DNA Structures

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Controlling RNA Digestion by RNase H with a Light-Activated DNA Hairpin**

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Many gene silencing technologies have focused on targeting mRNA by using ribozymes, DNAzymes, and complementary RNA or DNA oligonucleotides. It is attractive that mRNA is accessible during transcription, processing (e.g., 5′-capping, intro-exon splicing, polyadenylation, nuclear export), and ribosomal binding and translation. Hybridization of an "antisense" oligodeoxynucleotide (asODN) to a target mRNA inhibits translation by sterically blocking the ribosome and/or recruiting endogenous ribonucleases. In mammalian cells, mRNA/asODN duplex formation activates RNase H-mediated hydrolysis of mRNA. Antisense ODNs have proven effective in gene silencing[1] in many experimental systems and are being evaluated as treatments for cancer and other diseases in human clinical trials. It was recently reported that the stability of DNA hairpins relative to their corresponding RNA/DNA hybrids influenced the extent of RNA degradation by RNase H.[2] Based on this principle, we designed a light-activated DNA hairpin to control this enzymatic reaction (Figure 1 A).

Photoinduced generation of nucleic acid strand breaks has been reported by several groups.[3] However, most studies have focused on mimicking genomic lesions, whereas our strategy is based on the photorelease of DNA for controlling biological processes. Light-activated methods for controlling the binding of asODNs to target mRNA would permit the reduction of protein expression in a cell or tissue-specific fashion and thereby limit systemic toxicity.[4] Recent efforts to increase the specific activity of asODNs have focused on improving delivery to cells and nuclease resistance.[5] Stability within cells can be enhanced considerably by modifying the backbone of the oligonucleotide, as exemplified by phosphorothioate,[6] peptide,[7] morpholine,[8] and "locked"[9] nucleic acids. However, there are few reports about regulating the activity of asODNs.[10,11]

In a pioneering example, Matsunaga et al. recently applied the UV-mediated *trans*→*cis* isomerization of azobenzene to control RNA digestion by RNase H.[10] By attaching five azobenzenes to a 20-mer sense DNA strand, the binding of a complementary asODN decreased upon UV irradiation: *trans*, $T_m = 60.8\,^{\circ}C$; *cis*, $T_m = 42.6\,^{\circ}C$. The less conformationally extended *cis* isomer sterically blocked DNA duplex formation. Based on this difference in melting temperature, $\Delta T_m = 18\,K$, it was possible to regulate the hybridization of the asODN to a complementary RNA target.

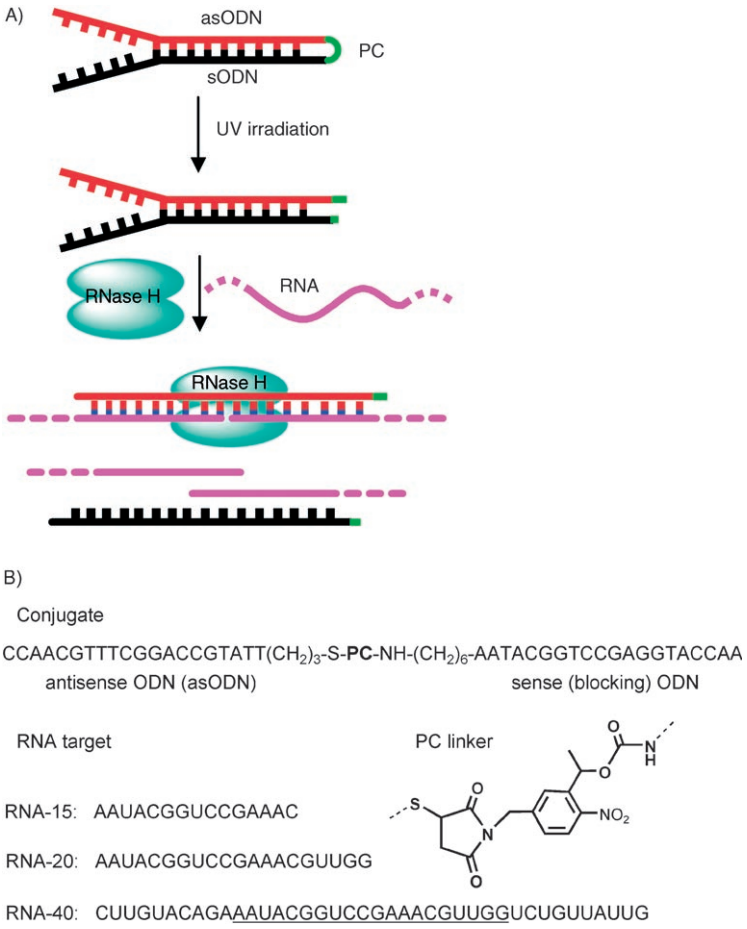

Figure 1. A) Strategy for regulating RNA digestion by RNase H by using a light-activated DNA hairpin. B) Sequences of the asODN-sODN conjugate and RNA targets; structure of the heterobifunctional photocleavable linker, PC. Underlined bases in RNA-40 are identical to RNA-20.

However, the photoefficiency was greatly reduced by the requirement for five azobenzenes. For DNA modified with n moieties, each with a photoquantum efficiency of 50%, the irradiation time required to generate the fully active molecule should scale as 2^n. In fact, prolonged UV irradiation leads to side reactions that lower the yield of the active molecule and cause cytotoxicity. For cell experiments, prolonged irradiation reduces temporal as well as spatial resolution due to diffusion. Moreover, the use of a reversible, photoisomerizable blocking group is problematic as mRNA is an abundant and persistent target.

In related studies, DNA hybridization was recently photomodulated between 14% (blocked) and 80% (activated) by using an average of 14–16 nitrophenyl adducts per 20-mer asODN.[11] Further precedent exists for the control of

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DNA and RNA activity with light.[12] However, the use of multiple photoactive groups in most cases has limited the utility of these methods for biological investigations. Our strategy for photoregulating DNA involves the incorporation of a single, maximally effective blocking group.

We synthesized a light-activated DNA hairpin by covalently attaching a 20-mer asODN to an ODN with 12 complementary bases through a heterobifunctional photocleavable linker (PC, Figure 1). This 1-(5-(*N*-maleimidomethyl)-2-nitro-phenyl)ethanol *N*-hydroxysuccinimide ester was designed to react with thiol and amine functionalities on opposite ends of the linker (Figure 1 B). PC was synthesized in four steps in a 14% yield. Its dual reactivity made it even more versatile for bioconjugation reactions than the 1-(5-(aminomethyl)-2-nitrophenyl)ethanol linker used previously.[13,14] Covalent attachments of the thiolated asODN to the maleimido group and the amino-ODN to the *N*-hydroxysuccinimide ester occurred in good yields. Purification of the conjugated DNA hairpin was achieved by ion exchange chromatography at 40 °C. This step was particularly important because any unreacted asODN would readily form a duplex with the target RNA and thus promote RNase H activity.

The DNA hairpin exhibited a high melting temperature, $T_m = 80$ °C. Upon UV illumination, the carbamate linkage was broken and the asODN/ODN duplex became much less stable, $T_m = 51$ °C. Photocleavage was confirmed by HPLC and MALDI-TOF mass spectrometry. This large difference in thermal stability, $\Delta T_m = 29$ K, allowed the photomodulation of RNA/asODN duplex formation, thereby regulating RNA hydrolysis by RNase H (Figure 1 A).

RNase H activity was studied with RNA oligomers of varied length, RNA-15 (15-mer), RNA-20 (20-mer), and RNA-40 (40-mer). Digestion of each RNA target was compared between solutions containing the control single-stranded asODN (CCAACGTTTCGGACCGTATT) or the DNA hairpin. Figure 2 shows hydrolysis of 45% of the total

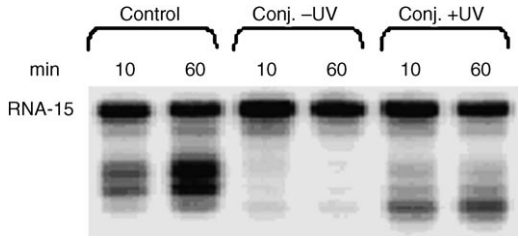

Figure 2. Denaturing PAGE (20%) analysis of RNA-15 digestion by RNase H at 37 °C. Lanes 1–2, asODN; Lanes 3–4, conjugate (conj.−UV); Lanes 5–6, UV-activated conjugate (conj.+UV).

RNA-15 target after photoactivation as compared with only 4.6% for the conjugate in a 1 h experiment with 1 unit of enzyme. Under the same conditions, 66% RNA degradation for the control single-stranded asODN was observed. The UV light used in these experiments had no effect on the enzyme or RNA stability. Control experiments confirmed that RNase H hydrolyzed RNA only when hybridized with asODN.

RNase H assays were also performed with RNA-20 and RNA-40. After photolysis of the DNA hairpin, RNA-20 and RNA-40 digestion increased by a factor of 1.3 ± 0.2 and 4.4 ± 0.4, respectively, versus a 9.0 ± 1.0-fold increase for RNA-15 (Figure 3). Three units of fresh RNase H gave nearly com-

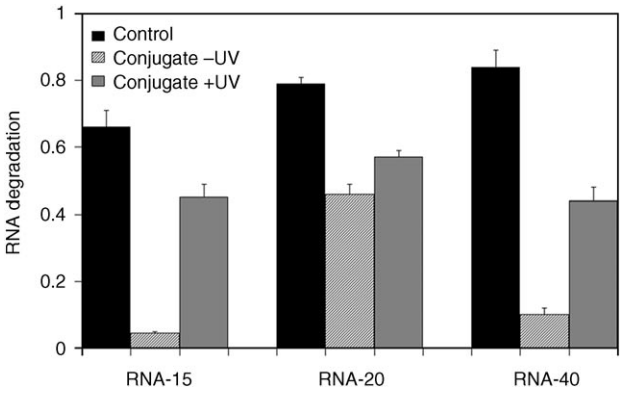

Figure 3. RNase H assays showing degradation of RNA-15, RNA-20, and RNA-40 target sequences in 60 min at 37 °C with 1 unit of RNase H. Error bars signify the variation from two separate trials. The control lanes show hydrolysis of RNA hybridized to asODN.

plete digestion of RNA-15 (98%), RNA-20 (88%), and RNA-40 (84%) in 1 h in the presence of the control asODN or photolyzed conjugate. This compared favorably with the consumption of only 49% of RNA-15, 70% of RNA-20, and 27% of RNA-40 by using the DNA hairpin (see the Supporting Information). RNA-40 is more representative of cellular mRNA, which has secondary structure that can limit nonspecific DNA hybridization and RNase H digestion. Within a cell, where each mRNA molecule can template the synthesis of several thousand proteins per hour,[15] the ability to photomodulate target mRNA between 73% and 16% of basal levels, as was shown for RNA-40, would produce a large effect.

The observed photomodulation efficiency was controlled predominantly by the relative thermodynamic stabilities of the conjugate, pre- and postphotolysis, and the RNA/asODN duplexes. The melting temperatures for duplexes of asODN with RNA-15 and RNA-20 were 63 and 70 °C, respectively. For these two RNA targets, the increases in RNase H activity upon photoactivation arose from the greater stability of the DNA hairpin ($\Delta T_m = 17$, 10 °C) compared with the RNA/asODN duplexes. Free-energy calculations gave ΔG values for asDNA/sDNA, asDNA/RNA-15, and asDNA/RNA-20 at 37 °C in RNase H buffer of −12.4, −14.5, and −16.3 kcal mol^{-1}, respectively.[16] For the conjugate, $\Delta G = -17.0$ kcal mol^{-1} was calculated by determining the changes in enthalpy and entropy upon duplex formation, ΔH_d and ΔS_d, respectively, from the concentration dependence of the melting equilibria by using the van't Hoff expression (see the Supporting Information). Binding RNA-15 or RNA-20 to the DNA hairpin was disfavored thermodynamically, $\Delta\Delta G = 2.5$ and 0.7 kcal mol^{-1}, respectively. The decrease in $\Delta\Delta G$ with increasing RNA oligomer length helps to explain the higher background levels of RNA-20 digestion, before photolysis.

RNase H has been shown to promote the formation and cleavage of RNA/DNA duplexes, even under conditions where RNA/DNA hybridization is thermodynamically disfavored.[17] After photolysis, the activated asODN/sODN hybrid readily converted to asODN/RNA-15 and asODN/RNA-20 ($\Delta\Delta G = -2.1$ and -3.9 kcal mol^{-1}, respectively).

In addition to thermodynamic considerations, the higher background level of RNase H activity for RNA-20 was owing to the nature of the blocking group. The 12 complementary bases on the sense ODN were designed to block the binding and degradation of RNA targets with the same 12-base recognition motif. However, the eight mismatched bases of the asODN in the DNA hairpin were able to base pair with RNA-20 and recruit RNase H. Although RNA-20 should be one of the most difficult sequences for this DNA hairpin to photodiscriminate against, the photomodulation efficiency (1.3 ± 0.2) was slightly greater than background levels.

The DNA hairpin exhibited the highest photomodulation efficiency towards the RNA-15 substrate. Contributing factors were the stability of the DNA hairpin relative to the asODN/RNA-15 duplex ($\Delta\Delta G = -2.5$ kcal mol^{-1}), proper complementarity of the blocking strand (slightly shorter than the target), and the structure of the RNA itself, which limited nonspecific DNA hybridization. The RNA melting temperatures of RNA-15, RNA-20, and RNA-40 were 51 °C, 54 °C, and 63 °C, respectively. The expected correlation between increasing RNA strand length and structure was interesting in view of the higher photomodulation efficiency for RNA-40 than RNA-20. These targets had the same 20 bases complementary to asODN and yet RNA-40 exhibited much lower background levels of hydrolysis. Evidently, for targets such as RNA-40, it is important that the asODN has a high degree of complementarity to compete with the stable RNA stem-loop structure. To test this hypothesis, a RNA 40-mer was employed with only 15 complementary bases (CUUGUACAGAAAUACGGUCCGAAACCAACCUCUGUUAUUG, underlined bases are identical to RNA-15). No RNase H activity was observed towards this substrate by using the photolyzed DNA hairpin, and only 13 % digestion was seen with the control asODN. The results with the four RNA substrates provide guidelines for designing photoactive DNA hairpins against a specific RNA target.

Notably, in the earlier azobenzene example, a small ΔT_m of 18 K required a 10-fold excess of sense ODN relative to asODN to limit RNA/asODN duplex formation.[10] This strategy is not practical for most biological applications as the sense ODN can diffuse away or become degraded inside the cell. Furthermore, introducing an organic chromophore in the middle of an ODN typically lowers the DNA duplex melting temperature only by a few degrees Celsius per blocking group.[10,11,14] We demonstrated herein that a much larger ΔT_m can be achieved by conjugating the sense ODN to the asODN through a single photocleavable linker. The PC linker plays a role in stabilizing the conjugate.

In conclusion, a DNA hairpin was designed that efficiently regulates DNA/DNA and DNA/RNA duplex formation by using a single photoactive group. The stability of the DNA hairpin, proper complementarity of the blocking group, and RNA structure were important factors affecting RNA hydrolysis by RNase H. This represents the most efficient method for photomodulating this enzyme activity. Oligonucleotide conjugates can be designed to photoregulate many other processes for biotechnological and cellular applications.

Experimental Section

The standard procedure for RNase H assay: The DNA conjugate was annealed in the 1 × ribonuclease H reaction buffer (Tris-HCl (20 mM), KCl (20 mM), $MgCl_2$ (10 mM), ethylenediaminetetraacetic acid (EDTA; 0.1 mM), dithiothreitol (DTT; 0.1 mM); pH 8.0) by heating to 95 °C, then slowly cooling to 70 °C. The temperature was held at 70 °C for 10 min to melt nonspecific DNA structures, before further slow cooling to 37 °C. [γ-^{32}P]-labeled RNA oligonucleotide was added and incubated at 37 °C for 20 min to allow RNA/DNA duplex formation. RNase H was added to the mixture and incubated at 37 °C. The total reaction volume was 10 μL and the final concentrations of conjugate or control (asODN), and RNA (RNA-15, RNA-20, or RNA-40) were 0.01 μM and 2 μM, respectively. Time points were taken at 10 and 60 min by sampling 4 μL of the reaction mixture, adding 6 μL loading buffer (EDTA (50 mM), formamide (90 %)), and heating to 95 °C for 3 min to terminate the reaction. Finally, 5 μL of the resulting solution was subject to electrophoresis on a polyacrylamide gel containing 7 M urea. RNA imaging was performed by using a Storm phosphorimager and quantified with IMAGEQUANT software (Amersham Biosciences). To measure RNA degradation after photoactivation, the annealed DNA hairpin was illuminated (Xe lamp with monochromator, 355 nm, 36 mW cm^{-2}, 10 min) and [γ-^{32}P]-labeled RNA oligonucleotide was then added and incubated for 20 min. RNA degradation by RNase H was determined as described above. See the Supporting Information for further experimental details.

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