Laser-mediated, site-specific inactivation of RNA transcripts

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ABSTRACT The biological function of specific gene products often is determined experimentally by blocking their expression in an organism and observing the resulting phenotype. Chromophore-assisted laser inactivation using malachite green (MG)-tagged antibodies makes it possible to inactivate target proteins in a highly restricted manner, probing their temporal and spatially resolved functions. In this report, we describe the isolation and in vitro characterization of a MG-binding RNA motif that may enable the same high-resolution analysis of gene function specifically at the RNA level. A well-defined asymmetric internal bulge within an RNA duplex allows high affinity and high specificity binding by MG. Laser irradiation in the presence of low concentrations of MG induces destruction of the MG-binding RNA but not of coincubated control RNA. Laser-induced hydrolysis of the MG-binding RNA is restricted predominantly to a single nucleotide within the bulge. By appropriately incorporating this motif into a target gene, transcripts generated by the gene may be effectively tagged for laser-mediated destruction.

The classical method for determining the function of a given gene is to isolate mutant organisms in which its expression has been abolished and to characterize its corresponding phenotype. The specific biological role of certain essential genes and genes with pleiotropic effects often cannot be readily determined by this method because their inactivation leads to inviability or to an early block in development. Conditional alleles are often more informative than null mutations but they occur much less frequently and in many screens have been impossible to obtain. As an alternative to conventional genetic methods, Jay and Keshishian (1) have developed the process of chromophore-assisted laser inactivation (CALI) (1). This technique uses microinjected, non-neutralizing antibodies covalently modified with the chromophore malachite green (MG). These antibodies act effectively as a vector to deliver the chromophore to its specific intracellular target. MG has the unique property of generating short-lived, but highly destructive, hydroxyl radicals upon irradiation by a high intensity laser of the appropriate wavelength (630 nm) (2). When antibody-tethered chromophore is exposed to a laser pulse, nascent radicals inactivate macromolecules in its immediate vicinity, including the antibody and the antibody’s target. By simply controlling where and when a laser pulse is applied, an experimenter thus is able to limit inactivation of the gene product to a specific time in development or to a specific subcellular location. Application of this technique in a range of cell types and organisms has yielded detailed information about protein function that could not be obtained by conventional methods (1, 3–6).

Although a powerful general method, CALI has some inherent technical limitations. Non-neutralizing antibodies must be available for the target protein and these must be microinjected into each targeted cell. Furthermore, MG-tagged antibodies target the protein products of genes, making it impossible to exert gene inactivation (and study gene expression) at the RNA level. In an effort to overcome these limitations, we are working to implement an extension of this technique outlined in Fig. 1 that targets the RNA transcript of a gene for inactivation. Central to this scheme is the isolation of a MG-binding RNA motif. Such a motif serves the same function as antibodies in the conventional CALI approach in that it provides a mechanism for localizing the reactive chromophore to the target. By introducing this motif into the targeted gene at the DNA level, every corresponding transcript is effectively tagged with a MG receptor. In the presence of low concentrations of MG (introduced into an organism or individual cells by feeding or infusion), this receptor binds the chromophore and sensitizes its own transcript to laser inactivation.

In vitro selection from pools of random sequence molecules, also known as SELEX (systematic evolution of ligands by exponential enrichment), has been shown to be a powerful method for isolating nucleic acids with well-defined binding properties. Previous experiments have yielded RNA aptamers for such diverse ligands as ATP, theophylline, vitamin B12, biotin, and assorted proteins and peptides (7–11). In the current paper, we describe in vitro selection of a specific MG-binding RNA. Binding by this molecule is specified by a large asymmetric bulge within an RNA duplex. We demonstrate that this motif induces site-specific cleavage upon laser irradiation under conditions where control RNAs are unaffected.

EXPERIMENTAL PROCEDURES

Materials. MG and adipic acid dihydrazide agarose were purchased from Sigma. MG isothiocyanate was purchased from Molecular Probes. Phosphorimidates for solid-phase DNA synthesis were provided by Prime Synthesis (Aston, PA).

MG Agarose Synthesis. Three milligrams of MG isothiocyanate solubilized in 300 μl of dimethylformamide (DMF) was coupled to 10 ml of adipic acid dihydrazide agarose previously equilibrated with 0.1 M NaHCO3 (pH 8.3). The reaction was allowed to proceed in darkness at room temperature overnight. Unreacted chromophore was removed by extensive washing with DMF and water.

Random Pool Construction. The random pool consisted of 5 × 1015 RNA molecules, all with a general structure consisting of 42- and 20-nt constant primer binding regions at both 5’ and 3’ ends, flanking a 72-nt random region (corresponding to the sequence 5’-GGAAAACATATCGGACATGGCAACC-N122-CCT-TGTTGTAGGATCC-3’). RNA was prepared essentially as described but starting from a 2 × 105 μmol initial solid-phase DNA synthesis of the sequence 5’-CGGGATCTCTAAATGA-CCAAGG-N72-GGTGCGAGTCTAGTGTCTCC-3’.

Abbreviations: CALI, chromophore-assisted laser inactivation; MG, malachite green; RRE, Rev-responsive element; SRP, signal-recognition particle.†To whom reprint requests should be addressed. e-mail: wilson@biology.ucsc.edu.

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This template strand was amplified by PCR using primers with the sequence 5'-TTCTAATACGACTCACTATAGGAACACTATCCGACTGGCACC-3' and 5'-CGGGATCCTAATTACGACCTATAGGAACACTATCCGACTGGCACC-3' as primers.

Selection Procedure. MG affinity chromatography was carried out by using 600 µl of MG agarose. Binding and washing was performed with selection buffer (0.1 M KCl/5 mM MgCl2, 10 mM Na-Hepes, pH 7.4). 32P-body-labeled RNA in selection buffer was applied to a MG column previously treated and washed with RNase-free Escherichia coli tRNA (Boehringer-Mannheim). The column then was washed with 15 volumes of selection buffer. Specifically bound RNAs were eluted by washing with five column volumes of selection buffer containing 1 mM MG. RNA was ethanol-precipitated after the addition of carrier glycogen and then enzymatically amplified or frozen at −80°C for future use.

Enzymatic Amplification and Characterization of Selected Molecules. Specifically eluted RNA molecules were reverse-transcribed with avian myeloblastosis virus reverse transcriptase (Promega) under standard conditions as described by the manufacturer by using the 3' primer (5'-CGGGATCCTAATTACGACCTATAGGAACACTATCCGACTGGCACC-3') and the 5' primer (5'-TTCTAATACGACTCACTATAGGAACACTATCCGACTGGCACC-3') at a final concentration of 2.5 µM. PCR was performed with the resulting cDNA template and primers with the same 3' primer and the 5' primer (5'-TTCTAATACGACTCACTATAGGAACACTATCCGACTGGCACC-3') at a final concentration of 0.5 µM. Half of the PCR was used as a template for subsequent in vitro transcription with recombinant T7 RNA polymerase. 32P-α-UTP (New England Nuclear/DuPont) was included in the transcription reactions to yield body-labeled RNA (facilitating analysis of MG agrose binding in the ensuing round of selection). Full-length transcript was obtained by purification on an 8% denaturing polyacrylamide gel. The isolated gel band was crushed, soaked, and rocked overnight with 0.3 M NaCl. After removing gel bits, RNA was concentrated by ethanol precipitation, resuspended in Milli-Q (Millipore) water, and stored at −20°C for later use.

The sequence complexity of the final selected pool was determined as described (14). PCR products corresponding to the enriched MG aptamers were cloned into the pT7 Blue vector (Novagen) and sequenced by using the Sanger method.

5' fragments of the aptamers were prepared by transcription of restriction enzyme-digested DNA template. Other constructs, including all site-specific mutants, were prepared by chemical synthesis of the appropriate DNA template strand followed by the bottom half of the T7 RNA polymerase promoter. The resulting oligonucleotides were gel-purified, annealed with T7 promoter top strand, and transcribed with T7 RNA polymerase. Transcription reactions were routinely purified by denaturing gel electrophoresis. 5' end-labeled RNA was prepared by treatment with Boehringer Mannheim calf intestinal phosphatase, followed by kinasing with 32P-γ-ATP (New England Nuclear/DuPont) and T4 polynucleotide kinase (New England Biolabs).

CALLI. A DCM [4(dicyanomethylene)-2-methyl-6-(p-dimethylamino-onyryl)-4H-pyran] circulating dye laser was pumped by a pulsed neodymium yttrium-aluminum garnet laser (10 Hz) to generate a 620-nm laser beam. This beam was focused to a spot size 2 mm with an optical lens and directed vertically into microcentrifuge tubes containing 0.1–1 µM RNA. The energy of each pulse was approximately 15 mJ. An 80-nt fragment of the Rev-responsive element (RRE) RNA prepared by in vitro transcription served as control RNA for the CALLI experiments (15). 5' end-labeled MG aptamer and/or RRE RNA was preincubated in affinity selection buffer containing 0–1 µM MG. Samples then were subjected to laser pulses of defined duration. Laser-exposed samples immediately were mixed with 2× gel loading dye (8 M urea, 1 mM EDTA, pH 8, 0.05% bromophenol blue) and analyzed by 20% PAGE. Fragments were analyzed by PhosphorImager and quantified by using the Molecular Dynamics ImageQuant software.

RNase T1 Digestion. 5' end-labeled and gel-purified RRE RNA and MG binding RNA were resuspended in 1.25× T1 buffer (25 mM sodium acetate, pH 5.0/8.75 mM urea/1.25 mM EDTA) in the presence of 5 mg tRNA. After denaturation at 90°C for 5 min, RNAs were treated with increasing amounts of RNase T1 and incubated at 55°C for 15 min. Digestion products immediately were loaded and analyzed on a prewarmed denaturing PAGE gel.

FIG. 1. RNA-CALI. A MG aptamer is engineered into the noncoding region of a transcript at the DNA level. The aptamer binds a free-floating molecule of MG. Subsequent laser irradiation induces free radical production by MG. Because of its proximity to the aptamer, MG preferentially targets destruction of the tagged transcript. If the transcript is a translated mRNA, laser inactivation separates the 5' cap or the poly(A) tail (depending on whether the aptamer is located in the 5' or 3' untranslated region) from the ORF, making the message untranslatable and targeted for complete nucleolytic degradation. If the transcript is a structural component of an RNP, laser inactivation cleaves the RNP into separate domains (whose continued association depends on the structure of the RNP).

630 nm laser irradiation

MG aptamer

malachite green

AAAAAA

5'-cap removal (no longer a substrate for translation)

rapid processing, degradation

AAAAAAAA
RESULTS

In Vitro Selection. By using MG agarose affinity chromatography as a basis for enrichment, we selected and amplified MG-specific RNA aptamers from a pool containing approximately 5 x 10^15 different random sequence molecules. Fig. 2 shows the steady increase in the population of specific chromophore binders with every round of selection. The fraction of MG agarose-binding RNAs appeared to plateau after eight cycles. Restriction digestion analysis performed with selected four-base cutters showed that this final pool was dominated by ~30–40 major species. This number agrees well with that calculated on the basis of the starting pool size and the fraction of sequences surviving each round of selection, indicating that the pool was not artifically limited by a bottleneck early in the selection experiment. With an apparent frequency of MG aptamers in the random sequence pool of approximately one every 10^16 nt, it seems extremely unlikely that high affinity aptamers in the random sequence pool of approximately 5 x 10^15 different random sequence molecules. Fig. 2 expected, and as described below, appears to partially define a consensus, CGAAXG, occurs four times more frequently than expected, and as described below, appears to partially define the binding motif for at least one aptamer.

Characterization of the Major Clone. Given its high efficiency binding, we selected clone MG-4 for further characterization. Partial alkaline hydrolysis suggested that nucleotides 1–56 of the original 112-nt molecule were sufficient for binding (data not shown). This hypothesis was tested directly by transcribing PCR-generated template DNA previously digested with TaqI (a TaqI recognition site appears fortuitously at position 54 in the aptamer sequence). The resulting RNA fragment bound efficiently to MG agarose, indicating that the 5' half of the molecule contains the complete binding motif. Sequence analysis of the TaqI-fragment revealed a short region of complementarity between its 5' and 3' ends (nucleotides 3–11 and 40–48) and low energy structures generated by the Zuker MFOLD algorithm consistently predicted a short hairpin for nucleotides 15–31 (12). Given these observations, we hypothesized that an asymmetric internal bulge flanked by RNA duplex was responsible for MG binding (indicated by the boxed region in Fig. 3). Similar motifs have been found for aptamers that specifically recognize ATP and FMN although high-resolution NMR studies have indicated few similarities between their three-dimensional structures (7, 13).

To test the proposed model for the aptamer and to obtain related molecules with improved binding, we prepared a series of modified minimal sequences and tested their ability to bind MG. As shown in Fig. 3, substitutions to the aptamer sequence that replace nucleotides in the predicted helical stems but that maintain base pairing are tolerated and actually can improve binding efficiency. Conversely, all point mutations tested in the internal bulges eliminate activity. It should be noted that the large 3' bulge is made up in part by the purine-rich motif common to several MG-aptamer sequences. A single potential translational start codon (AUG) is present in the aptamer but may be eliminated by adding a G:C base pair at the end of the first helix (indicated in the Fig. 5, Inset). A specific clone was isolated, sequenced, and assayed for binding. %, percentage RNA retained and affinity eluted from MG agarose; #, number of independent isolates of each clone.

Fig. 2. In vitro selection for MG aptamers. (A) Reaction of the isothiocyanate derivative of MG with adipic acid dihydrazide agarose yields a resin for affinity chromatography. (B) Binding to MG agarose in each round of selection. (C) Individual clones in the final selected pool were isolated, sequenced, and assayed for binding. %, percentage RNA retained and affinity eluted from MG agarose; #, number of independent isolates of each clone.
introduce the aptamer into the 5′ untranslated region of a transcript without changing the translation start site (D.G., unpublished observations).

To be useful for in vivo studies, the aptamer must recognize MG with high affinity and must function when embedded within other transcripts. The binding constant for MG can be inferred by measuring RNA-induced changes in its visible absorption spectrum. Unbound MG has an absorption maximum at 616 nm. The addition of increasing amounts of MG aptamer shifts \( \lambda_{\text{max}} \) to 630 nm. Control, nonbinding RNAs such as the RRE have no effect on MG absorbance. The RNA concentration dependence of the shift in \( \lambda_{\text{max}} \) indicates an apparent \( K_D \) of 1 mM (Fig. 3C). This affinity for soluble MG closely matches that measured for agarose-immobilized ligand by an equilibrium matrix binding assay (data not shown).

The lack of specific sequence requirements for the aptamer’s helical arms suggests that its bulge nucleotides may be introduced into the helical regions of another RNA to confer it with specific MG binding. To test this possibility, we have inserted the aptamer bulge nucleotides into the long central helix of the dog signal-recognition particle (SRP) RNA (Fig. 4). An unpaired CGA trinucleotide separating the Alu and S domains of wild-type SRP RNA fortuitously matches one side of the MG aptamer bulge. Modifications to the opposing bulge sequence were introduced to create a functional aptamer (MG-SRP) and a nonfunctional mutant (MG-SRP*, differing by inversion of a central pair of nucleotides in the aptamer sequence). As predicted, MG-SRP induces a significant red-shift in the MG absorbance maximum, indicating specific binding. Wild-type SRP RNA (not shown) and the mutant MG-SRP* have no effect on MG absorption.

**In Vitro CALI.** To test the ability of the MG-binding motif to confer instability upon an RNA via CALI, 5′ end-labeled control RNA (the RRE from HIV-1) and/or MG aptamer were preincubated with different concentrations of MG and then irradiated with a laser for varying times. Fig. 5 shows that MG-binding RNA is specifically targeted for destruction whereas control RNAs are not. The addition of 10 mM sodium azide (a hydroxyl radical scavenger) decreases cleavage efficiency by 40%, suggesting that laser-mediated destruction is the result of radical generation. As shown in Fig. 5B, cleavage rises as function of exposure time but is relatively unaffected by increases in MG concentration above 1 mM. It is worth noting the absolute extent of cleavage in these experiments is

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**Fig. 3.** Construction and analysis of a minimal MG aptamer. (A) Predicted folding of clone MG-4. Digestion of DNA template with Taq-I (arrowhead) yields a 5′ RNA fragment that retains high efficiency binding. Boxed region corresponds to the minimal element responsible for MG binding. (B) A minimal aptamer based on the MG-4 clone sequence with modified flanking helices was prepared and shown to bind with high efficiency. The introduction of single point mutations into the internal bulge as indicated eliminate binding. Swapping the last base pair in helix 1 (boxed) eliminates the only potential start codon within the minimal aptamer and has no significant effect on binding. (C) Addition of the minimal aptamer RNA induces a shift in the UV absorption maximum of MG from 616 nm to 630 nm. The concentration dependence of the RNA-induced shift suggests a \( K_D \leq 1 \) mM.

**Fig. 4.** Incorporation of the MG aptamer into the SRP RNA. (Upper) Predicted secondary structure of 7SL RNA from *Canis familiaris*. Replacement of 6 nt in the wild-type RNA (bold) yields MG-SRP, carrying the MG aptamer bulge, and MG-SRP*, carrying a mutant (italics), nonfunctional MG aptamer. (Lower) Binding of MG-SRP and MG-SRP* to 0.6 \( \mu \text{M} \) MG was assessed by measuring the change in the MG absorbance maximum.
likely limited by our apparatus because the cross section of the focused laser beam is somewhat smaller than that of the drop containing the RNA sample.

Laser irradiation of the MG aptamer yields essentially a single cleavage product corresponding to hydrolysis between uridine-25 and adenosine-26 (indicated in the Fig. 5A, Inset). Mutation of either nucleotide flanking the cleavage site completely abolishes ligand binding (Fig. 3), consistent with the possibility that MG is bound directly by these nucleotides and that cleavage results from their proximity to the ligand. With longer laser exposures, cleavage was detected at the immediately adjacent 5' and 3' nucleotides but at no other specific sites within the aptamer. The high specificity of RNA cleavage contrasts strongly with that observed for protein CALI in vitro.

Several factors, including the laser setup used for these experiments and misfolding of the aptamer, likely limit this extent of cleavage. If necessary, incorporation of tandemly repeated MG aptamers within a single transcript should straightforwardly make it possible to cleave target transcripts with higher efficiency. Initial control experiments with live yeast cells (Saccharomyces cerevisiae), worms (Caenorhabditis elegans), and flies (Drosophila melanogaster) indicate that these organisms readily tolerate MG in their media at the concentrations required for MG binding and that the dye freely diffuses into their cells (D.G., unpublished work).

DISCUSSION

We have shown that an in vitro-selected MG aptamer can be used to direct CALI cleavage of an RNA. The aptamer binds its ligand tightly ($K_D \approx 1 \mu M$), and laser treatment induces highly site-specific RNA cleavage. We have demonstrated in vitro that approximately half of the transcripts can be cleaved.

The ability to manipulate transcript cleavage in a temporally and spatially specific manner as outlined above should facilitate the analysis of RNA function in a variety of different settings.

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