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Template-directed photoligation of oligodeoxyribonucleotides via 4-thiothymidine

Jianquan Liu and John-Stephen Taylor*

Department of Chemistry, Washington University in St Louis, St Louis, MO 63130, USA

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ABSTRACT

Non-enzymatic, template-directed ligation of oligonucleotides in aqueous solution has been of great interest because of its potential synthetic and biomedical utility and implications for the origin of life. Though there are many methods for template-directed chemical ligation of oligonucleotides, there are only three reported photochemical methods. In the first report, template-directed photoligation was effected by cyclobutane dimer formation between the 5′- and 3′-terminal thymidines of two oligonucleotides with >290 nm light, which also damages DNA itself. To make the photochemistry of native DNA more selective, we have replaced the thymidine at the 5′-end of one oligonucleotide with 4-thiothymidine (s^4T) and show that it photoreacts at 366 nm with a T at the 3′-end of another oligonucleotide in the presence of a complementary template. When a single mismatch is introduced opposite either the s^4T or its adjoining T, the ligation efficiency drops by a factor of five or more. We also show that by linking the two ends of the oligonucleotides together, photoligation can be used to form circular DNA molecules and to 'photopadlock' circular DNA templates. Thus, s^4T-mediated photoligation may have applications to phototriggered antisense-based or antigene-based genetic tools, diagnostic agents and drugs, especially for those situations in which chemical or enzyme-mediated ligation is undesirable or impossible, for example inside a cell.

INTRODUCTION

Non-enzymatic, template-directed ligation of oligodeoxyribonucleotides and oligoribonucleotides in aqueous solution has been of great interest because of its potential synthetic and biomedical applications and its implications for the origin of life. Various template-directed chemical ligation reactions have been reported that join oligonucleotides by either a native phosphodiester bond (1–15) or non-native linkages (16–25). In contrast to the variety of chemical ligation methods, there are only a few reports of photochemical methods, the first of which was based on the photochemistry of native DNA (26) and two others which were based on the photochemistry of DNA containing appended coumarins (27) or stilbenes (23). In the first report (26), which explored the possibility that light may have served as a prebiotic ligase, ligation was effected by photochemically induced cyclobutane dimer formation between the termini of dT_{10} oligodeoxyribonucleotides in the presence of a poly(dA) template. Unfortunately, >290 nm UV light was required to effect photoligation, conditions which would also cause photoproduct formation at other sites. In contrast to the photochemistry of thymidine, 4-thiothymidine (s^4T) is photoreactive at 366 nm, a wavelength which does not significantly damage normal DNA or RNA and which is consequently not lethal or mutagenic to cells (28). Because of its selective photochemistry, s^4T has been extensively used as a photoaffinity probe or a crosslinker to study the secondary and tertiary structure of nucleic acids (29–33). Of particular relevance to photoligation, s^4T has been shown to form various covalent adducts with a 5′-flanking thymidine in a dinucleotide (Fig. 1) (34). Herein, we wish to report the use of s^4T in the template-directed photoligation of individual and tethered oligonucleotides.

MATERIALS AND METHODS

Acetonitrile (anhydrous), acetic anhydride/2,6-lutidine/THF (capping reagent A), 1-methylimidazole/THF (capping reagent B), tetrazole/acetonitrile (activation reagent), trichloroacetic acid/dichloromethane (DMT deprotection reagent) and DNA synthesis grade deoxynucleoside (A, G, C and T) CED phosphoramidites were from Glen Research. *S*-Pivaloyloxymethyl-4-thiothymidine CED phosphoramidite and hexaethylene glycol CED phosphoramidite were synthesized according to published procedures (35,36). Another 4-thiothymidine building block, *S*-cyanoethyl-4-thiothymidine CED phosphoramidite, is available commercially from Glen Research. Bacteriophage M13mp18 DNA, T4 DNA ligase and T4 polynucleotide kinase were purchased from New England BioLabs. Phagemid pSVK3 was from Pharmacia and [α-^{32}P]ATP was from Amersham. Reversed phase HPLC purifications were performed on a Rainin Dynamax C-18 column. UV spectral data were acquired on a Bausch and Lomb Spectronic 1001 spectrophotometer interfaced to an IBM personal computer with Bausch and Lomb wavelength scanning software. Solid-phase DNA syntheses were conducted on an Applied Biosystems 380B DNA Synthesizer. Irradiations were carried out with a Model UVGL-25 Mineralight Lamp (UVP Inc., San Gabriel, CA), which was rated as producing 720 $\mu W/cm^2$ 366 nm light at 3 inches. Radioactivity in gels was quantified by volume integration with ImageQuant v.3.3 software of digitized data acquired directly from

*To whom correspondence should be addressed. Tel: +1 314 935 6721; Fax: +1 314 935 4481; Email: taylor@wuchem.wustl.edu

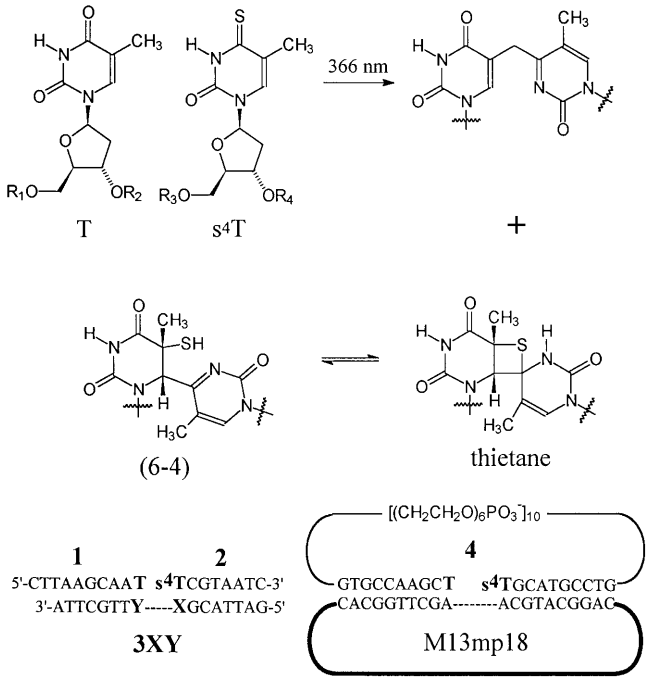

Figure 1. Photochemistry of Tps^{4}T (where R_1 = R_4 = H and R_2 and R_3 are internucleotide PO_2^- linkages) (34). Also shown are the photoligatable oligodeoxyribonucleotides **1**, **2** and **4** and their corresponding matched and mismatched templates **3XY** (where XY = AA, GA, CA, TA, AG, AC and AT) and single-strand circular bacteriophage M13mp18 DNA. Presumably, photoligation between the 3′-terminal T and 5′-terminal s^{4}T in **1** and **2** and in **4** will involve the formation of photoproducts similar to those produced in Tps^{4}T (i.e. where R_1 = R_4 = oligodeoxyribonucleotide, R_2 = H and R_3 = H or, if end-labeled, PO_3^{2-}).

a gel with a Molecular Dynamics PhosphorImager Model 4255 or from an autoradiogram.

Synthesis of oligodeoxyribonucleotides 1–4

Oligodeoxyribonucleotides were synthesized by automated DNA synthesis using 0.1 M CED phosphoramidites of normal bases (A, G, C and T), 0.2 M *S*-pivaloyloxymethyl-4-thiothymidine CED phosphoramidite and 0.2 M hexaethylene glycol CED phosphoramidite in anhydrous acetonitrile. For the synthesis of **2**, a 1 h coupling time was used for *S*-pivaloyloxymethyl-4-thiothymidine CED phosphoramidite, whereas for the synthesis of **4**, 30 min coupling times were used for both the *S*-pivaloyloxymethyl-4-thiothymidine and hexaethylene glycol CED phosphoramidites. The product of automated synthesis was cleaved from the CPG support and deprotected at room temperature with concentrated ammonia for 2 days. The mixture was then dried with a Savant Speedvac concentrator and purified by either reversed phase HPLC or 15% PAGE. Oligodeoxyribonucleotides **2** and **4** had peaks at 333 nm, characteristic of s^{4}T, and with respective intensities of 1/4 and 1/8 relative to the 254 nm maximum. The oligodeoxyribonucleotides (0.1 µM) were 5′-end-labeled by incubating with 4 U T4 DNA polynucleotide kinase and 10 µCi [α-^{32}P]ATP (6000 µCi/µmol) in 70 µM Tris–HCl (pH 7.6 at 25°C), 10 mM $MgCl_2$, 5 mM DTT at 37°C for 30 min, followed by heating for 5 min in boiling water.

Photoligation of oligodeoxynucleotides on matched templates

Unlabeled (0.25 µM) and ^{32}P-labeled oligonucleotides (0.1 µM) were mixed with or without the complementary template **3AA** (0.45 µM) in 35 mM Tris–HCl (pH 7.6 at 25°C), 5 mM $MgCl_2$ and 2.5 mM DTT. The mixtures were then warmed to 85°C for 5 min and then allowed to cool slowly to room temperature. The mixtures were irradiated on ice at 366 nm (140 µW) for 60 min. Two volumes of loading buffer (95% formamide, 20 mM EDTA) were added to each sample and then electrophoresed on a 15% (1:20 crosslinked) polyacrylamide gel.

Photoligation of oligodeoxynucleotides on mismatched templates

^{32}P-Labeled **2** (0.04 µM) was annealed to **3XY** (Fig. 1, XY = AA, AG, AC, AT, GA, CA or TA) (0.4 µM) together with **1** (0.2 µM) in 70 mM Tris–HCl (pH 7.6 at 25°C), 10 mM $MgCl_2$ and 5 mM DTT by warming up the mixture to 90°C for 5 min and allowing it to cool to room temperature slowly. Samples were irradiated with 366 nm light for the indicated time at 12°C in a cold room or at room temperature after which they were allowed to anneal for a further 30 min at 12°C. The reactions were diluted with 2 vol formamide loading buffer and then electrophoresed on a 15% (1:20 crosslinked) denaturing polyacrylamide gel.

Photopadlocking experiments

^{32}P-End-labeled oligonucleotide **4** (90 nM) and either single-strand M13mp18 (220 nM) or single-strand pSVK3 (140 nM) DNA were annealed at 85°C in 56 mM Tris–HCl (pH 7.6 at 25°C), 8 mM $MgCl_2$ and 4 mM DTT for 5 min and then allowed to cool slowly to room temperature. The mixtures were irradiated at 366 nm (140 µW) for 2 h at 0°C or treated with T4 DNA ligase and ATP in 10 µl 96 mM Tris–HCl (pH 7.5), 16 mM $MgCl_2$, 12 mM DTT, 0.8 mM ATP and 20 µg/ml BSA at 0°C overnight. Two volumes of formamide loading buffer were added to the mixtures and they were then separated by electrophoresis on either a 15% (1:20 crosslinked) denaturing polyacrylamide gel or 1% agarose gel. Yields of products were determined by volume integration of the radioactive bands in the polyacrylamide gel relative to a ^{32}P-labeled 25mer, which was used as an internal standard.

RESULTS AND DISCUSSION

To determine the feasibility of using a 5′-terminal s^{4}T to photoligate DNA, we synthesized oligonucleotides **1** and **2** and the complementary template **3AA** (Fig. 1) by standard automated synthesis and an s^{4}T phosphoramidite building block (37,38). When **1** and **2** were irradiated at 366 nm in the absence of template, no photoligation product was observed (Fig. 2, lanes a and b), but in the presence of template **3AA**, the expected 18mer photoligation product was produced in ~40% yield (Fig. 2, lanes d and f). The moderate photoligation yield may be due in part to competitive intrastrand reaction of the s^{4}T with the 3′-flanking C, as all bases are known to react with a relative order of T > U ≅ A > C > G (31) and photoreactions have been reported to occur with 3′-flanking nucleotides (29). Possible evidence for some sort of intramolecular photochemical reaction is evident from the formation of lower mobility bands migrating as 9mers and 10mers in the irradiation products of radiolabeled **2** which are not observed in the unirradiated oligodeoxyribonucleotide

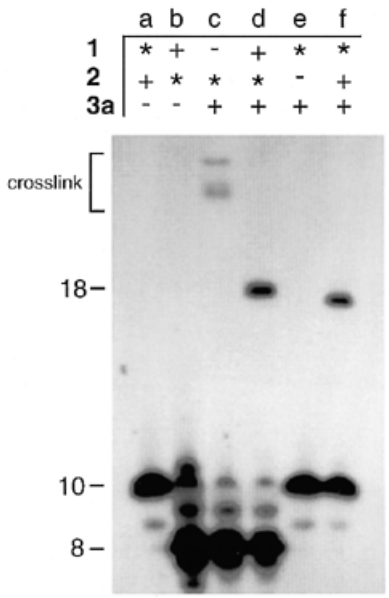

Figure 2. Autoradiogram of a denaturing 15% polyacrylamide electrophoresis gel of the photoproducts of various combinations of **1**, **2** and **3AA** following irradiation with 366 nm (140 µW) for 60 min on ice in 35 mM Tris–HCl, 5 mM $MgCl_2$ and 2.5 mM DTT. The ^{32}P-end-labeled oligodeoxyribonucleotide is indicated with an asterisk and was present at 0.1 µM, whereas the other of the pair, if present, was at 0.25 µM and the template, if present, was at 0.45 µM.

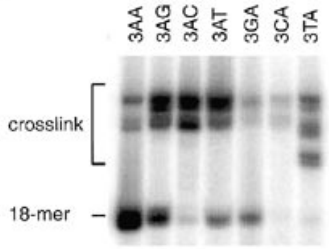

Figure 3. Autoradiogram of the photoligation and photocrosslinking products resulting from an 80 min irradiation with 366 nm light (140 µW) of 0.2 µM **1** and 0.04 µM ^{32}P-end-labeled **2** in the presence of 0.4 µM matched and mismatched templates **3XY** at 12°C.

(Fig. 2, lanes b–d). When **2** and **3AA** were irradiated in the absence of **1**, bands with higher mobility than an 18mer were produced in ~5% yield (Fig. 2, lane c), which can be attributed to photocrosslinking of s^4T to the template. Interstrand photocrosslinking with s^4T within duplex DNA is not observed, presumably due to the poor overlap between bases on both strands and the limited flexibility of the bases (39). Interstrand crosslinking has been observed in up to 53% yield, however, when s^4U is at the 5′-end of a blunt-end duplex (40). In our case, interstrand crosslinking takes place between a 5′-terminal s^4T and a 3′-overhanging end that can presumably make contact with s^4T in a number of places.

To determine the sensitivity of the photoligation reaction to mismatches at the photoligation site, photoligation was carried out at 12°C and room temperature with the fully complementary

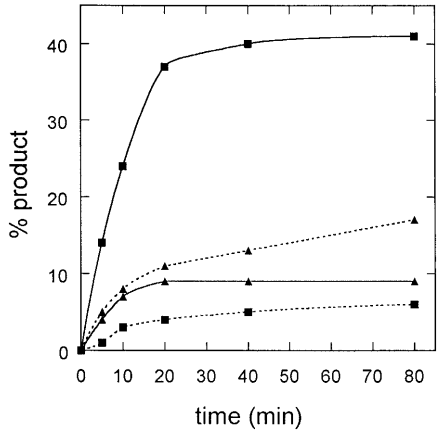

Figure 4. Plot of the yield of photoligation (solid lines) and photocrosslinking (dashed lines) products for **3AA** (squares) and **3AG** (triangles) as a function of irradiation time under the same conditions as described for Figure 3.

template **3AA** and all possible singly mismatched templates **3AY** and **3XA** (Fig. 1, XY = AA, AG, AC, AT, GA, CA or TA). As can be seen from Figure 3 and Table 1, single base mismatches opposite the ligation site have a dramatic effect on the photoligation yield. Whereas a 40% yield of photoligated product was observed with the fully complementary template **3AA**, a C·s^4T, C·T or T·s^4T mismatch at the ligation site (XY = AC, CA or TA) resulted in <1% photoligation (Fig. 3 and Table 1). When the templates contained a G·T, G·s^4T or T·T mismatch at the ligation site (XY = AG, GA or AT), the photoligation efficiencies were between 3 and 7%. Even in the least selective case, in which there was a G·T mismatch, the matched template was favored ~5:1 at 80 min irradiation. To see whether or not the photoligation reaction would be more selective at shorter time points, a time course study was carried out with the **3AA** and **3AG** templates under the same conditions as described for the 80 min photoreactions (Fig. 4). Under these conditions the photoligation yield plateaued at ~20 min and the selectivity at short times was similar to those at long times, possibly due to competing side reactions that deactivated the s^4T group, such as intrastrand photochemistry or crosslinking to the template.

Table 1. Photoligation and photocrosslinking efficiencies of **1** and **2** with matched and mismatched templates **3XY** at 12°C for the photoreactions shown in Figure 3 (given as a percentage of the total amount of radiolabeled products)

XY (template)	AA	AG	AC	AT	GA	CA	TA
Photoligation (%)	40	7	<1	3	3	<1	<1
Photocrosslinking (%)	6	16	18	18	2	2	10

The sensitivity of the photoligation reaction with s^4T to mismatches is very similar to that observed for ligation by T4 DNA ligase (41) and Tth DNA ligase (42,43), which are both least selective (>8:1) for G·T mispairs at the site of ligation. There is much less information on the effect of mismatches on chemical ligation methods. In one study, A·A and A·C mismatches slowed native phosphodiester bond formation by a water-soluble carbodiimide by only a factor of ~3 (44). In a study involving ligation by thiophosphate alkylation, single base mismatches 6 nt from the ligation site had an ~15-fold effect on the ligation yield

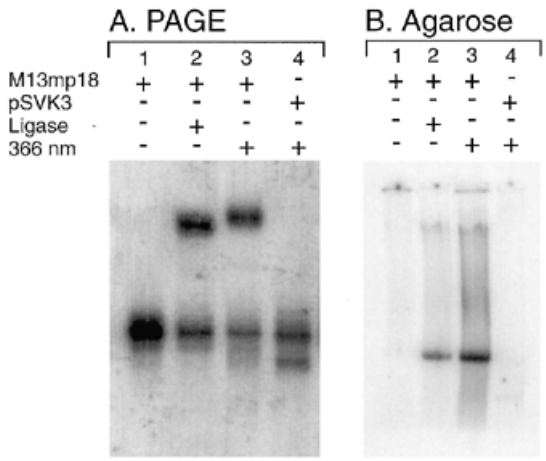

Figure 5. Autoradiograms of (**a**) 15% denaturing polyacrylamide or (**b**) 1% agarose electrophoresis gels of the overnight enzymatic or 2 h 366 nm (140 μW) photoligation products of 90 nM 5′-^{32}P-end-labeled **4** in the presence of complementary (M13mp18, 220 nM) or non-complementary (pSVK3, 140 nM) single-strand circular DNA vectors at 0°C.

of two octamers (21). Mismatches also had a substantial effect on the photoligation reaction by stilbenes, but no quantitative data was given (23).

Whereas ~6% photocrosslinking between **2** and **3** was observed for the matched template, photocrosslinking varied from 2% for **3CA** to ~18% for **3AC** and **3AT** for the mismatched templates (Fig. 3 and Table 1). The origin of this variability is not understood at the moment, but it is interesting to note that the highest yields of the crosslinking reaction are primarily associated with mismatches opposite the T and not opposite the s^{4}T. Unlike the photoligation reaction, photocrosslinking to either the **3AA** or **3AG** templates did not plateau at 20 min, but appeared to continue to increase with time (Fig. 4). Two major clusters of bands corresponding to photocrosslinked species were observed for all the templates, except for **3TA**, which showed an additional faster moving cluster which may indicate a photocrosslinking event unique to the presence of a T in the template at this position. When the photoligations were carried out at room temperature, very little photoligation was observed and the major products (<5%) appeared to be the interstrand photocrosslinking products of oligonucleotide **2** to the templates **3XY**.

We also examined the possibility of facilitating photoligation and 'photopadlocking' circular DNA molecules by tethering together the two oligonucleotides to be photoligated by analogy to the enzymatic version of the reaction (45). The tethered oligonucleotide **4** (Fig. 1), consisting of two decamers complementary to a 20 nt section of M13mp18, was synthesized by standard automated synthesis using a PEG building block (36). When **4** was irradiated in the presence of complementary single-strand M13mp18 DNA, a slower moving band was produced in ~50% yield that was similar in mobility to a band produced by enzymatic ligation (Fig. 5A, lanes 2 and 3) and is presumably the circularized product. The slight difference in mobility of the circularized products from photochemical and enzymatic ligation is probably due to the difference in the structure of the linkage. The circularized product was not observed when **4** was irradiated in the presence of a non-complementary template (Fig. 5A, lane 4) and instead a faster moving band was observed which might be the result of intrastrand crosslinking. Irradiation of **4** with single-strand M13mp18 DNA also produced a product that co-migrated with M13mp18 DNA, as did enzymatic ligation, which is presumably the topologically linked or 'photopadlocked' product (Fig. 5B, lanes 2 and 3). The yield of the linked product was <10% for both photochemical and enzymatic ligation reactions. The low yield observed contrasts with a previous report of the enzymatic padlocking (ligation) of two tethered 20mers for which the linked product was the major product (45). It may be that longer oligonucleotides are required to ensure linking of the two strands prior to ligation.

CONCLUSION

We have demonstrated that s^{4}T can be used to photochemically assemble a longer oligonucleotide from two smaller ones with an efficiency that is highly sensitive to a single base mismatch and at a wavelength that is not particularly injurious to nucleic acids. We have also demonstrated that template-directed photoligation can be carried out on tethered oligonucleotides, which affords a means for simultaneously delivering a pair of oligonucleotides to be photoligated and, additionally, can result in the 'photopadlocking' of circular DNAs. Thus, s^{4}T-mediated photoligation may have applications to phototriggered antisense- or antigene-based genetic tools, diagnostic agents and drugs, especially for those situations in which chemical or enzyme-mediated ligation is undesirable or impossible, for example inside a cell. Photoligation with s^{4}T may also find application to those situations in which it is necessary or desirable to maintain the native structure of DNA or when one of the pair of DNA molecules to be photoligated cannot be chemically modified.

ACKNOWLEDGEMENT

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