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A Single Amino Acid Substitution in Therminator DNA Polymerase Increases Incorporation Efficiency of Deoxyxylonucleotides

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Deoxyxylonucleic acid (dxNA) is a synthetic polymer that might have potential for heredity and evolution. Because of dxNA's unusual backbone geometry, sequence information stored in it is presumed to be inaccessible to natural nucleic acids or proteins. Despite a large structural similarity with natural nucleotides, incorporation of 2′-deoxyxylonucleotides (dxNTs) through the action of polymerases is limited. We present the identification of a mutant of the DNA polymerase Therminator with increased tolerance to deoxyxylose-induced backbone distortions. Whereas the original polymerase stops after incorporation of two consecutive dxNTs, the mutant is able to catalyse the extension of incorporated dxNTs with 2′-deoxyribonucleotides (dNTs) and the incorporation of up to four dxNTs alternates with dNTs, thereby translocating a highly distorted double helix throughout the entire polymerase. A single His-to-Arg substitution very close to the catalytic site residues is held to be responsible for interaction with the primer phosphate groups and for stabilizing nucleotide sugar-induced distortions during incorporation and translocation.

Introduction

Nucleic acids are a versatile class of compounds that can store genetic information or fold into three-dimensional structures such as aptamers and aptazymes that allow for complex molecular recognition or catalysis, respectively. Xeno nucleic acids (XNAs) are synthetic analogues with the potential to increase this chemical, structural and functional versatility and also to undergo Darwinian selection and evolution.[1,2] XNA might have a use, for example, in biosafety, as a "genetic firewall" that prevents sequence information transfer to DNA or RNA.[3] Such XNAs are said to be genetically orthogonal. For applications in which the XNA polymerase that catalyses the production of this XNA is present in vivo, enzyme orthogonality is also required in order to avoid interference with the endogenous DNA/RNA systems.

Some XNAs that have been produced differ from natural nucleic acids in modifications of the nucleobases, ranging from small changes to completely different scaffolds.[4–6] It is also possible to modify the universal pyrophosphate leaving group[7] (which would produce natural nucleic acids from xenonucleotides) or the sugar–phosphate backbone.[4,8,9] A large number of substitutes for the (deoxy)ribose sugar and/or the phosphate group have been explored. Even the backbone itself can be fundamentally changed, as in the cases of the glycerol–phosphate backbone of glycerol nucleic acids (GNAs)[10] or the *N*-(2-aminoethyl)glycine polyamide backbone of peptide nucleic acids (PNAs).[11]

One very relevant property of XNAs is the potential for structural and/or coding orthogonality in vivo, which applies when there is no interaction with biological processes such as replication, transcription or translation. There is a large group of potential alternative synthetic nucleotide building blocks to choose from, but currently only a fraction of these can be efficiently and accurately incorporated through the action of polymerase enzymes with broadened substrate spectra. Therminator DNA polymerase, for example, can transcribe DNA into threose nucleic acid (TNA) sequences[12] (much more readily than the closely related Vent polymerase[13]), up to five GNA[14] and up to seven flexible nucleic acid (FNA) nucleotides.[15] Evolved variants of TgoT DNA polymerase have allowed the enzymatic production of 1,5-anhydrohexitol nucleic acids (HNAs), arabinonucleic acids (ANAs), 2′-fluoroarabinonucleic acids (FANAs), cyclohexenyl nucleic acids (CeNAs), 2′-*O*,4′-*C*-methylene-β-D-ribonucleic acids or locked nucleic acids (LNAs), TNAs[16] and 5′-*O*-phosphonomethyl-threosyl nucleic acids (tPhoNAs).[17]

Most of the XNAs with altered backbones that have been produced with the aid of existing or evolved polymerases maintain canonical base pairing with DNA and/or RNA. Enzymatic production of a structurally orthogonal XNA that does not hybridize to natural nucleic acids has not been achieved so far, presumably because XNAs with large structural differences are hard to accommodate and translocate in an active site that is optimized for DNA or RNA, even in a polymerase with promiscuous activity. Enzyme engineering based on evolution

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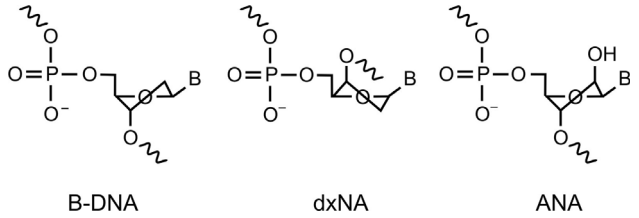
is also limited to polymerases that are dependent on primer–template complex formation and assumes that the distance in sequence space between a DNA polymerase and a XNA polymerase is not too large to be bridgeable by mutagenesis.

One such orthogonal nucleic acid would be 2′-deoxyxylonucleic acid (dxNA), which is identical to DNA except for the ribose C3′ stereochemistry, with 2′-deoxyribose thus being replaced by its epimer, 2′-deoxyxylose (Scheme 1). Because the



Scheme 1. Backbone configurations of 2′-deoxyribose-based DNA, 2′-deoxyxylose-based dxNA and arabinose-based ANA. The β-oriented 3′-hydroxy groups in dxNA drastically alter the nucleic acid backbone geometry.

C3′ oxygen atom is part of the backbone, this inversion drastically changes the backbone structure. It has been reported that, in vivo, reactive-oxygen-species-induced (ROS-induced) epimerization of 2′-deoxyribose to 2′-deoxyxylose in DNA can hinder replication and induce mutation.[18] Studies based on molecular dynamics[19] and on NMR and CD[20,21] have shown that a fully substituted dxNA strand forms extended, left-handed double helices with an antiparallel strand where Watson–Crick base pairing rules still apply. Complementary dxNA strands anneal with affinities similar to those of DNAs but show no hybridization either with RNAs or with DNAs. Comparison of dxNAs with multiple other XNA structures[9] shows that dxNAs constitute one of the XNA classes with the most drastically altered backbone conformations: the helix is much more elongated, with a very large major groove and the backbone tracing a zig-zag path. The large difference in structure makes dxNA a promising, truly orthogonal, but polymerase-challenging XNA candidate: even sequences with canonical bases would be invisible to natural replication, transcriptional or translational machinery because of a lack of recognition through strand hybridisation or backbone interactions.

Although chemical synthesis of dxNA (oligo)nucleotides is possible, enzymatically manipulating or sequencing dxNA strands is difficult and inefficient. Enzymes such as polymerases, ligases, endonucleases etc. that were specific for dxNAs would open up the same possibilities that exist for DNA and RNA but would need either to be designed or to be obtained through molecular evolution.[22] Precisely because of the orthogonal character of these 2′-deoxyxylonucleotides, no enzymes that efficiently catalyse their polymerization are available. Experiments with different polymerases have revealed dxNTs to be very resistant to incorporation and extension, with no more than three consecutive incorporation events on homopolymeric templates.[20]

In this study, we have attempted to improve on the existing promiscuous activity by using molecular evolution of Therminator DNA polymerase, derived from *Thermococcus* sp. 9°*N*-7 DNA polymerase with mutation A485L, and made exonuclease-deficient with mutations D141A and E143A.[23] With the aid of compartmentalized self-tagging (CST),[24] targeted libraries of this thermostable, archaeal family B polymerase were selected for incorporation of one or two 2′-deoxyxylothymidine nucleotides (dxTs), followed by a number of natural nucleotides (dNTs). We report here on the characterization of selected mutants that allowed us to pinpoint a single amino acid substitution that provided improved dxNT incorporation and translocation.

Results

Library construction, selection and screening

Mutant libraries were designed based on the crystal structure of the Therminator precursor, 9°N DNA polymerase bound to DNA (PDB ID: 4K8X). Six regions of the protein in close contact with DNA were defined and partially randomized in exonuclease deficient (*exo*$^-$) Therminator, so as to allow for a local high amount of mutations. Of the six libraries, two were used in this study (active site library and minor groove binding region library; see Experimental section). The polymerase catalytic Asp residues themselves were left unchanged.

These libraries were subjected to selection by compartmentalized self-tagging (CST)[24] for the incorporation of dxT. Two modifications of this CST protocol were made: primers were tagged by biotinylated dC incorporation, given that short and destabilizing extension was expected, and no Mn^{2+} was added. The template sequences were chosen such that nine nucleotides needed to be incorporated (the seventh of these being a dxT unit) in the first round of selection, and 18 in the second round (the 12th being a dxT unit), before incorporation of a first biotin-dC unit. This relatively low selection pressure was meant to select initially for mutants that can build one or two dxT units into DNA and translocate the dxT-induced backbone distortions along the polymerase. The next logical step would then be to further evolve distortion-tolerant mutants by gradually increasing the dxT incorporation load.

Resulting clones were screened by using a DNA-based enzyme-linked immunosorbent assay (ELISA) of cell lysate for the presence of polymerase variants capable of elongating a primer–template complex. Full elongation required the incorporation and elongation of seven natural non-T nucleotides, a single dxT nucleotide and 27 natural non-T nucleotides, consecutively. Elongation downstream of the introduced modification was probed with a complementary, fluorescently labelled oligonucleotide. Three out of 184 clones scored much more strongly in signal intensity than the wild type, with the strongest of these being clone E2 (Figure S1 in the Supporting Information). Several clones from the active site library and the minor groove binding region library were further tested with purified protein and more demanding templates requiring consecutive and intermittent incorporation of up to four dxT nucleotides. The clones E2 and G8 consistently showed higher ELISA activity than the wild type and the other mutants (Fig-

ure S2). DNA sequencing revealed E2 to contain a single amino acid substitution—H545R—and G8 to contain mutations R613H and E617R. The incorporation efficiencies of purified polymerase variants E2 and G8 were further characterized by analysis of primer elongation products by denaturing polyacrylamide gel electrophoresis (PAGE).

Homopolymeric templates

Three polymerases (Therminator, E2 and G8) were tested with different nucleotide types, concentrations and incubation times for comparison with previously published[20] dxNTP incorporation data on Therminator and other polymerases. Both Therminator *exo*$^-$ (for simplicity further denoted as Therminator or wild type) and mutant E2 are capable of incorporating up to two consecutive dxT units and up to three dxA units on homopolymeric polyA and polyT templates, respectively (Figure 1). Mutant G8, which had shown promise in the ELISA screening, performed similarly to E2 with dxA as a substrate; however, it catalysed the incorporation of virtually no dxT. Because the E2 mutant performed much better than G8, G8 was not tested further. Whereas both wild-type and E2 polymerases catalysed the extension of all primers with at least one dxNT within 5 min, the wild type took 45 min to catalyse the elongation of all primers with a second dxNT. In contrast, E2 catalysed only the partial addition of a second dxNT under the same conditions.

Incubation with different equimolar nucleotide concentrations of dNTPs and dxNTPs showed that the lowest concentration of 100 μM was optimal for evaluation of incorporation.

Use of increased concentrations up to 200 and 500 μM did not visibly improve the dxNT incorporation rate but did increase misincorporation rates in the case of the otherwise more accurate E2 polymerase. Therefore, all further elongation reactions were performed with 100 μM nucleotides. These were kept equimolar to minimize anticipated incorporation bias due to concentration differences.

To investigate the effects of dxA and dxT combination on primer elongation in the presence of wild-type and E2 polymerases, templates requiring the incorporation of different combinations of three dxA and dxT nucleotides were used (Figure 2). For all of these templates, only up to two dxNTs were incorporated, with no visible difference in incorporation efficiencies between dxA and dxT. The only exception was incorporation against the ATT template, in which case trace amounts of +3 product, quantifiable by digital image analysis, were possibly formed in the presence of E2. The presence or absence of dGTP and dCTP in the reaction mixture showed no significant difference; neither did the use of TAT or ATA templates (Figure S4).

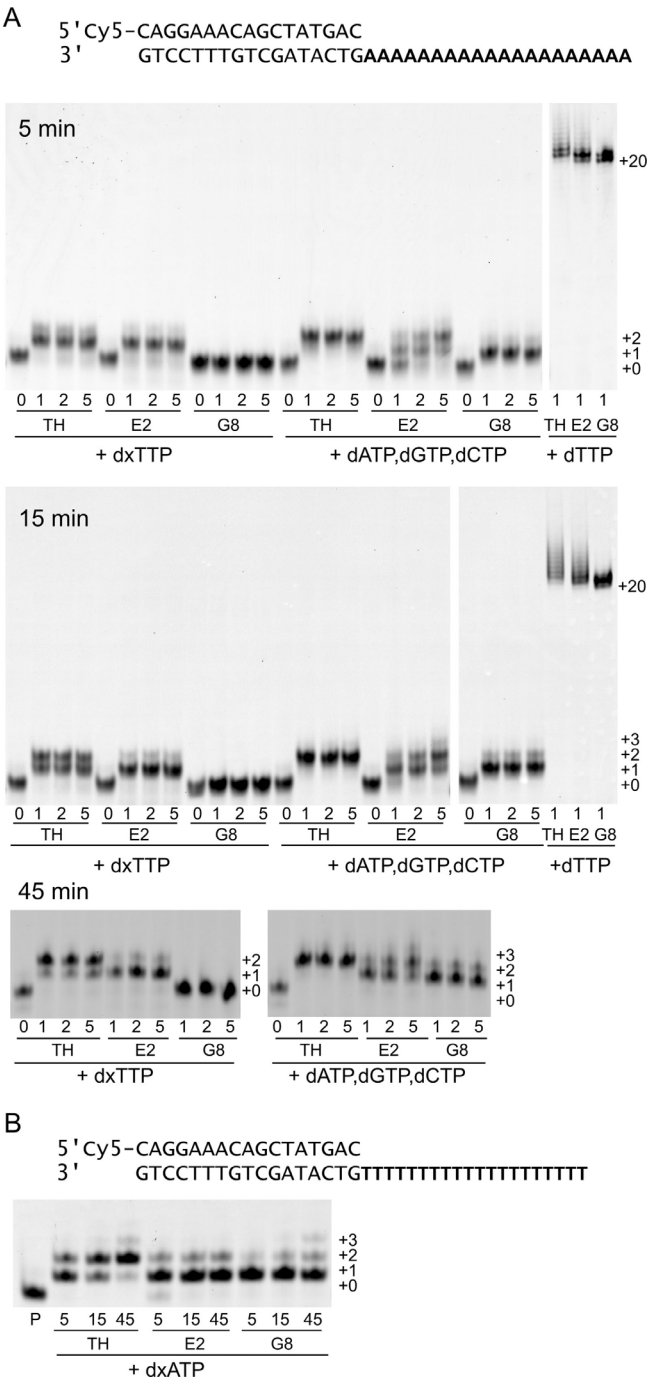


Figure 1. Effects of nucleotide type, nucleotide concentration and incubation time on primer elongation products from homopolymeric templates in the presence of Therminator *exo*$^-$ (TH) and mutant polymerases E2 and G8. A) Primer–template complex requiring incorporation of dxNT (template in bold). Elongation products after 5, 15 and 45 min. Lanes 0, 1, 2 and 5 are with dxTTP concentrations of 0, 100, 200 and 500 μM. Misincorporation controls are with dATP, dGTP and dCTP but without either dTTP or dxTTP. Positive controls (top right) with 100 μM dTTP. B) Primer–template complex requiring incorporation of dxNA (template in bold). Elongation products with 100 μM dxATP. Lanes 5, 15 and 45 are samples taken after 5, 15 and 45 min, respectively. P = unextended primer.

Our observation that extension was blocked after incorporation of two dxNTP units brought up the question of whether elongation stops because no more nucleotides can be incorporated beyond two consecutive dxNTs, or because the polymerase cannot catalyse the incorporation of three consecutive dxNTs. When templates that require not only the incorporation of two initial dxT units but also elongation with non-T dNTs

Elongation of two incorporated dxT units with dNTs

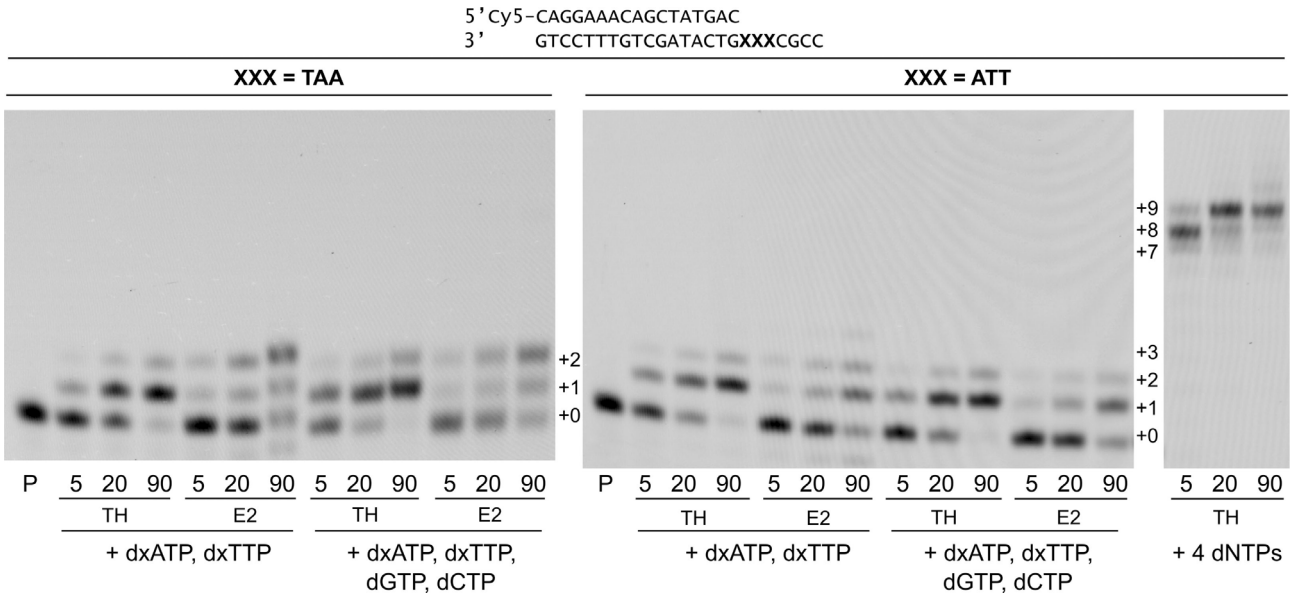


Figure 2. Primer elongation with both dxATP and dxTTP in the presence of wild-type Therminator *exo*⁻ (TH) and of mutant polymerase E2. The TAA template (XXX = TAA) was designed to allow incorporation of one dxA and two dxT units, the inverse with the ATT template. Reactions were performed with dxNTPs in the absence or in the presence of additional dGTP and dCTP. Samples were taken after 5, 20 and 90 min. Right: positive control with natural dNTPs. P = unextended primer.

were used, E2 showed a distinctly higher activity than Therminator *exo*⁻ (Figure 3). It took the wild type up to 45 min to catalyse the production of small amounts of full-length products, whereas with E2 some complete elongation was already evident within 5 min, with 38% of the primers being fully elongated after 45 min. However, full-length products were also produced in the absence of dTTP and dxTTP. This indicates that two consecutive mismatches did not stall either polymerase and raises the question of whether the full-length products observed in the presence of dxTTP were genuinely formed after dxT incorporation or were due to non-T mismatches (see further mass spectral analyses, which show the presence of dxNT-containing extended products).

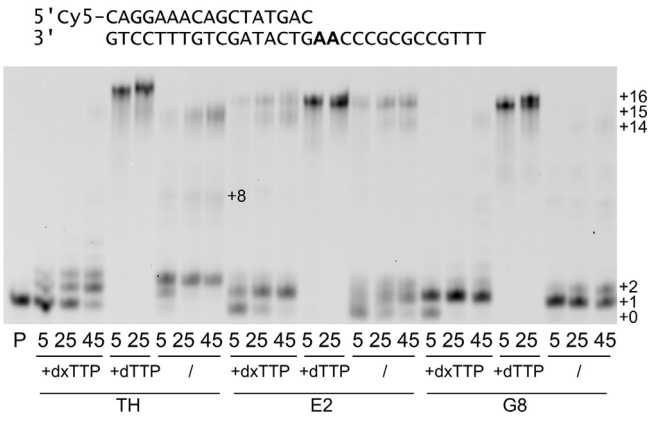


Figure 3. DNA elongation downstream of incorporated dxTTP in the presence variously of wild-type Therminator *exo*⁻ and of the E2 and G8 mutants. Samples were taken after 5, 25 and 45 min. P = unextended primer. All reactions were run in the presence of dATP, dGTP and dCTP. Either dxTTP, dTTP or no further nucleotides were added. Bold: templating bases requiring incorporation of dxNT.

Elongation with alternating dxNTs and dNTs

Because two consecutive dxNTs impede elongation with a third dxNT whereas extension with a large number of dNTs is possible, we tested how many local dxNT-induced distortions could be tolerated, and how closely they could be spaced. Elongation reactions that required the immediate incorporation of one or two dxNTs, followed variously by one, two, four or six natural nucleotides, another dxNT and a stretch of dNTs showed very little end product in the presence of the wild-type polymerase, whereas measurable amounts were indeed produced in that of E2 (Figure 4). Either dxTTP or dxATP was used as substrate, in combination with the other three normal dNTPs. dxA was incorporated and extended more efficiently, and the amount of full-length product was substantially increased, but the overall patterns were the same. Unextended +1 and +2 products were present in reactions catalysed by the E2 polymerase, but mostly in those catalysed by the wild-type polymerase, especially with dxTTP as substrate. Yields for the incorporation of a single dxNT were higher than those for the incorporation of two consecutive ones. However, increasing the distance to the next dxNT did not significantly affect full-length product yields; rather, the total number of dxNTs in the elongated sequence seems to be the determining factor for production of full-length products. Intermediate products accumulated at the first two incorporation steps, as well as at the step prior to incorporation of a subsequent dxNT.

To investigate the effects on polymerase activity of the number of nucleotides and of the distance between 2′-deoxyxylose-induced distortions, templates that required the incorporation of between one and five equally spaced dxNTs within a stretch of 10 nucleotides were used, followed by further incorporation of dNTs to a total length of +20 (Figure 4). Again,

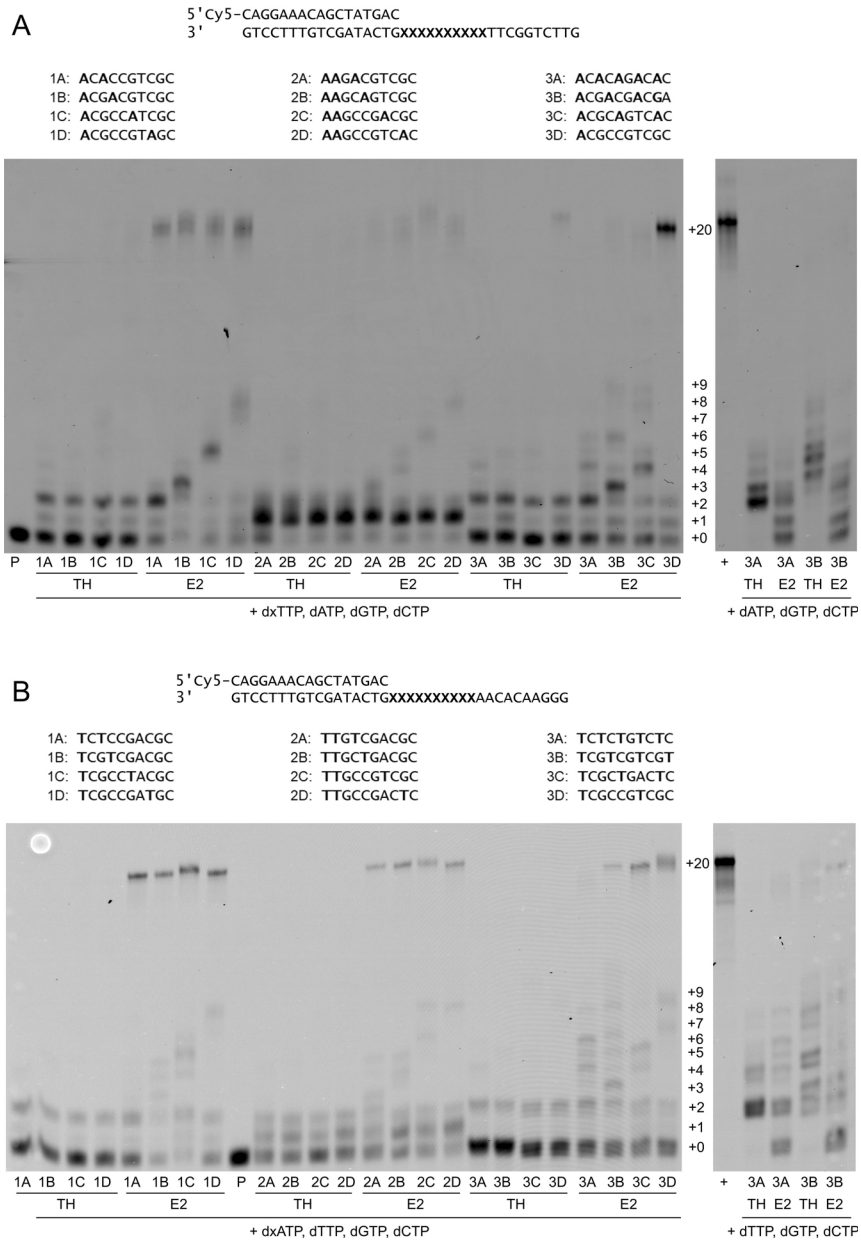


Figure 4. Elongation in the presence of polymerases Therminator *exo*⁻ (TH) and E2 with templates requiring mixed incorporation of dxNT and dNT units. Samples were taken after 90 min. A) Elongation with dxT. Templates 1A, 1B, 1C and 1D require incorporation of one dxT, followed by one, two, four or six dNT units, respectively, another dxT unit and dNT units to a total length of +20. Templates 2A, 2B, 2C and 2D require the same but with two initial dxT units instead of one. Templates 3A, 3B, 3C and 3D require incorporation of five, four, three or one dxT unit(s) over 10 bases, followed by 10 dNT units. B) Elongation with dxA, similarly to panel A, with the exception that template 3D requires two spaced dxA units instead of one. + positive control with TH and dNTPs. Misincorporation controls (right-hand gels in panels A and B) were performed without dxNTP in the presence of templates 3A and 3B.

dxATP appeared to be a better substrate than dxTTP. The main intermediate elongation products accumulated prior to incorporation of the second dxNT (roughly one third of the elongation products made through the action of E2 in the presence of dxTTP substrate after 90 min). For templates in which the dxNT spacing is close, intermediates were formed at nearly every incorporation step.

No full-length products were produced in the presence of the wild-type polymerase, except for small amounts (5%) from the least challenging template, requiring incorporation of one dxT unit, and neither were measurable amounts of intermediates longer than +5 produced. In contrast, full-length products were produced in the presence of E2 with all but the most demanding template (requiring five dxNT incorporations in close succession), although in very low amounts, probably in part the result of misincorporation elongation. Nevertheless, the synthesis of a dxNT-containing primer strand (as in reaction 3B, Figure 4B) implies the possibility of elongating and translocating a large amount of steric distortions. Although the data cannot conclusively show that this sequence is produced with high fidelity, they do show that E2 can nevertheless tolerate substantial distortions. In the case in which a single dxT unit

was to be incorporated, followed by 19 dNTs (Figure 4A, reaction 3D), the majority of primer molecules were fully elongated, thus showing that in a reaction very similar to that in CST selection, E2 readily catalyses the incorporation of a dxNT into DNA and clearly outperforms the wild type.

Terminal transferase activity

Because the full-length products often showed distinct bands that were longer than anticipated, and to find out if these polymerases are able to catalyse template-independent elongation, elongation reactions were performed with a blunt-ended primer–template complex (Figure 5). Reaction mixtures

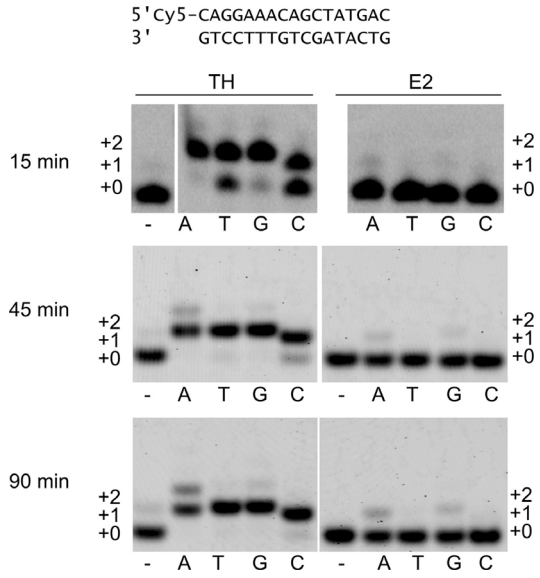


Figure 5. Terminal transferase activity in the presence of polymerase Therminator *exo*⁻ (TH) and of the E2 mutant with a blunt primer–template complex. dATP, dTTP, dGTP or dCTP (100 μM) was tested as substrate.

with each of the four natural nucleotides were incubated in parallel. In the presence of the wild-type polymerase the majority of the primers were already extended with an untemplated nucleotide within 15 min, the exception being dC, which was added to only half of the primers. In contrast, E2 took 45 min before visible +1 products were evident in the presence of dATP or dGTP, and these did not increase by much over the next 45 min. The wild-type polymerase, on the other hand, showed partial addition of a second purine nucleotide in the absence of a templating base after 90 min of incubation.

Specificity for 2′-deoxyxylose versus arabinose nucleotides

Successful selection on dxNT incorporation can result in increased specificity for dxNT but also in a more general broadening of promiscuous activity, while at the same time being constrained by the need still to be able to produce DNA. Previous results in our lab had shown promiscuous activity for ANA in the presence of Vent polymerase. We therefore tested incorporation of arabinonucleotides, which have a sugar pucker very similar to that of DNA (even though ANA is an RNA epimer). We expect that a tolerance mechanism that merely removes steric hindrance created by backbone distortions, thus making DNA–protein interactions less specific (e.g., by widening the groove through which the formed nucleic acid translocates), would also show improved activity towards other xenonucleotides that can be promiscuously incorporated through the action of the wild type. On the other hand, increasing specificity for dxNTPs can come at the expense of activity towards other (xeno)nucleotides. A homopolymeric polyT template (T_{20}) and a template with five T units alternating with single non-T units [$(TV)_5V_{10}$] were used to elongate a primer in the presence of arabinose adenosine triphosphate (araATP) and natural nucleotides as a control (Figure 6). With the template T_{20} in the presence of the wild-type polymerase, 15 to 18 araA units were readily incorporated within 15 min, whereas in the presence of E2 the process stalled after two, with incorporation of no more than four nucleotides. The ladder of products extending beyond the expected maximal length may be explained in terms of polymerase slippage through cycles of polymerase dissociation, followed by mismatched reannealing of the primer–template complex, polymerase reassociation and further elongation.[25,26] Reactions requiring either incorporation of five araA units alternated with a non-A dNT, or of araA units only, quickly yielded full-length products (both of expected length and slightly longer ones) in the presence of the wild type. On the $(TV)_5V_{10}$ template, E2 first showed accumulation of araA-containing +6 extension products, producing a majority of full-length products only after 90 min and being able to catalyse the incorporation of no more than four consecutive araA units on the T_{20} template.

dxNT versus mismatch incorporation

Mass spectrometry was used to distinguish genuine dxNT incorporation from mismatch incorporation. Templates required incorporation of one dxNT and nine dNTs. Because dxNTs and their natural isomeric counterparts cannot be distinguished by mass alone, none of the reaction mixtures in this study was incubated with both isomers combined. Therefore, masses consistent with the addition of a matching nucleotide in the absence of the natural one were used to verify incorporation of the correct dxNT indirectly. Elongation products were shorter than expected from PAGE gel results, presumably due to the much larger DNA-to-polymerase ratio in the reactions. Nevertheless, elongation products in which either dxA or dxT was incorporated and extended with the correct natural nucleotide were found (Figure S5). In the presence of an obligate dxTTP, the Therminator (*exo*⁻) polymerase catalyses purine misincorporations but in lower relative amounts than dxT incorporation (Figure S6). Such misincorporations were not observed with E2, or in the presence of an obligate dxATP with either polymerase. Mixtures of only three of the four dNTPs yielded misincorporation and elongation products to up to +7 nucleotides. All four bases were prone to misincorporation, with dA misincorporation dominating in the absence of d(*x*)TTP (Figure S6). Misincorporated dNTs were also less elongated in the presence

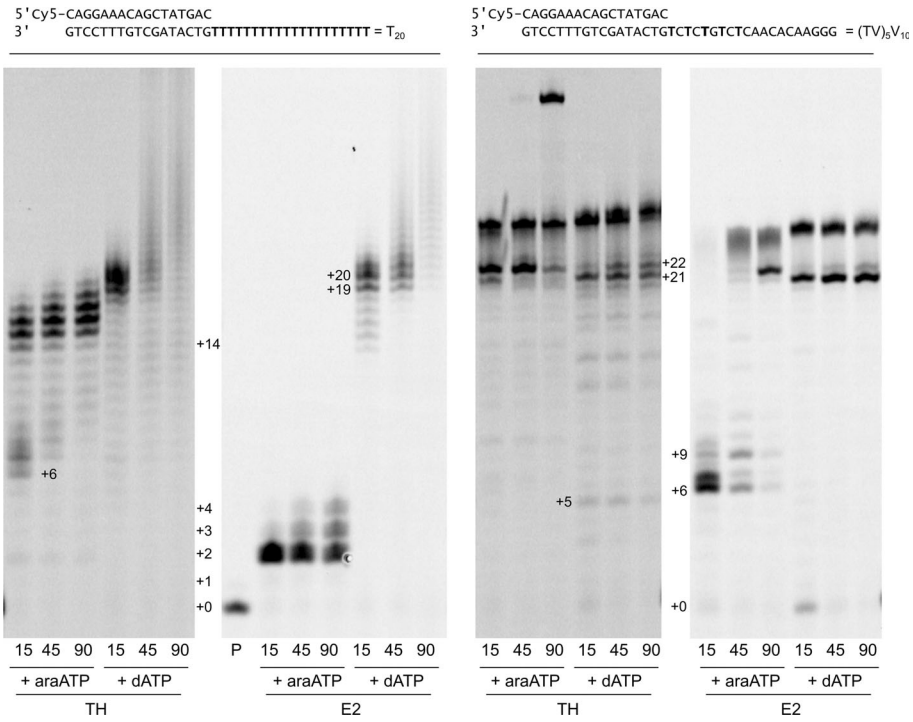


Figure 6. Specificity for arabinose adenosine triphosphate (araATP) versus natural dATP substrates of wild-type polymerase (TH) and the E2 mutant. dGTP, dTTP and dCTP were also added when templates $(TV)_5V_{10}$, with *V* = non-T, were used. Samples were taken after 15, 45 and 90 min. P = unextended primer.

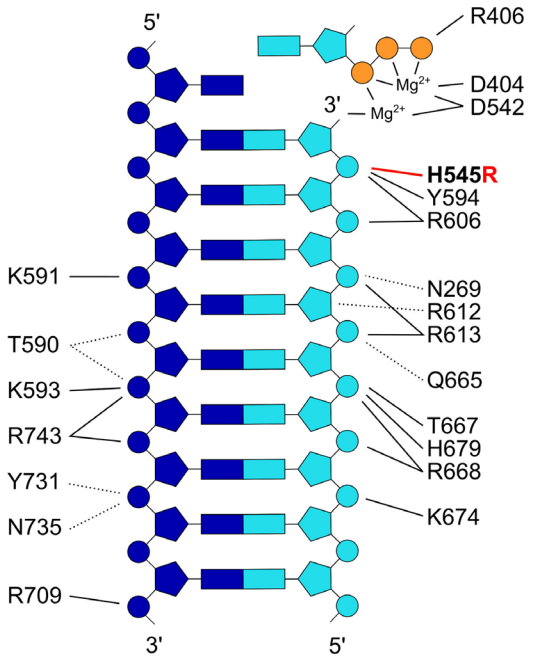
of E2 than in that of the wild type. Using the wild type in the presence of the four natural dNTPs produced mostly full-length product extended by one extra dA unit (Figure S6).

Discussion

Despite their chemical similarity to natural nucleotides, dxNTPs are challenging substrates for DNA polymerases. The sugar ring conformation in dxNA has an *N*-type/C3′-*endo* pucker, as in RNA and A-DNA, in contrast with the *S*-type/C2′-*endo* pucker of B-DNA.[27] The 3′-hydroxy group is inverted in relation to DNA (Scheme 1), and even though this structural difference from a dNTP is limited, they are still incorporated less efficiently in the presence of most tested polymerases.[20] We confirmed that the incorporation of a first dxT or dxA unit in the presence of the 9°N and related DNA polymerases is still relatively fast in comparison with the production of full-length products. The latter process can take up to 90 min to yield visible bands in PAGE, whereas substantial amounts of primer extended with at least one dxNT can already be observed in 5 min samples. Once incorporated and further extended by a second dxNT, these nucleotides would be expected to introduce a steric distortion in the backbone that would affect the position of the phosphate and the relative orientation of the sugar ring and nucleobase, potentially inducing additional distortions in upstream and downstream primer nucleotides through neighbour effects, as well as in the opposite template strand. This is presumably the main difficulty in elongation of a dxNA backbone through the action of a DNA polymerase (more so than the incorporation of the synthetic nucleotide substrates themselves). These distortions may interact with multiple residues in the DNA polymerase thumb domain during translocation. Because there are many points of contact between the thumb domain and the template and primer (Scheme 2), and the thumb domain wraps tightly around most of the duplex for roughly one helical turn, blocked translocation rather than incorporation of dxNTs can become the elongation-limiting step.

Bands corresponding to elongation products right below the dxNT incorporation band are often observed when a small number of dxNTs are spaced by at least three dNTs (Figure 4, templates 1C, 1D, 2C, 2D, 3C and 3D), thus indicating that elongation is limited by incorporation and not by extension of incorporated dxNTs. Stalling prior to dxNT incorporation would suggest poor nucleotide triphosphate binding due to the different sugar ring conformation and/or presence of a 3′-β-OH group. However, when the number of incorporated dxNTs increases and their spacing decreases to one or two dNTs (Figure 4, templates 3A and 3B), other intermediates become more abundant, even when dNTs are incorporated directly on dNTs. In this case, hampered translocation is also becoming a significant cause of stalling, due to increasingly larger helix backbone distortions and potential steric clashes. Intermediates formed at consistent distances from an incorporated dxNT could be explained in terms of steric checkpoints where a backbone distortion is most difficult to translocate.

E2 is capable of catalysing the incorporation of up to four dxA units if each is spaced apart by two dNTs, and still producing small amounts of full-length products, thus implying translocation of the distortions throughout the polymerase. Incorporation of a dxA-dNT tandem series stalled after extension of the fourth dxA unit, with failure to incorporate a fifth dxA unit.



Scheme 2. Diagram showing interactions between a primer–template DNA duplex and Therminator DNA polymerase, based on a 9°N crystal structure (PDB ID: 4K8X). Dark blue: template. Light blue: primer. Orange: phosphate groups of the nucleotide substrate. The primer 3′-OH group and the incoming nucleotide are organized by a network consisting of amino acid residues, catalytic Mg^{2+} ions, the triphosphate group and water molecules (not shown). The DNA duplex is tightly bound by the polymerase for roughly one helical turn, mostly by positively charged residues from the thumb domain. Solid lines: hydrogen bonds and ionic interactions. Dotted lines: polar contacts. The side chain of the mutated Arg residue (red) in the polymerase E2 mutant is appropriately positioned to produce a potent additional interaction with the primer phosphate backbone.

These results indicate that a double-stranded hybrid dxNA/DNA with at least four closely spaced backbone distortions can be produced. This is remarkable in view of the expected strong backbone distortions in this nucleic acid. After all, when in 9 bp DNA duplexes three deoxyribose moieties are replaced by 2′-deoxyxylose, the T_m becomes so low that there is no longer a melting curve for the duplex,[28] meaning that these strands do not hybridize in solution. Similar results, involving a polymerase being able to catalyse the elongation of a primer–template complex that is not stable/hybridized in solution, were obtained by Tsai et al. with Therminator for DNA polymerization on a GNA template[29] and by Liu et al. for tPhoNA synthesis and reverse transcription in the presence of a TgoT mutant.[17] It cannot be deduced from our data whether the distorted primer–template complex is translocated while bound to the polymerase by processive elongation, or whether the polymerase repeatedly dissociates and rebinds, forcing the reannealing of the primer 3′ end, although advanced dissociation is expected because of the low enzyme concentration used, the absence of processivity factors and the many instances of forced stalling. In either case, the polymerase has to provide strong stabilization of a deformed duplex.

In the incorporation of two consecutive dxNTs, formation of the +2 product would imply incorporation of a dxNT as well as its extension with a dxNT. Stalling at +2 is seen for every template that requires either one or two initial dxNTs; this implies that the last phosphodiester group of the primer (Scheme 2) is specifically affected by a newly incorporated and extended dxNT. We therefore hypothesize that it is a main steric checkpoint. In the crystal structure, this phosphate group is in very close contact with residues H545, R606 and Y594 (Figure 7). H545 is packed in a hydrophobic pocket with several aromatic residues and interacts indirectly with the backbone through hydrogen bonding with water molecules and residue Y594. In polymerase variant E2, the H545R substitution appears to tolerate larger backbone distortions, as well as to reduce misincorporation and template-independent elongation, in spite of slower initial incorporation of dxNTs than in the case of the wild type.

We presume that the positive charge of R545 is positioned very close to this primer phosphate group. Because R545 may undergo aromatic stacking with Y402, this could also change the interactions with other residues and water molecules near or in the active site. Because Arg has a longer side chain, offers better interaction geometry with phosphates and has a strong positive charge, it is presumed that this interaction with the most distortion-sensitive phosphate group stabilizes the +2 product by keeping the phosphate group, and thereby indirectly also the 3′-hydroxy group, closer to the conformation needed for extension, rather than directly improving specificity for dxNT binding and incorporation. The modelling of H545R suggests that it takes the place of two water molecules next to Y402, adopting their polar interactions but bringing the posi-

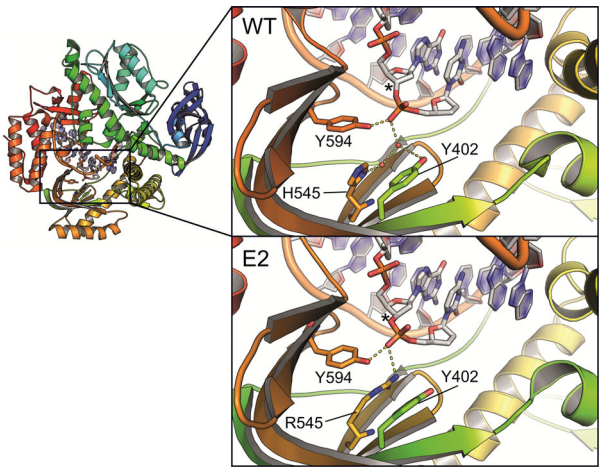


Figure 7. Diagram showing the interaction between the primer backbone and residue 545 near the active site of the 9°N DNA polymerase crystal (PDB ID: 4K8X) and the modelled structure of the dxNT complex, rainbow coloured from blue N-terminal domain to red C-terminal thumb domain. Top: interaction between the wild-type (WT) H545 and 2′-deoxyribose backbone. Bottom: interaction between the E2 mutant R545 and 2′-deoxyxylose backbone. An asterisk indicates the inverted chiral centre of the backbone sugar at position +2. This model was created with MOE (CCG, Canada) implemented with the AMBER:EHT force field. The dxNT was modelled by inverting the stereochemistry of the 3′-carbon–oxygen bond in the crystal structure of the wild-type complex (PDB ID: 4K8X). The H545R mutation was introduced, followed by conjugated gradient energy-minimization of the residues in a radius of 10 Å around the R545 guanidinium head.

tive charge much closer to the phosphate group. Whereas the wild type can efficiently catalyse the incorporation of two consecutive dxNTs but stalls before further extension, the H545R mutant catalyses the incorporation of dxNTs more slowly and is able to extend them, albeit more readily with natural nucleotides.

Other possible effects of the H545R substitution are a general change in protein dynamics that shifts the equilibrium more towards the closed state or facilitating translocation, or more large-scale secondary structure rearrangements that change the DNA/nucleic acid interface or active site geometry, especially in the thumb domain where most of the interactions between the DNA and protein are located. Whatever the mechanism of action, it must be noted that the H545R mutant remains an efficient, accurate and thermostable DNA polymerase.

In archaeal family B polymerases, residue 545 (relative to Therminator) is represented by a varying aromatic residue (His, Tyr or Phe) within an otherwise very strongly conserved active site (Figure S3). The precise aromatic residue varies both within and between clades of 51 family B polymerases over a large phylogenetic distance. This implies there is some mutational freedom at this position, as long as it does not destabilize existing interactions with the conserved residue Y594 and other nearby aromatic residues. When this residue is mutated to Lys (data not shown), polymerase activity assays show it to be still active as a polymerase, but less so than the E2 clone, and only for dNTs, thus implying that merely a positive charge on a long flexible chain is not sufficient to elicit the improved phenotype for dxNT incorporation and elongation. To the best of our knowledge, mutation of this residue in polymerases evolved to incorporate xenonucleotides has not been reported so far.

With regard to fidelity, the H545R mutant appears to be more accurate than the wild type, at least under the limited tested conditions. The latter readily catalyses the addition of one, and eventually a second, untemplated terminal nucleotide (with a large preference for purines), to a blunt-ended DNA duplex, whereas H545R is nearly inactive. Forced misincorporations (caused by the absence of one or more substrate nucleotide types) happen at lower relative rates in the H545R mutant, and although intermediates accumulate, it takes several consecutive misincorporations to stall the polymerases completely and prevent full elongation. Whether this lower mismatch incorporation rate in the E2 polymerase is due to an overall lower intrinsic rate of the enzyme, or whether mismatch incorporation rates have been lowered relative to canonical incorporation rates, cannot be deduced from these data because rates of dNT incorporation are comparatively fast and full-length products are formed within 5 min.

Although misincorporation was observed by MS in the presence of canonical dxNTPs as well (Figure S5), the spectra indicated that canonical dxNT incorporation dominates, especially for the E2 mutant and with dxATP as the modified substrate. Because a large fraction of primers reaches full elongation from templates requiring one dxNT to be incorporated, the majority of these full-length products are therefore presumed to contain a correctly templated and fully translocated dxNT rather than dxNTs acting as extension or elongation terminators. As expected, no misincorporation downstream of a correctly incorporated dxNT was observed, thus implying that the suboptimal orientation and pucker of the 2′-deoxyxylose sugar group slow down extension but do not negatively affect extension fidelity. It has been suggested that sugar conformation and mispair geometry are independent,[30] so subsequent nucleotides can still be accurately matched to the template and properly stack on the nucleobase of the previously incorporated dxNT.

The promiscuity of the E2 mutant for other xenonucleotides was tentatively explored by comparing incorporation of arabinose- with 2′-deoxyxylose-based nucleotides. The H545R mutation caused a substantial decrease in incorporation activity for araA both on homopolymeric dT templates and on templates with dT units spaced by a few natural nucleotides. This suggests that the tolerated distortions could be more specific to 2′-deoxyxylose, rather than tolerating distortions in general. Evolution towards 2′-deoxyxylose-based nucleotides and away from 2′-deoxyribose therefore presumably comes with a trade-off for arabinose too. This difference between distortion tolerances is consistent with an increased preference for dxNA (and potentially other, structurally similar XNAs), and contradicts a mechanism of merely opening the thumb and thereby avoiding steric clashes by creating a wide, nonspecific tunnel that indiscriminately assists extension with everything it can incorporate. This could be tested further with xenonucleotides that have pseudorotation phase angles which are different from both DNA and dxNA such as FRNA or TNA,[9] as well as those that have modified nucleobases rather than modified backbones.

Conclusion

In summary, a polymerase capable of catalysing improved elongation with accurately incorporated dxNTs, thanks to a single H545R amino acid substitution, was isolated by CST. Many substitutions in published polymerases that catalyse the incorporation of synthetic nucleotides with sugar-modified backbones are found in the thumb domain and, when present in or near the active site, are rarely in direct contact with the nucleic acid. This makes the residue at this position, and the other residues that directly or indirectly interact with it, promising candidates for further targeted mutagenesis to evolve polymerases that not only catalyse the incorporation of modified nucleotides but are also able to catalyse their extension, with the goal of efficient enzymatic transcription of DNA into long-chain dxNA.

Experimental Section

Materials used: All oligonucleotides and a codon-optimized gene for Therminator DNA polymerase *exo*$^-$ were purchased from Integrated DNA Technologies (IDT). dxTTP and dxATP were synthesized as described.[27] Anhydrotetracyclin (AHT) and pASK-IBA2 vector were purchased from IBA Life Sciences. Electrocompetent E. cloni

cells were purchased from Lucigen (Middleton, WI, USA). Biotinylated dCTP was purchased from Jena Bioscience (Jena, Germany).

Mutant libraries: The synthetic gene was subcloned in a pASK-IBA2 plasmid derivative kindly provided by Misha Soskine. Mutant libraries were constructed by use of inverse PCR with partially degenerate spiked primers. Every nucleobase within a targeted region had a 3% chance of being mutated into one of the other three bases. Seven positions were partially randomized; the two catalytic aspartate residues were not subject to targeted mutation. Nucleotide substitutions within the active site library (L537 to T547) were calculated to average around 2.4 per mutant, resulting in an average of 1.8 amino acid substitutions within this nine-amino acid region. Library diversity was estimated at 8×10^5. The minor-groove-binding region library spanned residues F588–V596 and T605–E617.

Compartmentalized self-tagging (CST): Selection was performed by use of CST as described by Pinheiro et al.,[24] but in the absence of misincorporation-promoting Mn^{2+} ions, and labelling by incorporation of biotinylated dC during extension. An oil phase [mineral oil with Span 80 (4.5%), Tween 80 (0.4%) and Triton X-100 (0.05%)] and aqueous phase [Thermopol buffer (New England Biolabs, 1×), $MgSO_4$ (1 mM), biotinylated dCTP (20 μM), equimolar dATP/dGTP mix (200 μM), dxTTP (200 μM), primer (0.38 μM), bovine serum albumin (BSA, 1 mg mL^{-1}), dithiothreitol (DTT, 1 mM), glycerol (10% *v/v*)] were emulsified in a 4:1 ratio by adding the aqueous phase dropwise to the oil phase while stirring with a small magnet.

Plasmid libraries were transformed in electrocompetent E. cloni and plated on lysogeny broth (LB) agar with ampicillin (Amp). Colonies grown overnight were harvested by adding LB medium and gently scraping them from the plates. The suspension (≈30 mL, ≈30 OD) was mixed with glycerol (60%) in a 3:1 ratio and flash-frozen in aliquots (100 μL). One aliquot was used to inoculate a starting culture (4 mL) grown overnight (37 °C, 200 rpm). Overnight culture (50 μL) in LB + Amp medium (4 mL) was grown at 37 °C, 200 rpm to an OD_{600} of 0.7, induced with AHT and grown at 25 °C overnight. Cultures were diluted to an OD_{600} of 1, cell suspension (100 μL) was centrifuged at 12000 *g*, and supernatant was removed. These cells were added to the aqueous phase before emulsification.

The elongation reactions were run for 10 min at 95 °C, 15 min at 58 °C and 15 min at 72 °C. The primer for the first round of CST required incorporation of GAAAAG**xT**GC-biotin. In the second round the selection pressure was increased by requiring incorporation of AAAAAGGGAA**xT**AAGGGC-biotin (Figure S7).

After CST, the emulsion was broken by centrifugation at 13000 *g* for 5 min and two water-saturated diethyl ether extractions. Primer–plasmid complexes were purified by use of spin columns (GE Healthcare S400 microspin columns) and incubated with magnetic beads (NEB streptavidin magnetic beads, 4 mg mL^{-1}) for at least 1 h at room temperature on a rolling platform. The beads were washed twice with binding buffer [NaCl (0.5 M), Tris·HCl (20 mM), EDTA (pH 7.5, 1 mM)] and once with low-salt buffer [NaCl (0.15 M), Tris·HCl (20 mM), EDTA (pH 7.5, 1 mM)] and eluted in elution buffer [25 μL of Tris·HCl (10 mM), EDTA (pH 7.5, 1 mM)]. Recloning was done by PCR amplification of the variable half of the Therminator gene with use of the eluted fraction (2 μL) as template and PCR amplification of the nonvariable half of Therminator and the rest of the plasmid backbone from the original wild type, followed by restriction digestion and ligation of both fragments to reconstitute the original plasmid.

Polymerase activity assay: After selection, libraries enriched in mutants with improved incorporation activity were screened by use of a previously described polymerase activity assay.[16] Recloned plasmid libraries were transformed in *E. cloni* (Lucigen) by use of electroporation and plated on LB + Amp agar plates. Colonies were inoculated in a 96-well plate with deep, rounded wells. Cells were grown in 400 μL cultures and induced to express the polymerase with AHT at 25 °C and 200 rpm overnight, and lysates were prepared for each mutant by heating for 20 min at 80 °C, centrifugation and resuspension in Thermopol buffer [1×, pH 8.8, Tris·HCl (20 mM), $(NH_4)_2SO_4$ (10 mM), KCl (10 mM), $MgSO_4$ (2 mM), Triton X-100 (0.1%)]. The elongation reaction uses the cell lysate as a source of polymerase, and a biotinylated primer is elongated with a template that requires incorporation of the sequence AAGGGAA**xT**AAGGGC with use of dATP, dGTP, dCTP and dxTTP, followed by a non-T sequence that will hybridize with a DNA probe (through an intermediary linker). Polymerase mutants that succeed in incorporation of dxTTP opposite the templating adenine and elongation past the resulting distortion produce elongated primers that can bind this DNA probe, which carries a digoxigenin group. After binding of the primers to a streptavidin-coated 96-well plate, duplex denaturation and washing with phosphate-buffered saline with Tween 20 [PBST, pH 7.2, NaCl (125 mM), Na_2HPO_4 (16.6 mM), NaH_2PO_4 (8.43 mM), Tween 20 (0.2% *v/v*)], they were incubated with an anti-digoxigenin antibody fused to horseradish peroxidase (HRP, dilution 1:10000). After nonspecifically bound probes and antibodies had been washed away with PBST, the chromogenic substrate 3,3′,5,5′-tetramethylbenzidine (TMB, 100 μL) was added; this turns blue in the presence of HRP. The OD_{450nm} is an indication of the amount of fully elongated primers, and stronger signals correlate with more active polymerase-containing lysates.

Polyacrylamide gel electrophoresis (PAGE): Elongation of 5′ Cy5-tagged fluorescent primers was performed with templates that introduce different or increasing requirements for the DNA/dxNA polymerases. Reaction mixtures [20 μL, Thermopol buffer (1×), $MgSO_4$ (1 mM), template (250 nM), primer (125 nM), d(x)NTPs (100 μM unless specified otherwise), purified polymerase (2 μL)] were incubated in a T300 Thermocycler (Biometra, Göttingen, Germany) at 72 °C, and samples (5 μL) were taken at indicated times after a short spin with a table-top centrifuge and quenched in quenching buffer [10 μL, formamide (95%), EDTA (18 mM), sodium dodecyl sulfate (SDS, 0.025%), Bromophenol Blue (0.05%)]. Denaturing polyacrylamide gels were prepared with acrylamide (15%) and urea (7 M) in Tris/borate/EDTA (TBE) buffer [pH 8, Tris (89 mM), boric acid (89 mM), EDTA (2 mM)] with ammonium persulfate (APS, 0.08%) and tetramethylethylenediamine (TEMED, 0.04%). Samples of 2 μL were loaded (estimated to contain roughly 70 μmol primer per sample). Constant currents were set at 25 (single gel) or 50 mA (two gels). The gel dimensions were 20 cm × 30 cm × 1 mm. Gels were imaged with a Typhoon FLA 9500 instrument (GE Healthcare Life Sciences) at 635 nm.

Protein purification: *Escherichia coli* cell cultures containing the pASK-IBA plasmid encoding the Therminator *exo*$^-$ wild type or mutant polymerase were grown in LB with ampicillin (4 mL) at 37 °C, 200 rpm up to an OD_{600} of 0.7. Expression was induced with AHT, and cultures (100 mL) were grown overnight in LB with ampicillin at 25 °C. These were then centrifuged at 3400 *g* in Falcon tubes (50 mL) for 12 min. The cell pellet was resuspended in phosphate buffer (pH 7.5, 50 mM, 30 mL) with $MgCl_2$ (1 mM) and DNaseI (20 μL, 1 U μL^{-1}), frozen at −80 °C for 20 min and thawed at 37 °C, lysozyme (≈10 mg) and Triton X-100 (60 μL) were added, and the mixture was incubated at room temperature for 30 min. This sus-

pension was then sonicated twice for 90 s and centrifuged at 4 °C at 3400 *g*. The supernatant was heated to 80 °C for 20 min and centrifuged (15 min at 4 °C, 3400 *g*). His-tag-carrying polymerases were purified from the supernatant by use of Ni-NTA resin in PD miniprep G-25 columns. Expression levels were estimated by use of SDS-PAGE, and relative concentrations (mass per volume) were normalized by dilution to roughly 25 μg mL^{-1}.

Mass spectrometry: Elongation reactions (each 80 μL) were run for six cycles of 30 min at 72 °C and 1 min at 30 °C, with primer (5′-CAGGA AACAG CTATG AC-3′, 20 μM), template (5′-CGCTG CCGCA GTCAT AGCTG TTTCC TGCC-3′ for dxTTP, 5′-CGCAG CCGCT GTCAT AGCTG TTTCC TGCC-3′ for dxATP, 20 μM), d(x)NTPs (100 μM), Thermopol buffer), $MgSO_4$ (1 mM) and purified polymerase (8 μL). Samples were purified twice by ethanol purification.

Electrospray ionization mass spectra were obtained in negative-ion mode with a quadrupole/time-of-flight mass spectrometer (Synapt G2, Waters, Milford, MA) equipped with a standard ionization source. The instrument was tuned to a resolution of 15 000 (full peak width at half maximum) and the mass accuracy of the instrument was 1 ppm or better with use of leucine enkephalin as internal calibrant (lock mass). Masses for the oligonucleotides were obtained by deconvolution of the spectra with use of the MaxEnt algorithm of the software (MassLynx 4.1, Waters).

High-performance liquid chromatography (HPLC): HPLC on a C_{18} reversed-phase column (PepMap 0.5×15 mm, LC Packings, Amsterdam, The Netherlands) was performed with a buffer containing *N*,*N*-dimethylaminobutane (DMAB, Acros, Geel, Belgium) as ion pairing reagent and 1,1,1,3,3,3-hexafluoropropan-2-ol (hexafluoroisopropanol, HFiP, Acros, Geel, Belgium). In brief, the solvent system consisted of acetonitrile (Fisher Scientific, Loughborough, UK, 84%, *v*/*v*) as organic phase and DMAB (0.05%, *v*/*v*) with HFiP (1%, *v*/*v*) in water as aqueous phase (pH 8.0). Oligonucleotides were eluted with a flow rate of 12 μL min^{-1} by application of a gradient starting at 2% organic phase and increasing by 2% min^{-1} for 15 min. The concentration of the oligonucleotide samples was around 10 μM, and between 30 and 480 pmol product was injected per run.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: deoxyxylonucleic acids · synthetic biology · transferases · xeno nucleic acids · XNA polymerases

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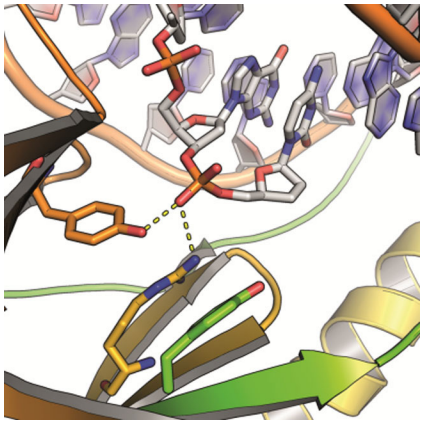
A Single Amino Acid Substitution in Therminator DNA Polymerase Increases Incorporation Efficiency of Deoxyxylonucleotides



Point of contact: A synthetic nucleic acid in which the deoxyribose component is substituted by deoxyxylose in the sugar–phosphate backbone poses a great barrier to replication through the action of natural DNA polymerases. A key mutation in the polymerase, near the active site, that gives rise to more efficient and specific incorporation of deoxyxylose-based nucleotides into a hybrid XNA/DNA duplex has been identified.