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A naturally occurring, noncanonical GTP aptamer made of simple tandem repeats

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Recently, we used in vitro selection to identify a new class of naturally occurring GTP aptamer called the CA motif. Here we report the discovery and characterization of a second class of naturally occurring GTP aptamer, the "CA motif." The primary sequence of this aptamer is unusual in that it consists entirely of tandem repeats of CA-rich sequence motifs such as CAACA arranged as three nucleotides. Members of this aptamer class contain multiple copies of CA-rich sequence motifs such as CAACA arranged in tandem repeats, which we have designated the "CA motif." Members of this aptamer class contain multiple copies of CA-rich sequence motifs such as CAACA arranged in tandem repeats. Although recent studies indicate that tandem repeats can play biological roles, including roles in human disease, the functional properties of such repeats are still not well understood. A second intriguing feature of the CA motif aptamer is that its sequence requirements are not consistent with those of a canonical nucleic acid structure. For example, CA motif variants with limited potential for Watson-Crick base pairing, including those made of repeats containing only cytidine and adenosine nucleotides, can still bind GTP. Circular dichroism (CD) analysis of the CA motif aptamer also supports the idea of an unusual structure: the characteristic CD spectrum of the CA motif aptamer is distinct from that of several common types of nucleic acid structures, including A-form RNA. Although much less widespread in nature than the previously described G motif, the CA motif was detected in 70 percent of the approximately 400 eukaryotic genomes analyzed, and several examples were identified whose ability to bind GTP has been conserved in evolution. Taken together, these results reveal the existence of a second type of naturally occurring GTP aptamer with a noncanonical structure, and reveal a new potential biochemical activity of naturally occurring RNA tandem repeats.

Results

GTP aptamers made of tandem repeats

Previously, we used in vitro selection to isolate GTP aptamers from a pool of phylogenetically diverse genome-derived RNA fragments (Fig. 1A). The majority of these aptamers had the potential to form G-quadruplex structures, and further analysis

Introduction

GTP plays a central role in diverse cellular processes, and at least three percent of the proteins in the human proteome bind GTP. The fundamental role played by GTP in cells, as well as the emerging realization that the functions of biological proteins and RNAs may not be as distinct as once thought, led us to hypothesize that RNA molecules that bind GTP might also be widespread in nature. To test this hypothesis, we used in vitro selection to search a pool of phylogenetically diverse genome-derived RNA fragments for new examples of naturally occurring GTP aptamers. Several active variants of the CA motif aptamer lack the ability to form conventional Watson-Crick base pairs in any register, while others consist of repeats containing only cytidine and adenosine residues, indicating that noncanonical interactions play important roles in its structure. The circular dichroism spectrum of the CA motif aptamer is distinct from that of A-form RNA and other major classes of nucleic acid structures. Bioinformatic searches indicate that the CA motif is absent from most archaeal and bacterial genomes, but occurs in at least 70 percent of approximately 400 eukaryotic genomes examined. These searches also uncovered several phylogenetically conserved examples of the CA motif in rodent (mouse and rat) genomes. Together, these results reveal the existence of a second class of naturally occurring GTP aptamer whose sequence requirements, like that of the G motif, are not consistent with those of a canonical secondary structure. They also indicate a new and unexpected potential biochemical activity of certain naturally occurring tandem repeats.
demonstrated that both RNA and DNA G-quadruplexes possess an intrinsic GTP-binding activity. Most of the remaining sequences isolated in the selection contained short CA-rich motifs such as CAACA that often occurred in the context of tandem repeats (Fig. 1B). To determine whether members of this second family of sequences were also GTP aptamers, three examples were synthesized and tested for the ability to bind GTP. Each example bound GTP-agarose at least 20-fold better than a
random sequence control RNA, and none bound agarose lacking GTP at significant levels (Fig. 1C).

The most effective GTP binder among these aptamers, GG(GCAACA)₆, was chosen for further characterization. This aptamer could be eluted from GTP-agarose columns with GTP, indicating that it binds free as well as immobilized GTP (Fig. 2A). Based on the concentration dependence of elution,¹²,¹³ we estimate that this aptamer binds free GTP with a dissociation constant (Kₐ) of 1.8 mM (Fig. 2B). Elution profiles were consistent with a single binding site for GTP (as was assumed in Fig. 2B), but could be described equally well by more complex models in which multiple, independent binding sites are considered. The aptamer could also be efficiently eluted from GTP-agarose columns with ddGTP, and somewhat efficiently eluted with GDP, but not with 7-methyl-GTP, 6-methylthio-GTP, ITP, ATP, CTP, or UTP (Fig. 2C). These observations indicate that the CA motif recognizes GTP primarily through interactions with its nucleobase (Fig. 2D).

Taken together, these results demonstrate that naturally occurring simple tandem repeats can form binding sites specific for biological small-molecule ligands such as GTP.

Unusual sequence requirements of the CA motif aptamer

To better understand the types of tandem repeats that can bind GTP, we used site-directed mutagenesis to characterize the sequence requirements of this aptamer in greater detail. We focused our efforts on the (GCAACA)ₙ aptamer, both because its sequence was the simplest among the tandem repeat aptamers identified and because it bound GTP the most efficiently (Fig. 1C). To determine the minimum number of repeats needed for efficient binding as well as the number required for maximal binding, a series of aptamer variants containing between two and 16 repeats were synthesized and tested for the ability to bind GTP-agarose. No significant binding was observed for variants containing two or four repeats (Fig. 3A). The ability to bind GTP increased dramatically for a variant containing six repeats, and continued to increase as repeat number increased to ten, before gradually decreasing (Fig. 3A). We hypothesize that at higher repeat numbers the expected increase in GTP-binding activity from additional binding sites is offset by less efficient aptamer folding.¹⁴

To identify nucleotides in the aptamer most important for its GTP-binding activity, variants containing each of the 18

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Figure 2. Characterization of the affinity and specificity of the CA motif aptamer using competitive column elution. (A) Elution of the CA motif aptamer from a GTP-agarose column with free GTP. (B) Elution of the CA motif aptamer from a GTP-agarose column as a function of free GTP concentration. (C) Competitive column elution data mapped onto the chemical structure of GTP. The CA motif construct GGG(GCAACA)₆ was used for these experiments. Values reflect the average of three independent experiments, and error bars indicate one standard deviation.
possible single-mutation changes in the GCAACA motif were synthesized and tested for the ability to bind GTP in the context of an aptamer containing six tandem repeats. These results confirmed the importance of the CA-rich portion of the sequence. While in some cases mutational changes at the first, second, or third positions had only small effects, all single-mutation changes at the fourth, fifth, and sixth positions either strongly decreased or abolished the GTP-binding activity of the aptamer (Fig. 3B). This analysis also revealed that, at least in the context of single-mutation changes, the optimal nucleotide at five out of six positions in the GCAACA repeat is either a cytidine or adenosine (Fig. 3B). Similar analysis of the GCCUCCCAGUGAUUGUA aptamer showed that the CA-rich motif CCAA is important for its activity, and that variants containing the CCAAGG motif bind GTP more effectively than the original isolate (Fig. 3C).

To further probe the sequence requirements of the CA motif, we analyzed possible secondary structures formed by this aptamer. As a consequence of its CA-rich sequence, the potential of this aptamer to form canonical base pairs is limited: only two of the 15 possible pairs of positions in each GCAACA repeat, G1-C2 and G1-C5, have the potential to form a Watson-Crick base pair (Fig. 4). Furthermore, only the G1-C2 pairing can generate secondary structures containing consecutive base pairs (Fig. 4A).

Two observations, however, suggest that the potential G1-C2 interaction is unlikely to be important for aptamer activity. First, of the five different single-mutation variants of this aptamer we generated in which this putative base pair was disrupted, the GTP-binding activity of only two were significantly lower than that of the reference sequence (Figs. 3B and 4A). Second, none of the three potential compensatory mutations tested at positions 1 and 2 rescued aptamer activity (Fig. 4A). In contrast, mutations that disrupted the putative G1-C5 pairing typically reduced aptamer activity as expected (Figs. 3B and 4B). Furthermore, in one case this loss of GTP-binding activity could be rescued by compensatory mutations consistent with a standard Watson-Crick base pair, although in two other cases such compensatory mutations did not restore activity (Fig. 4B). These results suggest that positions 1 and 5 interact in some way, although not necessarily in the context of a canonical Watson-Crick base pair. For example, the observed rescue patterns at these positions are more consistent with either trans-Hoogsteen/Hoogsteen base pairing geometries (in which G-C to C-G changes are isosteric but G-C to U-G or U-A are not) than with a standard cis-Watson-Crick/Watson-Crick pairing.13

CA motif aptamer repeats only require cytidine and adenosine

Taken together, these results suggest that the CA motif aptamer may not require canonical base pairs for its activity. To further explore this hypothesis, we investigated the extent to which aptamer variants with little or no ability to form canonical base pairs could bind GTP. Previous results suggested that the GG(GCAACA)6 aptamer was a good starting point for these experiments because, although its ACAACA repeats cannot form canonical base pairs in any register, this aptamer still binds GTP at low but detectable levels (Fig. 3B). As was the case for the reference GCAACA construct (Fig. 3A), the ability of the ACA/ACA/aptamer to bind GTP increased with repeat number, and constructs containing 18 or 24 copies of this motif bound GTP almost as well as the reference GG(GCAACA)6 sequence (Fig. 5A). Like other aptamer variants tested, GG(AACA)6, did not bind control agarose lacking GTP at significant levels under these conditions (Fig. 5B). These results show that noncanonical interactions involving cytidine and/or adenosine nucleotides play critical roles in the structure of the CA motif aptamer.

Figure 3. Sequence requirements of the CA motif aptamer. (A) Binding of the GG(GCAACA)6 aptamer to GTP-agarose as a function of repeat number. (B) Ability of all single-mutation variants of the GCAACA motif in the GG(GCAACA)n aptamer to bind GTP-agarose. (C) Ability of all single-mutation variants of the GCCUCCCAGUGAUUGUA aptamer to bind GTP-agarose. Percent bound values reflect the average of three independent experiments, and error bars indicate one standard deviation.
activity (majority of these mutants exhibited little or no GTP-binding synthesized and tested for the ability to bind GTP). Although the containing either a cytidine or adenosine at these positions were (on the separate effects of 2C to 2A and 6A to 6C mutations bound GTP up to 50-fold more efficiently than expected based in the absence of GTP, although some differences were observed for the 1U construct (Fig. 6A). In the presence of 1 mM GTP, spectra were similar to those measured (Fig. 4B). They also differed from typical spectra of several other important types of nucleic acid structures, including the B-form helix, the Z-form helix, the parallel strand G-quadruplexes, the antiparallel strand G-quadruplexes, and the i-motif.

The spectrum of the CA motif most closely resembles those of several sequences that adopt triple-stranded architectures, although we note that considerable heterogeneity in such spectra has been reported. Like the CD spectrum of the CA motif, the spectra of these triple sequences contain negative peaks at ~210 nm and ~250 nm and positive peaks at ~220 nm and ~270 nm. Taken together, these results provide additional evidence that the CA motif cannot form a canonical A-form duplex, and are consistent with an unusual structure such as a triple-stranded helix.

**Figure 4.** Evaluation of potential Watson-Crick base pairs in the CA motif aptamer. (A) Binding of single and double mutants at positions 1 and 2 in each repeat of the GG(GCAACA)₆ aptamer to GTP-agarose. (B) Binding of single and double mutants at positions 1 and 5 in each repeat of the GG(GCAACA)₆ aptamer to GTP-agarose. The antiparallel-strand model being tested is shown above each graph, with potential Watson-Crick base pairs indicated by dashes. For simplicity, only two copies of the GCAACA motif are shown. Percent bound values reflect the average of three independent experiments, and error bars indicate one standard deviation.

To more clearly elucidate the nature of these interactions, we used site-directed mutagenesis to search for compensatory mutational effects among positions 2, 3, 4, and 6 in the GG(GCAACA)₆ aptamer. Fifteen of the 16 possible variants differed significantly from that of tRNA (A-form RNA), which contains a negative peak at ~220 nm and a positive peak at ~260 nm (Fig. 6B). Although uncommon, parallel strand nucleic acid structures have been previously described, and they occur frequently in certain noncanonical folds such as G-quadruplexes.

**Circular dichroism spectrum of the GCAACA aptamer**

Different classes of nucleic acid structures often exhibit characteristic circular dichroism (CD) spectra. To investigate the relationship between the structure of the CA motif and those of other types of nucleic acid motifs, we obtained the CD spectrum of the GG(GCAACA), reference sequence and four variants of this aptamer. These variants differed from the reference sequence at six positions, and each bound GTP-agarose with an efficiency comparable to that of the GG(GCAACA), aptamer (Fig. 3B). The CD spectra of these five sequences were similar: each contained a negative peak at ~210 nm and ~250 nm, a positive peak at ~270 nm, and a peak that was usually positive at ~220 nm (Fig. 6A). In the presence of 1 mM GTP, spectra were similar to those measured in both the human and chicken genomes, but provided little information about other species in which this aptamer is found. To better characterize the phylogenetic distribution of the CA motif, we searched genomic sequence databases for additional examples using a sequence model derived from the site-directed mutagenesis experiments described in Figures 3A and B, 4B, and 5A. This model required sequences to contain at least six copies of the GCAACA motif or at least ten copies of the ACAACA motif, allowed for five mutational changes (1G to U, 2C to A, 2C to G, 3A to C, and 1G5C to 1C5G) observed in active variants of the GCAACA aptamer, and required that tandem repeats be perfect (model summarized in Fig. 7A).

A survey of approximately 430 phylogenetically diverse genomes using this sequence model failed to detect the CA motif aptamer in either bacterial or archaeal genomes. In contrast, this aptamer was identified in about 70 percent of the eukaryotic genomes examined. Approximately, 20 percent of these genomes contained a single example of the CA motif, while 5 percent of single and double mutants at positions 1 and 2 in each repeat of the GG(GCAACA)₆ aptamer to GTP-agarose. The antiparallel-strand model being tested is shown above each graph, with potential Watson-Crick base pairs indicated by dashes. For simplicity, only two copies of the GCAACA motif are shown. Percent bound values reflect the average of three independent experiments, and error bars indicate one standard deviation.
contained 35 or more examples. Examination of the species in which the CA motif was most abundant (Table S2) revealed several taxonomic groups in which this aptamer occurred frequently in all available genomes, including family Muridae (Mus musculus and Rattus norvegicus) and family Culicoidea (Anopheles gambiae, Aedes aegypti, and Culex quinquefasciatus). Analysis of gene expression databases such as the Gene Expression Omnibus indicated that at least some of these examples are also expressed. For example, of the 29 species in which the CA motif occurs 25 or more times, 19 contain at least one expressed example supported by EST evidence, and six more contain at least one example predicted to be expressed (Table S2). Because we have likely not identified all possible variants of the CA motif that can bind GTP, the results of these searches represent lower limits on the true number of CA motif aptamers in the genomes analyzed.

In addition to examining the overall abundance of the CA motif in different species, we also investigated the extent to which specific examples have been conserved in evolution. Because the CA motif is abundant in both mouse and rat, and the comparative genomics of this pair is well studied, we focused most of our efforts to identify conserved variants on these two species. Rat orthologs of mouse CA motif variants were identified using whole-genome alignments, manually examined to determine their similarity to the CA motif consensus sequence, and in some cases tested for the ability to bind GTP. Although conserved orthologs were not detected for most of the approximately 120 CA motif variants analyzed, two mouse/rat pairs were identified in which the CA motif was potentially conserved (Fig. 7B). For both pairs, the mouse sequence was an exact match to the CA motif consensus, while the rat ortholog was similar but not identical (Fig. 7B). One of these examples (mouse/rat 1) mapped to an intergenic region on chromosome 5, while the other (mouse/rat 2) occurred antisense to an intron in the Hid1 gene. Both mouse sequences as well as their rat orthologs bound GTP-agarose (Fig. 7B), indicating that in some cases the ability of the CA motif to bind GTP has been conserved in evolution.
Figure 6. For figure legend, see page 689.
Discussion

Although antiparallel, double-stranded, right-handed helices formed by canonical Watson-Crick base pairs are the most common structural elements in both RNA and DNA, numerous variations on this theme have been identified.26 In the most commonly observed case, noncanonical base pairs occur in the context of otherwise standard helical elements.27,28 Unusual helical architectures such as Z-DNA 26,29 and triplex DNA 20,30 have likewise been reported. Structures in which canonical base pairs are entirely absent are also known. Examples include the G-quadruplex, which contains guanosine tetrads rather than standard base pairs,17,18 and the i-motif, which contains hemiprotonated C-C base pairs organized into a four-stranded structure.31

The sequence requirements of the CA motif suggest that, like the G-quadruplex and the i-motif, the structure of this aptamer does not require canonical base pairs. Although our experiments do not provide direct information about the three-dimensional fold of this structure, they significantly constrain the possibilities. Variants of the CA motif made up of repeats containing only cytidine and adenosine can still bind GTP (Fig. 5A and B),
suggesting that the essential interactions made by the aptamer only require these two nucleotides. Compensatory mutations involving CA to AC changes (Fig. 5C and D) suggest that in some cases these interactions occur between cytidine and adenosine. The CD spectrum of the CA motif suggests that it does not form a standard A-form helix (Fig. 6B). Finally, if the 1–5 (Fig. 6B) and 2–6 interactions (Fig. 5C and D) reflect physical contacts, they are consistent with a structure containing parallel strands (Fig. 5E).

The structure of the CA motif may be related to that of a viral translational enhancer element called the omega sequence.27 This enhancer consists almost entirely of imperfect AAA repeats,26 although the number of repeats is not high enough to be expected to bind GTP under the conditions used in our study. The omega sequence by ultracentrifugation, thermal melting, and chemical probing indicates that, despite the inability to form canonical Watson-Crick base pairs, it adopts a compact and stable structure.35–36 The CA-rich region of this enhancer was proposed to form a triple helix containing both parallel and antiparallel strand topologies;37 and some details of this model are consistent with our data. For example, our site-directed mutagenesis experiments suggest that the CA motif aptamer could form a structure containing parallel strands (Figs. 4B and 5C–E). The CD spectrum of the CA motif (Fig. 6) is also consistent with a triple helix,35 although a high-resolution structure will be needed to confirm this hypothesis. A second known example of a stable structure formed by an RNA containing only cytidine and adenosine is a dimer formed by two molecules of CA and an intercalated proflavine molecule.38 Like the proposed fold of the omega sequence, the structure of this dimer contains parallel strands.

The results of our selection using a pool of genome-derived RNA fragments differ significantly from those of most previous studies in which functional RNAs were isolated from random sequence pools.39 The majority of the aptamers and ribosymes isolated in these studies form secondary structures containing standard helical elements. For example, 14 GTP aptamers previously identified using in vitro selection, including at least six distinct motifs isolated from random sequence pools, form secondary structures containing canonical Watson-Crick base pairs.40–41 In contrast, neither of the GTP-binding motifs we isolated from a pool of genome-derived RNA fragments adopts such a structure. This observation is consistent with the idea that nucleic acid motifs that form noncanonical structures may be more abundant in eukaryotic genomes than they are in random sequence pools. It also suggests that efforts to identify naturally occurring functional RNA elements should include methods capable of identifying noncanonical motifs, such as the selection-based approach used here, in addition to those designed to identify phylogenetically conserved RNA secondary structures containing Watson-Crick base pairs.42 Recent studies suggest that such motifs can bind a wide range of biological cofactors, providing further evidence of the functional diversity of noncanonical nucleic acid structures.43

RNA aptamers that bind small molecules are widespread in bacteria, and play important roles in the regulation of gene expression.44 The results described in this and a previous study45 are consistent with the possibility that such aptamers also occur more frequently in eukaryotes than is currently appreciated.46 We anticipate that direct selection-based approaches will continue to serve as a powerful approach to further explore this possibility.

Materials and Methods

In vitro selection

Pools were generated by fragmentation of genomic DNA using DNase I followed by gel purification of ~100–600 bp fragments on agarose gels. Fragments with 3’ adenosine overhangs were generated by incubating first with DNA polymerase I, and then with dATP and Taq DNA polymerase. These fragments were ligated into pGEM-T vectors and amplified by PCR using primers flanking the insertion site, one of which contained a T7 promoter at its 5’ end. Templates were transcribed using T7 RNA polymerase to generate starting pools for in vitro selection experiments.

GTP aptamers were isolated by incubating pool RNA with GTP-agarose, washing away unbound molecules with selection buffer, and eluting bound RNA with EDTA. Eluted molecules were subjected to RT-PCR and transcribed to generate RNA for the next round of selection. After four rounds of selection, the pool was cloned using the TOPO TA kit (Invitrogen) and sequenced. See our previous study for detailed descriptions of these protocols.47

GTP-agarose binding assays

Assays were performed as previously described44 using GTP-agarose from Innova Biosciences and micro-spin columns from Pierce (catalog number 89879). Following the addition of RNA (100 nM final concentrations with a trace amount of body labeled RNA in a volume of 170 µL), columns were incubated for 15 min and then washed four times with 170 µL 1 × binding buffer (20 mM MgCl₂, 200 mM KCl, 20 mM HEPES pH 7.1). Bound RNA was eluted by washing twice with 170 µL of 5 mM EDTA, pH 9.

To determine the percent of RNA bound to GTP-agarose, equivalent percentages of wash and elution fractions were ethanol precipitated, resuspended, and spotted on TLC plates. Plates were scanned using a Typhoon phosphorimager, and counts determined using ImageQuant software. For constructs for which the fraction bound to control agarose was also determined, this value was never significantly different from the fraction bound by a random sequence control pool (Fig. 3C).

Competitive columns elution

The GG(GCAACA)₆ construct of the CA motif aptamer was bound to GTP-agarose using the protocol described above. After washing twice with 170 µL of 1 × binding buffer to remove non-specifically bound RNA, 50 µL of elution buffer (20 mM MgCl₂, 200 mM KCl, 20 mM HEPES pH 7.1 and 5 mM GTP analog) was added to the column. After incubating for 5 min, the column was briefly spun in a benchtop centrifuge, and the flowthrough was transferred to a clean tube. After performing this elution protocol zero (Fig. 2C) or four (Fig. 2A) more times,
6. VeL calculate the volume required to elute 50 percent of the aptamer a volume of 100 mM HEPES pH 7.1, the solution was incubated for 30 min at room temperature and the circular dichroism spectrum was measured using a JASCO J-715 Spectropolarimeter.

**Bioinformatics**

CA motif variants were typically identified by BLAST (run from the DOE Joint Genome Institute website) and BLAST (run from the NCBI website). BLAST searches were performed on unmasked genomes, while BLAST searches were performed in the absence of filters and masking. Results obtained from BLAT were virtually identical to those obtained by “replace all” searches in Microsoft Word files of downloaded genomes (R² = 0.99), while results from BLAST searches were similar (R² = 0.86) but underestimated the number of aptamers in some genomes. In some cases, results of BLAST searches were also compared with those of BLAST searches of masked genomes (implemented using the UCSC Genome Browser). These two approaches typically gave similar results, although BLAST could not reliably detect the (ACAACA) aptamer in most masked genomes. Examples expressed of the CA motif were identified by BLAT and BLAST (as described above). Mouse/rat whole-genome alignments were analyzed using the UCSC Genome Browser.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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## Supplemental Material

**Supplemental material** may be found here: www.landesbioscience.com/journals/rnabiology/article/28798/

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