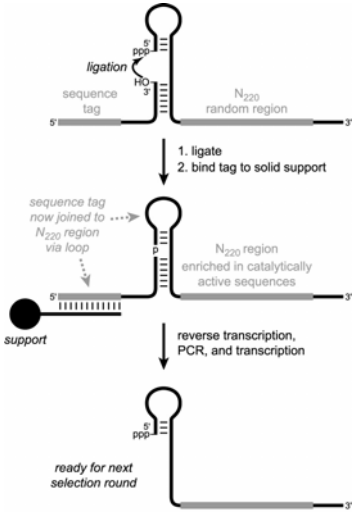

Fig. 5. Key selection step of the strategy for identifying the first RNA ligase ribozymes (Bartel and Szostak 1993).

Regardless of which separation method is used, once the "winning" sequences have been separated and subsequently amplified by RT-PCR, the selection rounds are iterated until the catalytic activity of the pool as a whole is satisfactory. Similar to aptamer selections, the number of rounds is typically 5-15, although in some efforts well over 20 rounds have been required. After selection rounds have concluded,

individual ribozymes are cloned and tested for catalytic activity. Often this is done before any sequencing is performed; the individual ribozymes may be prepared by transcription even without knowledge of their sequences. Once ribozymes with high catalytic activities have been identified, their sequences may be determined and optimized by more detailed follow-up experiments.

As is the case for aptamer selections, many incubation variables such as pH, temperature, buffer, organic cosolvent, and metal ions must be considered. In addition, the incubation time itself is a particularly important variable, because shorter incubation times impose selection pressure for faster ribozymes. For the original RNA ligase ribozymes (Bartel and Szostak 1993), the initial rounds of selection were performed at pH 7.4, 600 mM KCl, and 60 mM $MgCl_2$ at 25 °C for 16 h, although changes were made in the later selection rounds. This highlights the large number of variables to consider, each of which may have a wide range of values—and in most cases, the optimal values to favor catalysis are not known at the outset of selection. Therefore, several ribozyme selections are often performed in parallel, with numerous combinations of the variables explored in side-by-side experiments. Particularly when some of these combinations are more successful than others, the results provide information on which specific incubation conditions are essential to optimal catalytic activity.

The length of the random region used in ribozyme selections is an important consideration. Successful ribozyme selections have used random regions up to N_{228} (Unrau and Bartel 1998), for which sequence space encompasses $4^{228} \approx 10^{137}$ possibilities. For N_{228} selections that are initiated with 10^{15} molecules, only the incredibly small fraction 10^{-122} of sequence space is explored—yet these experiments have successfully led to active ribozymes. At least for the investigated catalytic activities, this success implies that the functional RNA sequences are quite common in sequence space (Bartel and Szostak 1993; Tarasow et al. 1997). More typically, random regions for ribozyme selections are between N_{40} and N_{80}. Complex catalytic motifs benefit from particularly large random regions (Sabeti et al. 1997), but excess sequence elements can inhibit catalysis, so larger is not always better. To account for the various uncertainties in the optimal length of random region, several selection experiments that each have a different length of random region may be performed in parallel.

6.1.2 Additional Considerations for Ribozyme Selections

Negative selection (Section 2.1.2) may be applied to eliminate RNA sequences that catalyze undesired reactions and therefore opportunistically survive the selection procedure (Tsang and Joyce 1996b). Of course, the best approach to success in ribozyme selection is to design the procedure with sufficient care such that the only way that sequences can survive is by catalyzing the reaction of interest. Nevertheless, "parasitic" sequences can survive the selection without actually catalyzing the

desired reaction. As just one example, if a selection for RNA ligation depends upon a gel shift to separate active from inactive sequences, those pool sequences that fold in such a way that they migrate at the position of the larger ligated product may opportunistically survive each round. In unfavorable cases, such parasitic sequences may come to dominate the overall effort, in which case it may be imperative to use either negative selection or multiple physical techniques for the key separation steps of successive rounds.

One approach to identify ribozymes is to begin with an RNA aptamer sequence, then add a random region and select for ribozymes that both bind the substrate and catalyze a reaction of the substrate. This approach has been used successfully (Lorsch and Szostak 1994a; Wilson and Szostak 1995), although it is not clear that the initially programmed aptamer subunits are functionally retained in the final ribozymes. Furthermore, anecdotal evidence and theoretical considerations suggest that this strategy may not work in all cases. For example, aptamer binding may occlude a site on the target molecule that must be accessible for catalysis to occur. A related approach to achieve a complicated catalytic activity is to start with a known artificial ribozyme and attach a random region to enable an even more challenging catalytic activity. As one example, this approach was used with spectacular success to identify an RNA polymerase ribozyme that is capable of processive nucleotide additions to a growing RNA chain (Johnston et al. 2001).

Because sequence space is too large to be covered thoroughly when the random region is N_{25} or larger, initially obtained ribozyme sequences are probably not optimal catalysts, and systematic methods to examine variant sequences are useful. Error-prone PCR (mutagenic PCR) is one such method (Cadwell and Joyce 1994). Appropriate PCR conditions (commonly inclusion of Mn^{2+}) is known to increase the Taq polymerase error rate, and this can be used to introduce variation into the enzyme pool during any particular selection round. Alternatively, nonstandard nucleotide analogs may be used to promote random mutations during the PCR step (Kore et al. 2000). Reselection (see Section 2.1.2) is a second approach that may be used to improve ribozyme activity. A new pool is prepared based on a known ribozyme sequence, but with partial randomization. For example, a typical reselection effort might use a pool in which each ribozyme nucleotide position has a 70% probability of being the nucleotide originally present in the ribozyme and a 10% probability (each) of being one of the other three nucleotides. It is straightforward to compute the distribution of expected mutations in the resulting pool (Breaker and Joyce 1994a; Tsang and Joyce 1996a; Flynn-Charlebois et al. 2003a; Knight and Yarus 2003a), although the optimal extent of mutation is difficult to know in advance.

Finally, many ribozymes are converted from a cis-acting (intramolecular) to a trans-acting (intermolecular) form; i.e., to a form of the ribozyme in which the substrate is not covalently attached to the ribozyme at the outset of the reaction. Of course, only trans-acting ribozymes are in principle capable of multiple-turnover catalysis, which is often a practical objective. For ribozymes that cleave or ligate

nucleic acid substrates, catalysis in trans is usually achieved simply by omission of a covalent phosphodiester linkage that is present between the ribozyme and one of its substrates during the selection process. For ribozymes with other catalytic activities, no general prescription exists for conversion into a trans-acting form. If during selection a substrate is attached to the ribozyme by a covalent (e.g., PEG) tether, then omitting some or all of the tether may permit multiple-turnover catalysis (Seelig and Jäschke 1999). However, this is not guaranteed to be successful. In some cases, one may anticipate that achieving multiple-turnover catalysis will require a method that inherently allows selection directly for this feature (see next section).

6.1.3 Alternative Methods for Ribozyme Selections

By analogy to efforts with catalytic antibodies, an early approach to ribozymes was to select for binding to transition-state analogues. An aptamer that binds to a transition-state analogue should be a catalyst for the reaction that proceeds via that transition state. Although this approach was successful in some instances (Prudent et al. 1994; Conn et al. 1996; Chun et al. 1999), other efforts were unsuccessful (Morris et al. 1994). Currently, the conventional wisdom is that selecting directly for catalysis is a superior approach (Bartel and Szostak 1993).

Drawbacks of most conventional in vitro selection procedures are their tedious and time-consuming low-throughput nature. As one alternative, continuous evolution has been developed. In this approach, self-replicating molecules are diluted serially (e.g., 1000-fold dilution) and at constant temperature, in contrast to employing discrete selection rounds and PCR temperature cycling. Many more cycles are possible with continuous evolution; more than 100 serial transfers are common. The early Spiegelman Qβ replicase experiments (Mills et al. 1967; Spiegelman 1971) are considered to be the first examples of continuous evolution. More recently, Joyce and coworkers have broadened the field to include evolution of catalytic function (Wright and Joyce 1997). For example, continuous evolution was used to evolve an RNA ligase ribozyme that resists the activity of an RNA-cleaving deoxyribozyme (Ordoukhanian and Joyce 1999). Despite some advantages of speed and amplification power, continuous evolution suffers from susceptibility to contamination and limitations on the types of reactions that may be catalyzed (Johns and Joyce 2005). Therefore, the true power of continuous evolution approaches to identify new ribozymes is uncertain.

A second alternative approach was spurred by recognizing a key limitation imposed by a covalent linkage between the RNA and its substrate during the selection process. This requirement often means that multiple turnover cannot be engineered into any resulting ribozymes, if the link between the RNA and substrate cannot be broken while retaining catalytic activity (see previous section). The fundamental problem is that the traditional selection procedure inherently cannot, in principle, select for multiple turnover. Therefore, procedures based on in vitro compartmentali-

zation (IVC) have been developed (Tawfik and Griffiths 1998; Miller et al. 2006). In IVC, the candidate ribozyme sequences are encapsulated within individual droplets in a water-in-oil emulsion. If each droplet contains just one ribozyme candidate, then the encapsulation is functionally equivalent to a covalent bond in terms of linking the RNA sequence information to catalytic ability. Indeed, IVC has been applied to develop several ribozymes that achieve multiple turnover (Agresti et al. 2005; Levy et al. 2005). Although such turnover may fortuitously be possible for ribozymes that are identified via the traditional methodologies, the use of IVC allows selection explicitly to achieve this property.

6.2 Chemical Reactions Catalyzed by Ribozymes

All natural ribozymes catalyze phosphodiester cleavage or ligation reactions, with the exception of the ribosome that creates peptide bonds (see Chapter 1). A number of artificial ribozymes also catalyze phosphodiester cleavage or ligation, although many ribozymes with other activities have been identified. Fig. 6 shows some representative examples of particular ribozymes, and Table 5 reveals the scope of ribozyme-catalyzed reactions. Although the available data do not suggest any limits on what kinds of reactions can be catalyzed by RNA, more experiments are needed to probe these limits.

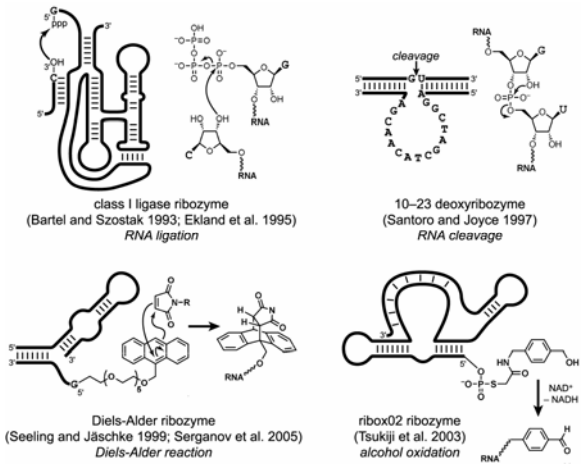

Fig. 6. Secondary structures and reactions of representative artificial ribozymes.

Reaction catalyzed	Bond	Rate enh	M^{2+} req	Reference
cyclic phosphate hydrolysis	O–P	50	Pb^{2+}	(Pan and Uhlenbeck 1992a)
RNA cleavage	O–P	80	Pb^{2+}	(Pan and Uhlenbeck 1992b)
RNA cleavage	O–P	200	Mg^{2+}	(Williams et al. 1995)
RNA cleavage	O–P	nd	None	(Jayasena and Gold 1997)
RNA ligation	O–P	7×10^{6}	Mg^{2+}	(Bartel and Szostak 1993)
RNA ligation	O–P	8×10^{8}	Mg^{2+}	(Ekland et al. 1995)
RNA ligation	O–P	5×10^{5}	Mg^{2+}	(Hager and Szostak 1997)
RNA ligation	O–P	250	Mg^{2+}	(Teramoto et al. 2000)
RNA branching	O–P	nd	Mg^{2+}	(Tuschl et al. 1998)
RNA phosphorylation	O–P	1×10^{5}	Mg^{2+}	(Lorsch and Szostak 1994a)
RNA phosphorylation	O–P	6×10^{6}	Mg^{2+}	(Curtis and Bartel 2005)
RNA capping	O–P	10^{3}–10^{4}	Mg^{2+}	(Chapman and Szostak 1995)
RNA capping	O–P	nd	Ca^{2+}	(Huang and Yarus 1997)
RNA capping	O–P	nd	Ca^{2+}	(Huang et al. 1998)
amino acid adenylation	O–P	nd	Ca^{2+}	(Kumar and Yarus 2001)
cofactor synthesis	O–P	nd	Mn^{2+}	(Huang et al. 2000)
RNA polymerization	O–P	nd	Mg^{2+}	(Ekland and Bartel 1996)
template-dir. polymerization	O–P	nd	Mg^{2+}	(Johnston et al. 2001)
RNA-protein conjugation	N–P	nd	Mg^{2+}	(Baskerville and Bartel 2002)
Diels-Alder reaction	C–C	800	Cu^{2+}	(Tarasow et al. 1997)
Diels-Alder reaction	C–C	1×10^{4}	Cu^{2+}+Ni^{2+}	(Tarasow et al. 2004)
Diels-Alder reaction	C–C	1×10^{4}	Mg^{2+}	(Seelig et al. 2000)
aldol reaction	C–C	4×10^{3}	Zn^{2+}	(Fusz et al. 2005)
alcohol oxidation	C–H	1×10^{7}	Mg^{2+}+Zn^{2+}	(Tsukiji et al. 2003)
aldehyde reduction	C–H	3×10^{6}	Mg^{2+}+Zn^{2+}	(Tsukiji et al. 2004)
pyrimidine nt synthesis	C–N	1×10^{8}	Mg^{2+}	(Chapple et al. 2003)
purine nt synthesis	C–N	nd	Mg^{2+}	(Lau et al. 2004)
N^{7}G alkylation	C–N	3×10^{6}	Mg^{2+}	(Wilson and Szostak 1995)
amide synthesis	C–N	1×10^{5}	Cu^{2+}	(Wiegand et al. 1997)
urea synthesis	C–N	1×10^{6}	nd	(Nieuwlandt et al. 2003)
peptide bond formation	C–N	1×10^{6}	Mg^{2+}	(Zhang and Cech 1997)
peptidyl-RNA synthesis	C–N	100	Ca^{2+}	(Illangasekare and Yarus 1999b)
acyl transfer	C–O	1×10^{10}	Mg^{2+}	(Suga et al. 1998b)
acyl transfer	C–O	nd	Mg^{2+}	(Jenne and Famulok 1998)
aminoacylation	C–O	2×10^{5}	Mg^{2+}+Ca^{2+}	(Illangasekare et al. 1995)
aminoacylation	C–O	nd	Mg^{2+}	(Lee et al. 2000)
aminoacylation	C–O	2×10^{5}	Mg^{2+}	(Saito et al. 2001)
aminoacylation	C–O	6×10^{7}	Ca^{2+}	(Illangasekare and Yarus 1999a)
carbonate hydrolysis	C–O	100	None	(Chun et al. 1999)
sulfur alkylation	C–S	2×10^{3}	Mg^{2+}	(Wecker et al. 1996)
Michael reaction	C–S	3×10^{5}	Mg^{2+}	(Sengle et al. 2001)
porphyrin metalation	Cu–N	500	Mg^{2+}+Cu^{2+}	(Conn et al. 1996)
Pd nanoparticle formation	Pd–Pd	nd	None	(Gugliotti et al. 2004, 2005)
biphenyl isomerization	None	88	Mg^{2+}	(Prudent et al. 1994)

Table 5. Artificial ribozymes. nd = not determined.

6.3 Biochemical Characterization of Ribozymes

6.3.1 Secondary Structures and Minimization of Ribozymes

As for aptamers (see Section 3.2.3), ribozyme secondary structures may be proposed on the basis of computer algorithms and sequence alignments. Then, experimental verification of secondary structures may be performed by enzymatic and chemical probing along with covariation analysis. Sequences and likely secondary structures of some representative ribozymes are illustrated in Fig. 6.

A practical goal is often to characterize the minimal version of a particular ribozyme that is still highly functional. Minimal ribozymes are important because smaller ribozymes are more readily synthesized and less susceptible to random degradation; they are also easier to study mechanistically. Minimization efforts are usually guided by the secondary structures mentioned above. One must be careful not to remove nucleotides that may be catalytically important, as shown by a number of studies on the natural hammerhead ribozyme (Uhlenbeck 2003). Nonhomologous random recombination may also be used in tandem with selection for direct minimization (Wang and Unrau 2005). One advantage of this approach is the lack of bias that would be introduced via any preconceived notions of which secondary structure elements are important for ribozyme activity.

6.3.2 Ribozyme Mechanisms and Rate Enhancements

Evidence has increasingly accumulated that natural ribozymes can rely mechanistically upon acid-base catalysis involving the nucleobases (Das and Piccirilli 2005; Bevilacqua and Yajima 2006). It seems likely that artificial ribozymes will be able to take advantage of similar mechanisms. However, little is known about the mechanisms of most artificial ribozymes. Rate parameters and metal ion requirements (Table 5) are usually determined as part of the overall characterization. In contrast, detailed mechanistic analyses are rare, although it is clear that relatively sophisticated physical organic approaches such as kinetic isotope effects may be applied (Unrau and Bartel 2003). The catalytic roles of metal ions are often unknown. Because metal ions are required simply for binding of targets by aptamers (Section 3.1), separating the binding and catalysis roles of metal ions for ribozyme activity is a tremendous experimental challenge. The mechanistic roles of metal ions have been explored most extensively for acyl-transferase ribozymes (Suga et al. 1998a; Flynn-Charlebois et al. 2001; Vaidya and Suga 2001; Saito and Suga 2002).

One aspect of ribozyme activity that is often quantified is the rate enhancement (Table 5). For any particular ribozyme, an appropriate background reaction with a relatively low rate can usually be identified. For a ribozyme that ligates two RNA substrates, the relevant background reaction could be taken as the analogous ligation reaction that occurs when the two reacting functional groups are held in together by a complementary splint that lacks an enzyme region, rather than the ribozyme itself

(which has both binding and catalytic regions). From the ratio of rates of the background and catalyzed reactions, the rate enhancement may be computed (Fig. 7). The observed ribozyme rate enhancements range from modest (10^2) to substantial (10^{10}). Although all of these values pale in comparison with rate enhancements for some proteins, the appropriate benchmarks are the natural ribozymes, against which artificial ribozymes compare favorably.

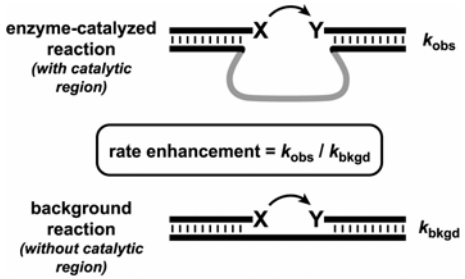

Fig. 7. Quantifying the rate enhancement of a ribozyme by comparison of its reaction rate to the rate of an analogous "splinted" background reaction.

The large rate enhancements of artificial ribozymes imply that they do more than passively hold their substrates in close proximity via "effective molarity". Consistent with this, almost all ribozymes form just one product, even when multiple products are chemically possible. For example, although the early RNA ligase ribozymes could have made multiple isomeric linkages during synthesis of the ligated RNA, each particular ribozyme was found to make just one type of linkage (Bartel and Szostak 1993). This is in sharp contrast to "effective molarity" considerations, which would ordinarily be expected to permit formation of a mixture of isomeric linkages. In most cases, the mechanistic explanation for ribozyme activity is not yet known. As for proteins, the two main possibilities are direct lowering of transition-state energies and precise positioning of the reactive groups (i.e., orientation effects). Given that these mechanistic issues have not been settled for most of the natural ribozymes, including the ribosome (Sievers et al. 2004; Weinger et al. 2004; Bieling et al. 2006), it is clear that mechanistic ribozymology is still a nascent field.

6.3.3 Ribozyme Structural Biology

X-ray crystal structures or NMR structures are available for many natural ribozymes (see Chapter 1). In contrast, artificial ribozymes have not yet been investigated widely by these structural methods. The Pb^{2+}-dependent RNA-cleaving "leadzyme" has been investigated (Hoogstraten et al. 1998; Legault et al. 1998; Wedekind and McKay 1999, 2003), revealing probable relationships between metal ion binding and structural rearrangements that are required to reach the catalytically active conformation. Separately, a Diels-Alderase ribozyme has been examined (Serganov et al. 2005), showing that the substrate binding pocket is hydrophobic and providing a rationale for the observed enantioselectivity. Beyond these two examples, much work remains to understand the structural basis of artificial ribozyme catalysis.

6.3.4 Evolutionary Considerations for Ribozymes

Certain features of artificial ribozymes have implications for prebiotic chemistry and the "RNA World" hypothesis. For example, ribozymes that incorporate three or fewer different nucleotides (rather than the conventional four) have been investigated. One effort omitted cytidine (C) from the enzyme region and identified ribozymes that ligate RNA (Rogers and Joyce 1999). Performing reselection with inclusion of C allowed the catalytic rate to improve 20-fold, which calibrates our understanding of the importance of structural variety in ribozyme function (Rogers and Joyce 2001). Remarkably, an RNA ligase ribozyme comprising only two different nucleotides was also identified (Reader and Joyce 2002). This "binary informational system" included only uridine (U) and 2,6-diaminopurine (D), which can form base pairs with each other (Fig. 8). Although this ribozyme was fairly inefficient, its identification demonstrates that a highly minimal system can encode relevant catalysis.

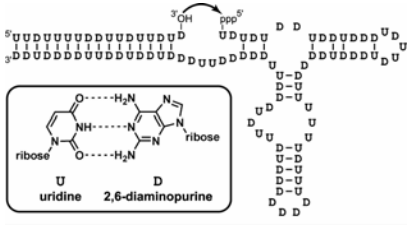

Fig. 8. A minimal RNA ligase ribozyme that has only two nucleotides, uridine (U) and 2,6-diaminopurine (D).

An interesting conceptual challenge is to evolve one ribozyme sequence into another. One study found evidence that a single RNA sequence can adopt either of two entirely distinct secondary structures, each of which has a different catalytic activity (Schultes and Bartel 2000). Furthermore, this bifunctional sequence could be accessed by a series of no more than two mutation steps from prototype ribozymes that catalyze only one of the two activities. Therefore, the collection of ribozymes forms a "neutral network" that has implications for understanding early RNA evolution.

6.4 Ribozymes with Chemical Modifications

Similar to aptamers (Section 3.3), the biochemical stability of ribozymes can be improved by judicious incorporation of nonstandard nucleotides. This approach frequently uses 2'-fluoro or 2'-aminopyrimidine nucleotides, which confers resistance to ribonucleases (Pieken et al. 1991).

To enhance catalytic activity, several ribozyme selection efforts have used modified nucleotides that introduce functional groups not found naturally in RNA. The first such effort sought ribozymes that catalyze the Diels-Alder reaction, with the assistance of pyridine-modified uridine derivatives (Tarasow et al. 1997; Tarasow et al. 2004). Most of the ribozymes required Cu^{2+}, suggesting a role for Lewis acid catalysis, and the pyridine groups were conjectured to participate in some combination of hydrogen bonding, hydrophobic interactions, dipolar interactions, and metal ion coordination. Because the pyridine was attached via the C5 of uridine, Watson-Crick base pairing interactions were not disrupted, and the modified nucleotides could be incorporated into RNA via T7 RNA polymerase. Other efforts with modified nucleotides have also been reported, involving 5-imidazolyl-U and amide synthesis (Wiegand et al. 1997), N^6-amine-modified adenosines and RNA ligation (Teramoto et al. 2000), or 5-imidazolyl-U and urea synthesis (Nieuwlandt et al. 2003).

An interesting series of experiments has identified ribozymes that catalyze crystallization of palladium nanoparticles (Gugliotti et al. 2004, 2005). These efforts used pyridine-modified U nucleotides, and these nonstandard nucleotides were required for catalytic activity. Different ribozymes create different crystal shapes, such as hexagonal plates or cubes. The mechanistic basis for the selective crystallization is not known.

7 In Vitro Selection of Deoxyribozymes

DNA differs from RNA solely by absence of the 2'-hydroxyl group at every nucleotide position of DNA. Because RNA has relatively few functional groups to begin with, the missing hydroxyl group has long been speculated to render DNA catalytically inferior to RNA (Cech 1987). This speculation was reinforced by the natural role of DNA, which is for genetic information storage as a double helix. Nevertheless, just like single-stranded RNA, single-stranded DNA has conformational flexibility that could permit intricate three-dimensional shapes and consequently catalytic activity. In 1994 the first deoxyribozyme was discovered by in vitro selection (Breaker and Joyce 1994b), and since that time an increasing variety of DNA catalysts have been identified.

7.1 Methodology to Identify Deoxyribozymes

Unlike for ribozymes, the DNA template does not need to be transcribed into RNA and then (after isolation of the catalytically active sequences) reverse-transcribed back into DNA. Instead, for deoxyribozymes the DNA functions as both information and catalyst throughout the selection process. Other than this procedural difference, the methodology to select deoxyribozymes is essentially the same as for ribozymes (Section 6.1). This includes several examples of nonstandard nucleotides used to enhance the chemical repertoire of DNA (Santoro et al. 2000; Perrin et al. 2001; Thum et al. 2001; Liu and Sen 2004; Sidorov et al. 2004; Keiper and Vyle 2006). Negative selection may also be used; for example, to improve the metal ion specificity of RNA-cleaving deoxyribozymes (Bruesehoff et al. 2002).

As for aptamers, single-stranded DNA needs to be separated from its complement after PCR during each round of selection. This can be achieved by incorporating a moiety such as a polyethylene glycol (PEG) spacer in one of the two PCR primers (Williams and Bartel 1995; Sheppard et al. 2000; Flynn-Charlebois et al. 2003b). The PCR enzyme—usually Taq polymerase—cannot extend past the spacer within the template strand, and therefore the two unequal-length single strands of the PCR product are separable by PAGE. Other approaches can be used; for example, based on removal of the biotinylated form of the undesired DNA strand (see Section 2.1.1.).

7.2 Chemical Reactions Catalyzed by Deoxyribozymes

Many fewer types of reactions have been explored using deoxyribozymes rather than ribozymes. This reflects the later discovery of deoxyrbozymes and also the emphasis placed in catalytic RNA due to the "RNA World" hypothesis. Nonetheless, deoxyribozymes (which are also termed DNA enzymes, DNAzymes, or catalytic DNA) have proven quite competent catalyticaly when they have been examined (Table 6). Most of the known DNA-catalyzed reactions involve phosphodiester exchange.

Reaction catalyzed	Bond	Rate enh	M^{2+} req	Reference
RNA cleavage	O–P	10^5	Pb^{2+}	(Breaker and Joyce 1994b)
RNA cleavage	O–P	10^5	Mg^{2+}	(Breaker and Joyce 1995)
RNA cleavage	O–P	50	Mg^{2+}	(Santoro and Joyce 1997)
RNA cleavage	O–P	1×10^8	None	(Geyer and Sen 1997)
RNA cleavage	O–P	nd	Zn^{2+}	(Santoro et al. 2000)
RNA cleavage	O–P	nd	None	(Perrin et al. 2001)
RNA cleavage	O–P	10^5	None	(Sidorov et al. 2004)
RNA ligation	O–P	300	Mg^{2+}	(Flynn-Charlebois et al. 2003b)
RNA ligation	O–P	2×10^4	Zn^{2+}	(Hoadley et al. 2005)
RNA ligation	O–P	10^4	Mg^{2+}	(Purtha et al. 2005)
RNA ligation	O–P	10^5	Zn^{2+}	(Purtha et al. 2005)
RNA branching	O–P	5×10^6	Mn^{2+}	(Wang and Silverman 2003)
RNA branching	O–P	10^5	Mg^{2+}	(Coppins and Silverman 2005)
RNA lariat formation	O–P	10^5	Mn^{2+}	(Wang and Silverman 2005)
DNA phosphorylation	O–P	10^9	Mn^{2+}	(Wang et al. 2002c)
DNA adenylation (capping)	O–P	2×10^{10}	Mg^{2+}+Cu^{2+}	(Li et al. 2000)
DNA ligation	O–P	3×10^3	Cu^{2+} or Zn^{2+}	(Cuenoud and Szostak 1995)
DNA ligation	O–P	10^5	Mn^{2+}	(Sreedhara et al. 2004)
oxidative DNA cleavage	C–O	10^6	Cu^{2+}	(Carmi et al. 1996; Carmi et al. 1998)
DNA deglycosylation	C–N	9×10^5	Ca^{2+}	(Sheppard et al. 2000)
thymine dimer photoreversal	C–C	3×10^4	None	(Chinnapen and Sen 2004)
phosphoramidate cleavage	N–P	10^3	Mg^{2+}	(Burmeister et al. 1997)
porphyrin metalation	Cu–N	1×10^3	Cu^{2+} or Zn^{2+}	(Li and Sen 1996)

Table 6. Artificial deoxyribozymes. nd = not determined.

7.3 Biochemical Characterization of Deoxyribozymes

Experimental data indicate that DNA is quantitatively as competent as RNA in terms of catalytic ability. For example, the RNA-cleaving 10–23 deoxyribozyme can achieve a k_{cat} of 10 min^{-1}, which rivals natural ribozymes, and its k_{cat}/K_m is ~10^9 M^{-1} min^{-1}, which is higher than that of the protein enzyme ribonuclease A (Santoro and Joyce 1997). Furthermore, deoxyribozymes that cleave RNA are at least as proficient as ribozymes that accomplish the same task (Silverman 2005).

The similarities between RNA and DNA enzymes may relate to common classes of mechanisms, although few deoxyribozymes have been investigated in detail. It is intriguing to note that RNA-cleaving deoxyribozymes can use cofactors such as ascorbate or histidine as cofactors (Carmi et al. 1996; Roth and Breaker 1998), thereby demonstrating that DNA can use noncovalently associated small molecules to expand its relatively limited repertoire of functional groups. Structural investigations of deoxyribozymes have also been limited. In the only experiment published to date, the 10–23 deoxyribozyme was crystallized but formed an inactive 2:2 complex

with its RNA substrate (Nowakowski et al. 1999). Although the resulting structure was an interesting four-way junction that has implications for understanding natural Holliday junction intermediates of recombination, no insights into DNA catalysis could be obtained.

It is interesting to ask if a ribozyme and deoxyribozyme can share the same nucleotide sequence (substituting U for T nucleobases) yet retain catalytic activity. In one study, a deoxyribozyme with hemin-dependent peroxidase activity was shown to retain some activity when all of its DNA nucleotides were exchanged for RNA (Travascio et al. 1999). In the other direction, selection was used to convert a known RNA ligase ribozyme into a deoxyribozyme (Paul et al. 2006). The ribozyme was catalytically inactive when made as DNA, and the evolved deoxyribozyme was inactive when made as RNA. If a single nucleotide sequence could be identified that is functional as either RNA or DNA, this would have implications regarding crossover between the two types of informational systems.

8 In Vitro Selection of Aptazymes

For practical applications of functional nucleic acids, an important goal is regulation of nucleic acid enzyme catalysis. A key regulatory mechanism for protein enzymes is allostery, in which binding of a target to an enzyme remote from the active site controls the catalytic activity. Similarly, experiments have focused on placing artificial nucleic acid enzymes under the control of targets that bind at sites distant from the active site. These efforts have combined aptamers for target binding with nucleic acid enzymes for catalysis, resulting in allosteric nucleic acid enzymes, or "aptazymes" (Robertson and Ellington 1999).

8.1 Aptazymes Obtained by Rational Fusion of Aptamers with Nucleic Acid Enzymes

In the basic design for an aptazyme, an aptamer that binds a particular target is connected via a "communication module" to a ribozyme. Binding of the target can lead either to an increase or a decrease in ribozyme activity. The first report of a rationally designed aptazyme was published in 1997, using ATP binding to its aptamer for inhibition of hammerhead ribozyme catalysis (Fig. 9) (Tang and Breaker 1997). Subsequent efforts with the hammerhead ribozyme were successful with other targets such as flavin mononucleotide (Araki et al. 1998; Soukup and Breaker 1999a), theophylline (Araki et al. 1998; Soukup and Breaker 1999a), and proteins (Vaish et al. 2002; Wang and Sen 2002). Additionally, many other ribozymes such as the HDV (Kertsburg and Soukup 2002), hairpin (Hartig et al. 2002), group I intron (Kertsburg and Soukup 2002; Thompson et al. 2002), and X-motif (Kertsburg and Soukup 2002) ribozymes have been converted into aptazymes. In general, a wide range of aptamer targets has been used with many ribozymes (Silverman 2003).

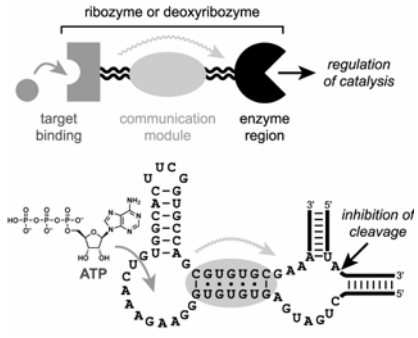

Fig. 9. Creating an aptazyme by rational fusion of an aptamer with a ribozyme. Schematic view (*top*); specific example of ATP-inhibited aptazyme (*bottom*).

In many of these cases, no selection at all was involved, in that known aptamer and ribozyme domains were simply joined via strategically placed nucleotides to create the aptazyme. Screening of candidates with different communication modules allowed identification of functional aptazymes. In this fashion, some designed aptazymes can respond to more than one target (Jose et al. 2001), presaging analogous discoveries involving natural riboswitches (see Chapter 1). In addition, more than one aptamer domain may be attached to a single enzyme (Cho et al. 2005). In a related approach to identifying aptazymes, the power of in vitro selection has been used to find optimal communication modules (Soukup and Breaker 1999b; Robertson and Ellington 2000; Soukup et al. 2000; Kertsburg and Soukup 2002; Srinivasan et al. 2004), including the use of negative selection to obtain maximal response to the target. In all of these experiments, the aptamer and ribozyme units themselves remain intact. In that context, aptazyme creation by fusion of aptamer and enzyme domains (without or with selection to optimize the communication module) may be considered a rational process.

Allosteric deoxyribozymes have been pursued to a limited extent. The sole unambiguous report of an allosteric deoxyribozyme for a small-molecule target (in the general design sense of Fig. 9) is an ATP-regulated RNA-cleaving deoxyribozyme, whose allosteric activation was optimized by selecting for functional communication modules (Levy and Ellington 2002). Using related design principles, studies have developed adenosine-activated RNA-cleaving deoxyribozymes (Wang et al. 2002b)

for practical applications (Liu and Lu 2004). Allosteric deoxyribozymes have also been developed by using competition between a regulatory oligonucleotide and the aptamer target to control deoxyribozyme catalysis (Achenbach et al. 2005).

8.2 Aptazymes Obtained by In Vitro Selection for Regulated Catalysis

In the above studies, the target-binding domain of the aptazyme was preprogrammed by using a known aptamer. Alternatively, a random region can be fused to a known ribozyme, and in vitro selection can then be used to identify aptazyme sequences that are regulated by the target in the desired fashion. The first report of such direct selection of aptazymes sought regulation of catalysis via binding of cGMP or cAMP (Koizumi et al. 1999). Subsequent experiments identified aptazymes that are controlled by a range of targets including divalent metal ions (Zivarts et al. 2005), caffeine and aspartame (Ferguson et al. 2004), and other small molecules (Piganeau et al. 2000; Piganeau et al. 2001; Seetharaman et al. 2001), in addition to proteins (Robertson and Ellington 2001). Experiments have also used partial randomization and reselection of a known aptamer (as part of an aptazyme) to improve aptazyme function (Soukup et al. 2000; Soukup et al. 2001).

Aptamer domains within aptazymes can be identified and validated separately for their target-binding ability (Soukup et al. 2001). Therefore, selection of aptazymes is an indirect way to select for aptamers themselves. One methodological advantage of this approach for identifying aptamers is that the target does not need to be covalently modified for immobilization on a solid support. This is particularly advantageous if the target compound is available in only small quantities or if its chemical modification is difficult. As a separate conceptual advantage of this approach, the aptamer domain can completely engulf the target [analogous to some natural riboswitches (Batey et al. 2004; Serganov et al. 2004)], which is not possible when a covalent tether must be attached to the target to enable the selection process (see Fig. 3, Section 3.1.1).

8.3 Mechanisms of Aptazyme Signal Transduction

The mechanism by which target binding to the aptamer leads to regulation of nucleic acid enzyme catalysis has been investigated in several studies. The known mechanisms include steric interference between the aptamer and enzyme domains (Tang and Breaker 1998), “slipping” of secondary structure involving the communication module (Soukup and Breaker 1999b; Suess et al. 2004; Hall et al. 2006), and stabilization of catalytically active enzyme structure (Araki et al. 1998; Soukup and Breaker 1999a; Atsumi et al. 2001). Other mechanisms are presumably possible. Oligonucleotide-dependent nucleic acid enzymes are regulated by different mechanistic principles that involve generally straightforward base-pairing interactions (Kuwabara et al. 1998; Robertson and Ellington 1999; Komatsu et al. 2000; Wang

and Sen 2001; Burke et al. 2002; Wang et al. 2002a; Najafi-Shoushtari et al. 2004; Penchovsky and Breaker 2005). Aptazymes that combine regulation by both small molecules and oligonucleotides have also been developed (Najafi-Shoushtari and Famulok 2005).

9 Perspective on Artificial Functional Nucleic Acids

What fundamental questions remain to be addressed for aptamers and nucleic acid enzymes? For aptamers, the wide range of targets that have been recognized—from very small molecules such as ethanolamine all the way to large proteins, some of which have been presented on the exterior of a living organism—lends credence to the notion that an RNA or DNA aptamer can be identified for essentially any desired molecular target. Two research areas are probably the most pressing for future development of aptamers. First, selectivity among closely related targets has been explored in numerous experiments, but the limits of selectivity have probably not been reached. An important goal is a full understanding of how to engineer high target selectivity as a reliable outcome of selection experiments. Second, aptamer selection as typically implemented is labor-intensive and time-consuming. Advances in streamlining selection procedures, including the growing use of automation, will likely prove important in bridging basic research and practical applications.

The in vitro selection of ribozymes and deoxyribozymes is less advanced than analogous studies of aptamers. The scope of chemical reactions that may be catalyzed by RNA and DNA still remains to be determined. The relatively small number of examples other than phosphodiester cleavage or ligation offers substantial hope that a much wider variety of reactions may be catalyzed, but in most cases the difficult work of achieving this new catalysis remains to be done. The payoff for reaching this goal should be the ability to use nucleic acid enzymes (and not just aptamers) in practical applications for which catalyzed chemical reactions are a critical design element.

As demonstrated by the other chapters in this book, the immense body of research on fundamental development of aptamers and nucleic acid enzymes has already enabled several practical applications. These examples of the translation of fundamental research into practical applications should becelebrated, particularly as scientists face ever-increasing pressure to connect their basic research with the challenges of the modern world.

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