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Fluorescence Property of Oxazole Yellow-linked Oligonucleotide. Triple Helix Formation and Photocleavage of Double-stranded DNA in the Presence of Spermine

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Abstract—Oxazole yellow is an intercalator that shows enhanced fluorescence upon binding to DNA. We prepared an oxazole yellow-linked oligonucleotide that can form triple helix with interleukin 2 receptor α chain promoter. The oxazole yellow-linked oligonucleotide showed linear increase of fluorescence by the triple helix formation with double-stranded DNA and also induced photocleavage of the targeted DNA in the presence of spermine upon visible illumination. Cleavage site of one strand was 7 or 8 bases away from the site of intercalation whereas the other strand was cleaved at the intercalated site.

Introduction

Autoradiography is commonly used for the detection of nucleic acids in clinical diagnosis as well as for the analysis of biochemical processes that involve nucleic acids. However, there still remain some inconveniences in autoradiography such as the undesirable use of hazardous isotopes and the need of separation steps that make the procedure troublesome. Improved safety and workability in the procedure have therefore been desired and, in fact, exploited by many research groups including Hélène and co-workers who reported the fluorescence detection of double-stranded DNA based on the energy transfer between donor- and acceptor-linked DNA probes.[1]

In general, it is known that some intercalators bind DNA to induce fluorescence enhancement. We were particularly interested in oxazole yellow (YO) that shows marked enhancement of the fluorescence upon binding to a double-stranded DNA.[2–5] We reported the usefulness of YO in the fluorescence monitoring and quantification of polymerase chain reaction.[6] Furthermore, considering that an oligonucleotide equipped with a YO would emit enhanced fluorescence on binding to a complementary strand, we prepared a duplex-forming YO-linked oligonucleotide probe and demonstrated the applicability to real-time monitoring of the production of a single-stranded messenger RNA by the in vitro transcription process of hepatitis C virus RNA[7] (Scheme 1).

Since DNA mostly exists as double strand in living system, it would be more useful if we could manipulate double-stranded DNA with sequence specificity. Thus, we attempted to extend our chemistry to double helical DNA and examined the triple helix-forming property of our designed YO-linked oligonucleotides (Scheme 2).

It was necessary to examine also the DNA-cleaving property of our YO-linked oligonucleotide in addition to the fluorescence property, because Åkerman and Tuite reported that YO and its homodimer cleave DNA upon visible illumination.[8] We describe herein detailed study on the fluorescent property and DNA-photocleaving activity of YO-linked oligonucleotide.

We selected interleukin 2 receptor α chain promoter sequence that contains a NFκB recognition sequence as our target[9] because of our recent interest in the signal transduction chemical biology.[10–13] Thus, we employed the following oligonucleotides and thiol-modified oligonucleotides for our study.

Key words: Oxazole yellow; fluorescence; DNA photocleavafge; triple helix; oligonucleotide.
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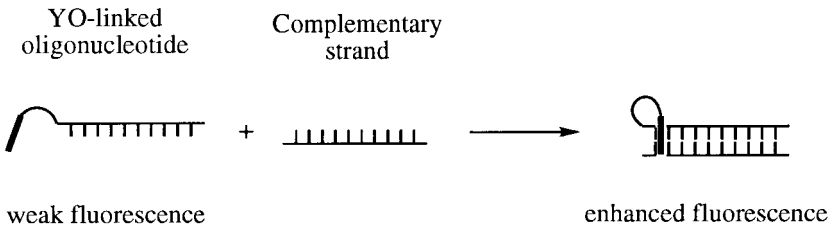
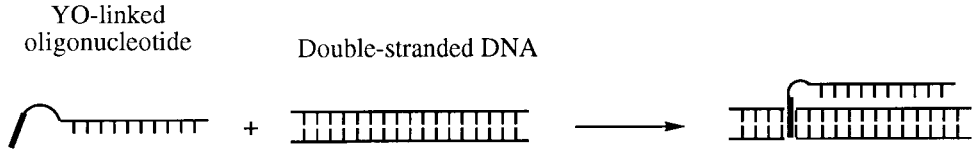


Scheme 1.



Scheme 2.

DS-1: 5′-TTTTCCTCTCCCTCT-3′
TH-1: 5′-GATCGGCA**GGGGAATCTC**CCTCTCCTTTTATGGGC-3′
TH-2: 5′-TCGAGCCCATAAAAGGAGAGG**GAGATTCCCC**TGCC-3′
TH-3: 5′-CGATCGTCTCCCTCTCCTTTTACCTAAGGGAAA-GAGGAAAGGCCTAG-3′
TH-4: 5′-CTAGGCCTTTCCTCTTTCCCTTAGGTAAAAGGA-GAGGGAGACGATCG-3′
PU-1: 5′-HS$(CH_2)_6$-OPO_3-AGAGGGAGAGGAAAA-3′
PY-1: 5′-HS$(CH_2)_6$-OPO_3-TTTTC*C*TC*TC*C*C*TC*T-3′
YO-PU-1: 5′-YO-$(CH_2)_3$-S$(CH_2)_6$-OPO_3-AGAGGGAGAGGAAAA-3′
YO-PY-1: 5′-YO-$(CH_2)_3$-S$(CH_2)_6$-OPO_3-TTTTC*C*TC*TC*C*C*TC*T-3′
[C* = 5-methylcytosine]

The κB site is shown by boldface and the triple helix site is underlined. DS-1 is complementary to PU-1 and YO-PU-1. TH-1 and TH-2 are complementary to each other and the resulting double-stranded DNA has a triple helix site matching with PY-1 or YO-PY-1. TH-3 and TH-4 also form a longer duplex that can bind PY-1 or YO-PY-1.

Materials and Methods

Preparation of oligonucleotides

Oligonucleotides DS-1, TH-1, TH-2, TH-3, and TH-4 were prepared on a DNA synthesizer. Thiol-modified oligonucleotides PU-1 and PY-1 were synthesized on a DNA synthesizer using C6-ThiolModifier (CLONTECH) according to the standard protocol including the procedure for the removal of the triryl group on the incorporated C6-ThiolModifier unit.

Preparation of YO-PU-1 and YO-PY-1

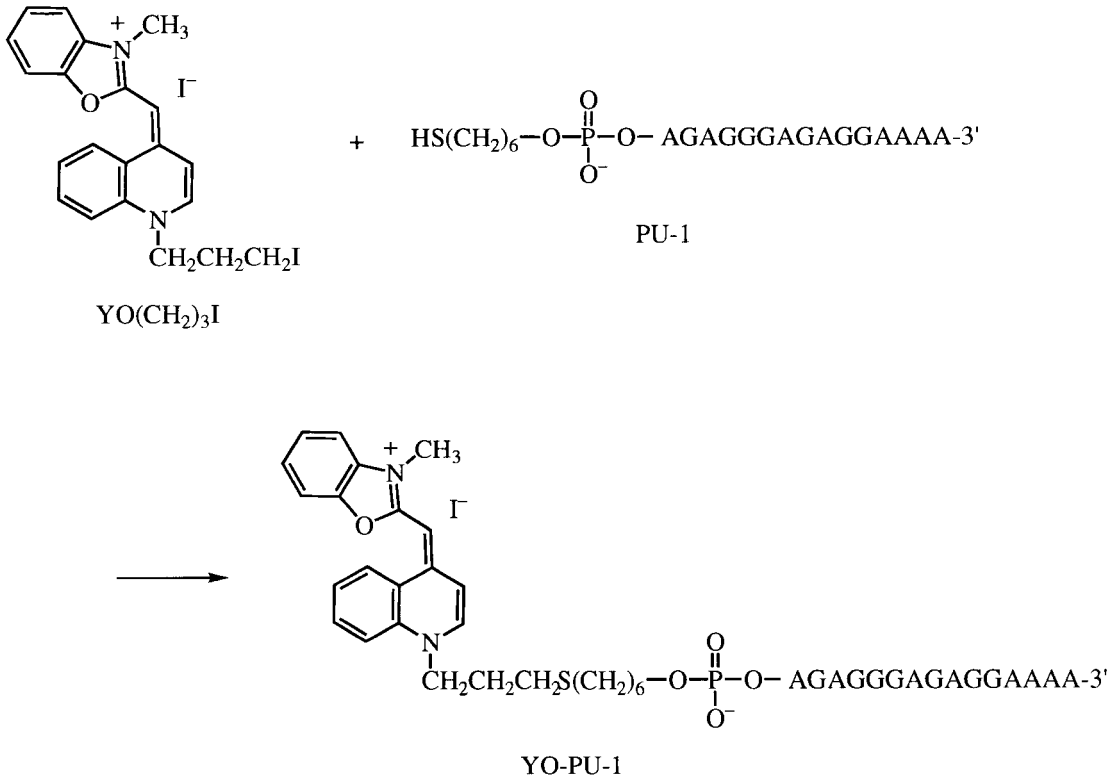
PU-1 prepared as above was subjected to HPLC purification according to the standard protocol (ULTRON VX-Nucleotide column, Shinwa Chemical Industries, Ltd.; eluted with a linear gradient of eluent A [5% acetonitrile in 0.1 M TEAA buffer] and eluent B [50% acetonitrile in 0.1 M TEAA buffer]; 100% eluent A to 100% eluent B in 30 min) to give a fraction containing purified PU-1. Aqueous dithiothreitol (10 μM, 20 μL) was added to the voltexed HPLC fraction containing PU-1 (solution A). $YO(CH_2)_3I$[7] was saturated in a mixture of DMF (200 μL), 1.0 M phosphate buffer (pH 10.0, 300 μL), and H_2O (500 μL) (solution B). Solutions A and B were mixed under argon (solution A: solution B = 3:1). The resulting mixture stood for 2 h and purified by Sephadex G-25 (eluted with 5% acetonitrile in 0.1 M TEAA buffer, pH 7.0). The purified fraction was concentrated, and the resultant was dissolved in distilled water and further purified by HPLC according to the above standard protocol. The fraction obtained was concentrated to dryness under reduced pressure to afford YO-PU-1 (Scheme 3). The concentration of YO-linked oligonucleotide was determined by measuring the absorption at 260 nm. YO-PY-1 was synthesized according to the same procedure starting with PY-1.

Hybridization and fluorescence measurement

DS-1 was added into a hybridization buffer (20 mM Tris HCl, pH 7.5, total volume 50 μL) containing YO-PU-1 (30 pmol). The mixture was annealed by heating up to 90 °C followed by cooling to room temperature. To the mixture was added 500 μL of the same buffer. The fluorescence spectrum of the solution was measured. The excitation wavelength was 490 nm and the emission spectrum was obtained at 509 nm.

Triple helix formation and fluorescence measurement

Varied amounts of TH-1 and TH-2 were annealed in the above hybridization buffer by heating up to 90 °C and cooling to room temperature. The annealed double helix (named TH-1,2) and YO-PY-1 (20 pmol) were added into a buffer (total volume 100 μL) containing 25 mM Tris acetate (pH 5.1), 50 mM NaCl, 20 mM $MgCl_2$ and no or 0.5 mM spermine. The mixture was incubated at 20 °C for 30 min. To the mixture was added 500 μL of the same buffer and the fluorescence of the mixture was measured.



Scheme 3.

Determination of the melting temperature of triple helix

TH-1,2 (500 pmol) and YO-PY-1 (500 pmol) were mixed in a buffer consisting of 25 mM Tris acetate (pH 5.1), 50 mM NaCl, 20 mM $MgCl_2$ and 0.5 mM spermine (total volume 1 mL). Melting curve was obtained by substracting the absorbance (measured at 260 nm) of the duplex alone from the absorbance of the three-strand mixture at each temperature. The T_m was determined as the temperature of half-dissociation of YO-PY-1 from TH-1,2.

DNA photocleavage by YO-linked oligonucleotide

TH-3 or TH-4 was 5′-end-labeled using T4 polynucleotide kinase and [γ-^{32}P]-ATP. The labeled strand and the corresponding complementary non-labeled single-stranded DNA were annealed by heating up to 90 °C and cooling to room temperature. Reaction samples contained 4 μM oligonucleotide (including ~100 K cpm of labeled oligonucleotide), 50 mM Tris HCl (pH 5.8), 50 mM NaCl, 20 mM $MgCl_2$, 0.5 mM spermine and 10 eq or 100 eq of YO-PY-1. Samples were incubated at 20 °C for 30 min and illuminated by a visible light[14] at 20 °C for 1 h. After illumination, the reacted DNA was precipitated with ethanol and dried in vacuo. The samples containing 20 K cpm of labeled oligonucleotide were loaded onto a 12% denaturing polyacrylamide gel and electrophoresed at 2000 V for 3.5 h. The gel was then transferred to a film and stored at −80 °C during autoradiography.

Results and Discussion

Fluorescence property of YO-linked oligonucleotide

Previously we reported that YO-$(dA)_{13}$ is virtually non-fluorescent, indicating no intramolecular interaction between the YO moiety and the $(dA)_{13}$ part presumably owing to the absence of internal folding in the $(dA)_{13}$.[7] Herein the fluorescence intensity of YO-PU-1 was measured at various concentrations in Tris HCl (pH 7.5, 20 mM) to examine the inter- versus intra-molecular interactions of the YO and the PU-1 moieties. As shown in Figure 1, the fluorescence intensity increased accordingly as the amount of YO-linked oligonucleotide

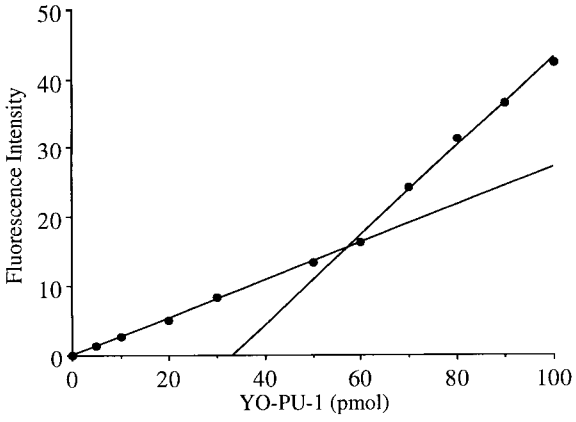


Figure 1. Concentration dependence of the fluorescence intensity of YO-PU-1.

increases and the mode of the increase biphasically changed at the concentration of 60 pmol. This suggests that the fluorescence enhancement below 60 pmol concentration is due to the intramolecular interaction of the PU-1 and the linked YO moiety, whereas the intermolecular interaction between the YO moiety and the PU-1 is predominant at the concentration above 60 pmol. Accordingly, the use of 30 pmol concentration of YO-linked oligonucleotide seemed suitably to avoid the intermolecular interaction in the hybridization and the triple helix formation.

Fluorescence enhancement of YO-linked oligonucleotide by duplex formation

We investigated whether the designed YO-linked oligonucleotide enhances the fluorescence by duplex formation with the complementary strand. As shown in Figure 2, YO-PU-1 showed evident fluorescence enhancement by hybridization with the complementary DS-1. The fluorescence linearly increased in proportion to the amount of DS-1 and the increase stopped when the ratio of DS-1 to YO-PU-1 reached unity showing the maximum fluorescence intensity. YO-PU-1 indicated no enhancement of fluorescence in the presence of non-complementary TH-2 (data not shown), demonstrating that YO-PU-1 recognizes the complementary strand DS-1 to form duplex that shows the enhanced fluorescence.

Fluorescence enhancement of YO-linked oligonucleotide by triple helix formation

We then extended our attention to the fluorescence change of YO-linked oligonucleotide accompanying the triple helix formation. Since triple helical DNA is known to be stabilized by spermine,[15] the influence of spermine on the fluorescence of the YO-linked triple helix was first investigated. Thus, the fluorescence intensity of YO-PY-1 was measured with increasing concentrations of double helical TH-1,2 in the presence of 0.5 mM spermine. As shown in Figure 3(A), the fluorescence linearly increased in proportion to the amount of TH-1,2. The fluorescence reached a plateau when the concentration of TH-1,2 became equal to that of YO-PY-1. In contrast, in the absence of spermine, the increase of the fluorescence was not linear and did not show plateau even after the concentration of TH-1,2 exceeded that of YO-PY-1 as shown in Figure 3(B). Thus, spermine facilitated the linear increase of fluorescence by the addition of the YO-linked third strand. YO-PY-1 exhibited no fluorescence enhancement when a duplex poly A-poly T was used (data not shown).

We also investigated the melting temperature of the triple helix of YO-PY-1 and TH-1,2. The T_m of the triple helix YO-PY-1-TH-1,2 was 36 °C and was higher than that of the corresponding unmodified triple helix PY-1–TH-1,2 that was 32 °C.[1] The observed higher T_m of the triple helix of YO-PY-1 and TH-1,2 is presumably due to the stabilizing effect of the intercalated YO moiety.

Thus, YO-PY-1 recognized the double-stranded DNA to form a stable triple helix in the presence of spermine and the recognition could be detected by measuring the fluorescence enhancement. These results suggest that the fluorescence enhancement depends on the ratio of triple helix to unbound YO-PY-1.

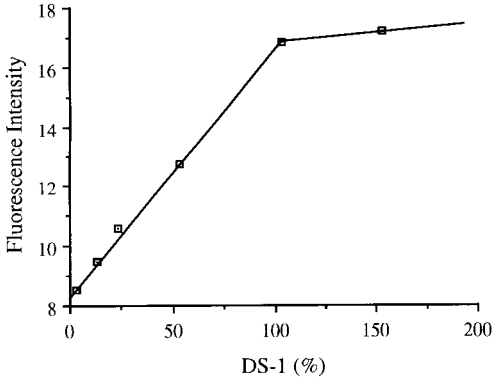


Figure 2. Fluorescence intensity of YO-PU-1 enhanced by the duplex formation with the complementary strand DS-1.

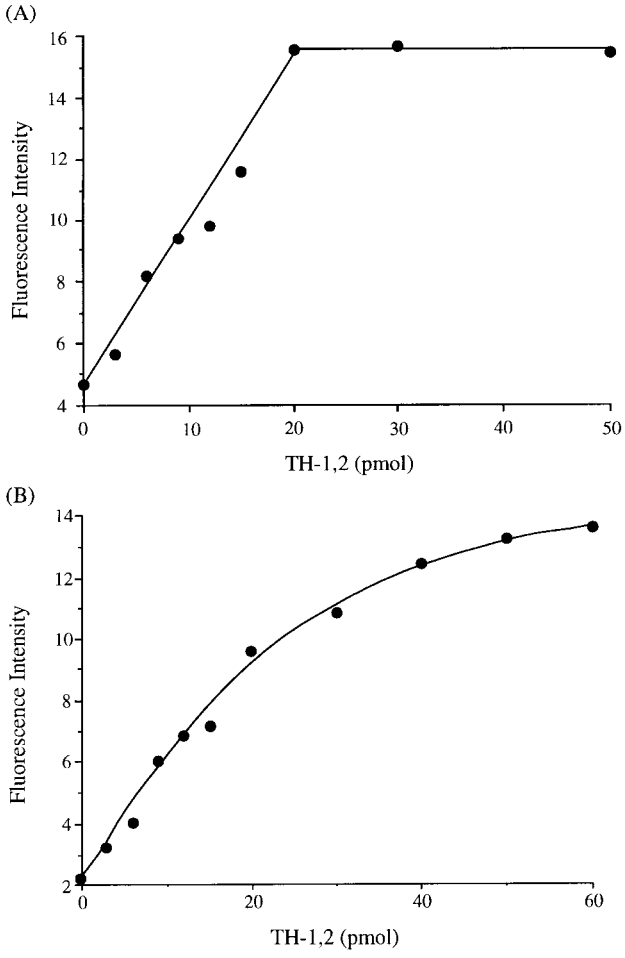


Figure 3. Fluorescence intensity of YO-PY-1. (A) Linearly enhanced by triple helix formation with double-stranded TH-1,2 in the presence of spermine, and (B) non-linearly enhanced with TH-1,2 in the absence of spermine.

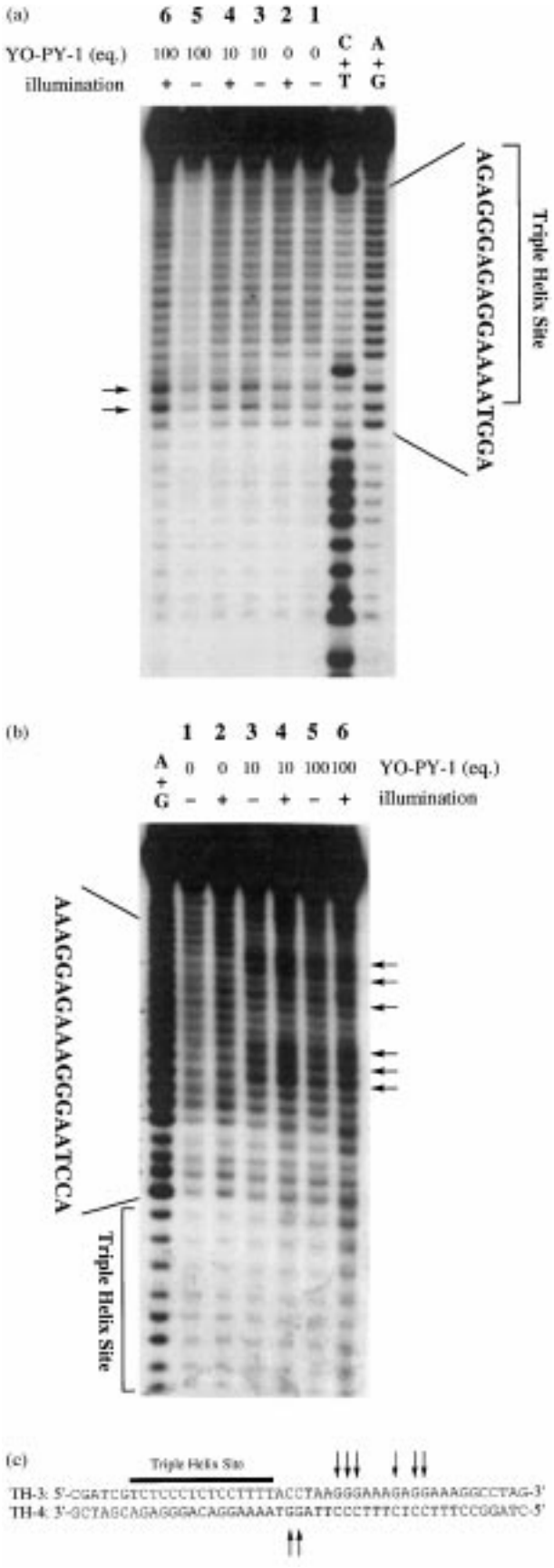


Figure 4. Photocleavage of double-stranded TH-3,4 with YO-PY-1. (a) Cleavage of TH-4-labeled duplex; (b) cleavage of TH-3-labeled duplex; (c) histogram showing the cleaved sites.

DNA photocleavage by YO-linked oligonucleotide

YO-PY-1 evidently enhanced the cleavage of double-stranded DNA of TH-3 and TH-4 in the presence of spermine upon visible illumination as shown in Figure 4. Figure 4(a) shows the result of TH-4-labeled duplex. Evident photocleavage was observed when 100 eq of YO-PY-1 was used (lane 6) and the cleavage was not significant without illumination (lane 5). The cleavage sites were guanine bases near to the site of triplex formation. The cleavage mode of TH-3-labeled duplex was different from that of TH-4-labeled duplex as shown in Figure 4(b), i.e. the cleavage sites were 5′-side guanine sites located at 7 or 8 base away from the presumed site of intercalation. Further similar cleavage was again observed at the 5′-side of the guanine cleavage site. The remote cleavage site of TH-3 strand may be explicable by assuming the energy transfer whereas TH-4 strand was cleaved at the intercalated site presumably by the generation of singlet oxygen.[16] Addition of spermine was required for detectable photocleavage (data not shown). In our previous fluorescence detection protocol using YO or YO-linked oligonucleotide, the possibility of photocleavage is ruled out because spermine was not used in those cases.[6,7] Further application of YO-linked oligonucleotide is currently under investigation.

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