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A New Approach for Reversible RNA Photocrosslinking Reaction: Application to Sequence-Specific RNA Selection

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microRNAs (miRNAs) are small (19–25 nucleotides) non-coding RNAs that regulate gene expression through mRNA degradation or translation inhibition.[1] miRNAs play key roles in regulatory pathways including development, apoptosis, cell proliferation and differentiation, organ development, and cancer.[2] RNA interference (RNAi) and antisense methodology have high potential for studying gene functions by controlling gene expression.[3] In particular, the crosslinking reaction can serve as a potential blocker of transcription and translation by covalent bond formation between DNA and RNA.[4] Biotechnological approaches based on the RNA crosslinking reaction have also been used in investigating RNA structures[5] and constructing RNA architecture.[6] The crosslinking reaction between transplatin and RNA has been used as a tool to investigate RNA structures and to modulate gene expression.[7] However, in this method, transplatin was irreversibly crosslinked to target RNA. Development of a rapid and reversible RNA photocrosslinking method is essential for modulating gene expression and manipulating RNA molecules. We have been studying artificial DNA bases as a tool for the photochemical DNA crosslinking method.[8] In our recent study, we reported that a modified oligodeoxynucleotide (ODN) that contains 3-cyanovinylcarbazole nucleoside (^{CNV}K) can be photochemically crosslinked with an adjacent pyrimidine base in a [2+2]-cycloaddition reaction by irradiating at 366 nm.[9] However, the RNA photocrosslinking method by using a modified ODN containing artificial DNA bases has not yet been investigated. Herein, we report a new approach for reversible RNA photocrosslinking reaction via an artificial DNA base such as ^{CNV}K in hybrid duplex DNA/RNA. We demonstrate that selection[10] of the target RNA sequence was performed by using the reversible photocrosslinking reaction through the modified ODN containing ^{CNV}K.

The phosphoramidite of ^{CNV}K was prepared according to a reported method.[9] The various modified ODNs, ODN(X^{CNV}K; X=A, G, C, or T), were prepared according to standard phosphoramidite chemistry on a DNA synthesizer by using the phosphoramidite of ^{CNV}K. ODNs containing ^{CNV}K were characterized by MALDI-TOF-MS. We determined the feasibility of the interstrand RNA photocrosslinking reaction via ODN containing ^{CNV}K as shown in Figure 1. When ODN(A^{CNV}K) (5′-d(TGCA^{CNV}KTCGT)-3′) and RNA(GU) (5′-r(ACGAGUGCA)-3′) were irradiated at

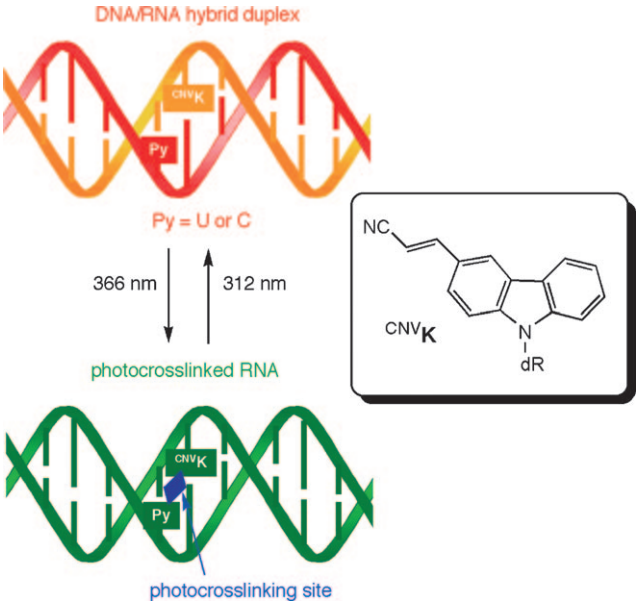

Figure 1. Schematic illustration of the reversible RNA photocrosslinking reaction.

366 nm for 1 s, the HPLC showed a peak relating to ODN(A^{CNV}K)–RNA(GU) at a 94% yield along with the disappearance of the ODN(A^{CNV}K) and RNA(GU) peaks (Figure 2). MALDI-TOF-MS indicates that the isolated ODN(A^{CNV}K)–RNA(GU) obtained from HPLC purification was a photocrosslinked product of ODN(A^{CNV}K) and RNA(GU) (*m/z* calcd: 5675.74 for $[M+H]^+$; found: 5675.25). The enzymatic digestion of isolated ODN(A^{CNV}K)–RNA(GU) showed the formation of rCyd, dCyd, rGuo, dGuo, dThd, rAdo, and dAdo in a ratio of 2:2:3:2:3:3:1, togeth-

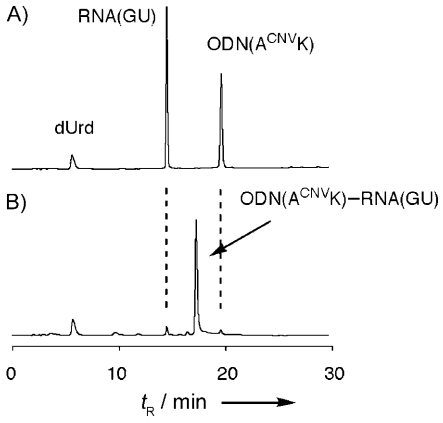

Figure 2. HPLC analysis of the irradiated ODN(A^{CNV}K) in the presence of RNA(GU): A) before irradiation; B) irradiated at 366 nm for 1 s, 94% yield. 2′-Deoxyuridine (dUrd) was used as an internal standard.

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er with the ^{CNV}K–rUrd photoadduct, which was confirmed by MALDI-TOF-MS (*m/z* calcd: 601.1911 $[M+Na]^+$; found: 601.1944). On the other hand, when ODN(G^{CNV}K) (5′-d(TGCG^{CNV}KTCGT)-3′) and RNA(GC) (5′-r(ACGAGCGCA)-3′) were used in the photocrosslinking reaction, the rCyd base reacted with photoexcited ^{CNV}K to produce a photocrosslinked product ODN(G^{CNV}K)–RNA(GC) efficiently.[11] Compared to the target DNA, the photocrosslinking rate between the rUrd base and ^{CNV}K on the target RNA such as RNA(GU) was almost equal to the corresponding target DNA, but the photocrosslinking rate between the rCyd base and ^{CNV}K on the target RNA such as RNA(GC) was threefold slower than the corresponding target DNA (see the Supporting Information). The photocrosslinking reaction between ODN(A^{CNV}K) and RNA(GU) was completed by 366 nm irradiation for only 1 s. This RNA photocrosslinking reaction proceeded efficiently compared with the traditional method by using the crosslinker such as transplatin and psoralen.[13]

To examine the effect of sequence contexts around the photocrosslinking site, we constructed 16 sequences of RNAs, RNA(ZY) (5′-r(ACGAZYGCA)-3′, Y, Z = A, G, C, or U), in the opposite strand. The photocrosslinking yield that was obtained by photoirradiation at 366 nm for 20 s was determined by HPLC and UPLC analysis. Among the various combinations of base pairs between ^{CNV}K and natural bases Z (Z = A, G, C, or U), we observed that ODNs containing ^{CNV}K produced a photocrosslinked product efficiently in each base pair (Table 1). Importantly, rAdo and rGuo bases on the photocrosslinking site are inactive toward a photocrosslinking reaction with ODNs containing ^{CNV}K (see the Supporting Information). Therefore, we can determine the difference between the pyrimidine and purine bases of the target RNAs by using a photocrosslinking reaction.

Table 1. Photocrosslinking yield obtained by photoirradiation at 366 nm for 20 s by using ODNs containing ^{CNV}K, RNA(ZU) and RNA(ZC). Z = A, G, C, or U.

	Z			
	A	G	C	U
ODN(A^{CNV}K)	93	94	90	91
ODN(G^{CNV}K)	92	75	81	76[a]

[a] This photocrosslinking reaction was performed by photoirradiation at 366 nm for 60 s at RT.

To examine the influence of the photocrosslinking reaction on thermal stability, the melting temperature (T_m) of the hybrid duplex ODN(A^{CNV}K)/RNA(GU) or ODN(A^{CNV}K)–RNA(GU) was determined by UV-monitored thermal denaturation. The hybrid duplex ODN(A^{CNV}K)/RNA(GU) showed a melting temperature of 21.9 °C, whereas ODN(A^{CNV}K)–RNA(GU) melted at 53.5 °C (Table 2). An example of this behavior has been seen for photocrosslinked ODNs by the ODN containing *p*-carbamoylvinyl phenol nucleoside.[8] The hybrid duplex ODN(G^{CNV}K)/RNA(GC) showed a melting temperature of 33.4 °C, whereas ODN(G^{CNV}K)–RNA(GC) melted at 63.1 °C. Thus, the photocrosslinking reaction increased the T_m of ODN, and resulted in a dramatic stabilization of the hybrid duplex.

Table 2. T_m values (°C) for hybrid duplexes.[a]

	T_m/°C[b]		T_m/°C[b]
ODN(A^{CNV}K)/RNA(GU)	21.9 ± 0.2	ODN(A^{CNV}K)–RNA(GU)	53.5 ± 1.3
ODN(AC)/RNA(GU)	47.1 ± 0.1	ODN(G^{CNV}K)/RNA(GC)	33.4 ± 0.2
ODN(G^{CNV}K)–RNA(GC)	63.1 ± 0.8	ODN(GC)/RNA(GC)	52.1 ± 0.1

[a] All T_m values of the hybrid duplexes (3.0 μM) were measured in 50 mM cacodylate buffer (pH 7.0) and 100 mM NaCl. ODN(AC) = 5′-d(TGCACTCGT)-3′, ODN(GC) = 5′-d(TGCGCTCGT)-3′. [b] Each experiment was repeated at least three times.

To confirm the photoreversibility of the photocrosslinked product, irradiation of the photocrosslinked ODN(A^{CNV}K)–RNA(GU) at 312 nm was examined. Rapid disappearance of ODN(A^{CNV}K)–RNA(GU) was observed with irradiation for 60 s at 312 nm, whereas the reverse photoreaction produced only ODN(A^{CNV}K) and RNA(GU) without any byproducts (Figure 3).

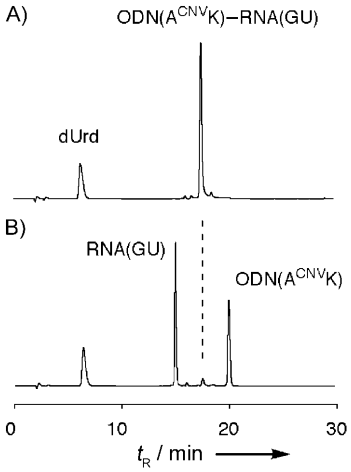

Figure 3. HPLC analysis of the photosplitting reaction of the ODN(A^{CNV}K)–RNA(GU): A) before irradiation; B) irradiated at 312 nm for 60 s, 92% yield. 2′-Deoxyuridine (dUrd) was used as an internal standard.

When the photocrosslinked ODN(G^{CNV}K)–RNA(GC) was used in the reverse photoreaction, rapid disappearance of ODN(G^{CNV}K)–RNA(GC) was observed with irradiation at 312 nm for 3 min, to give ODN(G^{CNV}K) and RNA(GC). Therefore, we succeeded in the reverse reaction by irradiation at 312 nm, without damaging the normal RNA. To the best of our knowledge, this is the first approach for the reversible RNA photocrosslinking reaction by using an artificial DNA base.

We examined molecular modeling studies of the hybrid duplex between ODN(A^{CNV}K) and RNA(GU). The vinyl group of ^{CNV}K is stacked on the C5–C6 double bond of the rUrd base of RNA(GU) (Supporting Information). The molecular weight of ODN(A^{CNV}K)–RNA(GU) was equal to the sum of the molecular weights of ODN(A^{CNV}K) and RNA(GU). As judged from the molecular modeling, the photoreversibility, and UV spectrum of the ^{CNV}K–rUrd photoadduct, there is a strong suggestion that

the photocrosslinking reaction proceeded by [2+2] cycloaddition between the double bond of ^{CNV}K and the C5–C6 double bond of the rUrd base to give a cyclobutane structure.[14]

We determined the feasibility of RNA selection by using the reversible RNA photocrosslinking reaction through an ODN that contained ^{CNV}K (Figure 4). Four RNA sequences (RNA(10), RNA(15), RNA(20) and RNA(25)) were irradiated at 366 nm at

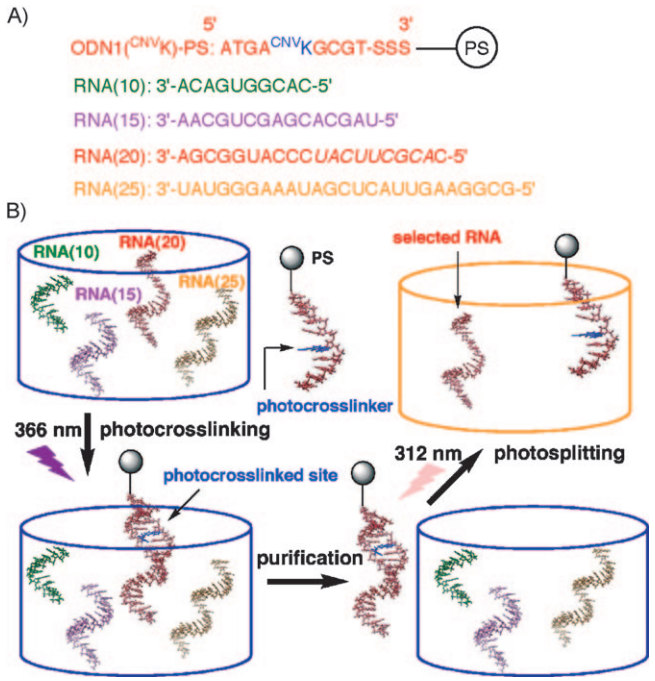

Figure 4. Sequence and progress of the reversible interstrand RNA photocrosslinking reaction. A) Capture and target RNA sequences used in this study. S corresponds to a hexa(ethylene glycol) linker fragment. PS corresponds to a polystyrene/poly(ethylene glycol) copolymer. Italic characters indicate a matched sequence with a capture strand. B) Strategy for selection of a target RNA sequence.

0 °C in the presence of the capture strand (ODN1(^{CNV}K)-PS) for 30 min in 50 mM sodium cacodylate buffer (pH 7.0) and 1 M sodium chloride. After the capture strand was washed with deionized water, a solution of CH_3CN/H_2O (1:1) containing urea (2 M) was added to the capture strand, and the capture strand was irradiated at 312 nm at 25 °C for 15 min. As shown in Figure 5, capillary gel electrophoresis (CGE) analysis showed disappearance of the target RNA(20) after irradiation at 366 nm, and showed only the target RNA(20) sequence with the completely complementary case after the operation of RNA selection. We also conducted a traditional RNA selection by using only hybridization selectivity for the same target RNA to verify the potential selectivity of our reversible RNA photocrosslinking reaction for RNA selection. In the RNA selection by the traditional method, CGE analysis showed four RNA sequences after the operation of RNA selection (see the Supporting Information). Therefore, this photocrosslinking reaction is shown to proceed with high sequence specificity. Finally, to demonstrate the sequence-specific selection of miRNAs, we conducted a set of three closely related target miRNAs (hsa-

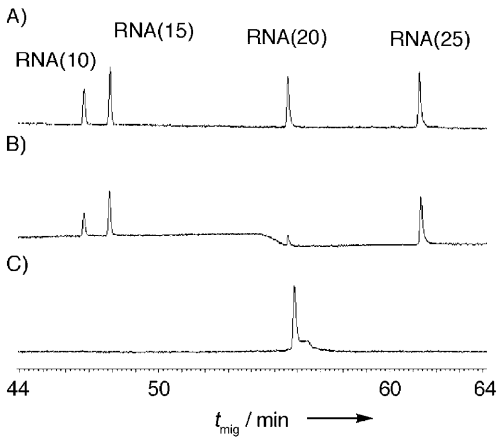

Figure 5. CGE analysis for each operation: A) before RNA selection; B) the washed solution after irradiation at 366 nm; C) after RNA selection.

let-7a, hsa-let-7f, hsa-let-7g) with a single variable base (G, A, U), along with 7-mer capture strand.[15] Three miRNA sequences were irradiated at 366 nm at 37 °C in the presence of the capture strand (ODN2(^{CNV}K)-PS) for 30 min in 50 mM sodium cacodylate buffer (pH 7.0) and 1 M sodium chloride. After the capture strand was washed with deionized water, a solution of CH_3CN/H_2O (1:1) containing urea (2 M) was added to the capture strand, and the capture strand was irradiated at 312 nm at 25 °C for 15 min. As shown in Figure 6, CGE analysis showed disappearance of the target hsa-let-7a after irradiation at 366 nm, and showed only the target hsa-let-7a with the com-

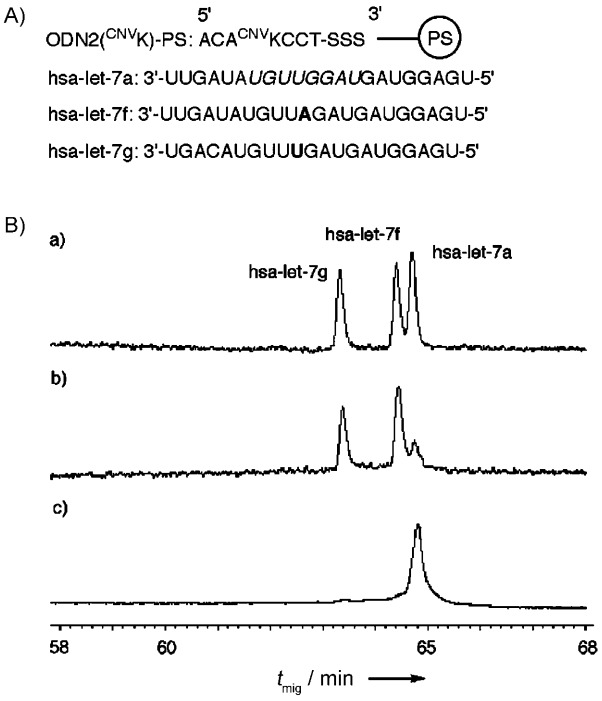

Figure 6. A) Capture and miRNA sequences used in this study. Italic characters indicate a matched sequence with a capture strand. Bold characters indicate a single mismatch. B) CGE analysis for each operation: a) before RNA selection; b) the washed solution after irradiation at 366 nm; c) after RNA selection.

pletely complementary case after the operation of RNA selection. Therefore, this photocrosslinking reaction showed a high degree of single nucleotide specificity.

In conclusion, we have demonstrated that a modified ODN containing ^{CNV}K can be crosslinked by irradiation at 366 nm with an adjacent RNA pyrimidine base in a [2+2] manner. The photocrosslinked ODN–RNA reverts to the original RNA after irradiation at 312 nm. The photocrosslinking reaction of ODN containing ^{CNV}K is ultrafast (with an irradiation time of only 1 s) and clean. Furthermore, this photocrosslinking reaction can be widely used for the selection of target RNA sequences. RNA is a labile material, but RNA materials were stabilized by this photocrosslinking method, and a reliable RNA manipulation method can be developed. The reactivity, selectivity, and reversibility of the novel photocrosslinking reaction will be beneficial to the inhibition of transcription, the pinpoint fluorescent labeling of target RNA, and the quantification of a multitude of noncoding RNAs.

Experimental Section

Photocrosslinking of RNAs as monitored by HPLC: The reaction mixture (total volume 30 μL) containing ODN(A^{CNV}K) and RNA(GU) (each 20 μM, strand concentration) in sodium cacodylate buffer (50 mM, pH 7.0) and NaCl (100 mM) was irradiated with a UV-LED (366±15 nm light at 1600 mW cm^{-2}) at a distance of 1.5 cm at 0 °C for 1 s. After irradiation, the progress of the photoreaction was monitored by HPLC. The yield was calculated based on RNA(GU). The quantum yield of the formation of photocrosslinked product was measured at 366 nm, based on the disappearance of RNA(GU) by employing valerophenone as an actinometer. The formation of ODN(A^{CNV}K)–RNA(GU): $\Phi = 0.25$.

Photosplitting of photocrosslinked RNAs as monitored by HPLC: A solution (total volume 60 μL) containing ODN(A^{CNV}K)–RNA (GU) (20 μM, strand concentration) in CH_3CN/H_2O (1:1) containing urea (2 M) was irradiated with a 15 W transilluminator (312 nm) at 25 °C for 60 s. After irradiation, the progress of the photoreaction was monitored by HPLC. The yield was calculated based on ODN-(A^{CNV}K)–RNA(GU).

RNA selection as monitored by capillary gel electrophoresis: The experiments were conducted on a Beckman P/ACE System MDQ (Beckman Coulter, Fullerton, CA) equipped with a UV absorbance detector. Separations were performed at an applied voltage of 20 kV and at a temperature of 30 °C. RNAs were detected by monitoring their absorbance at 254 nm.

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Keywords: DNA · gene technology · nucleosides · photochemical reaction · RNA

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