N^6-methyladenosine-sensitive RNA-cleaving deoxyribozymes

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Abstract: *Deoxyribozymes are synthetic enzymes made of DNA that can catalyze the cleavage or formation of phosphodiester bonds and are useful tools for RNA biochemistry. Here we report new RNA-cleaving deoxyribozymes to interrogate the methylation status of target RNAs, thereby providing an alternative method for the biochemical validation of RNA methylation sites containing N^6-methyladenosine, which is the most wide-spread and extensively investigated natural RNA modification. Using in vitro selection from random DNA, we developed deoxyribozymes that are sensitive to the presence of N^6-methyladenosine in RNA near the cleavage site. One class of these DNA enzymes shows faster cleavage of methylated RNA, while others are strongly inhibited by the modified nucleotide. The general applicability of the new deoxyribozymes is demonstrated for several examples of natural RNA sequences, including a lncRNA and a set of C/D box snoRNAs, which have been suggested to contain m^6A as a regulatory element that influences RNA folding and protein binding.*

Among the more than one hundred different types of modified nucleotides present in natural RNA, N^6-methyladenosine (m^6A) is one of the most intensively studied modifications in eukaryotic mRNA and non-coding RNA.[1] The majority of m^6A sites is installed either by a methylation complex containing the METTL3, METTL14 and WTAP proteins,[2] or by the METTL16 methyltransferase,[3] and can be removed by the demethylases FTO[4] or ALKBH5.[5] RNA methylation is suggested to play crucial regulatory roles in many biological processes. For example, m^6A was found to influence the splicing,[6] translation[7] and decay of mRNAs that carry this modification.[8] In these and other studies, m^6A is usually detected by sequencing using m^6A-seq,[1b] MeRIP-seq,[1a] PA-m^6A-seq[9] and miCLIP[10] techniques, which combine immunoprecipitation of target RNA with m^6A-specific antibodies and deep sequencing. These transcriptome-wide techniques are prone to artefacts and cannot reveal quantitative information on modification levels and stoichiometry.[11] Therefore, candidate m^6A sites must be further analyzed with robust biochemical or biophysical methods. To this end, a multistep procedure called SCARLET (site-specific cleavage and radioactive labeling followed by ligation-assisted extraction and TLC) was successfully employed to validate m^6A sites in mRNAs and lncRNAs.[12] Recently, additional techniques were suggested, based on reverse transcription in the presence of SeTTP,[13] or using engineered DNA polymerases that are optimized to respond to m^6A in the RNA template by enhanced misincorporation rates.[14] Moreover, the sensitivity of the *E. coli* MazF RNA endonuclease to m^6A in the ACA recognition sequence was reported.[15] However, MazF is not site-specific and can only be applied to a limited set of m^6A RNAs, as the majority of m^6A sites in mRNAs and lncRNAs are present within the consensus DRACH motif.[10, 16]

RNA-cleaving deoxyribozymes combine endonuclease activity with site-specificity and are promising tools for the examination of RNA modifications. DNA enzymes of the 10-23 and 8-17 families can cleave different dinucleotide junctions,[17] although the cleavage rates for diverse RNA substrates span several orders of magnitude.[18] DNA enzymes have been used for the site-specific analysis of ribose methylations in *S. cerevisiae* 25S rRNA, by harnessing the effect that 2'-*O*-methylation at the cleavage site prevents DNA-catalyzed cleavage.[19] Helm and coworkers analyzed tRNA modifications by DNA enzymes that liberated the modified nucleotide at the 5'-end of the cleavage fragment.[20] Analysis was achieved by 5'-^{32}P-labeling, digestion and TLC analysis of the cleavage product. In this approach modified and unmodified RNAs must be cleaved and labeled with equal efficiency. However, the activities of 8-17 and 10-23 DNA enzymes on modified RNA have not been systematically investigated, and only RNA modifications with unperturbed Watson-Crick base-pairing sites, such as 5-methylcytidine and pseudouridine, could be analyzed.[20a] In a third approach, DNA enzymes were used to excise a modified RNA fragment of defined length from a larger RNA, by designing DNA enzymes to cleave at unmodified positions several nucleotides upstream and downstream of the modification.[20a] The resulting small RNA fragments were then isolated and subjected to further analysis.

Here we addressed the question if DNA enzymes could directly report the presence of RNA modifications and site-specifically detect m^6A in RNA. This would require that the modified nucleotide at or near the cleavage site significantly alters the activity of the deoxyribozyme, i.e. accelerates or inhibits the DNA catalyst such that modified and unmodified RNA can be discriminated. With this goal in mind, we examined the influence of m^6A on DNA-catalyzed RNA cleavage and developed new DNA enzymes for the site-specific analysis of m^6A in cellular RNA.

Known 8-17 variants[17a] were first examined for cleavage of synthetic RNA substrates that contained m^6A in the consensus GGACU motif, or in other known natural m^6A sequence contexts, including fragments of 18S rRNA and U6 snRNA. Depending on the substrate and chosen cleavage site, we found significantly different effects, which ranged from almost no influence of m^6A on the activity of 8-17NG, to reduced efficiency and undesired loss of specificity for cleaving m^6A RNA by 8-17NA next to m^6A

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(Figure S1). In contrast, an 8-17NA DNA enzyme targeting a synthetic fragment of the human 18S rRNA one nucleotide upstream of m[6]A1832 reproducibly showed slightly accelerated cleavage of the methylated RNA compared to the unmodified RNA (Figure S2). However, high concentrations of Mg^{2+} or a combination with transition metal ions such as Mn^{2+}, or long incubation times, are undesirable conditions for RNA isolated from biological samples. Therefore, the goal of this study was to develop RNA-cleaving deoxyribozymes that robustly differentiate m[6]A-containing and unmodified RNA, and do not require transition metal ions for catalytic activity.

Gel-based *in vitro* selection experiments were carried out with m[6]A-modified RNA as a substrate (R1), to identify modification-specific DNA catalysts (Figure 1, sequences given in Table S1). Negative selection rounds were performed with an unmodified RNA (R2) to increase the specificity by eliminating DNA enzymes that can cleave both modified and unmodified RNA (red, clockwise direction in Figure 1b). In parallel, DNA enzymes were sought that cleave only unmodified RNA and are inhibited by the m[6]A modification. In this case, positive selection used R2, and negative selection was performed with R1 (green, anticlockwise direction in Figure 1b).

The synthetic RNA substrates (R1 and R2) contained a GG(m[6]A/A)CU motif (the most frequent representative of the DRACH motif in mRNAs and lncRNAs). The deoxyribozyme library contained twenty random nucleotides flanked by two RNA binding arms and an extension at the 3'-end that was used for covalent attachment to the RNA substrate by splinted ligation. Four *in vitro* selection experiments were performed in parallel, two starting with m[6]A-modified RNA R1 (selections named VMA and VMB) and two were initiated with unmodified RNA R2 (VMC and VMD). The RNA-DNA conjugates were incubated at pH 7.5 with 5 mM Mg^{2+} at 37°C (VMA, VMC) or 45°C (VMB, VMD). Active members of the library underwent self-cleavage and were separated by polyacrylamide gel electrophoresis (PAGE) using appropriate size markers. After amplification by PCR and separation of single strands, the enriched DNA library was again ligated to the substrate RNA and introduced to the next selection round. The gel bands corresponding to cleavage products were first observed in round 6 and the intensity increased to 37% cleavage for VMA in round 8 (Figure S3). In alternating negative selection rounds, the deoxyribozyme pool was ligated to the non-cognate RNA substrate and the gel bands corresponding to the intact (non-cleaved) species were isolated. The selection pressure was increased by gradually shortening the incubation time in positive selection rounds. After 16 rounds, the resulting deoxyribozyme pools were cloned, and 60 clones were chosen for sequencing. Overall, we identified 23 unique DNA sequences (Table S2) which were then individually examined for their ability to cleave 3'-fluorescently labeled RNA substrates *in trans* (i.e. RNA substrate not covalently ligated to the DNA), and to discriminate modified from unmodified RNA. After removing the 5'-overhang and the 3'-loop region, 15 DNA enzymes retained *trans*-cleaving activity.
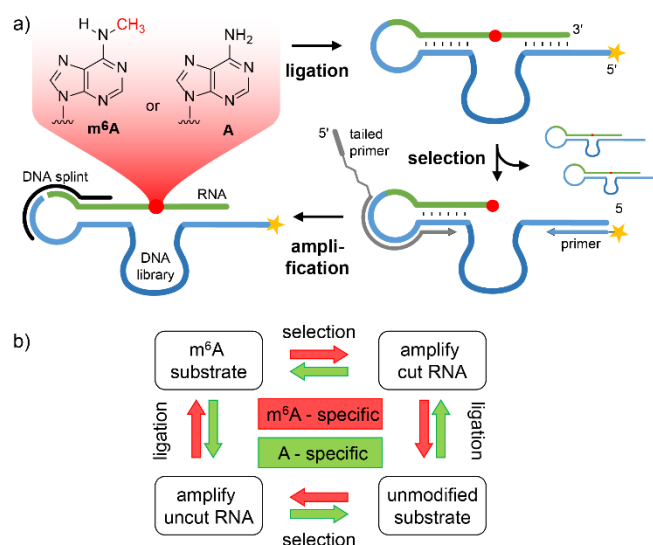

Figure 1. a) Schematic presentation of *in vitro* selection for identification of m[6]A-sensitive RNA-cleaving deoxyribozymes. The active fraction of the DNA library was isolated by PAGE and amplified using a 5'-fluorescently labeled primer and a tailed primer containing an ethylene glycol spacer. b) Experimental strategy for alternating modified and unmodified RNA substrates in positive and negative selection rounds. Red arrows indicate the direction for selections VMA and VMB starting with m[6]A RNA (R1), green arrows indicate the direction for selections VMC and VMD starting with unmodified RNA (R2).

Using a T1 digestion and an alkaline hydrolysis ladder for comparison, the cleavage site of each DNA enzyme was identified (Figure S4). Recapitulating the design of the selection experiment, most DNA enzymes from the VMA and VMB selections favored cleavage of m[6]A RNA, while VMC and VMD enzymes preferentially cleaved unmodified RNA, but individual DNA enzymes used different cleavage sites near the m[6]A modification. This could be expected as the size of the cleavage product was not strictly enforced during the selection.

The initial tests were supported by kinetic assays, in which none of the VMB or VMD DNA enzymes showed preferential behaviour compared to VMA and VMC. Therefore, VMA8, VMA15 and VMC10, which were the most frequently found clones during sequencing, were chosen for detailed examination (Figure 2). Both VMA DNA enzymes cleaved the m[6]A-containing RNA substrate R1 faster than the unmethylated RNA R2: VMA8 showed a 7-fold enhanced rate, and VMA15 cleaved R1 three times faster than R2. In contrast, VMC10 has a strong preference for cleaving the unmodified RNA R2. Cleavage of the m[6]A RNA R1 was negligible under all conditions tested, and at 20 mM Mg^{2+}, an 85-fold faster k_{obs} was observed for R2. All three DNA enzymes maintained the selectivity for their cognate RNA substrate under a range of Mg^{2+} concentrations (from 5 to 50 mM Mg^{2+}, Figure S5). Moreover, endpoint measurements of cleavage yield for defined mixtures of R1 and R2 generated calibration curves for m[6]A content (Figure S6).
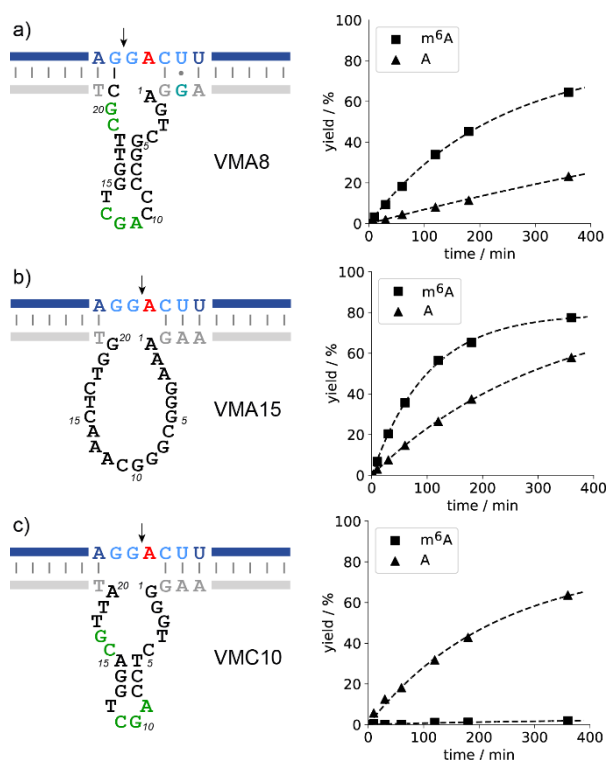
Figure 2. Deoxyribozyme sequences and kinetic plots for cleavage of the GG(m^{6}A/A)CU substrates R1 and R2 with a) VMA8 (k_{obs}(R1) = 0.27 h^{-1}, CY (cleavage yield after 6 h) ≈ 65%; k_{obs}(R2) = 0.038 h^{-1}, CY ≈ 25%), b) VMA15 (k_{obs}(R1) = 0.60 h^{-1}, CY ≈ 80%; k_{obs}(R2) = 0.19 h^{-1}, CY ≈ 55%) and c) VMC10 (k_{obs}(R1) = 0.003 h^{-1}, CY ≈ < 3%) k_{obs}(R2) = 0.26 h^{-1}, CY ≈ 65%). The arrow indicates the cleavage site, and each data point represents the mean from three independent experiments. Incubation conditions: 1 μM RNA, 10 μM deoxyribozyme, 20 mM MgCl$_2$, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5, 37 °C. Cleavage products were resolved on polyacrylamide gels and pseudo-first order rate constants were obtained by fitting to a monoexponential model (Table S3).

Analysis of the sequences and predicted secondary structures revealed that VMA8 and VMC10 contain a short 3-4 bp stem flanked by an AGC triple in the apical loop and a CG dinucleotide in the 3' direction of the stem (Figure 2). These structural elements resemble known characteristics of the 8-17 family,[17b] but both deoxyribozymes show unique features concerning cleavage site and m^{6}A selectivity not previously observed for 8-17 analogues. The binding arms of the DNA library were designed to keep the first three nucleotides of the GGACU motif unpaired. During selection, the left binding arm of VMA8 acquired an extra C to form a base pair with the first G of the motif, while a mutation in the right binding arm changed an original U-A base pair into a U-G wobble pair. Restoring the Watson-Crick base pair resulted in significantly reduced activity, suggesting a crucial role of the mismatch for the formation of the catalytic core of VMA8 (Figure S7a). On the other hand, a variant of VMC10 in which A20 of the catalytic core was mutated to C showed enhanced activity (Figure S7b), suggesting a favourable role for base-pairing the first G of the GGACU motif for VMC10. The catalytic sequence of VMA15 is not related to any known RNA-cleaving DNA enzyme and secondary structure

predictions do not reveal any preferred Watson-Crick base-pairs in the catalytic core.

The substrate scope of the new DNA enzymes was next explored by testing their activity on mutants of the RNA selection substrate containing alternative DRACH motifs, i.e. UG(m^{6}A/A)CU (R3/R4), AG(m^{6}A/A)CU (R5/R6), and GA(m^{6}A/A)CU (R7/R8). Varying degrees of sensitivity to sequence changes close to the cleavage sites were observed (Figure S8, Table S3). VMA15 and VMC10, which both cleave after the second G of the DRACH motif (GG|(m^{6}A/A)CU), tolerated changes to the first G. In contrast, the G to A transition (R5/R6) was less well accommodated by VMA15, while VMC10 retained much of its activity. Transition of the second G (R7/R8) had the most dramatic effect, and none of the three tested DNAs yielded detectable amounts of cleavage products with the GA(m^{6}A/A)CU motif. Therefore, the collection of new DNA enzymes can be used to examine m^{6}A in DGACH motifs.

Next, we changed the sequence context flanking the m^{6}A motif, and demonstrated that the sensitivity to m^{6}A RNA is not limited to the selection substrate. Using synthetic fragments of natural RNA sequences for which m^{6}A in DRACH motifs had been experimentally validated,[12,21] including m^{6}A7414 from Rous Sarcoma Virus (RSV) RNA[22] and m^{6}A1216 in human ACTB mRNA,[12] the cleavage experiments confirmed the substrate generality of the VMA and VMC DNA enzymes outside of the DGACH motif (Table S3, Figure S9, Figure S10).

To explore the response of these m^{6}A-sensitive DNA enzymes on longer RNA substrates, we chose a family of C/D box snoRNAs for which the influence of m^{6}A on snoRNP assembly has been suggested.[23] We investigated the DNA-catalyzed cleavage of full-length non-coding RNAs, including human SNORD29, SNORD41, and SNORD44, addressing the adenosine of the key *trans* Hoogsteen-sugar A:G base-pair that is essential for folding of the k-turn structure and binding of the 15.5k protein, and that is reported to be N^6-methylated.[23] The VMA15 and VMC10 DNA enzymes were designed to cleave upstream of the putative m^{6}A (shown in red in Figure 3a), and the cleavage of unmodified and methylated RNA was compared (Figure 3, S11).[24] The results indicate that the methylation-sensitive DNA enzymes can faithfully discriminate m^{6}A-containing SNORD RNAs and their unmodified derivatives. In particular, VMC10 is a robust DNA enzyme, which cuts unmodified RNA efficiently (> 80% cleavage for the SNORD RNAs in Figure 3) and is strongly inhibited by m^{6}A (<10% cleavage). VMA15 is more sensitive to sequence context and RNA secondary structure, such that the accelerated cleavage of m^{6}A RNA is less pronounced in some cases.

In a similar fashion, we analyzed mouse SNORD2 RNA, which contains overlapping GGACU and UGACC motifs with two putative m^{6}A sites at positions 31 and 35 that were both found by single-nucleotide resolution m^{6}A sequencing (Figure 4a).[10] As expected, VMC10 was inhibited by m^{6}A31 as well as m^{6}A35, and the cleavage yield linearly correlated with the methylation level (Figure 4b,c). However, VMA15 showed different behavior for the two target sites. The results were validated with a SNORD2 RNA containing a single m^{6}A at position 35 that was

prepared by splinted ligation of synthetic RNA fragments, and ruled out any cumulative effect of multiple m^{6}A modifications in the transcript (Figure S12). To examine the m^{6}A level in endogenous SNORD2, total RNA was extracted from mouse liver, and SNORD2 was enriched by pulldown with a 5'-biotinylated DNA oligonucleotide
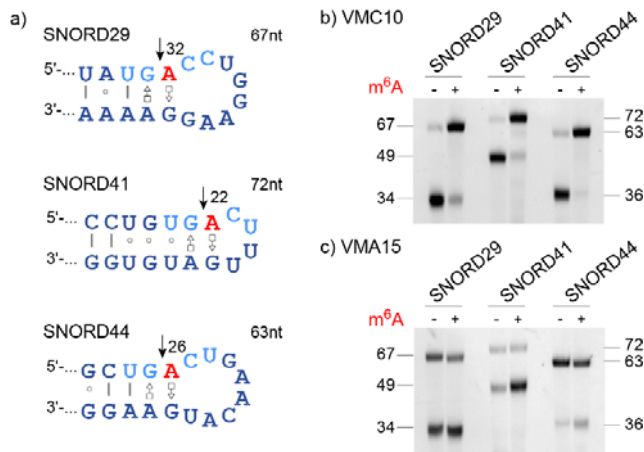

Figure 3. DNA-catalyzed cleavage of snoRNA transcripts by VMC10 and VMA15. a) Excerpt of RNA sequence with proposed regulatory m^{6}A in *trans* Hoogsteen-sugar A:G base pair in red. b), c) PAGE analysis of DNA-catalyzed cleavage of methylated and unmethylated transcripts with VMC10 (b) and VMA15 (c) DNA enzymes. Transcripts were 3'-fluorescein labeled, incubation with DNA enzymes at 37°C for 6 h. Corresponding kinetic curves in Figure S11.

complementary to the 3'-terminal region of SNORD2. Aliquots of the enriched RNA were incubated with VMA15 and VMC10 respectively, the cleavage reaction were analyzed by denaturing PAGE and visualized by northern blotting (Figure 4d). For comparison, *in vitro* transcribed RNA was assayed in parallel (Figure S13). Mouse SNORD2 RNA was efficiently cleaved at A31 by VMC10 but not by VMA15, and both enzymes cleaved the RNA at A35. Together, these results suggest a low level of m^{6}A at either position that can only be detected upon enrichment by m^{6}A-specific antibodies prior to sequencing.[10]

Finally, the DNA enzymes can be used to examine the methylation status of long endogenous RNAs. Using RT-qPCR, this was shown for A2577 in the human lncRNA MALAT1, which is known to contain m^{6}A.[12,21] The target RNA was enriched from total HeLa RNA and was treated with VMC10, and an unmodified *in vitro* transcribed fragment was treated under the same conditions. The qPCR data show that efficient cleavage of the unmodified transcript resulted in a 14-fold change in the amount of intact RNA. In contrast, the amount of amplifiable RNA remained constant upon treatment of the endogenous RNA with VMC10, consistent with high methylation of the target site (Figures S14, S15).

In summary, we have identified new RNA-cleaving deoxyribozymes that are sensitive to the presence or absence of the modified nucleotide m^{6}A, and can be used to examine the methylation status of DG(m^{6}A/A)CH motifs. The methyl group

can have a significant impact on the formation of a functional active site that was optimized for unmodified RNA (as in VMC10), likely because the methyl group introduces a steric clash or prevents the formation of stabilizing hydrogen bonds. Conversely, a deoxyribozyme that was developed to accommodate m^{6}A in its active site is not completely inactive in the absence of this small modification (as in VMA8 and VMA15). However, the kinetic differences are significant and can therefore be used to examine the modification state at selected target sites. Conceptually, the discrimination of methylated versus unmethylated RNA can be
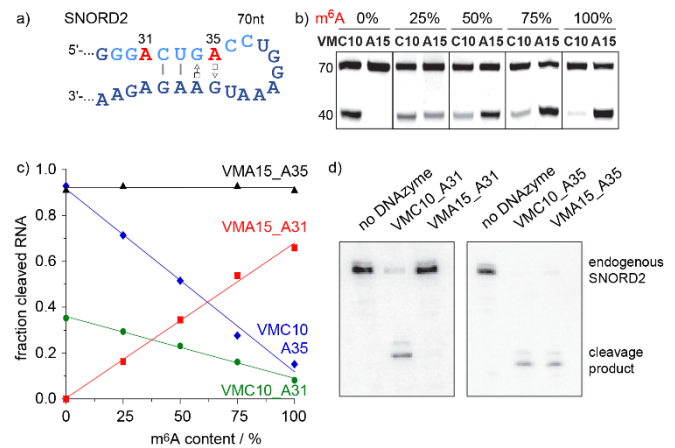

Figure 4. DNA-catalyzed cleavage of mouse SNORD2 RNA by VMC10 and VMA15. a) Excerpt of SNORD2 RNA sequence with proposed m^{6}A sites in red and overlapping DRACH motifs in light blue. b) PAGE analysis of cleavage of fluorescently labeled transcripts at A31, containing defined amounts of m^{6}A-modified RNA. c) Calibration curves for analysis of the methylation level in SNORD2 at m^{6}A31 and m^{6}A35. d) Analysis of endogenous SNORD2 RNA with VMC10 and VMA15 by northern blot. Full gel images including replicates and northern blots of control transcripts in Supplementary Figure S13.

compared to the *in vivo* recognition of m^{6}A-modified RNAs by proteins, e.g. those containing YTH domains, where the presence of m^{6}A enhances protein binding due to an energetically favorable interaction with aromatic amino acids in the protein binding site.[25] In contrast, m^{6}A is also known to repel RNA binding proteins that specifically bind unmodified RNA.[26] For the deoxyribozymes described in this work, it remains to be identified which interactions prevail in the active sites that enable a favorable organization of the catalytic DNA residues and/or magnesium ions to discriminate methylated from unmodified RNA. Given the recent success in structure determination of catalytic DNA,[27] the structural basis for the m^{6}A-sensitivity of RNA-cleaving deoxyribozymes can likely be revealed in the near future. These experiments are currently under way in our laboratories. Moreover, a similar *in vitro* selection approach can be used to develop DNA enzymes for analysis of other epitranscriptomic RNA modifications.

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Entry for the Table of Contents

Meet the methyl mark: Site-specific RNA-cleaving deoxyribozymes are reported that respond to the presence of N^6-methyladenosine close to the cleavage site in RNA with enhanced or strongly diminished cleavage rates, and thereby directly reveal the presence of the epitranscriptomic m6A modification in the target RNA.
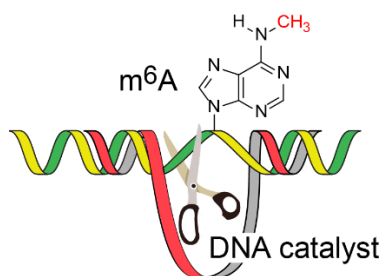
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