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Authors

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Tang, Feng
Zhao, Linlin

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Facile Preparation of Model DNA Interstrand Cross-Link Repair Intermediates Using Ribonucleotide-Containing DNA

Jin Tang[†], **Feng Tang**[†], **Linlin Zhao**[†,§,*]

[†]Department of Chemistry, University of California, Riverside, Riverside, California, 92521, United States

[§]Environmental Toxicology Graduate Program, University of California, Riverside, Riverside, California, 92521, United States

Abstract

DNA interstrand cross-links (ICLs) are lesions with a covalent bond formed between DNA strands. ICLs are extremely toxic to cells because they prevent the separation of the two strands, which are necessary for the genetic interpretation of DNA. ICLs are repaired via Fanconi anemia and replication-independent pathways. The formation of so-called unhooked repair intermediates via a dual strand incision flanking the ICL site on one strand is an essential step in nearly all ICL repair pathways. Recently, ICLs derived from endogenous sources, such as those from ubiquitous DNA lesions, abasic (AP) sites, have emerged as an important class of ICLs. Despite the earlier efforts in preparing AP-ICLs in high yield using nucleotide analogs, little information is available for preparing AP-ICL unhooked intermediates with varying lengths of overhangs. In this study, we devise a simple approach to prepare model ICL unhooked intermediates derived from AP sites. We exploited the alkaline lability of ribonucleotides (rNMPs) and the high cross-linking efficiency between an AP lesion and a nucleotide analog, 2-aminopurine, via reductive amination. We designed chimeric DNA/RNA substrates with rNMPs flanking the cross-linking residue (2-aminopurine) to facilitate subsequent strand cleavage under our optimized conditions. Mass spectrometric analysis and primer extension assays confirmed the structures of ICLs substrates. The method is straightforward, requires no synthetic chemistry expertise, and should be broadly accessible to all researchers in the DNA repair community. For step-by-step descriptions of the method, please refer to the companion MethodsX paper.

Graphical Abstract

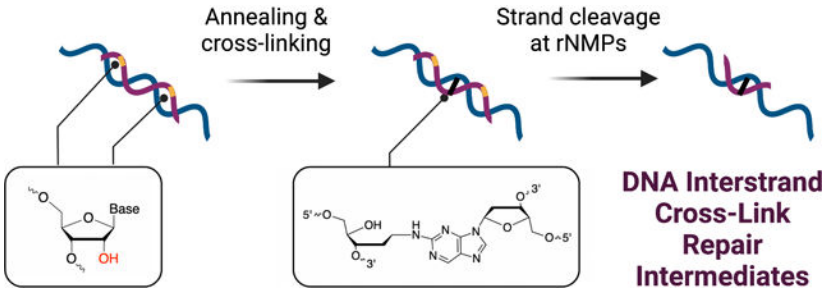
[*]Corresponding author: Linlin Zhao, Department of Chemistry and Environmental Toxicology Graduate Program, University of California, Riverside, California 92521-0403, United States, Phone: 951-827-9081, linlin.zhao@ucr.edu.

Author Contributions

L.Z. conceived the idea; J.T. and F.T. performed the experiments; L.Z. administered the project; J.T. and L.Z. wrote the manuscript.

Disclosure statement

The authors report no declarations of interest.



Keywords

DNA damage; DNA interstrand cross-links; DNA repair; RNase H; translesion synthesis (TLS)

1. Introduction

DNA is susceptible to chemical and physical factors, generating a plethora of DNA lesions [1]. DNA interstrand cross-links (ICLs) are among the most deleterious DNA lesions, because the covalent linkage between the two complementary strands can alter the structure and enzymatic read-out of DNA, potentially leading to genomic instability and cell death [2–6]. It is estimated that one ICL lesion is sufficient to kill a bacterial or yeast cell [7], and approximately 40 ICLs can kill a repair-deficient mammalian cell [8]. ICLs can be repaired via replication-coupled and replication-independent pathways [6]. In almost all known ICL repair pathways, the generation of so-called unhooked repair intermediates by dual incision of ICLs via endonucleolytic activities is an essential step. Critical to the endonucleolytic activities and initiation of ICL repair are endonuclease XPF-ERCC1 and the scaffolding protein SLX4 [3,9,10], along with a number of other endonculeases, such as FAN1, MUS81-EME1, SLX1, and SNM1A [3,9,11–14]. The resulting ICL remnant can be bypassed by translesion synthesis (TLS) DNA polymerases followed by homologous recombination (HR) in replication-dependent ICL repair pathways [6].

Historically, ICLs have been studied in the context of chemical warfare and chemotherapeutic agents such as nitrogen mustards, mitomycin C, psoralens, and cisplatin [15–18]. Recently, ICLs derived from endogenous chemicals have emerged as an important class of ICLs [5]. In particular, abasic (AP) sites, one of the most abundant endogenous DNA lesions, have been shown to form ICLs with a guanine residue on the 5'-side of the nucleotide opposite to an AP site on the complementary strand [19,20], or an adenine on the 3'-side of the nucleotide opposite to an AP site on the complementary strand [21,22]. Subsequent studies using DNA substrates containing ICLs derived from an AP lesion and an adenine residue discovered a specialized NEIL3-mediated repair pathway and supported circumstantially the biological relevance of AP-derived ICLs (AP-ICLs) [23–25].

The advancement of the understanding of biological consequence and repair pathways of ICLs is driven in part by the development of approaches to prepare site-specifically modified oligodeoxynucleotides containing a chemically defined ICL. However, the preparation of ICL-containing substrates is not trivial. Traditional methods using cross-linking agents, such as formaldehyde or nitrogen mustards, to react with a double-stranded (ds) DNA substrate

suffer from poor site selectivity and low yield [26–28]. The resulting mixtures of cross-linked products require laborious purification and can complicate data interpretation. Several solid-phase synthetic methods have been developed over the years [29–33]; nonetheless, the requirement of stringent reaction conditions and synthetic chemistry expertise has hindered broader applications of these methods. A number of elegant methods have been devised by Gates and colleagues to prepare AP-ICLs [13–16], and two methods achieved high cross-linking yield using nucleotide analogs, N^4-amino-2'-deoxycytidine [34] and 2-aminopurine (P) [35]. The dearth of information on preparing AP-ICL unhooked intermediates and the need for additional mechanistic insights into AP-ICL repair motivated us to develop a simple and accessible approach to prepare such substrates.

Herein, we developed a simple approach to prepare site-specifically modified model AP-ICL substrates to mimic unhooked repair intermediates. We exploited the alkaline lability of ribonucleotides (rNMPs) in chimeric DNA/RNA substrates [36] and the high cross-linking efficiency between AP and a nucleotide analog (P) via reductive animation [35]. We designed rNMPs on the 3'-side and 5'-side of the cross-linking residue (P) to facilitate strand cleavage under alkaline conditions. We optimized the rNMP cleavage conditions using NaOH or RNase HII to prepare two model ICL substrates with five or nine nucleotide overhangs. Primer extension assays using a prototypical TLS DNA polymerase and a replicative DNA polymerase demonstrate that both substrates are strong blocks to bypass synthesis. All DNA substrates used in this study are commercially available, which ensures the accessibility of the approach by most labs interested in ICL repair or nucleic acid modifications in general.

2. Materials and Methods

2.1 Materials

Chemicals were purchased from Fisher Scientific and are either analytical grade or molecular biology grade. Uracil-DNA glycosylase (UDG), RNase HII and *B. subtilis* DNA polymerase I (Pol I, lacking the exonuclease activity) were from New England Biolabs. *S. solfataricus* P2 DNA polymerase IV (Dpo4) was expressed and purified based on our previous protocol [37]. HPLC-purified unmodified and modified oligodeoxynucleotides were purchased from Integrated DNA Technologies.

2.2 Preparation of ICL substrates

AP site-containing oligodeoxynucleotides were prepared by excising the uracil residue from a deoxyuridine-containing oligodeoxynucleotide with UDG followed by phenol/chloroform extraction, as described previously [38]. An AP-containing DNA oligodeoxynucleotide was annealed with a P-containing oligodeoxynucleotide. The resulting DNA substrate was incubated in 750 mM NaOAc (pH 5.4) and 250 mM $NaCNBH_3$ at 37°C overnight to form the P-AP ICLs substrate precursors. The formation of ICL was monitored by 18% denaturing PAGE.

2.3 Strand cleavage at rNMPs

Strand cleavage reactions at rNMPs with P-AP ICL precursors were performed under NaOH or RNase HII. The NaOH reactions contained 0.1 M, 0.2 M, or 0.3 M NaOH and were conducted at 55°C for up to 2 h followed by neutralization using HCl and gel analysis or purification. The RNase HII reactions were with larger amounts of RNase HII than the manufacture suggested conditions, as specified in the Figure 2 caption. For reactions with ICL-R1, the optimal condition was 10 pmol ICL-R1 and 10 units of RNase HII. For reactions with ICL-R2, the optimal condition was 10 pmol ICL-R2 and 5 units of RNase HII at 37°C for 18 h. The manufacture defines one unit as the amount of enzyme required to yield a fluorescence signal comparable to nicking of 100 pmol of synthetic dsDNA substrate containing a rNMP near the quencher of a fluorophore/quencher pair in 30 minutes at 37°C in 1X ThermoPol Buffer.

2.4 Primer extension assays with ICLs

The primer extension assay was conducted with ICL1 and ICL2 that cleaved via alkaline strand cleavage reaction. The reaction was performed with 100 uM dNTPs and 1 uM ICLs with 0.5 unit/uL Pol I or 71 nM Dpo4 with 50 mM Tris-HCl pH 7.4, 5 mM DTT, 5 mM $MgCl_2$, 50 mM NaCl, 50 ug/mL BSA and 5% Glycerol at 37°C. The reaction aliquots were taken at various times and quenched with 20 mM EDTA (pH 9.0) in 95% (v/v) formamide.

3. Results and Discussion

3.1 Design of ICL substrates and cross-linking reactions

As outlined in Figure 1a, the preparation of model ICL substrates consists of several straightforward steps, i.e., (1) annealing two complementary single-stranded (ss) DNA oligomers and the cross-linking reaction, (2) cleaving the precursor substrate at rNMPs, and (3) purifying the model ICL by PAGE. We designed oligodeoxynucleotides containing a P modification and two rNMPs on the 5'- and 3'-side of P (Figure 1e, the top strand of ICL-R1 and ICL-R2) to facilitate subsequent strand cleavage at these sites via alkaline transesterification reactions. Although Couvé et al. had used chimeric RNA/DNA oligonucleotides followed by RNase A digestion to construct psoralen-induced ICL repair intermediates [39], the applicability of their method has not be tested for other ICL substrates and digestion by RNase A could be limited by the cross-linking chemistry, the spacing of rNMP residues, and steric hinderance. In this study, we seek to develop a method based on the alkaline lability of rNMPs to generate strand cleavage at rNMPs sites (Figure 1b). We exploited the high cross-linking yield between P and AP sites (Figure 1c) [35] to generate covalent cross-links between two complementary oligonucleotides, which are referred to as ICL precursors. The sequences of oligomers containing P or AP modifications are shown in Figure 1e. The ICL precursors with rNMPs at different locations are referred to as ICL-R1 and ICL-R2 (Figure 1e). The cross-linking yield was approximately 90% after an overnight reaction for ICL-R1 and ICL-R2 (Figure 1d), indicating that the presence of rNMPs did not alter the cross-linking reactions. ICL-R1 and ICL-R2 were cleaved at rNMPs to generate model ICL substrates to mimic unhooked repair intermediates of different sizes. One ICL substrate contains a 5-nt overhang cross-linked to the AP site, and the other contains a 9-nt overhang (hereinafter referred to as ICL1 and ICL2, respectively).

3.2 NaOH-catalyzed cleavage at rNMPs

We optimized two types of rNMP cleavage conditions, i.e., NaOH- and RNase HII-catalyzed reactions. rNMP cleavage under 0.3 M NaOH at 55 °C for 2 h has been used to map the rNMPs embedded in the genomic DNA [40,41]. We first investigated the suitability of this condition with ICL-R1 and ICL-R2. PAGE analysis revealed that the highest yield of the desired ICL products peaked at 60% (indicated by green squares in Figures 2c, Figure S1) after a 1-h reaction followed by further conversion to side products (indicated by red triangle in Figures 2c). After a 2-h reaction, the desired ICL products accounted for only 27% with the majority of products being cleaved fragments. Such products are likely due to the cleavage of the phosphoester bond 5' of the AP lesion, considering that the migration is slower than the 5'-remnant after cleavage at AP sites and the difference in migration patterns for products formed in ICL-R1 and ICL-R2 reactions. To optimize the yield of ICL1 and ICL2, we decreased the concentration of NaOH, i.e., 0.1 M and 0.2 M NaOH, at 55°C for varying times (Figures 2a and 2c, Figure S1). We compared the yield of the desired ICL products under different reaction conditions (Figure 2c) and selected the condition under 0.2 M NaOH at 55°C for 60 min to produce the highest yield of ICL1 (62%) and ICL2 (67%) (Figure 2c). Further, ICL1 and ICL2 were purified by denaturing PAGE (Figure S2) to obtain products with 95% and 99% purities (Figure S2), respectively. NaOH-mediated cleavage yields a primarily products with a phosphate group at the 5'-end of the unhooked strand, as evidenced by phosphatase treatment (Figure S2) and mass spectrometry analysis (Figure S3).

3.3 RNase H-catalyzed at rNMPs

RNase H enzymes cleave the RNA in RNA/DNA hybrids and remove ribonucleotides from DNA to maintain the stability of genome [42,43]. RNase HII selectively cleaves the phosphodiester bond 5' to the ribonucleotides (Figure S4a), generating terminal nucleotide structures different from the NaOH cleavage reaction (Figure 2b and Figure S4b). Indeed, when comparing products in NaOH-catalyzed reactions with those from RNase HII-mediated cleavage, a clear difference was observed in the product migration patterns (lane 4 vs. lane 7 with ICL1 and ICL2 in Figure 2b). RNase HII exhibited better specificity relative to reactions with NaOH, as evidenced by the absence of shorter fragments. Nonetheless, the reactions required a considerable amount of RNase HII (approximately 10-fold higher than the manufacturer suggested stoichiometry) and longer reaction time (18 h) to achieve complete cleavage at both rNMPs. In particular, the reaction was sensitive to the spacing of the two rNMPs. Under the same condition, the cleavage of ICL-R2 was more efficient than that of ICL-R1. The ICL-R2 reaction was nearly complete after an overnight reaction, whereas the ICL-R1 reaction yielded only 25% of the desired product ICL1 (lane 7 of Figure 2b left panel). We were able to improve the product yield with ICL1 by using more RNase HII (Figure S4c). Together, our results demonstrate that NaOH-mediated rNMP cleavage serves as an efficient and robust method to produce the desired model ICL substrates, whereas RNase H-catalyzed reactions offer a means to generate ICL products in a higher yield with optimization needed for different substrates. Notably, we tested the cleavage efficiency at rNMPs with a reaction developed for cleaving the rNMP sites in DNA protein cross-links (DPCs) [44]. The reaction exhibited low cleavage efficiency with considerable amounts of side products (Figure S4d). Therefore, the condition reaction was not pursued.

3.4 DNA polymerase bypass reactions with model ICLs

To test the replication-blocking effects of ICL1 and ICL2, primer extension assays were performed with primer-template substrates containing ICL1 and ICL2 (Figure 3a) and a model TLS DNA polymerase, Dpo4, or a replicative DNA polymerase, *B. subtilis* Pol I. The primer-template substrates containing ICL1 and ICL2 mimic the replication intermediates prior to bypass by TLS polymerases. Such substrates have been used by a number of earlier studies to understand the role of specific TLS polymerases in bypassing ICL lesions [45–48]. With unmodified substrates, both enzymes yielded full-length extension products. Dpo4 showed different reaction efficiency when it was incubated with two ICL-containing substrates (Figures 3b). With ICL1, DNA synthesis by Dpo4 stalled 2 nt before the cross-linking site. ICL2 exhibited a stronger inhibitory effect, with stalling occurring at 4 nt to 5 nt prior to the cross-linking site, consistent with the longer overhang present in ICL2 compared to ICL1. Reactions with Pol I produced extended primers with 7 nt added within 3 min with both ICL1 and ICL2-containing substrates (Figure 3c), indicating that Pol I can extend the primer up to 1 nt prior to the cross-linking site but cannot bypass the ICL lesion. Longer reaction times up to 30 min did not improve the primer-extension reactions by Pol I. Compared to reactions with Dpo4, the overall longer extension products observed with Pol I are consistent with the known processivity and strand displacement activity of Pol I [49]. Together, data with two model DNA polymerases support the successful preparation of two model ICL structures and demonstrate strong replication-blocking effects of AP-derived model ICL substrates.

4. Conclusions

The ICL unhooked intermediates form in nearly all replication-dependent and replication-independent ICL repair pathways. It has been shown that AP-dA ICLs are unhooked by NEIL3; however, in mammalian cells lacking NEIL3, these lesions rely on Fanconi anemia (FA) pathway [24,50], suggesting the likelihood of formation of AP-ICL unhooked intermediates. Therefore, simple and straightforward approaches to prepare such model AP-ICL substrates are warranted to facilitate the delineation of AP-ICL repair mechanisms and the substrate specificities of different endonuclease, exonucleases, and accessory proteins [13,14].

We devised a simple method to prepare model ICL unhooked intermediates derived from AP sites. The method exploits the alkaline lability of rNMPs in chimeric DNA/RNA substrates to generate unhooked strands of different sizes. We showed that rNMP cleavage under NaOH serves as a robust means regardless of the spacing of two rNMPs, whereas cleavage using RNase H may offer a higher product yield with optimization required for specific substrates. We envision that creative modification of the method by using one or two rNMPs will aid the preparation of relevant substrates for further elucidation of AP-ICL repair mechanisms. The method can also serve as an alternative means to other methods where enzyme processing may be limited by the spacing of the residues (e.g., cleavage of deoxyuridine by UDG) [51].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations:

AP	abasic sites
AP-ICLs	AP-derived ICLs, Dpo4, *S. solfataricus* P2 DNA polymerase IV
ICLs	DNA interstrand cross-links
FA	Fanconi anemia
HR	homologous recombination
P	2'-aminopurine
Pol I	*B. subtilis* DNA polymerase I
TLS	translesion synthesis
UDG	uracil-DNA glycosylase

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Highlights

- DNA interstrand cross-links (ICLs) are mutagenic and cytotoxic DNA lesions.
- Abasic site(AP)-derived ICLs are important class of ICL lesions.
- A method is developed to prepare AP-derived model ICL unhooked repair intermediates.
- The method exploits the alkaline lability of ribonucleotides to yield structurally defined ICLs.
- The method is expected to facilitate the understanding of ICL repair mechanisms.

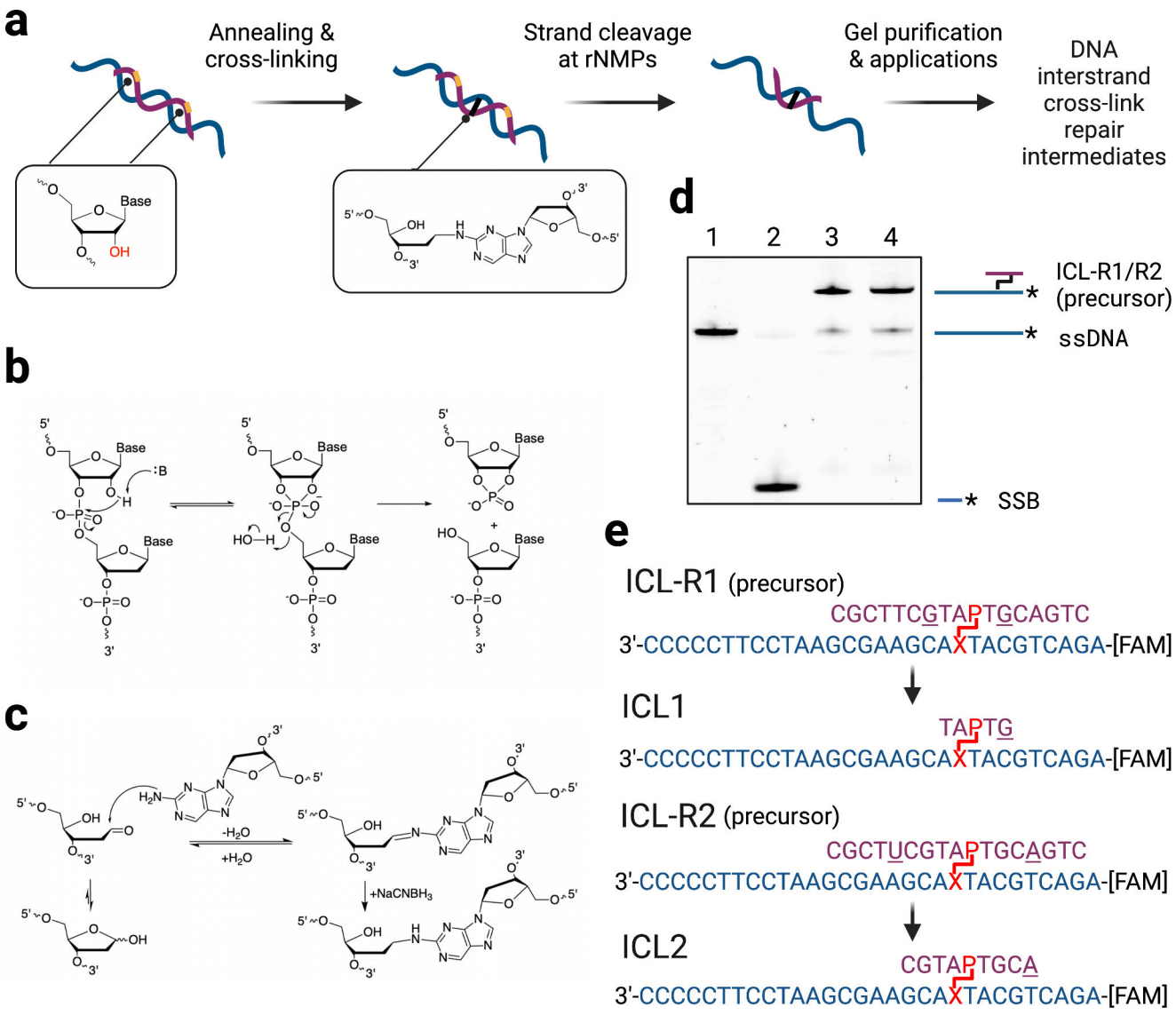

Figure 1.

Design and preparation of ICL substrates. (a) Preparation of model ICL mimics. The workflow consists of annealing and P-AP crosslinking reaction, rNMP cleavage, and PAGE purification. (b) rNMP cleavage via alkaline transesterification reactions. (c) Cross-linking reaction between P and AP sites to form P-AP ICLs under reductive amination. (d) 18% denaturing PAGE analysis of the product after the P-AP cross-linking reaction in the presence of 250 mM $NaCNBH_3$. Lane 1 is 30-mer uracil-containing DNA oligomer (the sequence is shown in the bottom strand of ICL-R1 in Fig1e); lane 2 is 9-mer oligomer product after NaOH-induced cleavage at the AP site with AP-containing 30-mer DNA oligomer; lane 3 and 4 show cross-linking yields are 90% and 87% for ICL-R1 and ICL-R2, respectively. (e) Sequences of rNMP-containing ICL precursors and model ICL structures upon cleavage at rNMPs.

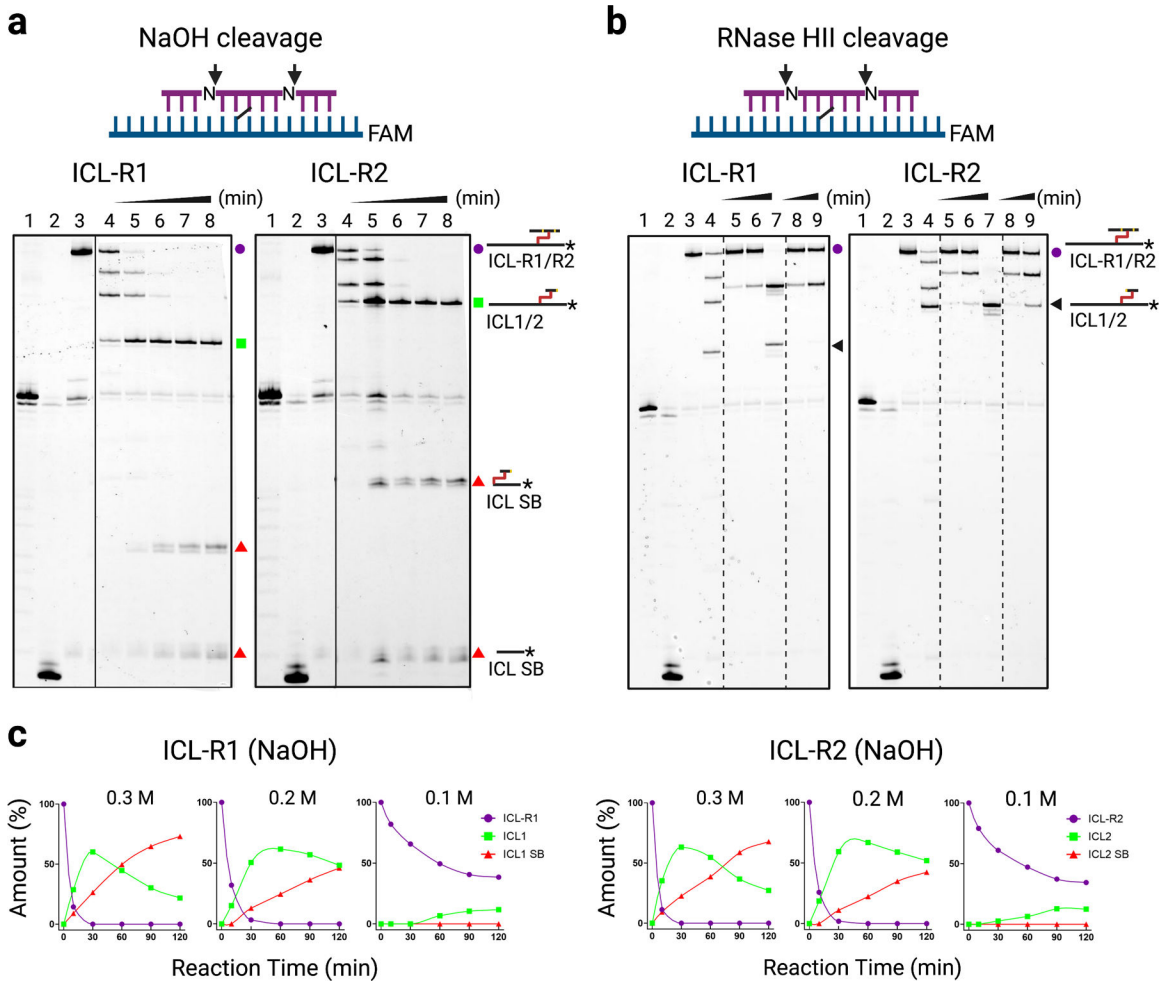

Figure 2.

Time course of rNMP cleavage reactions with ICL-R1 and ICL-R2. (a) Reactions with ICL-R1 or ICL-R2 under 0.2 M NaOH. Black arrows indicate locations of phosphoester bond cleavage. Lane 1 is fluorescein-labeled template DNA oligomer with a deoxyuridine; lane 2 is the NaOH-cleaved product of AP-containing template DNA oligomer; lane 3 is ICL-R1 or ICL-R2 precursor. Lanes 4–8 are reactions with ICL-R1 or ICL-R2 under 0.2 M NaOH for 10, 30, 60, 90, and 120 min. The ICL precursors are indicated by purple spheres. The desired ICL products are indicated by green squares. Side products correlating to strand cleavage at AP sites or degradation of the P-AP cross-links are indicated by read triangles. Reaction intermediates that migrated in between the ICL precursors and the desired ICL products correspond to cleavage at one rNMP residue. (b) Strand cleavage reactions with ICL-R1 or ICL-R2 using RNase HII. Lanes 1–3 contain the same samples as described in (a). Lane 4 represents cleavage products of ICL-R1 or ICL-R2 under 0.3 M NaOH for 10 min. Lanes 5–7 are reactions with 10 pmol ICL-R1 or ICL-R2 incubated with 5 units RNase HII for 1, 3, and 18 h. Lane 8 and 9 are reactions with 10 pmol ICL-R1 or ICL-R2 incubated with 10 units RNase HII for 1 and 3 h. Black triangles indicate the desired ICL products. (c) Quantification of reaction products from the denaturing PAGE analysis in Figure 1a and Figure S1. The percent intensity of starting materials in lane 3 were defined as 100%.

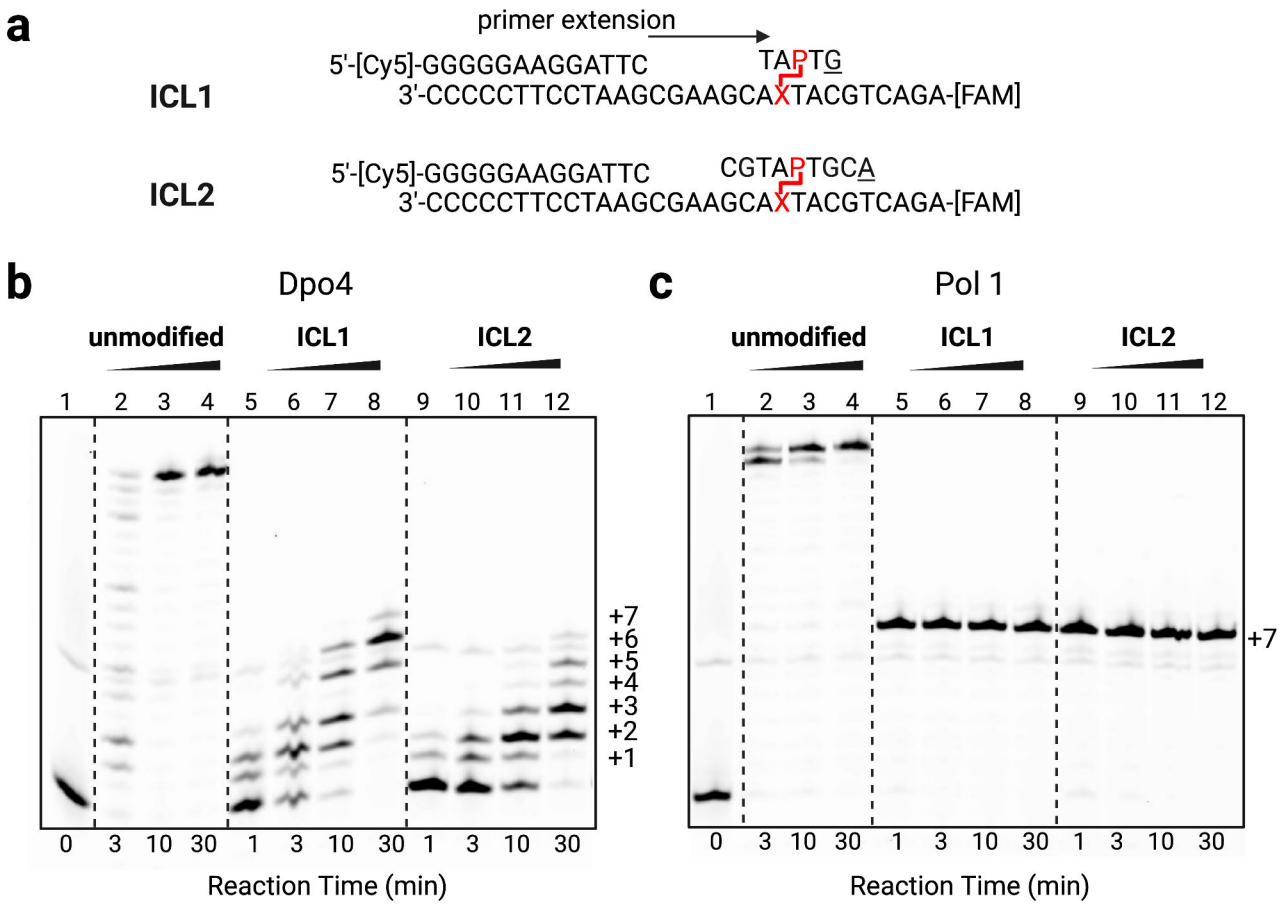

Figure 3.

Primer-extension reactions with ICL1 and ICL2 substrates catalyzed by Dpo4 and Pol I. (a) Primer-template substrates containing ICL1 and ICL2 for primer extension reactions. (b) Reactions with Dpo4 and an unmodified substrate, ICL1 or ICL2. (c) Reactions with Pol I and an unmodified substrate, ICL1 or ICL2.