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Description	

Reversible Photopadlocking on Double-Stranded DNA

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We describe a highly efficient method for reversible photocircularization of oligonucleotide (ODN) on a double-stranded DNA template. 5-Carboxyvinyl-2'-deoxyuridine-containing ODN was reversibly circularized around the target sequence of the double-stranded plasmid DNA resulting in formation of a catenated plasmid.

As more of the sequence data for the human genome becomes available, new approaches for drug discovery for the treatment and diagnostics of human diseases caused by genetic mutations are being developed. Previous studies have demonstrated that the expression of specific genes at the level of transcription and translation can be controlled based on chemical approaches.[1] Designed modified oligonucleotides (ODNs) including triple helix-forming ODN[2] and synthetic molecules such as polyamides[3] and PNA[4] have been shown to bind sequence-specifically to double-stranded DNA owing to their ability to hybridize with their complementary sequences and to be useful in genetic regulations because the resulting triple helix structures prevent DNA-binding proteins from recognizing the target sequences and initiating the transcriptional process. Recently, circular ODNs as binding agents have attracted considerable interest because of their unique binding properties and high-affinity recognition of target sequences.[5] Circular ODN probes are also promising reagents for genetic analysis by forming a circle onto the target.[6] Padlock probes can act as inhibitors to block the DNA-protein interaction required for the transcriptional initiation at sites where padlock probes bind to the plasmid DNA site-specifically. They also discriminate gene sequences with single base mutations due to the high sequence specificity of the padlock[7] and serve as templates for rolling circle replication.[8]

Padlock probes catenated with the target sequences of the single-stranded form of the circular cloning vector M13mp18 have been synthesized by both enzymatic and photochemical methods.[6,9] However, these methods are not suitable for a circular double-stranded DNA target because enzymatic reactions are undesirable under conditions where a triple helix is formed and because the photochemical method is not efficient. Escudé *et al*. reported that a linear 89-mer ODN was circularized around a double-stranded DNA in the presence of a 20-mer template via a triple helix formation by using T4 DNA ligase.[10] The use of a photoreaction instead of an enzymic reaction is more desirable for synthesizing circular ODN around double-stranded DNA. Furthermore, the reversibility of the circularization[11] is suitable for controlling gene expression by a photochemical switch. We previously investigated an efficient and reversible template-directed photoligation in a *cis-syn* [2+2] manner via a vinyl group of modified ODN without using chemical reagents.[12] We report here the synthesis of circular ODN onto a double-stranded template and the formation of padlock probes onto plasmid DNAs by reversible photoligation via 5-carboxyvinyl-2'-deoxyuridine (^{CV}U) (Fig. 1). We also demonstrate that padlock probes inhibit the access of a restriction enzyme for recognizing the target sequences.

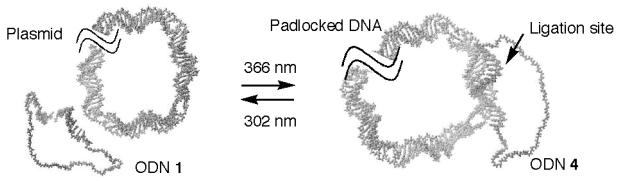

Fig. 1 Schematic representation of reversible photopadlocking.

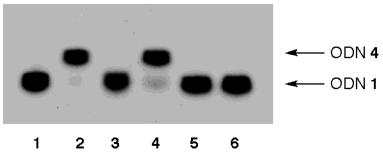

Fig. 2 Autoradiogram of a denaturing 20% polyacrylamide gel electrophoresis of photoreaction of ^{32}P-5'-end-labeled ODN **1** (2 μM) and ODN **2** and ODN **3** (10 μM) in a solution of NaCl (100 mM) and $MgCl_2$ (20 mM). Lane 1, before reaction; Lane 2, irradiation at 366 nm, 1 h (sodium acetate 50 mM, pH 5.0); Lane 3, irradiation at 302 nm, 1 h of Lane 2; Lane 4, irradiation at 366 nm, 1 h (sodium cacodylate 50 mM, pH 6.0); Lane 5, irradiation at 366 nm, 1 h (sodium cacodylate 50 mM, pH 7.0); Lane 6, irradiation at 366 nm, 1 h (Tris-HCl 50 mM, pH 8.0).

We synthesized ODN **1** (5'-d(^{CV}UTTCCCCTCTT)-S_{10}-d(CCTCTTC)-3'; here S corresponds to a hexa(ethylene glycol) linker fragment) containing ^{CV}U at its 5'-end and a triplex-forming sequence with a target duplex formed by ODN **2** (5'-d(GAATTCGGAGAAGAAAGGGGAGAATTC)-3') and ODN **3** (5'-d(GAATTCTCCCCTTTCTTCTCCGAATTC)-3') by standard automated DNA synthesis using PEG building blocks and examined an intrastranded circularization of ODN **1** on the duplex by photoirradiation. When 5'-end-labeled ODN **1** was irradiated at 366 nm for 1 hour at pH 5.0 in the presence of ODN **2** and ODN **3**, ODN **1** disappeared quantitatively and a slowly migrating band was observed on a denaturing 20% polyacrylamide gel (Fig. 2, Lane 2). The ratio of the migration of the slower band relative to that of the

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original 34 mer ODN **1** (Lane 1) was *ca.* 0.9. This result indicates that the slower band in Lane 2 was a photocircularized product of ODN **1**.[12] This circular ODN **4** was converted reversibly to the original linear ODN **1** by photoirradiation at 302 nm for 1 hour (Lane 3). Although the circularization efficiency at pH 6.0 was similar to that observed at pH 5.0, no circular product was observed under the conditions under which the triple helix was not formed (Lane 5 and 6). The reactivity dependency on pH shown in Fig. 2 suggests that this photocircularization proceeded through a triple helix formation.

To examine the sensitivity of the photocircularization reaction to mismatches, photoreaction was carried out with various duplexes that contained mismatches for ODN **1** at the ligation site as well as at other sites (Fig. 3, and 4). In the case of one or two mismatches at the ligation site, a dramatic decrease of the yield was observed (< 2% yield, Lane 3, 4, and 5). When duplex **8** and duplex **9** which were matched at the ligation site and had several mismatches at other sites were used, circular ODN **4** was also not formed (Lane 6, and 7). These results clearly showed that the photocircularization was highly sensitive to mismatched sequences.

duplex **5**	5'-GTA CTG AAT TCG GAG AA**T** - AAA GGG GAG AAT TCT ACT C-3' 3'-CAT GAC TTA AGC CTC TT**A** - TTT CCC CTC TTA AGA TGA G-5'
duplex **6**	5'-GTA CTG AAT TCG GAG AAG - **T**AA GGG GAG AAT TCT ACT C-3' 3'-CAT GAC TTA AGC CTC TTC - **A**TT CCC CTC TTA AGA TGA G-5'
duplex **7**	5'-GTA CTG AAT TCG GAG AA**T** - **T**AA GGG GAG AAT TCT ACT C-3' 3'-CAT GAC TTA AGC CTC TT**A** - - TT CCC CTC TTA AGA TGA G-5'
duplex **8**	5'-GTA CTG AAT TCG GAG **CC**G - AAA GGG GAG AAT TCT ACT C-5' 3'-CAT GAC TTA AGC CTC **GG**C - TTT CCC CTC TTA AGA TGA G-3'
duplex **9**	5'-GTA CTG AAT TCG **TC**G **CC**G - AAA GGG GAG AAT TCT ACT C-5' 3'-CAT GAC TTA AGC **AG**C **GG**C - TTT CCC CTC TTA AGA TGA G-3'

Fig. 3 ODNs used in this study. Bold bases indicate the mismatched bases.

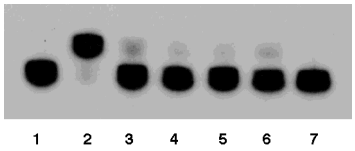

Fig. 4 Autoradiogram of a denaturing 20% polyacrylamide gel electrophoresis of photoreaction of ^{32}P-5'-end-labeled ODN **1** (2 μM) and ODN **2** (10 μM) and ODN **3** (10 μM) in sodium acetate buffer (50 mM, pH 5.0) containing NaCl (100 mM) and $MgCl_2$ (20 mM). Photoirradiation was conducted in a quartz capillary cell with transilluminator at 366 nm for 1 h at 0 °C. Lane 1, before reaction; Lane 2, ODN **1** + ODN **2** + ODN **3**; Lane 3, ODN **1** + duplex **5**; Lane 4, ODN **1** + duplex **6**; Lane 5, ODN **1** + duplex **7**; Lane 6, ODN **1** + duplex **8**; Lane 7, ODN **1** + duplex **9**.

To investigate further the possibility of the formation of padlocked DNA on a circular double-stranded DNA target by photocircularization of ODN **1**, a duplex consisting of ODN **2** and ODN **3** was treated with a restriction enzyme EcoRI and the resulting fragment was incorporated into pUC 18 plasmid DNA by T4 DNA ligase. Modified plasmids **10** and **11** for photopadlocking experiments were constructed that contained the target sequences one and five times, respectively (Fig. 5).[13] When ODN **1** and the modified plasmid **10** containing the target sequence at one site were photoirradiated at 366 nm at pH 5.0 for 3 h, the band failed to migrate on the gel and padlock probes catenated to the plasmid **10** target sequences was obtained in 47% yield (Fig. 6a, Lane 2). This padlocked DNA **12** was quantitatively converted to linear ODN **1** by photoirradiation at 302 nm (Lane 3). The padlock probes were not detected in the use of pUC 18 which did not contain the target sequences (Lane 4).

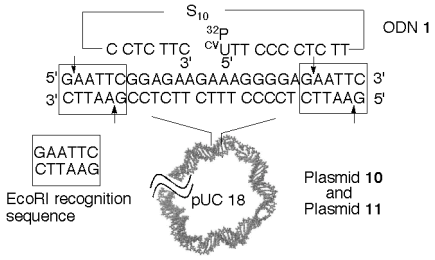

Fig. 5 Description of the plasmids and ODN **1** used in padlocking experiments. Modified plasmids were constructed by insertion of duplex consisting of ODN **2** and ODN **3** after treatment with EcoRI.

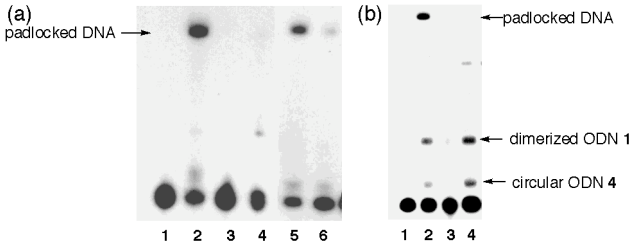

Fig. 6 Autoradiogram of a denaturing 12% polyacrylamide gel electrophoresis of photoreaction of ^{32}P-5'-end-labeled ODN **1** (2 μM) and plasmid **10** (0.125 μg/μL) or plasmid **11** (0.125 μg/μL) in sodium acetate buffer (50 mM, pH 5.0) containing NaCl (100 mM) and $MgCl_2$ (70 mM). (a) The formation of padlocked DNA on plasmid **10**. Lane 1, before reaction; Lane 2, ODN **1** + plasmid **10**, irradiation at 366 nm, 3 h; Lane 3, irradiation at 302 nm, 1 h of Lane 2; Lane 4, ODN **1** + unmodified pUC 18, irradiation at 366 nm, 3 h; Lane 5, EcoRI treatment (37 °C, 1 h) of Lane 2; Lane 6, HindIII treatment (37 °C, 1 h) of Lane 2. (b) The formation of padlocked DNA on plasmid **11**. Lane 1, before reaction; Lane 2, ODN **1** + plasmid **11**, irradiation at 366 nm, 3 h; Lane 3, irradiation at 302 nm, 1 h of Lane 2; Lane 4, EcoRI treatment (37 °C, 1 h) of Lane 2.

This result demonstrates that the photocircularization proceeded when ODN **1** specifically recognized the target sequences via a triple helix formation to form circular ODN **4**, which was catenated to plasmid **10**. When the reaction mixture after photoirradiation at 366 nm for 1 h was treated with EcoRI under appropriate conditions, the plasmid was not cleaved because the circular ODN **4** bounded to the recognition sequence of EcoRI prevented the access of the restriction enzyme at the target site (Lane 5). When the mixture was treated with restriction enzyme HindIII, which recognizes *ca.* 50 bases upstream from the EcoRI recognition site, the plasmid was then cleaved without inhibition by the padlock probes (Lane 6). With the use of modified plasmid **11** containing the five repeated target sequences, padlocked DNA was obtained in 20% yield (Fig. 6b, Lane 2). In this case, the plasmid was cleaved by EcoRI treatment because the enzyme recognized the sites where circular ODN **4** did not bind (Lane 4). Based on these results, it was concluded that the padlock probes formed by photocircularization actually inhibit the activity of the restriction enzyme. Reversible photopadlocking as shown above may find applications for controlling DNA-protein interactions.

In this study, we demonstrated that ^{CV}U is very useful for reversible photocircularization on double-stranded DNA with high efficiency. We also showed that reversible photopadlocking on the target DNA duplex via photocircularization was realized to block the access of the restriction enzyme.

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