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Investigation of the DNA-dependent cyclohexenyl nucleic acid polymerization and the cyclohexenyl nucleic acid-dependent DNA polymerization

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ABSTRACT

DNA polymerases from different evolutionary families [Vent (exo−) DNA polymerase from the B-family polymerases, *Taq* DNA polymerase from the A-family polymerases and HIV reverse transcriptase from the reverse transcriptase family] were examined for their ability to incorporate the sugar-modified cyclohexenyl nucleoside triphosphates. All enzymes were able to use the cyclohexenyl nucleotides as a substrate. Using Vent (exo−) DNA polymerase and HIV reverse transcriptase, we were even able to incorporate seven consecutive cyclohexenyl nucleotides. Using a cyclohexenyl nucleic acid (CeNA) template, all enzymes tested were also able to synthesize a short DNA fragment. Since the DNA-dependent CeNA polymerization and the CeNA-dependent DNA polymerization is possible to a limited extend, we suggest CeNA as an ideal candidate to use in directed evolution methods for the development of a polymerase capable of replicating CeNA.

INTRODUCTION

CeNA (cyclohexenyl nucleic acid) is a DNA mimic in which the deoxyribose is replaced by a six-membered cyclohexene ring (1–4) (Figure 1). Although CeNA is a larger molecule than DNA (as the oxygen atom of every sugar moiety of the nucleotide is replaced by an ethylene group), the flexibility of the cyclohexene ring system is comparable to that of the natural (deoxy)ribose furanose ring system. A cyclohexenyl nucleoside can adopt two half-chair conformations, the $^3H_2$ and the $^2H_3$ conformation, similar to the C3′- and C2′-endo conformations found in natural (deoxy)ribose, with a preference of the cyclohexenyl monomer for the $^3H_2$ conformation (1). The cyclohexenyl nucleoside is able to adopt different conformations when incorporated in different double-stranded DNA sequences, which proves the flexibility of the cyclohexene ring system (5). Since cyclohexenyl nucleosides lack the 2′-OH they can be considered as a DNA mimic. Based on their preference for the $^3H_2$ conformation they might be considered as an RNA mimic. Hybridization of CeNA with both DNA and RNA has been observed (1,6) and introducing cyclohexenyl nucleotides into the DNA strand of a DNA/RNA hybrid increases the thermal stability of the duplex. CeNA is also stable against degradation in serum (1). These observations were the basis for the investigation of CeNA in siRNA experiments and encouraged us to synthesize the triphosphates of cyclohexenyl nucleosides and test them as a substrate for different DNA polymerases.

DNA polymerases incorporate their natural substrates with high specificity and fidelity. However, different research groups have shown that polymerases can also tolerate a broad range of modified nucleotides as a substrate. The enzymatic incorporation of nucleotide analogs has proven useful for DNA labeling (7), to tag DNA with additional functionality (8,9) or for DNA sequencing (10). Efforts made to expand the genetic alphabet have led to the investigation of different unnatural base pairs as substrates for polymerases (11,12). Several nucleotides modified at the sugar part or the sugar–phosphate backbone have also been reported to be recognized and replicated by DNA polymerases (13–16), RNA polymerases (17,18) and reverse transcriptases (19–22). However, the enzymatic synthesis of modified nucleic acids of any substantial length has proven to be difficult due to the high substrate specificity of natural polymerases. Previously several researchers also reported enzymatic DNA synthesis when a non-standard nucleotide was incorporated in the template strand (23–25).

Here, we investigated the recognition of CeNA by DNA polymerases on different levels. We used cyclohexenyl nucleoside triphosphates as a substrate for polymerases and we checked whether DNA or CeNA polymerization is possible when a CeNA template was used. These assays were

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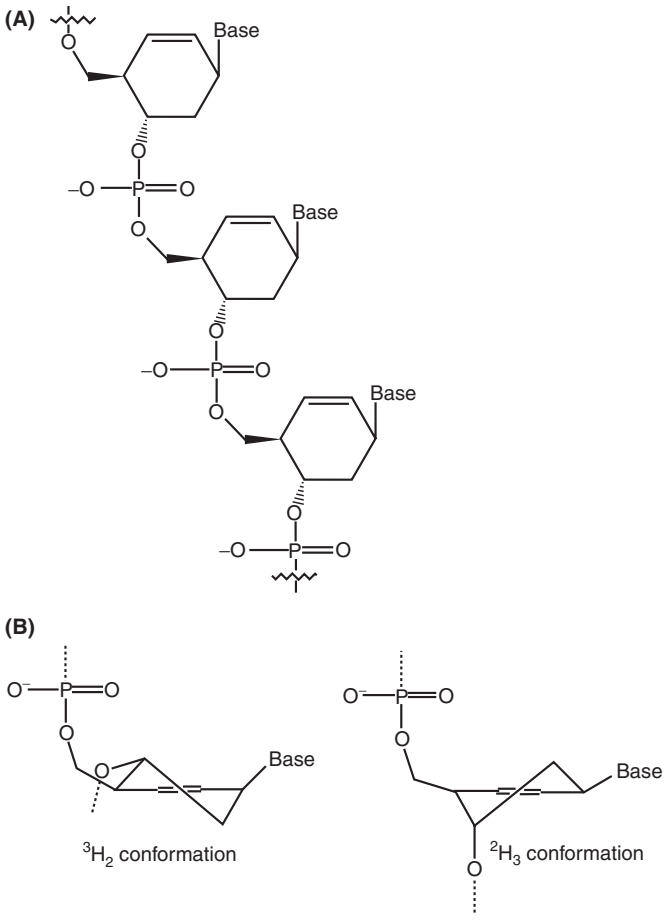

Figure 1. Cyclohexenyl nucleic acid. (**A**) Chemical structure of CeNA (bases are either adenine, guanine, cytosine or thymine). (**B**) $^3H_2$ and $^2H_3$ conformation of cyclohexenyl nucleosides.

performed to investigate whether CeNA replication could be possible. The *in vitro* synthesis of CeNA oligomers starting from a DNA template or the DNA synthesis starting from a CeNA template would be the first step toward the generation of an *in vitro* replicating system based on an artificial genetic system. We propose an artificial nucleic acid structure modified at the sugar moiety, as the base pairing system is too crucial for the function of a genetic heritable system. Earlier directed evolution methods have been used to generate a polymerase capable of using 2′-*O*-methyl-modified nucleotides as a substrate (26). However, we prefer to establish the enzymatic synthesis of a novel nucleic acid structure in which the five-membered ring is replaced by a six-membered ring. By introducing a larger difference in the sugar moiety, we eventually hope to establish a full self-autonomic system (and not a polymerase that recognizes both CeNA and DNA). Hereto, we first want to investigate the substrate capacity of CeNA by natural polymerases, to identify candidate polymerases that in future experiments will be used for the directed evolution of a CeNA polymerase. The replication of a third type of nucleic acid could lead to numerous advances in various domains. The bulk production of CeNA for diagnostic or therapeutic purposes would become available and polymerases that recognize non-canonical substrates can play a role in different techniques used in molecular biology.

MATERIALS AND METHODS

DNA polymerase reactions, kinetic measurements, analysis by NMR spectroscopy and mass spectrometry, as well as high-performance liquid chromatography purification, PAGE and radioactive labeling of oligonucleotides were performed as described previously (27).

Oligodeoxyribonucleotides

Oligodeoxyribonucleotides were purchased from Eurogentec. Oligodeoxyribonucleotides containing a cyclohexenyl nucleotide were synthesized on an Applied Biosystems 392 DNA synthesizer at a 1 μmol scale using phosphoramidites that were synthesized according to the published procedures (28).

Synthesis of cyclohexenyl nucleoside triphosphate

Cyclohexenyl-guanine nucleoside triphosphate and cyclohexenyl-adenine nucleoside triphosphate were synthesized according to the one-pot synthesis described by Ludwig (29). The nucleoside triphosphates were purified on a DEAE Sephadex-A25 column using a triethylammonium bicarbonate gradient.

Cyclohexenyl-guanine triphosphate. ^{31}P NMR (CeGTP) δ (ppm) (D_2O) −10.331 (d, γ-P), −10.402 (d, α-P), −22.778 (t, β-P). Exact mass calculated for $C_{12}H_{18}N_5O_{11}P_3$ [M − H] = 516.00865; found 516.0079.

Cyclohexenyl-adenine triphosphate. ^{31}P NMR (CeATP) δ (ppm) (D_2O) −10.262 (α- and γ-P), −22.401 (t, β-P). Exact mass calculated for $C_{12}H_{18}N_5O_{12}P_3$ [M + H] = 502.02938; found 502.0296.

DNA polymerase reactions

Vent (exo−) DNA polymerase (New England Biolabs), HIV reverse transcriptase (Amersham Biosciences) and Super *Taq* DNA polymerase (HT Biotechnology Ltd) were used in the primer extension reactions. For Vent (exo−) and *Taq* DNA polymerase, the supplied reaction buffers were used. For Vent (exo−) DNA polymerase: 20 mM Tris–HCl, pH 8.8, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100. For *Taq* DNA polymerase: 10 mM Tris–HCl, pH 9, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.01% (w/v) stabilizer. For HIV reverse transcriptase: 50 mM Tris–HCl, pH 8.3, 25 mM KCl, 5 mM $MgCl_2$, 0.5 mM spermidine and 5 mM DTT. The reactions were incubated at 55°C for Vent (exo−) DNA polymerase and *Taq* DNA polymerase and incubated at 37°C for HIV reverse transcriptase.

If indicated, the reaction buffer was supplemented with 1 mM $MgCl_2$. An overview of the primers and templates used in the primer extension reactions is given in Table 1.

Kinetic experiments

The single completed hit model described by Creighton *et al*. (30,31) was used to relate the intensity of the bands on the gel to the kinetic parameters for DNA polymerase incorporation. For incorporation kinetics of a first cyclohexenyl nucleotide, a DNA primer and DNA template were used (hybrid P_1T_5 for dATP and CeATP incorporation kinetics, hybrid P_1T_2 for dGTP and CeGTP incorporation kinetics). For dNTP incorporation kinetics 0.0005 U/μl Vent (exo−) DNA polymerase

Table 1. Sequences of the primers and templates used in the polymerase incorporation experiments

Primers	5′	CAGGAAACAGCTATGAC	3′	P_1
	5′	CAGGAAACAGCTATGACT*	3′	P_2
Templates	3′	GTCCTTTGTCGATACTG**TTTTTTT**	5′	T_1
	3′	GTCCTTTGTCGATACTG**CTTTT**	5′	T_2
	3′	GTCCTTTGTCGATACTG**CCTTT**	5′	T_3
	3′	GTCCTTTGTCGATACTG**T*T*T*T*T*T***	7′	T_4
	3′	GTCCTTTGTCGATACTG**TCCCC**	5′	T_5
	3′	GTCCTTTGTCGATACTG**ATCCCC**	5′	T_6

T* indicates a CeT nucleotide.

was used, and for CeNTP incorporation kinetics 0.002 U/μl was used. For incorporation kinetics of a second cyclohexenyl nucleotide, we used a chimeric DNA/CeNA primer with at the 3′ end a cyclohexenyl nucleotide (hybrid P_2T_6). For dATP incorporation kinetics 0.001 U/μl Vent (exo−) DNA polymerase was used; for CeATP incorporation kinetics 0.005 U/μl enzyme was used. In all experiments, triphosphate building block concentrations were chosen in a range from 0.39 to 12.5 μM and reaction times were between 1 and 8 min.

Molecular modeling

A model was constructed starting from the HIV-1 reverse transcriptase crystal structure, PDB entry 1rtda (32). After generation of the charges using gamess (33) and the RESP method (34), the two CeNA nucleotides having an adenine base were fitted onto the original unbound triphosphate nucleotide TTP and the first nucleotide of the primer (residue F22) using quatfit (Dave Heisterberg, quatfit, CCL archives) and both nucleotides were covalently linked by a phosphate group. Hydrogen atoms were added and Amber 8.0 was used to calculate the molecular mechanics energy of the system (in vacuum) (34–36). Minimization and molecular dynamics with restraints on all atoms, except the new residues, was used to minimize the molecular mechanics energy of the system. Finally, all restraints were removed and the energy of the system was further minimized until a minimum was reached.

RESULTS

DNA-dependent CeNA polymerization

Incorporation of CeATP into a DNA hybrid. We checked whether the triphosphate of cyclohexenyl-A could serve as a substrate for different DNA polymerases. We tested enzymes from different polymerase families, Vent (exo−) DNA polymerase from the B-family polymerases, *Taq* DNA polymerase from the A-family polymerases and HIV reverse transcriptase from the reverse transcriptase family. Elongation experiments using *Taq* DNA polymerase and Vent (exo−) DNA polymerase were carried out at 55°C (and not at the expected 72°C). A lower temperature was chosen since the ultimate goal of our research is the establishment of an artificial genetic system, based on CeNA, which also functions in bacterial cells. Ultimately, we hope to find a polymerase that is capable of using CeNA as a substrate at 37°C. We chose to use 55°C since at this temperature the thermostable polymerases retain some activity (as indicated by the positive control that is carried out in parallel with our assays). For these assays, we

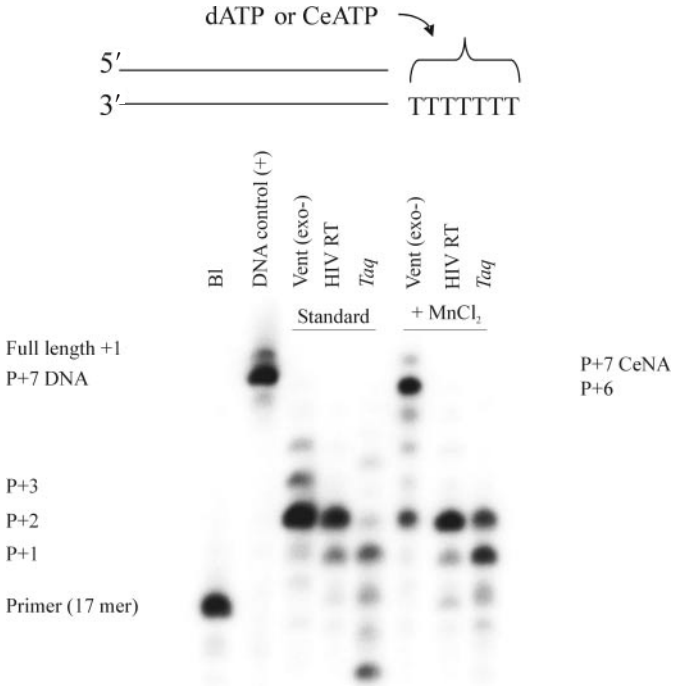

Figure 2. Recognition of CeATP by different polymerases. Phosphorimage of the enzymatic incorporation of CeATP into a 50 nM hybrid P_1T_1 in the presence of 0.05 U/μl enzyme and 100 μM CeATP under standard conditions or under conditions supplemented with 1 mM Mn^{2+}. Reaction time is 120 min. Bl indicates the reaction in the absence of dNTPs. For the positive control dATP was used instead of CeATP.

designed a template (T_1) with a 5′ homopolymeric sequence to check the successive incorporation of CeATP (Table 1). Our goal was to identify those reaction conditions and those enzymes that gave the best incorporation performance. To this order, we used different enzymes (in different concentrations, indicated in units as determined by the supplier) and conditions with or without manganese as a cofactor. All enzymes tested were capable of using the cyclohexenyl nucleotides as a substrate in a primer extension experiment.

In a primer extension assay using 0.05 U/μl of polymerase (Figure 2), we see for Vent (exo−) DNA polymerase a weak band corresponding to the incorporation of four consecutive CeATPs, although the major band corresponds to P+2. HIV reverse transcriptase is also able to incorporate two CeATPs. For *Taq* DNA polymerase, incorporation stops after one cyclohexenyl nucleotide. In order to improve the efficiency of the polymerization reaction, Mn^{2+} was added to the reaction buffer. Mn^{2+} ions are known to reduce the discrimination against different analogs with modifications in the furanose ring, the base or the phosphate linkage (37). In the presence of Mn^{2+}, we see the incorporation of six consecutive CeATPs for Vent (exo−) DNA polymerase, although we also see a band corresponding to the incorporation of two cyclohexenyl nucleotides. It seems to be difficult for the polymerase to incorporate more than two modified nucleotides, but once the polymerase crossed this barrier, the synthesis of a longer stretch of cyclohexenyl nucleotides is possible. HIV reverse transcriptase still stalls after the incorporation of a second CeA nucleotide and *Taq* DNA polymerase also incorporates two CeA nucleotides opposite the natural dT building block in the

template. It should be noticed that the CeNA incorporation pattern on a gel differs from the incorporation pattern of natural dNTPs in a way that the incorporation of CeNA nucleotides is characterized with a lower mobility (corresponding bands are positioned higher on a gel).

In a primer extension assay using 0.5 U/μl of polymerase, we noticed that HIV reverse transcriptase can fully extend a DNA primer with cyclohexenyl nucleotides (Figure 3). For Vent (exo−) DNA polymerase, a weak band corresponding to the extension of the primer with six CeATP nucleotides can be seen. For both enzymes, the major extension product, however, corresponds to the elongation of the primer with two nucleotide analogs. For *Taq* DNA polymerase, a weak spot is seen for P+4, with the major band corresponding to P+1. Adding Mn^{2+} to the reaction mixture also gives full primer extension for Vent (exo−) DNA polymerase. At the same time, the percentage of primer that is extended with multiple CeATPs increases for both HIV reverse transcriptase and Vent (exo−) DNA polymerase. Mn^{2+} does not affect the incorporation of CeATP by *Taq* DNA polymerase.

In general, we see that the polymerase holds after the incorporation of a second CeA nucleotide, but incorporation of multiple CeA nucleotides is possible for Vent (exo−) and HIV reverse transcriptase although not very efficient.

Enzymatic synthesis of mixed CeNA/DNA sequences. We checked whether it was possible to extend a primer with dNTPs after the incorporation of one cyclohexenyl nucleotide (Figure 4). Vent (exo−) DNA polymerase could easily incorporate one dATP after incorporation of a cyclohexenyl-G. After a reaction time of 2 h a weak band corresponding to the full extension of the primer could be seen. Also, HIV reverse transcriptase was only able to fully extend a primer, after incorporation of a cyclohexenyl-G, after a reaction time of 2 h. When *Taq* DNA polymerase was used, we were not able to detect incorporation of a cyclohexenyl-G nucleotide without, at the same time, the detection of misincorporation of a natural dA nucleotide opposite dC in the template. Using conditions in which no misincorporation of a natural dA nucleotide is seen, we were not able to detect any CeG incorporation. It is therefore not possible to draw any conclusions about the possibility of *Taq* DNA polymerase to use mixed dNTPs/CeNTPs substrates. In a following experiment, we also investigated whether extension of a primer after the incorporation of two cyclohexenyl-G nucleotides was possible (Figure 5). Both Vent (exo−) DNA polymerase and HIV reverse transcriptase failed to extend the primer with dATP after incorporation of two consecutive CeGTPs.

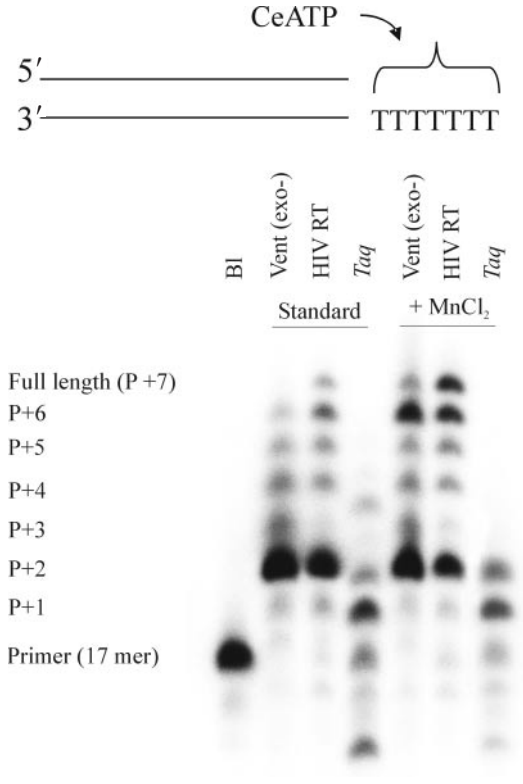

Figure 3. Recognition of CeATP by different polymerases using high polymerase concentrations. Phosphorimage of the enzymatic incorporation of CeATP into a 50 nM hybrid P_1T_1 in the presence of 0.5 U/μl enzyme and 100 μM CeATP under standard conditions and under conditions supplemented with 1 mM Mn^{2+}. Reaction time is 120 min. Bl indicates the reaction in the absence of dNTPs.

CeNA-dependent DNA polymerization

The efficiency of a CeNA-dependent DNA polymerization was also examined using a primer extension assay. We used a chimeric DNA/CeNA template (T_4) consisting of a DNA primer binding site followed by a stretch of CeT nucleotides (Table 1). Elongation products in the presence of each of the four nucleoside triphosphates are shown in Figure 6A.

For the correct base all the enzymes tested could fully extend the primer. For the three incorrect base pairs, misincorporation of one base opposite a cyclohexenyl-T is seen. Vent (exo−) DNA polymerase is the most efficient in using stretches of cyclohexenyl nucleotides as a template. Full elongation of the primer at low enzyme concentration and after 10 min reaction time is seen. For HIV reverse transcriptase and *Taq* DNA polymerase, we needed to increase the enzyme concentration to get a full elongation pattern. *Taq* DNA polymerase was the least efficient in templating from CeNA stretches, only after 2 h reaction time the primer could be fully extended.

CeNA-dependent CeNA polymerization

It was also investigated whether DNA polymerases were able to synthesize short CeNA fragments using a CeNA template (Figure 6B). It seems that polymerases have difficulty in accommodating both a CeNA template and a cyclohexenyl triphosphate in their active site. *Taq* DNA polymerase was not able to incorporate a single CeA nucleotide opposite CeT in the template. For Vent (exo−) DNA polymerase incorporation holds after the incorporation of a second cyclohexenyl nucleotide; HIV reverse transcriptase can incorporate a single cyclohexenyl nucleotide opposite cyclohexenyl-T. Adding manganese to the reaction buffer did not improve the CeNA-dependent CeNA polymerization (data not shown).

Cyclohexenyl nucleotide incorporation kinetics

To obtain an idea of the efficiency of incorporation of cyclohexenyl nucleotides by Vent (exo−) DNA polymerase, the kinetic parameters K_m and k_{cat} were determined. This

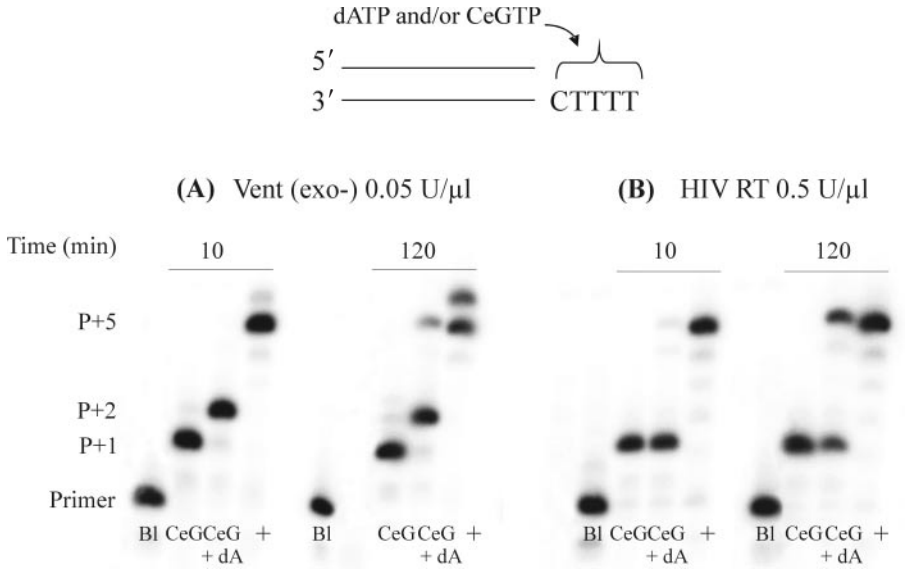

Figure 4. Further elongation with dATP after incorporation of one cyclohexenyl nucleotide. Phosphorimage of the enzymatic incorporation of CeGTP and dATP into a 50 nM hybrid P_1T_2 in the presence of (**A**) 0.05 U/µl Vent (exo−) DNA polymerase, (**B**) 0.5 U/µl HIV reverse transcriptase. The concentration of CeGTP was 100 µM and the concentration of dATP was 1 µM. Bl indicates the blanc reaction in the absence of dNTPs. For the positive control (indicated with +) dGTP + dATP was used.

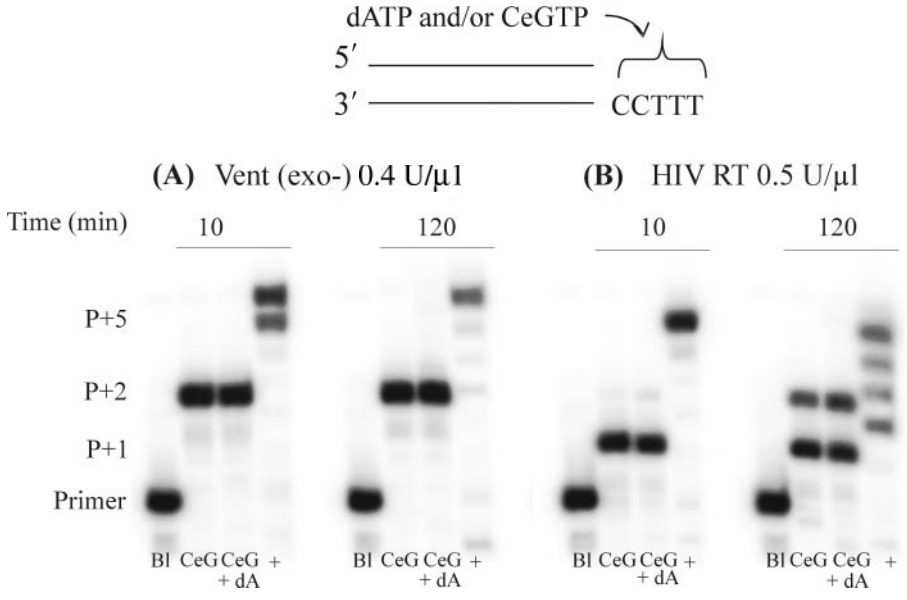

Figure 5. Further elongation with dATP after incorporation of two cyclohexenyl nucleotides. Phosphorimage of the enzymatic incorporation of CeGTP and dATP into a 50 nM hybrid P_1T_3 in the presence of (**A**) 0.4 U/µl Vent (exo−) DNA polymerase, (**B**) 0.5 U/µl HIV reverse transcriptase. The concentration of CeGTP was 100 µM and the concentration of dATP was 1 µM. Bl indicates the blanc reaction in the absence of dNTPs. For the positive control (indicated with +) dGTP + dATP was used.

experiment was performed according to the standing start assay as described by Creighton *et al*. (31,38). Quantification of radioactive spots, representing polymerized product and remaining primers, allowed calculation of the initial velocity of the reaction. Plotting these data against the substrate concentration gives the Michaelis–Menten curve.

Table 2 lists the kinetic parameters (average of three experiments) for CeGTP and CeATP incorporation by Vent (exo−) DNA polymerase in comparison with dGTP and dATP.

The catalytic efficiency (k_{cat}/K_m) is only reduced by a factor 1.6 if CeATP instead of dATP is used as a substrate for Vent (exo−) DNA polymerase. This is due to a decrease in the k_{cat} value. The K_m value for CeATP incorporation is decreased in comparison with dATP, so the affinity of the polymerase for the nucleotide has not decreased by changing the deoxyribose by a cyclohexene sugar unit. The same is seen for the incorporation of CeGTP, where there is no difference in K_m values compared with dGTP. The k_{cat} value for the incorporation reaction of a CeGTP is decreased with a factor 4.5 in comparison with dGTP, indicating that the incorporation of a modified adenosine triphosphate is easier than the incorporation of a guanosine triphosphate analog.

Since the incorporation of a first cyclohexenyl adenosine triphosphate is almost as efficient as the incorporation of the

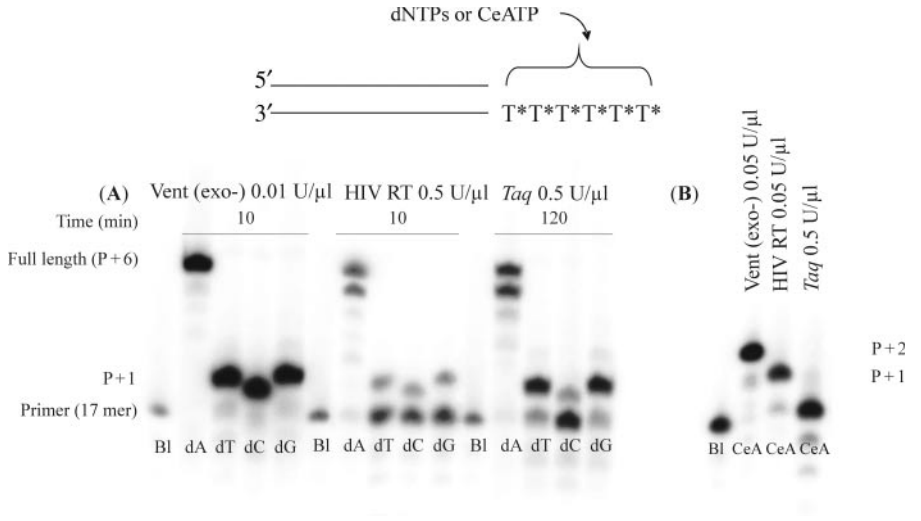

Figure 6. Phosphorimage of the primer extension reaction using a chimeric DNA/CeNA template. Phosphorimage of the enzymatic incorporation of dNTPs (**A**) or CeATP (**B**) into a 50 nM hybrid P_1T_4 in the presence of Vent (exo−) DNA polymerase, HIV reverse transcriptase or *Taq* DNA polymerase. Reaction time and enzyme concentrations are as indicated in the figure. Bl indicates the reaction in the absence of dNTPs. (A) CeNA-dependent DNA polymerization. dNTP concentration is 10 µM. (B) CeNA-dependent CeNA polymerization. CeATP concentration is 100 µM. T* indicates CeT.

Table 2. Kinetic parameters for incorporation of a first cyclohexenyl nucleotide by Vent (exo−) DNA polymerase

	K_m (µM)	k_{cat} (min^{-1})	k_{cat}/K_m
dA	1.46 ± 0.19	16.90 ± 0.65	11.6
CeA	0.98 ± 0.08	7.08 ± 0.15	7.2
dG	1.39 ± 0.20	59.81 ± 0.88	43.03
CeG	1.39 ± 0.17	13.30 ± 0.23	9.57

Table 3. Kinetic parameters for the incorporation of a second cyclohexenyl nucleotide by Vent (exo−) DNA polymerase using CeATP or dATP as a substrate

	K_m (µM)	k_{cat} (min^{-1})	k_{cat}/K_m
dA	3.73 ± 0.62	17.15 ± 0.94	4.6
CeA	3.66 ± 0.44	9.41 ± 0.37	2.5

natural substrate, we also measured the kinetic parameters for incorporation of a second CeATP (Table 3). For this experiment, we used a primer ending with a 3′ cyclohexenyl nucleotide (P_2) annealed to template T_6.

The K_m values are the same for incorporation of the cyclohexenyl adenosine triphosphate and for the natural dATP. In comparison with the K_m value for incorporation of a first nucleotide, the K_m value is increased with a factor 2.6 for dATP and 3.7 for CeATP. This indicates that after the incorporation of a first cyclohexenyl nucleotide, the affinity of the polymerase for a following nucleotide is lowered. The catalytic efficiency for the incorporation of a second cyclohexenyl nucleotide is decreased by a factor 1.8 in comparison with the incorporation of dATP after a cyclohexenyl nucleotide. This is comparable with the catalytic efficiency for the incorporation of the first nucleotide.

We can conclude that the kinetic parameters for the incorporation of the first or the second cyclohexenyl adenosine triphosphate are not changed much in comparison with dATP and that the incorporation of cyclohexenyl nucleotides is almost as efficient as the incorporation of the natural nucleotide. Kinetic parameters indicate the facile insertion of a second CeNA nucleotide; these results are in line with the results obtained from primer extension experiments where we noticed for Vent (exo−) DNA and HIV reverse transcriptase almost full primer extension with two consecutive cyclohexenyl nucleotides. The critical issue in the polymerization reaction, however, seems to be the slow addition of a third residue, processive incorporation of cyclohexenyl nucleotides seems to be compromised, as the polymerase seems to hold after the incorporation of a second cyclohexenyl nucleotide.

DISCUSSION

The establishment of an *in vitro* artificial genetic system based on CeNA requires the conversion of DNA into CeNA and the faithful conversion of CeNA templates into DNA. An *in vitro* artificial genetic system would allow the bulk production of nucleic acid-like polymers, which can be used as nucleic acid-based therapeutics, such as aptamers, ribozymes or siRNA, or have benefits in the emerging fields of bionanotechnology and nucleic acid computing. This is also the first step toward the generation of additional information into living cells based on sequences encoded by an alternative nucleic acid structure.

During the last decade, intense efforts in a number of laboratories have focused on expanding the coding potential of nucleic acid by a third base pair with the ultimate goal of constructing an artificial synthetic organism with an expanded genetic alphabet and ultimately an expanded genetic code (11,25,39–42). This would allow the incorporation of various unnatural, functional components into proteins at desired positions. Many different potential solutions have been put forward for synthetic nucleobases and alternative base pairs. However, nucleic acids based on these will suffer from an inability to communicate their informational content

with the rest of the genome. An alternative design for an artificial genetic system might therefore be preferably based on an alternative backbone structure. The chemical nature of the backbone for such a genetic enclave should meet certain requirements: if it resembles the (deoxy)ribose too closely, it would be difficult to prevent interference of the novel nucleic acid monomers with DNA and RNA biosynthesis; if it is too different from (deoxy)ribose it might prove difficult to recruit polymerases and other replicating enzymes, which could process the alternative polynucleotides and their monomeric building blocks. We propose to build an artificial genetic system based on CeNA, since these oligomers are particularly attractive alternatives to the ribose nucleic acids, because of their conformational analogy, helix-forming properties and resistance to enzymatic degradation.

The first step toward the generation of an alternative genetic system is the investigation of the CeNA-dependent DNA polymerization and the enzymatic synthesis of CeNA starting from DNA. This would enable us to identify the best enzyme to use as a candidate for the directed evolution of a polymerase capable of replicating CeNA.

Hereto, the ability of different enzymes to use CeNA as a template and to use cyclohexenyl nucleoside triphosphates as a substrate was investigated. Primer extension assays show that all enzymes tested can incorporate one cyclohexenyl nucleoside triphosphate opposite a natural counterpart in the template strand. HIV reverse transcriptase and Vent (exo−) DNA polymerase can even easily incorporate a second cyclohexenyl analog in a DNA hybrid. Extension of a primer with CeNTPs after the incorporation of two cyclohexenyl nucleotides, however, is limited and an increasing enzyme concentration or error-prone reaction conditions are needed for the synthesis of longer CeNA stretches by Vent (exo−) DNA polymerase and HIV reverse transcriptase. The difficulty of polymerases to further extend a primer with cyclohexenyl nucleotides after the incorporation of two building block analogs might either be due to steric hindrance in the active site (since the cyclohexenyl nucleotides contain a larger six-membered cyclohexenyl sugar moiety in comparison with the five-membered ribose ring of natural nucleotides) or due to the inability of the sugar ring to undergo the necessary conformational changes during the catalytic process. For family A polymerases and HIV reverse transcriptase, it was observed that the DNA is in the B-form throughout the polymerase but adopts an A-form conformation within the active site (43–46). For DNA polymerases from family B the primer/template DNA duplex maintains a B-form conformation throughout the whole incorporation system (47). Since cyclohexenyl nucleosides are flexible DNA analogs that can adopt two half-chair conformations (the $^{3}H_{2}$ and the $^{2}H_{3}$ conformation, comparable with either the C3′-endo conformation seen in A-form duplexes or the C2′-endo conformation seen in B-form duplexes), we assume that the cyclohexenyl nucleotides can accommodate to the different conformations of the DNA duplex at the polymerase active site. Although we do not exclude that the conformational flexibility of a nucleotide is an important factor defining the possibility of building block analogs to serve as a substrate for polymerase, we rather hypothesize that steric effects also play a role in the case of the cyclohexenyl nucleotides. This hypothesis was investigated using molecular modeling. A model was constructed starting from HIV-1 reverse transcriptase crystal structure with two CeNA adenine nucleotides built in the primer strand (Figure 7). Probably, during translocation, the six-membered sugar rings, that are larger than the five-membered deoxyribose rings in DNA, will have steric hindrance with residues A184 and/or A185, resulting in a stop of the build in process of new nucleotides (48). We assume that translocation is not hindered after incorporation of a first cyclohexenyl nucleotide, since the nucleotide in the post-insertion site is still a natural nucleotide and only the nucleotide in the insertion site is a non-canonical nucleotide.

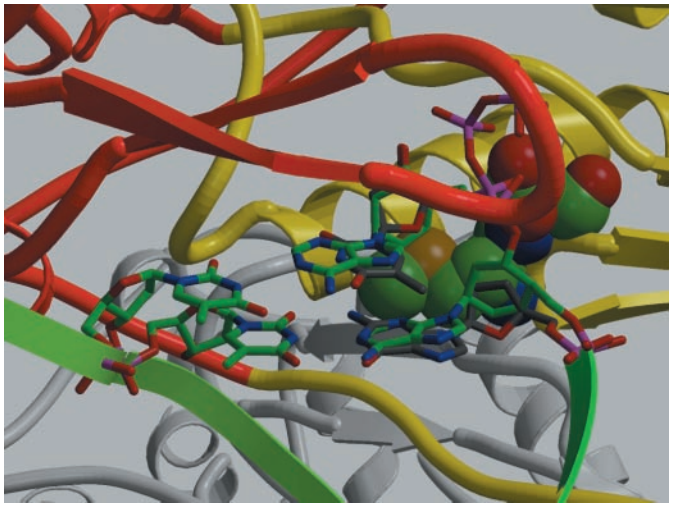

Figure 7. Close-up of the active site of HIV-1 reverse transcriptase with two CeNA adenine nucleotides built in the primer strand. The CeNA and base pairing residues are shown in sticks (carbon, green). The original terminal primer DNA nucleotide and triphosphate are also shown (carbon, grey). The double bonds in the CeNA rings are located at the bottom of the six rings. Residues A184 and A185 are shown in CPK. Pictures are generated using molscript, bobscript and raster3d (50–52).

Investigating the possibility to synthesize mixed DNA/CeNA sequences, it was clear that elongation of the primer after incorporation of one cyclohexenyl building block is possible, while extension after incorporation of two consecutive cyclohexenyl building blocks is hindered for both HIV reverse transcriptase and Vent (exo−) DNA polymerase. This might also be explained by the steric hindrance at the active site of the polymerase during translocation after the incorporation of two consecutive cyclohexenyl nucleotides. *Taq* DNA polymerase has already difficulties in full primer elongation with one cyclohexenyl nucleotide. This hinders the examination of the possible extension with natural dNTPs after cyclohexenyl nucleotide incorporation. In the conditions used it was not possible to detect incorporation of CeGTP without misincorporation of dATP opposite dC in the template strand. Using a mixture of dNTPs and CeNTPs, it can be expected that *Taq* DNA polymerase would rather misincorporate a natural nucleoside triphosphate than the correct cyclohexenyl nucleoside triphosphate.

The CeNA-dependent DNA polymerization was more efficient, since all enzymes tested are able to copy a stretch of six cyclohexenyl nucleotides into a DNA strand. The incorporation of a cyclohexenyl nucleoside triphosphate opposite a cyclohexenyl nucleotide in the template strand is more difficult.

Vent (exo−) DNA polymerase was able to incorporate two CeA building blocks opposite CeT in a template, while HIV reverse transcriptase can only incorporate one cyclohexenyl nucleoside triphosphate opposite CeT, and *Taq* DNA polymerase cannot incorporate CeNA triphosphates opposite a CeNA template base.

In the applied reaction conditions, we found that Vent (exo−) DNA polymerase displayed a superior ability to use CeNA as a substrate (both as a triphosphate as in the template strand) in comparison with *Taq* DNA polymerase or HIV reverse transcriptase. These results are in agreement with previously reported data comparing *Taq* DNA polymerase and Vent (exo−) DNA polymerase for the incorporation of various nucleotides labeled with reporter-molecules, which indicated that Vent (exo−) displays superior incorporation efficiency for the modified nucleotides (49). In our assays, the increased performance of Vent (exo−) DNA polymerase is also due to an increased enzyme activity at 55°C in comparison with *Taq* DNA polymerases, as was seen with the natural substrate dATP (data not shown). Since polymerase activity at a temperature <72°C was the aim of the study, we suggest Vent (exo−) DNA polymerase as the ideal candidate polymerase for directed evolution experiments, because of its higher intrinsic activity at 55°C as well as its broader substrate specificity (49).

Since natural polymerases were already able to copy a short CeNA strand into a DNA strand and, in conditions in which manganese ions were added to the reaction mixture, were able to synthesize a short CeNA strand starting from a DNA template, we expect to be able to evolve a CeNA polymerase by mutagenesis experiments for the development of an *in vitro* replicating system based on CeNA.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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