Articles

Enzymatic Incorporation in DNA of 1,5-Anhydrohexitol Nucleotides†

Karen Vastmans,‡ Sylvie Pochet,§ Annemie Peys,‡ Luc Kerremans,‡ Arthur Van Aerschot,‡ Chris Hendrix,‡ Philippe Marlière,|| and Piet Herdewijn*,‡

Laboratory of Medicinal Chemistry, Rega Institute, Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium, and Unité de Chimie Organique URA CNRS 2128 and Unité de Biochimie Cellulaire (URA CNRS 1129), Institut Pasteur, 28 rue du Docteur Roux, 75015 Paris, France

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ABSTRACT: The ability of several DNA polymerases to catalyze the template-directed synthesis of duplex oligonucleotides containing a base pair between a nucleotide with anhydrohexitol ring and its natural complement has been investigated. All DNA polymerases were able to accept the chemically synthesized anhydrohexitol triphosphate as substrate and to catalyze the incorporation of one anhydrohexitol nucleotide. However, only family B DNA polymerases succeeded in elongating the primer after the incorporation of an anhydrohexitol nucleotide. In this family, Vent (exo$^-$) DNA polymerase is the most successful one and was therefore selected for further investigation. Results revealed that at high enzyme concentrations six hATPs could be incorporated; however, a selective incorporation proved only feasible under experimental conditions where no more than two analogues could be inserted. Also the synthesis of a mixed HNA−DNA sequence was examined. Kinetic parameters for incorporation of one anhydrohexitol adenine nucleoside were similar to those of its natural analogue.

During the past years, our knowledge has significantly increased with respect to the structure of nucleotide polymerases and their interactions with nucleic acids. X-ray crystallographic studies have revealed the details of the three-dimensional structure of class I DNA polymerases (*1*−*7*) and of class II DNA polymerases (*8*, *9*). These studies, together with biochemical and genetic studies, revealed a general shape of the polymerase domain, linked to a "right hand" consisting of three distinct subdomains designated as fingers, palm, and thumb. Analysis of protein sequences (*10*) showed that, although there is little discernible resemblance between the sequences of polymerases of different families, it appears that the palm domains, which contain the conserved catalytic aspartate residues, show a similar topology among all families, except polymerase β. These observations led to the hypothesis that all polymerases share a common nucleotidyl transfer mechanism. Recent structural studies of an enzyme−DNA−dNTP ternary complex (*11*−*13*), together with site-directed mutagenesis experiments (*14*−*18*), measuring the effect of the resulting mutations on K_m(dNTP), have offered valuable insights in the molecular mechanism of template-directed DNA synthesis and revealed amino acid side chains making contact with the primer−template and the incoming dNTP. It is assumed that nucleotide incorporation starts by binding of a dNTP to the enzyme−DNA complex. Binding of a correct dNTP induces a rate-limiting conformational change, which results in the formation of a tight ternary complex positioning the triphosphate residue in such a way as to allow for phosphoryl-transfer. Following this chemical reaction, a second conformational change occurs that relaxes the tightly bound enzyme−product complex, allowing PP_i release and translocation of the DNA (*13*). Discrimination, necessary for DNA replication fidelity, occurs at two levels during this process. Initially it takes place during the dNTP substrate binding, serving to bring the dNTP into the polymerase site and involving base-pairing with the template strand. A second stage involves an enzyme coformational change that is only induced by a correct base pair (*19*), which geometry fits the pocket of the active site formed in the enzyme−primer−template complex.

To study in more detail the factors important in these two stages of selection, a sizable number of nucleotide analogues, modified in their base (*20*−*31*, *41*−*43*), sugar (*32*−*38*), and phosphate (*39*, *40*) components, have been used in primers (*20*−*24*) or templates (*23*−*31*) or as triphosphate building blocks (*25*−*43*). By use of modified nucleoside triphosphates, information becomes available about the interactions that are involved between sugar, base, and phosphate parts of the triphosphate building block and the enzyme−DNA complex.

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* To whom correspondence should be addressed: Phone +32-16-337387; fax +32-16-337340; e-mail Piet.Herdewijn@rega.kuleuven.ac.be.

‡ Katholieke Universiteit Leuven.

§ Unité de Chimie Organique (ERS 558), Institut Pasteur.

|| Unité de Biochimie Cellulaire (URA CNRS 1129), Institut Pasteur.

[1] Abbreviations: hNTP, 1,5-anhydrohexitol nucleoside triphosphates; HNA, hexitol nucleic acids; DEAE, diethylaminoethyl; TEAB, triethylammonium bicarbonate; dPTP, pyrene nucleoside triphosphate.

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Modifications of the nucleotide in the base were the subject of many studies. For example, Kool and co-workers (*44*, *45*) studied the incorporation of nonpolar isosteres of thymine and adenine, having the same size, shape, and conformation and thus differing from their natural counterparts only in their inability to form hydrogen bonds. Lutz et al. (*46*) studied the incorporation efficiency of the C-nucleoside analogue of thymidine, only differing from its natural counterpart by the presence of a C−C bond between sugar and base. These experiments indicated that shape complementarity is an important factor for acceptance as substrate. This idea was further confirmed in another experiment with dPTP offered as substrate in the incorporation assay (*47*). Likewise, it was indicated that conformation restraints in the active site of polymerases during primer elongation are important (*46*). Some base-modified analogues have proven to be extremely useful and have been commercialized. 8-Oxo-dGTP, for example, is used to generate random transitions and transversion mutations during PCR (*48*). Deaza-dGTP, deaza-dATP, and dITP are used in sequencing reactions to reduce band compressions.

To get an insight into which parts of the sugar moiety are necessary for recognition and incorporation by DNA polymerases and to reveal the importance of sugar conformation and conformational flexibility for DNA synthesis, several sugar-modified analogues have been analyzed the past few years. Especially analogues altered in the 3′-position (*32*−*34*, *37*), 2′-position (*35*, *36*, *38*), or both (*35*) have been studied. But also acyclic analogues (*49*, *50*) and analogues with modifications in the 4′-position of the ring (*51*) were the subject of incorporation assays. Results from these experiments support a hypothesis that modified nucleosides and nucleotides can be accepted on the conditions that they possess a partly flattened sugar residue of limited flexibility (*52*). The hypothesis that some conformational restriction is needed for efficient incorporation was also suggested in mechanistic studies based on the crystal structures obtained for T7 DNA polymerase, *Bacillus* DNA polymerase I large fragment, and *Taq* DNA polymerase with a primer−template complex and a nucleoside triphosphate in the active site (*11*−*13*). In the last decade, much research has been performed concerning the fidelity of DNA replication with respect to misincorporation of ribonucleotides into DNA. The traditional classification of nucleic acid polymerases as either DNA polymerase or RNA polymerase is based, in large part, on their fundamental preference for the incorporation of either deoxyribonucleotides or ribonucleotides during chain elongation (*15*). However, the affinity of DNA polymerases for the traditional 2′-deoxyribonucleoside triphosphates is not absolute. Ide et al. (*53*) described the incorporation of ribonucleoside triphosphate building blocks into DNA by DNA polymerases in place of the corresponding dNTP, although under stringent conditions and when present at high concentrations. rNTPs differ from dNTPs by the presence of an extra hydroxyl group in the 2′-position of the sugar ring. A recent experiment investigated the use of hTTP, the anhydrohexitol triphosphate analogue of dTTP, as substrate in enzymatic replication assays with deoxyadenosine or its anhydrohexitol analogue in the template. Specific incorporation was achieved in the presence of *Taq* DNA polymerase and Klenow DNA polymerase I (*54*). hNTP building blocks have conformational similarity with ribonucleoside triphosphates. Examination of the structure of a 1,5-anhydrohexitol nucleoside suggested a good fit with a natural furanose nucleoside in its 3′-endo conformation rather than in its 2′-endo conformation (*55*, *56*). In contrast to rNTPs, however, they do not possess an adjacent hydroxyl group analogous to the hydroxyl group at the 2′-position of the sugar residue in rNTPs. Incorporation assays with hNTPs as substrates can lead to a better understanding of the conformational requirements and of the necessity of a 2′-hydroxyl group in the sugar part of a triphosphate building block in the discrimination between dNTPs and rNTPs by DNA polymerases.

MATERIALS AND METHODS

Chemicals and DNA. DEAE-Sephadex A-25 was obtained from Sigma; highly purified 2′-deoxynucleoside triphosphates used in DNA polymerase reactions and the Mono Q 5/5 and 10/10 columns were purchased from Pharmacia.

Synthesis of Anhydrohexitol Nucleotides. The anhydrohexitol nucleosides were synthesized according to previously reported procedures (*57*, *58*). These analogues were converted to their respective anhydrohexitol nucleoside monophosphates as described by Yoshikawa et al. (*59*, *60*). The respective triphosphates were synthesized by a published procedure (*61*). The reaction mixture was loaded on a DEAE-Sephadex A-25 column in the HCO_3^- form. The column was washed with 0.1 M triethylammonium bicarbonate (TEAB), pH 7.5, and the deoxynucleotide triphosphate fraction was eluted with a 0−1 M TEAB gradient. TEAB was removed by repeated evaporations with methanol. Subsequently, the triphosphates were converted into their sodium salts by addition of a 1 M solution of sodium iodide in acetone. The precipitate was washed and dried overnight in vacuo over phosphorus pentoxide at room temperature. Before use in DNA polymerase reactions, hNTPs were freshly purified by preparative ion-exchange HPLC on a Mono Q 10/10 column (Pharmacia) with a linear gradient of TEAB (pH 7.5) from 0 to 0.5 M in 40 min, with a retention time of about 35 min. The overall yield for the isolated hNTPs was about 40%. ^{31}P NMR (hATP) δ (ppm) (D_2O) −6.13 (d, γ-P), −9.90 (d, α-P), −21.28 (t, β-P). The following extinction coefficients ϵ (λ = 260 nM) of hATP (15 000 M^{-1} cm^{-1}), hTTP (8400 M^{-1} cm^{-1}), hCTP (7000 M^{-1} cm^{-1}), and hGTP (12 000 M^{-1} cm^{-1}) were used to determine the respective concentrations.

HPLC. Ion-exchange HPLC was performed by means of a L-6200 A Merck−Hitachi pump with UV monitoring. For analytical purpose, samples were separated on a Mono Q 5/5 column with a TEAB gradient (pH = 7.5) at a flow rate of 1.0 mL/min. The TEAB percentage in the gradient was increased from 0 to 0.5 M TEAB in 30 min. For preparative separation, a semipreparative Mono Q 10/10 column was used with a flow rate of 2 mL/min and an analogous gradient (0−0.5 M TEAB in 40 min). Reversed-phase HPLC on a C_{18} column (self-packed, 12−40 μM, 250 × 9 mm) was performed with solvents A (0.1 M TEAB and 1% CH_3CN in H_2O) and B (90% CH_3CN in 0.1 M TEAB) and a linear gradient of 1−15% solvent B in 30 min at 2 mL/min.

Spectroscopy. Samples for NMR spectroscopy were prepared in nonbuffered solution of 99.96% D_2O. The ^{31}P NMR spectra were recorded with a Varian Unity 500 spectrometer with 85% H_3PO_4 in H_2O as internal standard.

Oligodeoxyribonucleotides. All oligodeoxyribonucleotides were purchased from Eurogentec. The primer oligonucleo-

tides were 5′-^{32}P-labeled with [γ-^{32}P]ATP (4500 Ci/mmol, 10 mCi/mL) (Pharmacia) using T4 polynucleotide kinase (Life Technologies) following standard procedures. Labeled oligonucleotides were further purified on a NAP-5 column (Pharmacia).

DNA Polymerase Reactions. End-labeled primers were annealed to their template by combining primer and templates in a molar ratio of 1:2.5 and heating the mixture to 70 °C for 5 min, followed by slow cooling to room temperature over a period of 2.5 h.

The reaction mixtures were prepared by adding 6 μL of a solution containing 5′-^{32}P-labeled primer−template complex in their respective reaction buffer (supplied with the polymerase) to 7.5 μL of an aqueous solution containing the triphosphate building blocks. Incorporation was started upon addition of 1.5 μL of enzyme dilution.

For the incorporation of one, two, or three hATPs, the primer P**1** (Figure 2) was annealed to template T**1** (Figure 2), template T**2** (Figure 2), and template T**3** (Figure 2), respectively. A series of 15 μL reactions was performed for each of eight DNA polymerases: Klenow fragment of exonuclease free (KF exo$^-$), Klenow DNA polymerase (KF), DNA polymerase I, T4 DNA polymerase, *Pfu* DNA polymerase (all from Promega), T7 Sequenase version 2.0 (Pharmacia), *Taq* DNA polymerase (Pharmacia), and Vent exo$^-$ DNA polymerase (Vent exo$^-$) (New England Biolabs). The final mixture contained either 250 nM (first series of experiments) or 50 nM (second series of experiments) primer−template complex, 100 μM triphosphate building blocks (dATP or hATP or dGTP or dGTP + dATP or dGTP + hATP) and 0.05 unit/μL enzyme. The mixture was incubated at 37 °C (DNA polymerases) or 55 °C (thermostable DNA polymerases), and aliquots were quenched after 3 and 10 min.

For the incorporation of consecutive anhydrohexitol triphosphates of adenine (hATP), the same 5′-end labeled primer P1 was annealed to template T**5** (Figure 2). The final mixture contained 50 nM primer−template complex, 10 μM or 1000 μM triphosphate building blocks (dATP or hATP) and 1 unit/μL Vent exo$^-$ DNA polymerase. The mixture was incubated at 55 °C and aliquots of the reaction were quenched after 5, 20, 60, or 120 min.

For elongation of a DNA primer consecutively with hATP and hCTP, the labeled primer P**3** (Figure 2) was annealed to template T**6** (Figure 2). The final reaction mixture contained 50 nM primer, 100 μM triphosphate building blocks (dATP or hATP or dCTP or hCTP or dATP + dCTP or hATP + hCTP or dATP + hCTP or hATP + dCTP) and 0.05 unit/μL Vent exo$^-$ DNA polymerase. The mixture was incubated at 55 °C, and the reaction was quenched after 120 min.

For the determination of the selectivity of incorporation, primers for template−primer P**1**−P**4** (Figure 2) were annealed to template T**4** (Figure 2) as described above. A series of 15 μL reactions was performed in the presence of Vent exo$^-$ DNA polymerase. The final mixture contained 50 nM primer−template complex, 10 μM each of the triphosphate building blocks (for hybrid P**1**/T**4,** dT or hT or dG or hG or dT + dG or hT + hG or dT + dG + dA or hT + hG + hA or dT + dG + dA + dC or hT + hG + hA + hC; for hybrid P**2**/T**4,** dG or hG or dA or hA or dG + dA or hG + hA or dG + dA + dC or hG + hA + hC or dG + dA + dC + dT or hG + hA + hC + hT; for hybrid P**3**/T**4,** dA or hA or dC or hC or dA + dC or hA + hC or dA + dC + dT or hA + hC + hT; for hybrid P**4**/T**4,** dC or hC or dT or hT or dC + dT or hC + hT) and 0.005 unit/μL enzyme. The mixture was incubated at 55 °C and the reaction was quenched after 5 min.

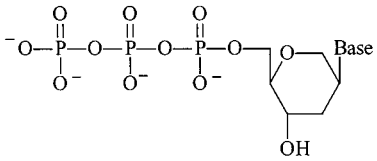

FIGURE 1: Structure of anhydrohexitol nucleoside triphosphates [(base = adenin-9-yl (hATP), guanin-9-yl (hGTP), cytosin-1-yl (hCTP), or thymin-1-yl (hTTP)].

Kinetic Experiments. To determine the kinetic parameters of the incorporation of hATP or dATP, a steady-state kinetic assay (*62*) was performed. The reaction mixture was prepared by adding Vent exo$^-$ DNA polymerase to primer P**3** (Figure 2) annealed to template T**4** (Figure 2), buffer, and dATP or hATP. The final mixture (20 μl) contained 0.0005 unit/μL Vent exo$^-$ DNA polymerase, the commercially supplied buffer, 250 nM primer−template complex, and various concentrations of dATP or hATP. A concentration range of 0.625−10 μM was used. Reactions were incubated at 55 °C. Reaction times were between 1 and 15 min.

Electrophoresis. All DNA polymerase reactions were quenched by the addition of a double volume of loading buffer (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol, and 50 mM EDTA). Samples were heated at 70 °C for 5 min prior to analysis by electrophoresis for 2−3 h at 2000 V on a 0.4 mm 20% denaturing gel in the presence of a 89 mM Tris−borate and 2 mM EDTA buffer, pH 8.3. Products were visualized by phosphorimaging. The amount of radioactivity in the bands corresponding to the products of enzymatic reactions was determined with Optiquant image analysis software (Packard).

RESULTS

Synthesis and HPLC Purification of the Anhydrohexitol Nucleoside Triphosphates. The anhydrohexitol nucleoside triphosphates of adenine (hATP), thymine (hTTP), cytosine (hCTP), and guanine (hGTP) (Figure 1) were prepared for the evaluation of their recognition potential by DNA polymerases and their incorporation rate into a DNA hybrid. These nucleotide analogues (hNTPs) were synthesized from their respective anhydrohexitol nucleosides in a two-step synthesis. The monophosphates were obtained according to the method of Yoshikawa et al. (*59*, *60*) and further derivatized to the anhydrohexitol triphosphates according to Moffat and Khorana (*61*). The hNTPs were purified on a DEAE-Sephadex A-25 column but needed further purification right before use on a Mono Q ion-exchange column. Ion-exchange HPLC showed better separation profiles compared to reversed-phase HPLC, with well-separated hNMP, hNDP, and hNTP peaks, whereas these triphosphates could not be resolved in a reversed-phase mode deliberately. A mobile phase composed of volatile components (TEAB) was used. The final hNTP thus obtained proved to be highly pure as could be seen from an analytical HPLC profile (data not shown). ^{31}P NMR showed the expected signals for the

5' CAGGAAACAGCTATGAC 3' P**1**

5' CAGGAAACAGCTATGACT 3' P**2**

5' CAGGAAACAGCTATGACTG 3' P**3**

5' CAGGAAACAGCTATGACTGA 3' P**4**

3' GTCCTTTGTCGATACTG**TCCCC** 5' T**1**

3' GTCCTTTGTCGATACTG**TTCCC** 5' T**2**

3' GTCCTTTGTCGATACTG**TTTCC** 5' T**3**

3' GTCCTTTGTCGATACTG**ACTGAAAAA** 5' T**4**

3' GTCCTTTGTCGATACTG**TTTTTTT** 5' T**5**

3' GTCCTTTGTCGATACTG**ACTGTGTGTGTG** 5' T**6**

FIGURE 2: Sequences of primers (P) and templates (T) used in the DNA polymerase experiments.

respective α-, β-, and γ-phosphates, confirming the structure and purity of the hNTPs (data not shown).

Incorporation of hATP in a DNA Hybrid and Extension by DNA Polymerases. The ability of several commercially available DNA polymerases to accept hATP as a substrate for DNA synthesis was assayed in incorporation experiments. Likewise, further elongation with a natural triphosphate building block of guanine (dGTP) following the insertion of the anhydrohexitol nucleoside triphosphate of adenine was investigated. Three primed templates T**1**, T**2,** and T**3** (Figure 2) were used for studying the insertion potential of, respectively, one, two, and three building blocks of adenine and further elongation with a natural deoxyguanosine. Two series of experiments were performed, differing in their hybrid concentration bya factor of 5, to examine whether incorporation patterns were similar at another ratio of enzyme to hybrid. Insertion profiles seemed to be similar, although a higher efficiency of incorporation for hATP could be achieved in the presence of a 5-fold lower hybrid concentration (data not shown). Aliquots of the enzymatic incorporation assays were quenched after, respectively, 3 and 10 min by mixing samples with loading buffer. Polymerized products were visualized by phosphorimaging following separation on a denaturing polyacrylamide gel. Qualitative data for the incorporation of hATP in the presence of several commercially available DNA polymerases and hybrid P**1**/T**1** and P**1**/T**2** are listed in Table 1. Results in the presence of hybrid P**1**/T**1** reveal that all considered DNA polymerases are able to incorporate at least one hA nucleotide into the DNA hybrid. The capability for further elongation, however, is different for the considered enzymes. DNA polymerase I KF(exo$^-$) and KF succeed in extending the DNA primer with four dGs,whereas T4 DNA polymerase, *Pfu* DNA polymerase, and Vent exo$^-$ DNA polymerase could insert only one dG following insertion of one hA. In the presence of T7 Sequenase version 2.0 and *Taq* DNA polymerase, elongation of the primer did not take place. In the presence of hybrid P**1**/T**2** (Table 1), the incorporation by means of KF(exo$^-$), KF, DNA polymerase I, Sequenase version 2.0, and *Taq* DNA polymerase was restricted to one anhydrohexitol adenine. Further extension with either an unnatural hA or a natural dG proved not possible. T4 DNA polymerase, *Pfu* DNA polymerase, and Vent exo$^-$ DNA polymerase succeeded in elongating the DNA primer with two hA nucleotides. Further elongation, however, did not occur with T4 DNA polymerase or *Pfu* DNA polymerase. Incorporation assays in the presence of hybrid P**1**/T**3** (Figure 3) and hATP reveal insertion profiles for KF(exo$^-$), KF, DNA polymerase I, Sequenase version 2.0, and *Taq* DNA polymerase similar to those in the presence of hybrid P1/T**2**: maximal incorporation of one hA (Figure 3A−C,E,F, lane 3) without further elongation with a natural dG (Figure 3A−C,E,F, lane 6). With dATP as substrate, four building blocks are incorporated (the fourth resulting from a misincorporation of dA opposite dT in the template) (Figure 3A−C,F, lane 2). This misincorporation was not seen for T7 Sequenase version 2.0 (Figure 3E, lane 2). With two different natural triphosphates, dATP and dGTP (Figure 3A−C,E,F, lane 5), maximal incorporation (primer + 5) was achieved. In the presence of T4 DNA polymerase and *Pfu* DNA polymerase, three natural dAs are inserted (Figure 3D,G, lane 2). With hATP as substrate, elongation of the DNA primer with only two building blocks was noticed (Figure 3D,G, lane 3). With a mixture of adenosine and guanosine triphosphate building blocks, full-length product was achieved in the presence of the natural triphosphates (Figure 3D,G, lane 5), in contrast to insertion of only two nucleotides with a combination of dGTP and hATP as the substrates (Figure 3D,G, lane 6). The incorporation pattern in the presence of Vent exo$^-$ DNA polymerase (Figure 3H) showed a weak incorporation of three hAs (insertion of the third hA indicated by the arrow) (lane 3), in comparison to incorporation of four deoxyadenylates with dATP as the substrate (lane 2). Further elongation with a natural dG was not possible in combination with hATP as the substrate (lane 6). Maximal incorporation was seen with two natural triphosphate building blocks (lane 5). Incorporation in the presence of dGTP as substrate results in misinsertion for all tested DNA polymerases (Figure 3A−C,F, lane 4),except for T4 DNA polymerase (Figure 3D, lane 4), T7 Sequenase (Figure 3E, lane 4), and *Pfu* DNA polymerase (Figure 3G, lane 4).

Incorporation of Multiple hN Nucleotides. Since Vent exo$^-$ DNA polymerase was the only enzyme capable of incorporating three hAs consecutively, it was selected for further investigation. In a first experiment, homopolymer formation was examined with hA opposite its natural counterpart in the template of a DNA hybrid. An incorporation assay was therefore performed in the presence of hybrid P**1**/T**5** (Figure 4), where the insertion of the natural dATP and of its anhydrohexitol analogue were determined under different experimental conditions. Extension products were analyzed by denaturing PAGE and phosphorimaging. The resulting insertion profile (Figure 4) indicated that, in the presence of dATP, maximal incorporation is achieved with high efficiency under all considered reaction conditions. At a dATP concentration of 1000 μM, an additional adenylate is inserted (lanes 6−9). Replacing the natural building block by its anhydrohexitol analogue at a concentration of 10 μM resulted in elongation of the primer with two building blocks (lanes 11−14). Upon increasing the concentration 100 times, insertion increased from two (lane 15) to six hA nucleotides under the most drastic conditions (lane 18). This incorpora-

Table 1: Quantitative Data for the Enzymatic Incorporation of hATP and Further Elongation in the Presence of Hybrid P**1**/T**1** and P**1**/T**2** by Means of Different DNA Polymerases[a]

	hybrid P**1**/T**1**		hybrid P**1**/T**2**	
	incorporation of one hATP	further elongation	incorporation of two hATPs	further elongation
KF (exo$^-$)	+++	+++ (p + 5)	−	−
Klenow	+++	+++ (p + 5)	−	−
DNA polymerase I	+++	+++ (p + 5)	−	−
T7 Sequenase version 2.0	+	−	−	−
T4 DNA polymerase	++	++ (p + 2)	++	−
Taq DNA polymerase	+	−	−	−
Pfu DNA polymerase	+++	+++ (p + 2)	++	−
Vent exo$^-$ DNA polymerase	+++	++ (p + 2)	+++	++ (p + 3)

[a] +++, complete primer extension; ++, extension >50% of the primer; +, extension <50% of the primer; −, no extension.

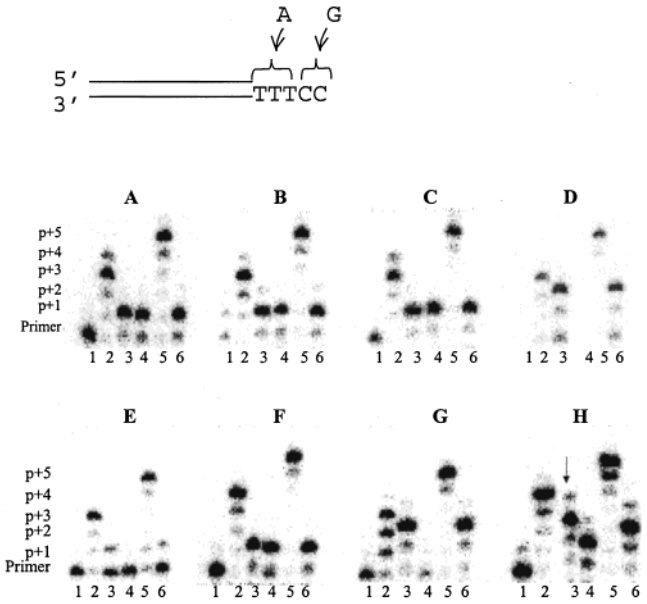

FIGURE 3: Phosphor images of the enzymatic incorporation of three hAs into 50 nM hybrid P**1**/T**3** in the presence of 0.05 unit/μL KF(exo$^-$) (A), KF (B), DNA polymerase I (C), T4 DNA polymerase (D), T7 Sequenase version 2.0 (E), *Taq* DNA polymerase (F), *Pfu* DNA polymerase (G), or Vent (exo$^-$) (H) and 100 μM NTPs: dATP (lane 2), hATP (lane 3), dGTP (lane 4), dATP + dGTP (lane 5), or hATP + dGTP (lane 6). The arrow indicates the insertion of the third hA. Lane 1 is the blank reaction in the absence of NTPs. The reaction time was 10 min.

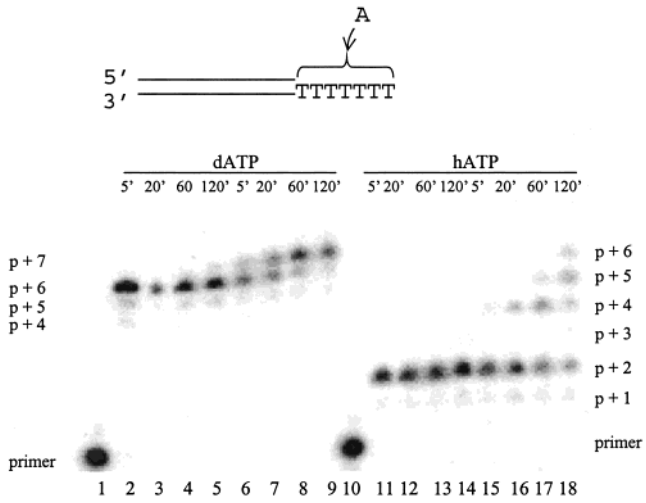

FIGURE 4: Phosphor image of the enzymatic incorporation of consecutive hAs into hybrid P**1**/T**5** in the presence of 1 unit/μL Vent exo$^-$ and dATP, 10 μM (lanes 2−5) or 1000 μM (lanes 6−9), or hATP, 10 μM (lanes 11−14) or 1000 μM (lanes 15−18). Lanes 1 and 10 are the blank reactions in the absence of NTPs. Reaction times are indicated at the top in minutes.

tion is not very efficient and clear pauses are seen after the addition of two, four, and five analogues. Because of the difference of conformation between dATP and hATP, the positions of the polymerized products on the gel are not similar with dATP and hATP as substrates.

The following experiment investigated the ability of Vent (exo$^-$) to produce a HNA decamer chain containing alternating A and C building blocks. Enzymatic insertion was performed in the presence of hybrid P**3**/T**6** (Figure 5) with a 10-mer DNA overhang allowing analysis of the incorporation of the separate triphosphate building blocks dATP, hATP, dCTP, and hCTP and of several combinations of two of these triphosphates. Polymerized products were subjected to polyacrylamide gel electrophoresis and visualized by means of phosphorimaging. The incorporation pattern (Figure 5) revealed incorporation of three deoxyadenosine nucleotides (lane 1), in contrast to the insertion of two building blocks with the anhydrohexitol analogue (lane 2). Addition of the cytosine building block resulted in elongation with two nucleotides, either the natural triphosphate (lane 3) or its anhydrohexitol analogue (lane 4). By combining two different building blocks, the expected maximal incorporation is achieved with the natural triphosphates (lane 5). Extension with six nucleotides, paused at position 4, is achieved with two anhydrohexitol nucleotides (lane 6) or a combination of hATP and dCTP (lane 8). A more efficient production of the primer +7 product occurred upon adding the alternative mixture of dATP and hCTP as the substrate (lane 7).

Selectivity of Incorporation. To evaluate the selectivity of incorporation of hNTPs opposite their natural analogues in the template in the presence of Vent exo$^-$ DNA polymerase, four separate incorporation assays were performed with four different hybrids, P**1**/T**4**, P**2**/T**4**, P**3**/T**4,** and P**4**/T**4** (Figure 2), consisting of the same template hybridized with, respectively, 17-, 18-, 19-, and 20-mer DNA primers. Each hybrid was used in separate reactions in the presence of different triphosphate building blocks. This way, insertion of the anhydrohexitol nucleotides in comparison to their natural analogues was investigated. Reaction conditions were determined in preliminary experiments, being a compromise between the lowest degree of misincorporation and an acceptable degree of incorporation for the anhydrohexitol analogues. The insertion patterns resulting from these incorporation assays are shown in Figure 6. Figure 6A reveals

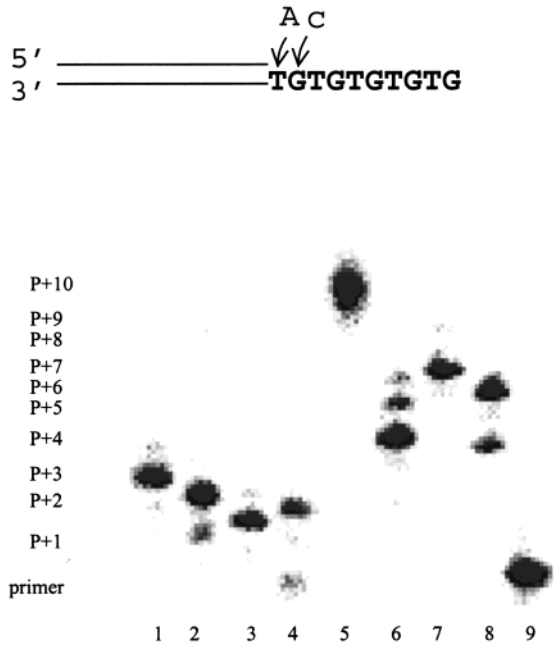

FIGURE 5: Phosphor image of the alternating enzymatic incorporation of ATP and CTP into hybrid P**3**/T**6** (50 nM) in the presence of 0.05 unit/μL Vent exo$^-$ and 100 μM dATP (lane 1), hATP (lane 2), dCTP (lane 3), hCTP (lane 4), dATP + dCTP (lane 5), hATP + hCTP (lane 6), dATP + hCTP (lane 7), or hATP + dCTP (lane 8). Lane 9 is the blank reaction in the absence of NTPs. The reaction time was 120 min.

the incorporation of two building blocks upon adding the natural thymidine nucleotide (lane 2) (the second incorporation resulting from misinsertion of dT opposite dC in the template). This misincorporation was not seen when dTTP was replaced by its anhydrohexitol analogue (lane 3). Adding dGTP (lane 4) or hGTP (lane 5) to the reaction mixture produced no elongation of the DNA primer. By combining two different bases, incorporation of two nucleotides was achieved with either two natural triphosphates (dTTP + dGTP) (lane 6) or their anhydrohexitol analogues (hGTP + hTTP), although in the last case insertion was less efficient (lane 7). Addition of three and four triphosphate building blocks, respectively, resulted in the expected maximal incorporation for the natural triphosphates (lanes 8 and 10) and in the insertion of two nucleotides for their anhydrohexitol analogues (lanes 9 and 11). Incorporation profiles in the presence of hybrid P**2**/T**4** (Figure 6B) were similar, although misincorporation of the first nucleotide opposite the second base in the template was not detected here (lane 2). However, misinsertion opposite dC in the template was seen with the anhydrohexitol triphosphate of adenine (lane 5), in contrast to its natural analogue (lane 4). Evaluation in the presence of hybrid P**3**/T**4** (Figure 6C) showed the expected incorporation of one nucleotide in the presence of the natural dA triphosphate (lane 2) as well as its anhydrohexitol analogue (lane 3). With dCTP (lane 4) or hCTP (lane 5) as the substrate, no elongation of the primer was obtained. By adding two or three different building blocks to the reaction mixture, a similar incorporation profile was noticed for dNTPs (lanes 6 and 8) as well as for the hNTPs (lanes 7 and 9). The incorporation pattern in Figure 6D revealed insertion into hybrid P**4**/T**4** of one dC (lane 2) and one hC (lane 3), although the last one showed lower efficiency. No incorporation was seen with dTTP (lane 4) or hTTP (lane 5). Full-length product was achieved in the presence of dCTP and dTTP (lane 6), in contrast to incorporation of only two nucleotides in the presence of hCTP and hTTP (lane 7).

Steady-State Kinetic Analysis of hATP Insertion in a DNA Hybrid. To quantify the insertion of hATP by Vent exo$^-$ DNA polymerase, a steady-state kinetic assay (*62*) was used. To compare kinetic parameters for incorporation of hATP and dATP, a 19-mer DNA primer (P**3**) and a 24-mer DNA template (T**4**) were used. The target site, T at position 2 in the template, was placed at the first position downstream of the primer. This allowed determination of the kinetic parameters for incorporation of either dA or hA from a standing start as described by Boosalis et al. (*62*). The primer was ^{32}P-labeled, enabling radiography of the polyacrylamide gel and subsequent quantification of the radioactivity of the remaining primer and the extended products, which allowed calculation of the initial velocity of the reaction. Kinetic parameters were determined as described under Materials and Methods. The Michaelis−Menten plot is shown in Figure 7, and the parameters (average of three experiments) are listed in Table 2. These data indicate that dATP and hATP have similar K_m and V_{max} values.

DISCUSSION

This study demonstrates that all investigated DNA polymerases can recognize the unnatural anhydrohexitol nucleotides and incorporate them correctly opposite their natural complements into a DNA hybrid. Although all DNA polymerases studied in this paper were able of accepting one modified nucleotide, their capacities for inserting more than one anhydrohexitol nucleotide were quite different. On the basis of the capability for chain elongation with another hexitol analogue after incorporation of the first hexitol nucleotide, a classification of the tested DNA polymerases can be made. T7 Sequenase version 2.0, *Taq* DNA polymerase, DNA polymerase I, KF, and KF(exo$^-$) are hampered in their DNA replicative function following the insertion of one building block analogue. With T4 DNA polymerase and *Pfu* DNA polymerase on one hand and Vent (exo$^-$) on the other hand, chain termination was achieved after the insertion of two or three hA nucleotides, respectively (Figure 3). A few years ago, the sequences of several DNA polymerases were analyzed to identify conserved and variable regions of the different DNA polymerases (*10*, *63*). On the basis of this research, four families (A, B, C, and X) of DNA polymerases can be distinguished. Each family can be further subdivided into several classes. This classification is in agreement with our results, since the enzymes capable of inserting only one hA nucleotide in our research all belong to the A family (also called pol I family). The enzymes capable of incorporating more than one hA [T4 DNA polymerase, *Pfu* DNA polymerase, and Vent (exo$^-$)] are members of the B family (also called pol II family or α family). This observation shows that the anhydrohexitol triphosphate building block of adenine might be an interesting tool for functionally classifying DNA polymerases.

The 1,5-anhydrohexitol nucleotide has a natural base moiety. The difference with a natural nucleoside is the size and the restricted conformational mobility of the sugar moiety. In the anhydrohexitol triphosphate an extra methylene group is inserted between carbon-1′ and the ring

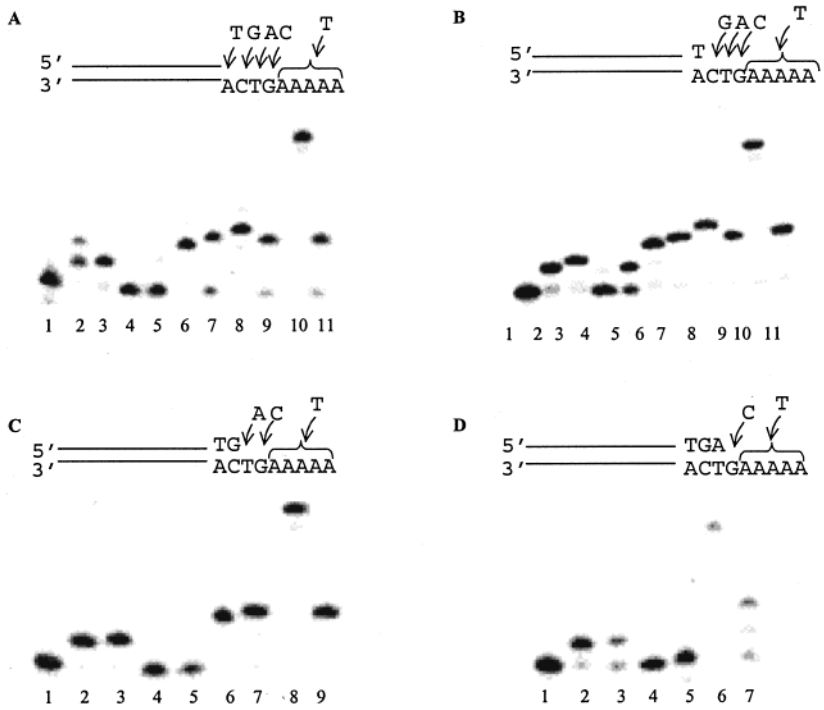

FIGURE 6: Phosphor images of selectivity tests in the presence of 0.005 unit/μL Vent exo$^-$ and 50 nM hybrid P**1**/T**4** (A), hybrid P**2**/T**4** (B), hybrid P**3**/T**4** (C) and hybrid P**4**/T**4** (D). The concentration of building blocks is 10 μM. (Panel A) dTTP (lane 2), hTTP (lane 3), dGTP (lane 4), hGTP (lane 5), dTTP + dGTP (lane 6), hTTP + hGTP (lane 7), dTTP + dGTP + dATP (lane 8), hTTP + hGTP + hATP (lane 9), dTTP + dGTP + dATP + dCTP (lane 10), hTTP + hGTP + hATP + hCTP (lane 11). (Panel B) dGTP (lane 2), hGTP (lane 3), dATP (lane 4), hATP (lane 5), dGTP + dATP (lane 6), hGTP + hATP (lane 7), dGTP + dATP + dCTP (lane 8), hGTP + hATP + hCTP (lane 9), dGTP + dATP + dCTP + dTTP (lane 10), hGTP + hATP + hCTP + hTTP (lane 11). (Panel C) dATP (lane 2), hATP (lane 3), dCTP (lane 4), hCTP (lane 5), dATP + dCTP (lane 6), hATP + hCTP (lane 7), dATP + dCTP + dTTP (lane 8), hATP + hCTP + hTTP (lane 9). (Panel D) dCTP (lane 2), hCTP (lane 3), dTTP (lane 4), hTTP (lane 5), dCTP + dTTP (lane 6), hCTP + hGTP (lane 7). The reaction time was 5 min.

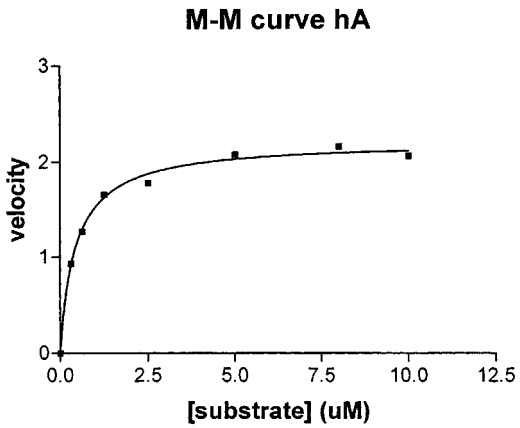

FIGURE 7: Plot of initial velocities [as defined by Boosalis et al. (*62*)] versus [dNTP] for the incorporation by Vent exo$^-$ DNA polymerase of hATP opposite T in the template T**4**.

Table 2: Kinetic Parameters of Vent Exo$^-$ from a Steady-State Kinetic Analysis of hATP in a DNA Hybrid

substrate	V_{max} (% min^{-1})	K_m
dATP	2.31 ± 0.07	0.63 ± 0.03
hATP	2.20 ± 0.09	0.44 ± 0.03

oxygen atom, resulting in a more rigid six-membered sugar moiety. Examination of the conformation of a 1,5-anhydrohexitol nucleoside suggested a good fit with a natural furanose nucleoside in its 3′-endo conformation, rather than in its 2′-endo conformation (*55*, *56*). DNA polymerases use deoxyribonucleotides as their substrate to elongate a DNA primer hybridized with a DNA template. A 2′-deoxynucleotide may occur in the 2′-endo as well as in the 3′-endo conformation, and the energy of interconversion is low. It is therefore not self-evident that hexitol nucleotides could serve as substrates for all natural DNA polymerases. On the other hand, it has been shown that the affinity of DNA polymerases for the traditional 2′-deoxyribonucleoside triphosphates is not absolute, as indicated by Ide et al. (*53*).

Recent structural studies of an enzyme−DNA−dNTP complex (*11*−*13*) and site-directed mutagenesis experiments (*14*−*18*) have contributed to our current state of knowledge concerning interactions between amino acids in certain regions of DNA polymerases and the sugar part of dNTPs. More information is available for class I DNA polymerases than for class II DNA polymerases. A region on the polymerase I cleft containing three amino acids is supposed to be involved in interactions with the sugar part of an incoming dNTP, namely, Phe 762, Tyr 766, and Glu 710 (all positions in *Escherichia coli* DNA polymerase I) (*64*). It is assumed that Phe 762 (*16*, *64*, *65*) monitors the 3′-substituent and therefore is responsible for discrimination between deoxy- and dideoxyribonucleotides. Glu 710 (*16*, *64*, *65*), likewise scanning the 3′-substituent in the sugar part, forms a steric gate preventing rNTPs from being accepted by DNA polymerases. A mutation of Glu 710 of KF polymerase I changes the rate of incorporation of rNTP. Tyr 766 (*16*, *64*, *65*), monitors the 2′-sugar position of an incoming rNTP by playing a role in positioning the DNA primer−template duplex. In class II DNA polymerases, it is

assumed that Tyr 412 in region II could potentially play a role in 2′-deoxyribonucleotide/ribonucleotide discrimination (*17*). Decreasing the size of the Tyr 412 side chain facilitated incorporation of rNTP but did not influence ddNMP incorporation. This amino acid residue is analogous to Tyr 766 in *E. coli* DNA polymerase I. In addition, Ala 488 and Tyr 499 have been reported to play a role in sugar discrimination of class II DNA polymerases (*17*). Replacing Ala 488 by a more bulky group facilitates incorporation of NTPs as well as ddNTPs. Until now, no analogue for Phe 762, present in class I polymerases, could be identified in class II polymerases. Since class II polymerases differ in many structural features from class I polymerases, no valuable explanation (based on the presence of Glu 710/Tyr 766 and Tyr 412) can be given for the difference in their ability to incorporate hAs. Earlier published data concerning the ability of different DNA polymerases to accept sugar analogues in DNA synthesis do not correspond with our observation that Vent (exo$^-$) is perhaps an enzyme more flexible in accepting sugar-modified analogues since in none of these studies was Vent (exo$^-$) more successful than other polymerases (*29*). These findings suggest that, on the basis of amino acid sequences, there may not be general rules for the recognition of modified substrates by polymerases. This observation has already been mentioned in other publications (*27*, *28*) and is further confirmed by our experiments with polymerases belonging to a same family. For example, in class II polymerases, T4 DNA polymerase and *Pfu* DNA polymerase showed termination after the insertion of two building block analogues, while Vent (exo$^-$) could incorporate three hAs. From class I polymerases, it could be noticed that T7 Sequenase version 2.0 seemed significantly less efficient than the other polymerases. Interestingly, Johnson (*66*) earlier noticed already that the rate of conformational change of T7 polymerases is extremely sensitive to proper base geometry. In addition, elongation following the insertion of one hA nucleotide differs between individual enzymes and depends on whether a natural or an anhydrohexitol building block is offered for insertion.

Since K_m and V_{max} values for the incorporation of one hAMP and one dAMP are very similar, it can be assumed that binding of a hNTP to the enzyme−hybrid complex is not disfavored for a larger ring structure with a stiff conformation. The absence of a hydroxyl group in the pseudo-2′-position of the sugar part reduces steric clash at this location, implying no or slight discrimination between hATP and dATP. Previously, it was published that when DNA is bound to DNA polymerases, the duplex tends to adopt a more A-like conformation close to the active site (*4*). This suggests indeed that hNTPs, with their 3′-endo sugar mimic, could be incorporated efficiently, which is proven now experimentally. Following incorporation of four or five residues, however, a conformational switch is seen from A-form to B-form DNA (*4*). Since an anhydrohexitol nucleotide has a rigid six-membered carbohydrate residue, a conformational switch in oligomerized anhydrohexitol nucleotides is disfavored. This might be a reason the extension of the primer with either a natural or an anhydrohexitol nucleotide is slowed after several hA residues have been incorporated. It was experimentally observed that, even with Vent (exo$^-$), the insertion of a third building block after two consecutive hA incorporations is very difficult. This phenomenon was already seen in other experiments with modified analogues (*40*, *41*) and was suggested to be due to a distortion of the DNA after incorporation of the sugar analogue. With HNA as a model, this observation is not surprising. Previously, we demonstrated that the conformations of HNA−DNA and HNA−RNA hybrids (*56*, *67*, *68*) showed remarkable similarities to that of double-stranded RNA and that HNA adopts an A-form-like structure. No B-form structures have been observed with HNA as a complement of natural nucleic acids. It might therefore be expected that, after incorporation of several residues, the newly synthesized HNA−DNA primer forms an A-like helix with the template and that the polymerase is therefore blocked in its elongation function. The synthesis of a homopolymer consisting of exclusively hA building blocks by means of Vent (exo$^-$) showed termination following the addition of two nucleotide analogues when a low concentration of hATP (10 μM) was used. Under extreme reaction conditions, the enzyme succeeded in elongating the primer with six nucleotides; however, clear pauses at several positions (+2, +4, and +5) were observed. Selectivity tests likewise confirmed that further elongation following two anhydrohexitol nucleotides was not possible under conditions necessary for selective incorporation.

CONCLUSION

It is proven that nucleotide analogues with an unnatural 1,5-anhydrohexitol ring can be incorporated by all DNA polymerases. DNA polymerases belonging to the B-family are more efficient in this capacity. Hexitol nucleoside triphosphates prove therefore to be excellent substrates to functionally classify different DNA polymerases. Vent (exo$^-$) is considered to be the most promising DNA polymerase for oligomerization reactions with hNTPs as substrates. Kinetics of incorporation of one hexitol nucleotide or one natural deoxynucleotide are very similar. Under less stringent reaction conditions, this enzyme proved capable of elongating a primer with six anhydrohexitol adenine nucleotides and even seven nucleotides when alternating adenine and cytosine analogues were used. Selectivity of incorporation, however, is only achieved under more stringent experimental conditions under which no more than two consecutive anhydrohexitol building block analogues can be inserted.

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