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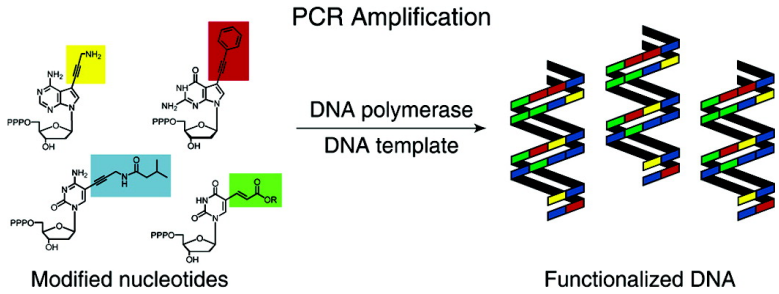

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A Versatile Toolbox for Variable DNA Functionalization at High Density

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Abstract: To broaden the applicability of chemically modified DNAs in nano- and biotechnology, material science, sensor development, and molecular recognition, strategies are required for introducing a large variety of different modifications into the same nucleic acid sequence at once. Here, we investigate the scope and limits for obtaining functionalized dsDNA by primer extension and PCR, using a broad variety of chemically modified deoxynucleotide triphosphates (dNTPs), DNA polymerases, and templates. All natural nucleobases in each strand were substituted with up to four different base-modified analogues. We studied the sequence dependence of enzymatic amplification to yield high-density functionalized DNA (fDNA) from modified dNTPs, and of fDNA templates, and found that GC-rich sequences are amplified with decreased efficiency as compared to AT-rich ones. There is also a strong dependence on the polymerase used. While family A polymerases generally performed poorly on "demanding" templates containing consecutive stretches of a particular base, family B polymerases were better suited for this purpose, in particular *Pwo* and Vent (exo-) DNA polymerase. A systematic analysis of fDNAs modified at increasing densities by CD spectroscopy revealed that single modified bases do not alter the overall B-type DNA structure, regardless of their chemical nature. A density of three modified bases induces conformational changes in the double helix, reflected by an inversion of the CD spectra. Our study provides a basis for establishing a generally applicable toolbox of enzymes, templates, and monomers for generating high-density functionalized DNAs for a broad range of applications.

Introduction

The nanosciences currently face a growing interest in applying nucleic acids and analogues as building blocks for the bottom-up construction of self-assembling functional objects in nanometer dimensions. The advantage of utilizing nucleic acids for these purposes lies in the logics and predictability of nucleobase pairing through complementary hydrogen-bond donors and acceptors. The encoded self-assembly of complementary nucleic acid strands to geometrically well-defined duplex structures has led to the generation of a variety of two- or three-dimensional topologies, supramolecular assemblies, and nanomechanical objects.[1] Moreover, nucleic acids possess supreme template properties that allow for their enzymatic replication and amplification by polymerases and the application of in vitro evolution technologies to select for nucleic acid-based sensors,[2] diagnostics,[3] therapeutics,[4] and catalysts.[5]

For further acceleration of these developments and to expand the scope of applications of nucleic acids in nanobiotechnology, it is necessary to introduce additional functionalities into oligonucleotides. This can be achieved either by utilizing bound cofactors[6] or by direct chemical derivatization of the building blocks. It has been shown that the introduction of modified DNA-monomers can significantly alter the physical and geometrical properties of nucleic acids.[7] Furthermore, additional chemical diversity can result in oligonucleotides containing sequence- and site-specific modifications that might exert additional functions or interactions that go beyond simple Watson−Crick pairing. These can be used, for example, as probes for elucidating functional and structural properties of DNA polymerases,[8] for RNA structure−function analysis,[9] for

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template-directed synthesis,[10] for controlled self-assembly,[11] for facilitating charge transfer in DNA,[12] to detect the presence of abasic sites in a DNA,[13] and other purposes.[14] Another interesting field of application of functionalized nucleic acids is the in vitro evolution of aptamers, DNAzymes, or ribozymes for molecular recognition and catalysis.[5] The goal in this research area is to generate molecules that are both easy to copy and vary, like DNA, and chemically adept, like proteins.

Chemical modifications can be incorporated into oligonucleotides via the heterocyclic nucleobases, in the sugar unit, or at the backbone level. While short highly modified DNA strands with less than 50−60 nucleotides are accessible through chemical solid-phase synthesis, the generation of longer modified oligonucleotides following this route is either very difficult, or impossible. In particular, the construction of longer DNA strands that contain various different modifications throughout the molecule remains elusive. Thus, strategies that allow one to introduce a large variety of different modifications into the same nucleic acid sequence at once might further expand the applicability of chemically modified DNAs. The ability to enzymatically replicate a DNA or RNA molecule that is functionalized at high density in a template-directed fashion would provide access to longer sequences that are difficult to obtain synthetically. This is useful, for example, for generating combinatorial libraries of DNA molecules with expanded chemical functionality from which aptamers and catalysts with expanded structural and functional properties can be isolated by in vitro evolution.[5] Such methodology is also required for multiplex sequencing,[15] and other purposes such as the incorporation of artificial base pairs for extending the genetic alphabet.[16]

It has been shown that up to two different modified nucleotides can be enzymatically polymerized by primer extension reactions or polymerase chain reactions (PCR) on a DNA template.[15,17] Earlier, we have reported the first enzymatic synthesis of a high-density functionalized DNA (fDNA) in which every base position was equipped with a different functional group. This fDNA was generated by primer extension using a natural model template of almost equal base distribution.[18] Recently, we could show that a high-density fDNA could in turn serve as a template for the polymerase-mediated generation of a fully functionalized DNA double-strand and can be amplified by PCR under certain conditions.[19]

To further expand the scope of the enzymatic generation and amplification of functionalized DNA and to also explore potential limitations of this approach, we have now synthesized an increased set of modified dNTPs and investigated their template-directed enzymatic incorporation into fDNA. We provide a systematic analysis of the sequence requirements by investigating more demanding DNA templates that consist of multiple nucleotide repetitions. Likewise, we assayed a variety of polymerases for their capability to incorporate modified dNTPs in different sequence contexts and identified the most generic ones. We show that a broad variety of chemical modifications can be tolerated by the identified DNA polymerases under certain conditions, regardless of their chemical nature. We further investigated whether functionalized DNA double-strands can be amplified from the demanding templates by PCR. We also performed an analysis of possible structural alterations of functionalized DNA duplexes as a function of the nature and density of the modification. With this analysis, we have established a platform for the generation and amplification of DNAs functionalized at various densities with different chemical modifications for a large variety of potential applications.

Results and Discussion

1. Design and Synthesis of Modified Nucleotides. We synthesized various deoxynucleoside triphosphate (dNTP) analogues of all four natural nucleotides bearing basic, acidic, or

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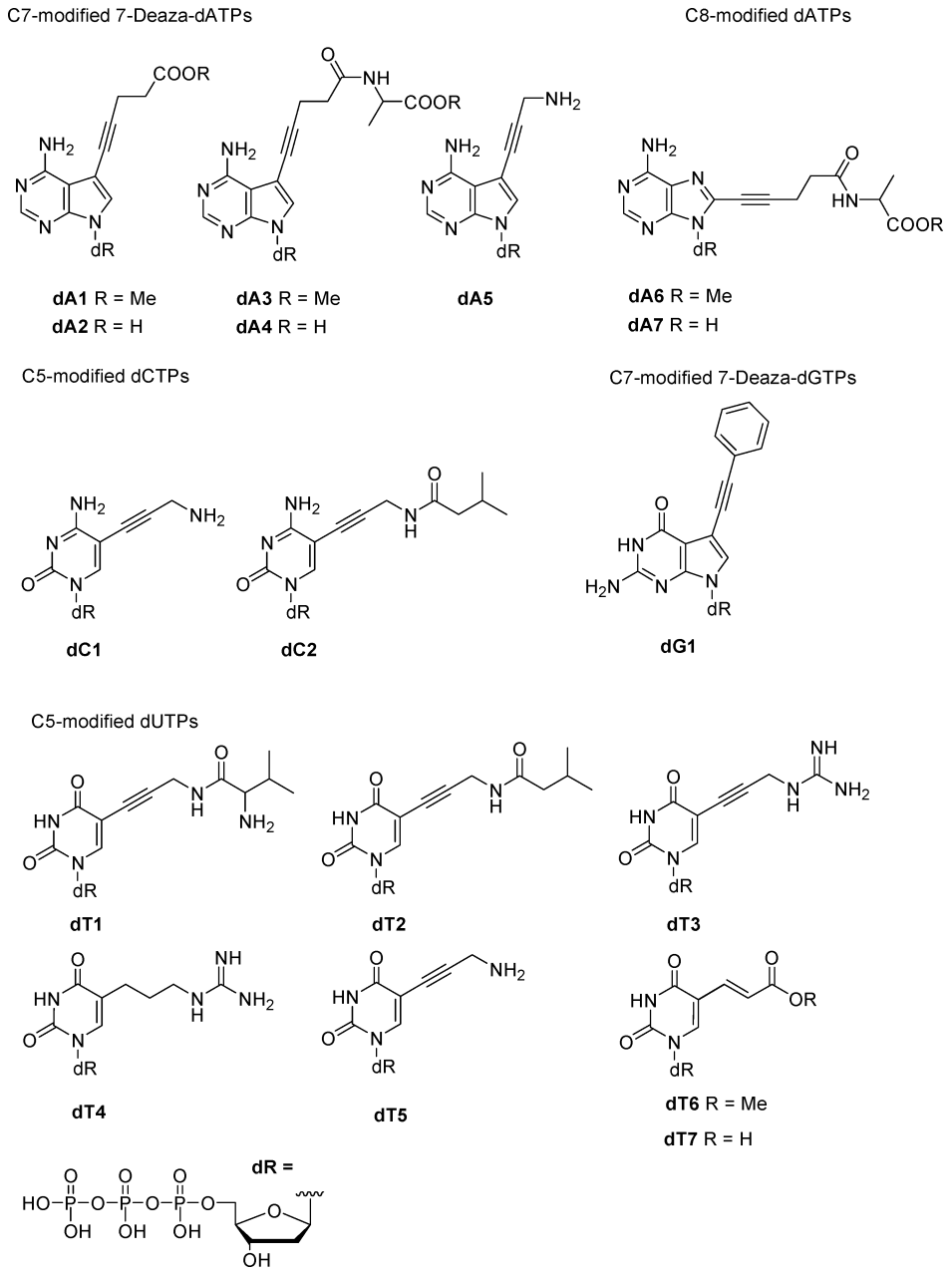

Figure 1. Chemical structures of the synthesized modified nucleosid-5′-*O*-triphosphates.

lipophilic groups. The functionalities were attached to the nucleobases via palladium-catalyzed coupling of the corresponding halogenated nucleoside with unsaturated alkynes or alkenes (Figure 1). Scheme 1 shows a representative synthesis of the purine **dA2** and the pyrimidine **dT2**. The base-modified nucleosides were transferred to the 5′-*O*-triphosphates. Full reaction schemes and spectroscopic data can be found in the Supporting Information.

The modifications of the functionalized 2′-deoxynucleotides have to be positioned so as to ensure compatibility of these derivatives with enzymatic transformations necessary for template-directed replication of the corresponding oligonucleotides, independent of their sequence and base composition. For the pyrimidine derivatives, position C5 had been shown to be optimal for positioning additional functional groups without disturbing the helical structures.[20]

For the purines, the N7- and C8-positions appear to be suitable for modification. 8-Modified deoxyadenosines are known to destabilize dsDNA secondary structure, presumably due to favoring the *syn*-conformation of the nucleotide.[21] However, an 8-modified adenosine has been used as surrogate for dATP in polymerase chain reaction.[5b,17b] Furthermore, a CD- and UV-study showed that a C8-alkyne derivative of 2′-deoxyadenosine causes only a slight decrease in the melting temperature of dsDNA and does not disturb the overall B-DNA conformation.[22] On the other hand, the substituents of 7-modified 7-deazapurines

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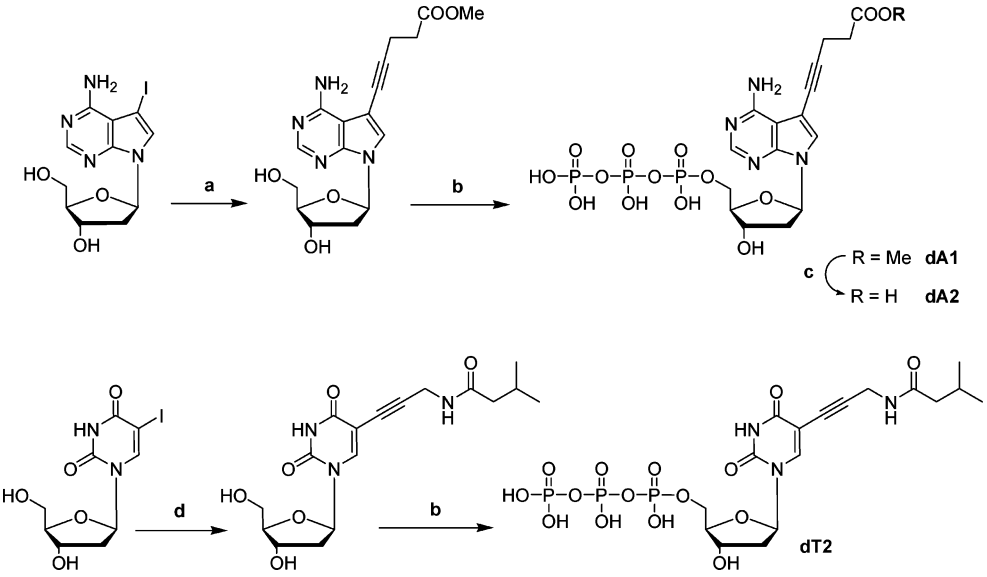
Scheme 1. Synthesis of a C7-Modified N7-Deaza-2′-deoxyadenosine Triphosphate and a C5-Modified Deoxyuridine Triphosphate[a]



[a] Conditions: (a) pent-4-ynoic acid methyl ester, $Pd(PPh_3)_4$, CuI, Et_3N, DMF; (b) (i) $POCl_3$, proton-sponge, $OP(OMe)_3$; (ii) $(n Bu_3NH)_2H_2P_2O_7$, $n Bu_3N$; (iii) 1 M TEAB; (c) aqueous NaOH; (d) 3-methyl-*N*-prop-2-ynylbutyramide, $Pd(PPh_3)_4$, CuI, Et_3N, DMF (DMF = *N*,*N*-dimethylformamide; TEAB = triethylammonium bicarbonate).

M79: 5′-A TGC CGA TGA CTA GTC GTC ACT AGT GCA CGT AAC GTG CTA -3′

A79: 5′-**A** CGT **AA**T GC**A** **AA**G CT**A** **AAA** TCG **AAA** **A**CT G**AA** **A**GT C**AA** CGT -3′

C79: 5′-**C** AGT **CC**T GA**C** **CC**G AT**C** **CCC** TAG **CCC** **C**AT G**CC** **C**GT A**CC** AGT -3′

G79: 5′-**G** ACT **GG**T CA**G** **GG**C AT**G** **GGG** TAC **GGG** **G**AT C**GG** **G**CT A**GG** ACT -3′

T79: 5′-**T** ACG **TT**G CA**T** **TT**C AG**T** **TTT** GAC **TTT** **T**AG C**TT** **T**CG A**TT** ACG -3′

P79: 5′-N NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN -3′

Figure 2. Sequences of the middle part of the templates (40 nt) that are flanked by a 19 nt and a 20 nt long constant primer region.

Table 1. Summary of the Product Formation by Family A and B Type DNA Polymerases in Primer Extension Experiments with Modified Nucleotides (**dA2**, **dC1**, **dG1**, **dT3**) and Different Templates (**M79**, **A79**, **C79**, **G79**, **T79**, **P79**)[a]

		templates					
polymerases		**M79**	**A79**	**C79**	**G79**	**T79**	**P79**
family A	*Taq*	(+++)	(−)	(++)	(++)	(+)	(−)
	Tth	+++	−	+++	+++	−	+
	KF (exo-)	+++	−	+++	+	+	+
family B	*Pwo*	+++	+++	+++	+++	+++	+++
	Tgo	+++	+++	+++	+++	+++	+
	Pfu (exo-)	+++	+++	+++	+++	+++	+
	Vent (exo-)	+++	+++	+++	+++	+++	+++

[a] Amount of full-length product generated with the modified nucleotides as compared to a control reaction with four natural nucleotides and the designated template: (−) < 5%, (+) = 5−40%, (++) = 40−70%, (+++) > 70%. The values for Taq polymerase are shown in parentheses because the length of the primer extension product is slightly shorter than the full-length product (see Figure 3A).

are well accommodated in the DNA major groove[23] and their triphosphates can efficiently replace natural dNTPs in PCR.[17c] Moreover, 7-modified purine dideoxy derivatives are used as chain terminators in cycle sequencing.[24]

2.1. Suitability of Polymerases for High-Density fDNA Synthesis by Primer Extension. Encouraged by our previous finding that *Tth* DNA polymerase is able to generate a modified DNA single strand in which every base is substituted by the modified nucleotides **dA2**, **dC1**, **dG1**, and **dT3** on a model template,[18] we investigated the range of other available DNA polymerases with this ability. Therefore, we initially performed primer extension experiments with a 5′-radiolabeled primer **P3′** using a 79 nucleotide long model template DNA **M79** (Figure 2) of almost equal base distribution.

We tested DNA polymerases from the A and B polymerase families (Table 1), specifically the family A polymerases from *Bacillus stearothermophilus* (*Bst*), *Thermus aquaticus* (*Taq*), *Thermus thermophilus* (*Tth*), the Klenow fragment (exo-), and Sequenase V 2.0. Among the family B DNA polymerases, we

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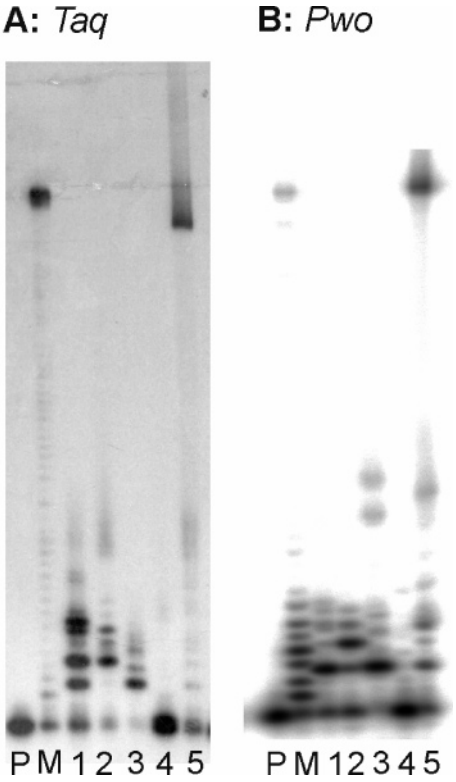

Figure 3. Primer extension experiments with 5′-[^{32}P]-labeled primer **P3′** and template **M79** with modified dNTPs and the denoted DNA polymerase, analyzed by 8% denaturing PAGE. (A) Reactions with *Taq* DNA-polymerase. Lanes: P, primer **P3′**; M, primer extension with four natural dNTPs and template **M79**; 1, reactions with modified nucleotides **dC1**, **dG1**, **dT3**; 2, **dA2**, **dG1**, **dT3**; 3, **dA2**, **dC1**, **dT3**; 4, **dA2**, **dC1**, **dG1**; 5, **dA2**, **dC1**, **dG1**, **dT3**. (B) Reactions with *Pwo* DNA polymerase: lanes as described for (A).

used the ones from *Pyrococcus furiosus* (*Pfu*), *Pyrococcus woesi* (*Pwo*), *Thermococc*us gorgonarius (*Tgo*), and *Thermococcus litoralis* [Vent (exo-)].

In our hands, *Bst* DNA polymerase and Sequenase did not accept the modified deoxynucleotides as substrates because no primer extension to full-length product was obtained (data not shown). However, as shown in Table 1, all other enzymes were able to generate full-length fDNA from modified nucleotides **dA2**, **dC1**, **dG1**, **dT3** (Figure 1) using template **M79** at levels of more than 70% as compared to positive control primer extensions with dNTPs.

Figure 3 shows a representative example of these experiments, including the negative control experiments in which one dNTP was left out. We also did the same experiment with natural dNTPs to compare the length of natural and modified DNAs. The quantity of elongated product (lane 5) is similar to the amount of DNA generated with natural dNTPs (lane M). No full-length product was observed in control experiments in which one of the nucleotides was omitted from the reaction mixtures (lanes 1−4). As expected, the polymerase stops either directly at the position where the missing base is encountered in the template, or one base later. With *Pwo* polymerase, a product was obtained that shows electrophoretic mobility similar to that of the DNA yielded with natural dNTPs (Figure 3B, lane 5). The observed smearing of the fDNA product band (Figure 3B, lane 5) is likely due to secondary structures or aggregates that were not resolved under the conditions of denaturing PAGE. This might be attributed to the incorporation of positively

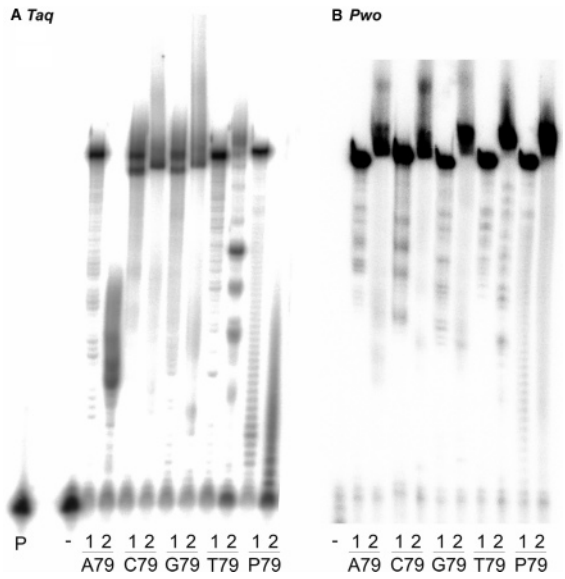

Figure 4. Generation of fDNA by primer extension using 5′-[^{32}P]-labeled primer and (A) *Taq* or (B) *Pwo* DNA polymerase with the designated template, analyzed by 8% denaturing PAGE. Lane P, primer; lane (−), reactions with **dA2**, **dC1**, **dG1**, **dT3**, and no template; lane 1, the natural dNTPs and the designated template; lane 2, **dA2**, **dC1**, **dG1**, **dT3**, and the designated template.

charged amino and guanidinium groups of **dT3** and **dC1** that can potentially form highly stable Coulomb interactions with the phosphate backbone or with the carboxyl group of **dA2**. Highly stabilized secondary and tertiary interactions can be expected in these oligonucleotides. In contrast, *Taq* polymerase repeatedly yielded a product with enhanced electrophoretic mobility, indicating that extension to full-length product was not achieved by this polymerase (Figure 3A, lane 5).

2.2. Suitability of Templates: Is Polymerization Sequence Dependent? Having shown that polymerases from both families A and B are able to accept functionalized dNTPs of all four DNA bases when using a template with almost equal base distribution, we next investigated more deeply whether the template-directed polymerization of modified nucleotides is dependent on the sequence context of the DNA template. Therefore, we designed more demanding templates that include repetitions of small stretches of the same base in their sequence. This template design results in an accumulation of steric and electronic requirements of one analogue component during the polymerase-mediated synthesis of the fDNA. Four 79-mer templates were used containing 40 nt long A- (**A79**), C- (**C79**), G- (**G79**), or T-rich (**T79**) regions with consecutive repetitions of the same nucleobase from two to four adjacent positions. The fifth template (**P79**) was composed of a fully randomized region of 40 bases, mimicking a combinatorial pool, typically used for in vitro selection experiments (Figure 2). We also used the previously described[19] shorter template **M59** as a positive control (data not shown).

We performed primer extensions using these five templates and the seven DNA polymerases from the A and B families. In this experiment, we used **dA2**, **dC1**, **dG1**, and **dT3** or the corresponding natural nucleotides for the control reaction. Figure 4 shows two representative examples of primer elongation experiments using *Taq* (Figure 4A) and *Pwo* DNA polymerase (Figure 4B), respectively; Table 1 summarizes the results for all tested DNA polymerases and templates.

In contrast to the results obtained when using the **M79** DNA as template, the family A DNA polymerases *Taq*, *Tth*, and KF (exo-) were not able to generate full-length fDNA on all templates. In the case of *Taq* DNA polymerase, the elongation was inhibited when three or four consecutive adenosines were present in the template (Figure 4A, **A79**, lane 2). Because *Taq* polymerase is highly Mg^{2+}-dependent, we repeated the reaction at higher Mg^{2+} concentrations to exclude possible quenching of Mg^{2+}-ions by modified dNTPs. However, even at higher Mg^{2+} concentrations in the reaction buffer, formation of full-length product could not be observed (data not shown). In primer elongations using the T-rich **T79**, *Taq* polymerase paused significantly after encountering the three or four nucleotide long T-stretches in the sequence (Figure 4A, **T79**, lane 2). Overall, the primer elongation with modified nucleotides resulted in significantly decreased product yields as compared to the reactions with natural dNTPs. *Tth* polymerase showed a similar behavior, and no full-length product could be observed for the A- and T-rich template (Table 1). In addition, KF (exo-) failed to elongate the primer to full-length with the A-rich template.

These data show that family A polymerases performed acceptably well with the C- and G-rich templates as compared to the control reactions. The T-rich **T79** and the random template **P79** were moderately accepted, whereas **A79** was not. This finding is in accordance with a previous result from the Benner laboratory, which showed that polymerases from the sequence family A were unable to incorporate a C5-modified dUTP-derivative carrying a protected thio-group via an alkynyl linker at multiple adjacent adenosine positions in a template.[25] Whether these distinctive behaviors of polymerases are due to differences in the structure and/or to different incorporation kinetics of individual modified dNTPs remains to be investigated.

In contrast, the polymerase of sequence family B: Vent (exo), *Tgo*, *Pfu* (exo-), and *Pwo* DNA polymerase performed much better and produced full-length product from the A-, C-, T-, and G-rich template in yields comparable to those obtained with natural dNTPs (Figure 4B and Table 1). While *Tgo* and *Pfu* (exo-) DNA polymerases showed only weak elongation of the primer with the randomized template **P79**, *Pwo* and Vent (exo-) DNA polymerases led to almost complete extension of the primer (Figure 4B, **P79**, lane 2 and Table 1). Thus, *Pwo* and Vent (exo-) DNA polymerases proved to be the best enzymes for the generation of a broad range of fDNA sequences.

Importantly, the integrity of the resulting **fM59** ssDNA, generated by primer extension with Vent (exo-) DNA polymerase, was confirmed by MS analysis (Supporting Information, part F, Figure S4A). The integrity of the modified **M79** template,[18] and the modified templates **A79**, **T79**, and **G79**, generated by primer extension with Vent (exo-) DNA polymerase was confirmed by Sanger sequencing (Supporting Information, part F, Figure S4B).

Taken together, these data show that family A polymerases exhibit a marked sequence dependence in their ability to yield full-length oligonucleotides when incorporating modified dNTPs into the random template or templates that contain multiple consecutive nucleotides. Among the family B polymerases, which generally performed better, only Vent (exo-) and *Pwo* DNA polymerases show virtually no sequence dependence.

(25) Held, H. A.; Benner, S. A. *Nucleic Acids Res.* **2002**, *30*, 3857−3869.

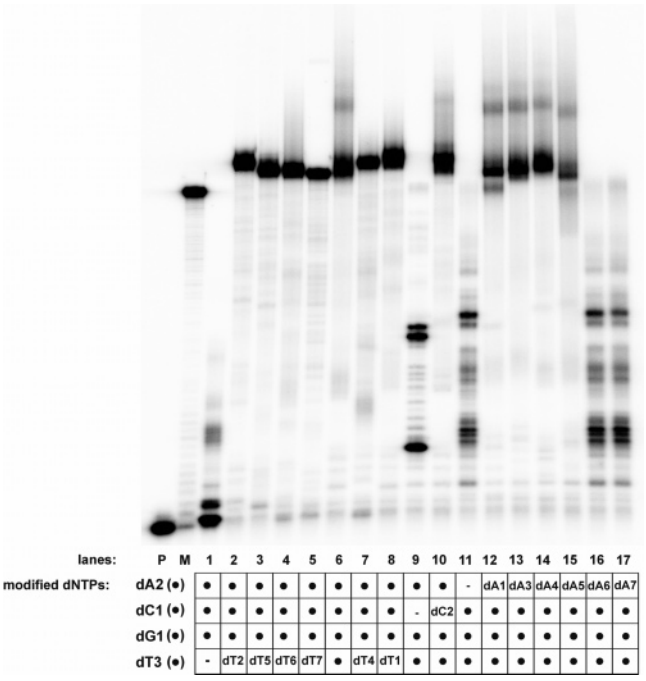

Figure 5. Primer extension reactions with Vent (exo-) DNA polymerase using 5′-[^{32}P]-labeled primer **P3′**, template **M79**, and different combinations of modified nucleotides. Lanes: lane P, primer **P3′**; lane M, positive control with four natural nucleotides dATP, dCTP, dGTP, TTP; lanes 1−17, analogues denoted below the lane.

2.3. Enzymatic Polymerization of Different Combinations of Modified dNTPs. Our aim was to establish a "toolbox" containing various functionalized building blocks for the modular construction of high-density functionalized DNA. So far, we could show that *Pwo* and Vent (exo-) DNA polymerases are optimal enzymes for generation of fDNA with one set of modified nucleotides (**dA2**, **dC1**, **dG1**, **dT3**). To explore the modularity of this approach, we investigated whether other combinations of modified dNTPs could be enzymatically polymerized, despite their different electronic and steric properties, in a template-directed fashion.

Therefore, we performed primer extension experiments with Vent (exo-) DNA polymerase, template **M79**, using the modified analogues **dA2**, **dC1**, **dG1**, **dT3** as basis for further incorporation studies with the other analogues. Specifically, we tested each modified TTP (**dT1**−**dT7**) in combination with **dA2**, **dC1**, **dG1**; the modified **dC2** in combination with **dA2**, **dG1**, **dT3**; and the modified ATP derivatives (**dA1**−**dA7**) in combination with **dC1**, **dG1**, **dT3** (Figure 5). To rule out the possibility of full elongation of the primer due to misincorporation under the applied conditions, appropriate control reactions were performed in the absence of the respective nucleotide (Figure 5, lanes 1, 9, 11). As a positive control, a reaction was carried out with the four natural dNTPs (Figure 5, lane M).

We found that every tested set with modified TTPs (Figure 5, lanes 2−8), modified dCTPs (lane 10), and 7-deaza-modified dATPs (lanes 12−15) produced full-length product. The yields were comparable to the control performed with natural dNTPs (Figure 5, lane M). In fact, the simultaneous incorporation of various charged residues (**dA2**, **dA4**, **dA5**, **dA7**, **dC1**, **dT1**, **dT3**, **dT4**, **dT5**, **dT7**), or nonpolar (**dA3**, **dA6**, **dC2**, **dG1**, **dT2**) and bulky modifications (**dC2**, **dT1**, **dT2**), did not appear to exhibit a substantial influence on product formation.

Interestingly, all full-length products derived from modified dNTPs (Figure 5, lanes 2−8, 10, 12−15), show a lower electrophoretic mobility as compared to natural DNA (lane M). This might be due to the increase in molecular weight of the functionalized oligonucleotides. Remarkably, the incorporation of the derivative **dT3**, which bears a guanidinium group with a rigid propynyl linker appears to lead to bands with decreased resolution and showed a strong tendency to smear (Figure 5, lanes 6, 10, 12−15). However, oligonucleotides containing the same guanidinium functionality attached via a more flexible, saturated linker to TTP (**dT4**) showed a band with improved resolution (Figure 5, lane 7). This observation is also supported by Roig et al., who reported recently that nucleotide **dT3** had a strong stabilizing effect on a DNA duplex.[26]

The C8-modified deoxyadenosines **dA6**, **dA7** could not be incorporated in these primer extension experiments (Figure 5, lanes 16, 17). The band pattern looks similar to the termination band of the reaction in which dATP derivatives were omitted (Figure 5, lane 11). Primer elongation experiments using Vent (exo-) DNA polymerase with C8-modified deoxyadenosine derivatives and natural dCTP, dGTP, TTP showed a band pattern (data not shown) similar to that of reactions without any dATPs. This suggests that 8-alkynylated derivatives were not recognized as substrates by the enzyme.

3. PCR Amplification of fDNA. To achieve fully modified systems that are replicated and amplified by DNA polymerases, we further investigated the performance of functionalized templates and functionalized dNTPs in PCR.

3.1. PCR Amplification of Natural dsDNA, Using Fully Functionalized Templates and Natural dNTPs. The easiest way to retransform the sequence information of fDNA back to natural DNA is by a PCR with natural dNTPs. In this case, a polymerase would have to incorporate a natural dNTP opposite to a complementary modified base in the functionalized template. Earlier, we provided an example showing that a fully functionalized DNA sequence could in turn serve as a template in a PCR with natural dNTPs for the sequence-specific amplification of natural DNA with *Pwo* DNA polymerase.[18]

To further explore the sequence dependence of fDNA-amplification, we generated additional fDNA templates for use in PCR experiments. The single-stranded modified templates were prepared by primer extension with 5′-biotinylated natural DNA templates **A79**, **C79**, **G79**, **T79**, **P79** (Figure 2) using 5′-[^{32}P] labeled primer **P3′** and the modified nucleotides **dA2**, **dC1**, **dG1**, **dT3 (**Figure 6). The resulting fDNA/DNA-hybrids were subsequently immobilized on streptavidin-agarose. The radio labeled fDNA was then recovered by elution with 0.1 N NaOH and used as a template in PCRs with natural dNTPs. As a positive control, single-stranded natural DNA was generated in the same way by primer extension using natural dNTPs. As additional controls, we immobilized biotinylated templates on streptavidin-agarose, washed and eluted as described above, and performed a PCR with the eluate.

As mentioned before, standard PCR conditions, using different DNA polymerases, natural dNTPs, and an fDNA template, did not result in any PCR-product,[18] presumably due to incomplete melting of the fDNA/DNA-hybrid under the conditions employed. Others have also observed that the presence of alkynyl-modified 7-deaza purines[23] or alkynyl C5-modified pyrimidines[26] in dsDNA increases the melting temperature. For these reasons, we increased the temperature of the denaturing step from 95 to 99 °C and used the extremely thermostable *Pwo* DNA polymerase in the presence of PCR additives that facilitate amplification of GC-rich DNAs sequences. To exclude the unintended amplification of contaminating biotinylated natural DNA templates, which might be present in small amounts as elution byproducts from the streptavidin column, the first 10 cycles of the PCR were performed only with primer **P5′**. In fact, this primer anneals only to the generated fDNA templates, excluding any unintentional amplification of unmodified DNA contaminations. Next, 5′-[^{32}P]-labeled primer **P3′** was added, and 8 cycles of PCR were performed. Equal amounts of fDNA or DNA templates were used in each experiment.

As shown in Figure 6B (lanes 2), the amplification of DNA from all different fDNA templates was successful (lanes 2) although with a lower efficiency than that from natural DNA templates (lanes 1). This might be attributed to an extensive formation of secondary structures within the single-stranded fDNAs or to an increased melting temperature of the corresponding fDNA/DNA hybrid.[23,26] Interestingly, the amplification of G-rich and C-rich templates (**C79**, **G79**, lane 2) appears to be somewhat less efficient than that of the A- and T-rich templates (**T79**, **A79**, lane 2). A similar correlation between sequence and efficiency of amplification is also observed in the natural DNA control reactions, although the effect appears to be less pronounced.

Nevertheless, the data in Figure 6 show that there is a nonnegligible degree of sequence dependence in the in vitro replication of fDNA templates. Whether this can have an effect on the outcome of SELEX experiments when using high-density modified oligonucleotides remains to be investigated. In this context, it is interesting to note that the pool template **P79** yielded a significantly higher amount of PCR product (∼60%) than the amount obtained on average with the A-, C-, G-, and T-rich templates (∼40%). This indicates that the sequences in the "demanding" templates might reflect fairly extreme situations of oligonucleotide compositions in an average template library.

3.2. PCR Amplification of High-Density Modified dsDNA, Using Natural Templates and Modified dNTPs. The ultimate step is the generation and direct amplification of functionalized dsDNA. The most difficult case for a DNA polymerase would be to copy and to directly amplify a functionalized DNA strand with the corresponding modified dNTPs as the only monomer source in a PCR. Previously, we could show that the *Pwo* DNA polymerase can amplify a double-stranded functionalized DNA from template **M79** in the presence of nucleotides **dA2**, **dC1**, **dG1**, **dT2** under distinct PCR conditions.[19]

Here, we investigated how generally applicable these conditions are by using the more demanding sequences (Figure 2) as templates. We performed PCR experiments with the templates **A79**, **C79**, **G79**, **T79**, **P79**, **M79**, the modified nucleotides **dA2**, **dC1**, **dG1**, **dT2**, and primers **P3′** and 5′-radiolabeled primer **P5′** (Figure 7A). As a positive control, a parallel set of PCRs was performed under the same conditions but in the presence of natural dNTPs instead of the modified ones. Reactions were stopped and analyzed after 18 PCR cycles.

(26) Roig, V.; Asseline, U. *J. Am. Chem. Soc.* **2003**, *125*, 4416−4417.

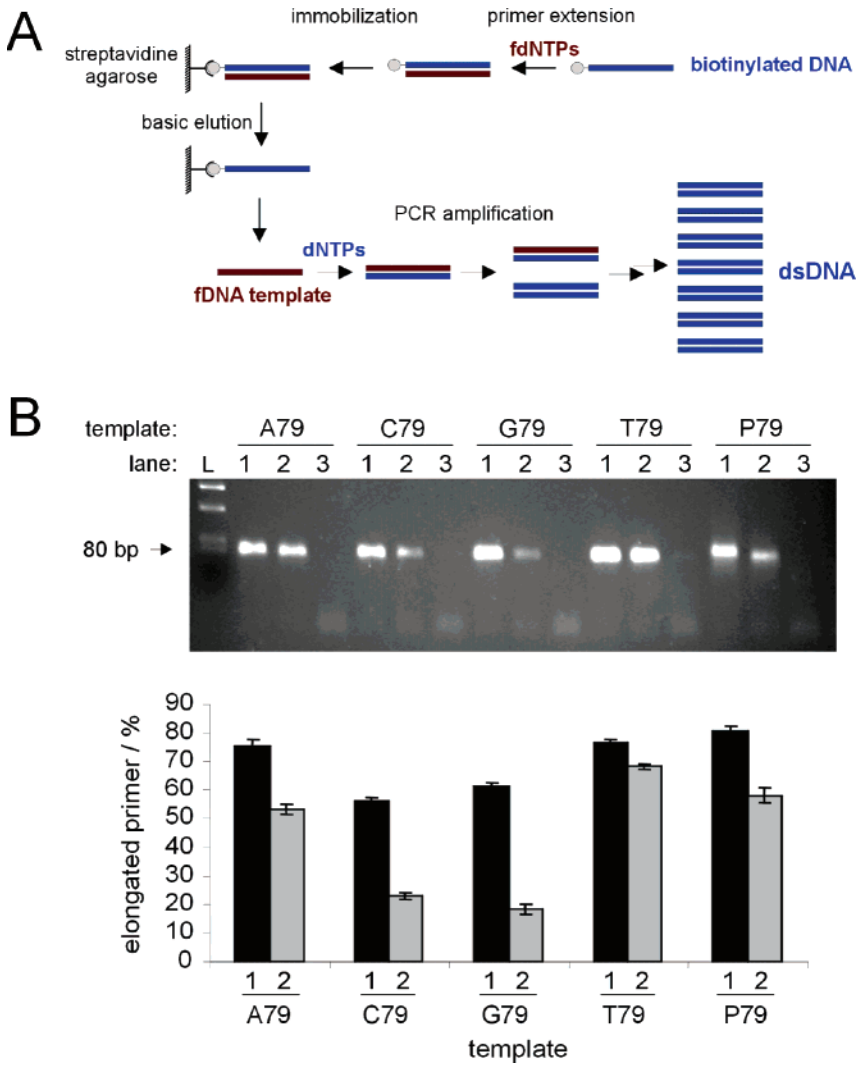

Figure 6. PCR amplification from fully functionalized templates and natural dNTPs yielding natural dsDNA. (A) Schematic for the generation and separation of functionalized DNA single strands (red) from biotinylated natural DNA templates (blue) with modified dNTPs (fdNTPs) and their PCR amplification with natural dNTPs. (B) PCR experiments with natural single-stranded DNA or fDNA templates, derived from primer extension with templates **A79**, **C79**, **G79**, **T79**, **P79**, primers **P5′**, 5′-[^{32}P]-labeled **P3′**, and *Pwo* DNA polymerase, analyzed by 2.5% agarose gel stained with ethidium bromide. Lane L, DNA ladder; lane 1, amplification from natural DNA templates; lane 2, amplification from fDNA templates; lane 3, negative control, amplification of eluates from immobilized biotinylated template. The same samples were loaded on a 10% denaturing polyacrylamide gel, and the bands were quantified (% of elongated primer) by phosphorimaging.

As shown in Figure 7, PCR experiments with C- and G-rich templates **C79** and **G79** gave the lowest yields (<10%). Experiments with A-rich template **A79** and the random sequence template **P79** yielded a comparable amount of product (∼20%), while T-rich template **T79** showed a 2-fold increase in product formation (∼50%). In general, a decrease in amplification products was observed in comparison to using natural dNTPs and the fDNA template. This is not surprising because the reaction is performed under conditions that are far from standard. Obstacles might be, for example, the possible formation of extensive secondary structures that can interfere with primer annealing or hamper the primer elongation of the polymerase, or a decreased processivity of the polymerase depending on the modified dNTP and the sequence context. Another likely reason for reduced amplification efficiency might be incomplete melting of the fDNA/fDNA double strands during the denaturing PCR step. This is supported by the data in Supporting Information Figure S3 (Supporting Information, Part E).

The integrity of the PCR products was investigated by sequencing (Supporting Information, Part G). For the A- and T-rich templates, sequences resulting from both modified strands were correct, demonstrating that the PCR amplification occurs without detectable replication errors. However, the results we obtained for the C- and G-rich templates were different. When the C-rich template was sequenced using the 3′-primer P3′, the same sequence as for the unmodified control DNA was obtained. With the 5′-primer P5′, however, sequence information is lost within a stretch of ∼14−15 bases. For the G-rich template, both primers P3′ and P5′ indicated the same loss of sequence information in this region (Supporting Information, Part G). This loss of sequence information in a central part of the C- and G-rich sequences correlates with the low yields for these templates during PCR with modified nucleotides (Figure 7).

Overall, these data show that while amplification of a completely functionalized DNA double strand is possible under

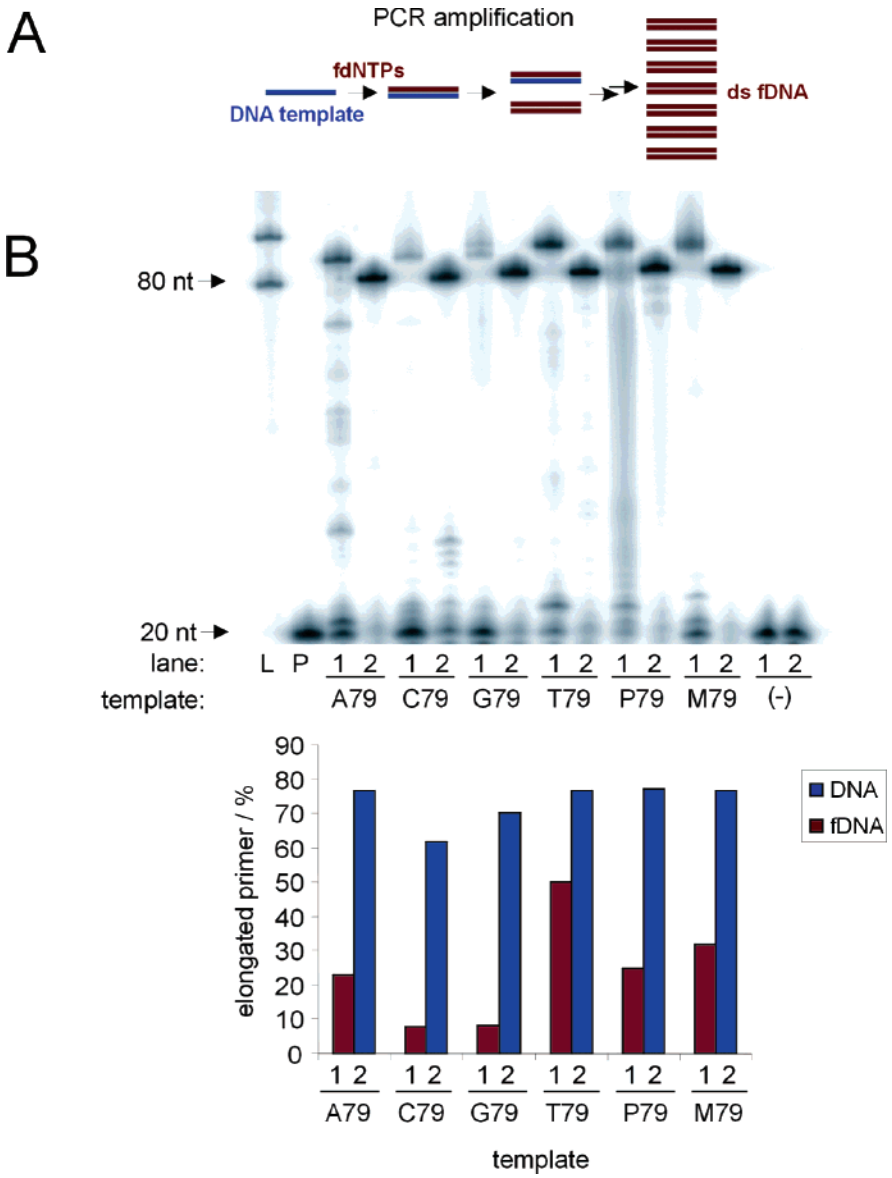

Figure 7. PCR amplification from natural templates and modified dNTPs yielding fully functionalized dsDNA. (A) Schematic for the generation of double-stranded fDNA (red) from natural DNA templates (blue) and modified dNTPs (fdNTPs). (B) PCRs with various templates **A79**, **C79**, **G79**, **T79**, **P79**, **M79**, primer **P3′**, 5′-radiolabeled primer **P5′**, PCR-additives, and *Pwo* DNA polymerase analyzed by 10% denaturing PAGE. Lane L, DNA ladder; P, primer **P5′**; lane 1, PCR with modified dNTPs **dA2**, **dC1**, **dG1**, **dT2** with the respective template; lane 2, PCR with natural dNTPs; (−), control reactions without a template with modified dNTPs (lane 1) or natural dNTPs (lane 2). Quantification of the product bands was done by phosphorimaging (% of elongated primer).

certain PCR conditions, it is also obvious that amplification efficacy, and maintenance of sequence information, strongly depends on the sequence context. Sequences that are extremely rich in G or C are amplified with about 8-fold reduced efficiency with some loss of sequence information as compared to the positive control using natural dNTPs, and with 1.5−3-fold reduced efficiency with A- or T-rich sequences.

4. Circular Dichroism Analyses of fDNAs Modified at Different Densities. Recently, we have observed that the CD spectrum of high-density modified **M59** dsDNA is inverted, as compared to a standard B-type DNA double helix.[19] The same effect was observed in the Brakmann laboratory when all of the pyrimidine residues in a double helix were substituted with fluorescently labeled analogues.[15] These results suggested that high-density incorporation of modifications into double-stranded DNA enforces a transition from the right-handed B-type DNA to a left-handed form, possibly resembling structures similar to a Z-type DNA.

To elucidate this effect in greater depth, and to study the possible structural alterations of functionalized DNA duplexes as a function of the nature and the density of modifications, we prepared a series of fDNA duplexes (**ds fM59**, duplexes **A**−**E**, **G**) in which the natural dNTPs are substituted with modified ones. For example, in duplex **A**, we replaced all A's by **dA2**, in duplex **B**, all C's were replaced with **dC1**, and so on (Figure 8). We also prepared an additional duplex (**E**), in which three bases, A, C, and T, were replaced with their modified analogues **dA2**, **dC1**, **dT2**. The **ds fM59** duplexes were synthesized by PCR amplification from a 59mer DNA template (**M59**) with about equal base distribution, primers **P3′**, **P5′**, and natural or modified dNTPs (**dA2**, **dC1**, **dG1**, and **dT2**), using *Pwo* DNA polymerase.

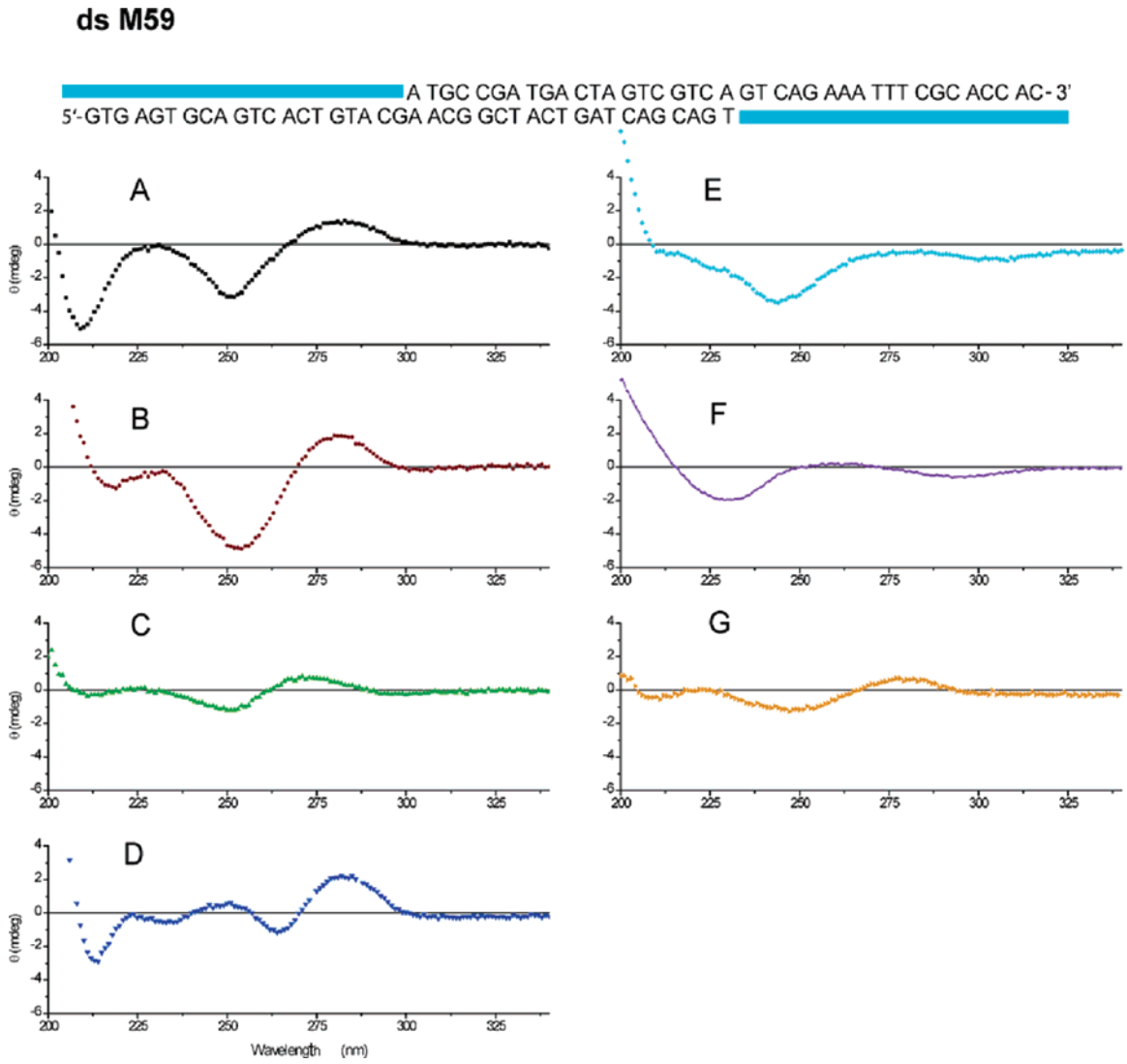

Figure 8. CD spectra of DNA double strands with the sequence of dsM59 containing different densities of modifications. Duplex **G** is the unmodified dsDNA **M59**; in duplex **A**, all As were substituted with **dA2**; and, respectively, for duplex **B**, **dC1**; duplex **C**, **dT2**; duplex **D**, **dG1**; duplex **E**, **dA2**, **dC1**, **dT2**; and duplex **F**, **dA2**, **dC1**, **dT2**, **dG1**.[19] Measured in phosphate buffer 10 mM, pH 7.0.

CD spectra of the duplexes containing one of the bases substituted with modified nucleotides (duplexes **A**−**C**) are presented in Figure 8. As can be seen, there are positive bands in the range of 270−280 nm as well as negative bands at 250 and 210−220 nm. The general appearance of the CD signal between 200 and 300 nm resembles that of the unmodified M59, which shows a typical B-DNA spectrum (Figure 8G). The CD spectrum of duplex D (containing **dG1**) includes additional maxima at 250 nm, which may result from electronic transitions of the extended π-system of the **dG1** phenyl residue interacting with the transitions of the chirally oriented surrounding bases (Figure 8D). Similar CD behavior, in particular around 250 nm, was observed by He and Seela[27] when incorporating various base pairs with propynyl residues into oligonucleotide duplexes. Nevertheless, the spectra of **D** seem to resemble a right-helix DNA with the typical B-type bands shifted to a longer wavelength.

Interestingly, at a density of three modifications, **dA2**, **dC1**, and **dT2**, different CD spectra (**E**) were obtained, which vary considerably in comparison to the nonmodified M59 duplex (**G**) or to duplexes modified at lower density (**A**−**D**). The band pattern of **E** includes mainly two minima (low intensity band at 304 nm and high-intensity band at 244 nm). The same band pattern is also obtained in the duplex in which all nucleotides were modified (**F**),[19] except that in **E** the bands are shifted to longer wavelengths. These alterations in the CD spectra appear to correlate with the density of functionalization.

In general, the differences in the CD spectra appear to originate from the effect that modified nucleobases have on base pairing and base stacking. The major groove is presumably filled with up to 4 types of modifications all of which contain the hydrophobic propynyl moiety with some also having altered overall base polarizability. In this study, we demonstrate that incorporation of one type of modified base does not alter the overall B-type DNA structure of the **M59** duplex, regardless of the type of modification used. However, three modifications are already enough for inducing a conformational change, that is, apparently, a step toward the manifestation of the inversion of the fully functional duplex.

(27) He, J. L.; Seela, F. *Nucleic Acids Res.* **2002**, *30*, 5485−5496.

Conclusions

Chemical modifications of nucleic acids present vast opportunities for extending the structural and functional properties of these biomolecules. In this study, we report the synthesis of an expanded set of modified nucleotides. We also provide a detailed investigation of the scope and limits for the enzymatic generation of DNA molecules that are functionalized at high density, by primer extension and PCR. On the basis of a large variety of chemically modified deoxynucleotide triphosphates, we can now define reaction conditions that allow the substitution of the natural nucleobases in each strand with up to four different base-modified analogues. We show that every base position, in various sequence contexts, can be addressed by different modifications and thus may facilitate a precise control of the three-dimensional placement of additional chemical functionalities.

Among the various polymerases used here, only family B polymerases were able to replicate "demanding" templates that contained consecutive stretches of a particular base, regardless of whether the template was a natural DNA or a high-density modified one. Nevertheless, it is remarkable that natural DNA polymerases exist that are able to recognize and incorporate a modified base opposite the complementary functionalized base in the template. This result suggests that it might be possible to increase the diversity of functional groups in a single DNA strand even further, by using modified artificial base pairs, as accomplished by the groups of Benner,[28] Schultz,[29] and Romesberg.[30] With this study, we have established a versatile toolbox of enzymes, templates, and monomers for generating high-density functionalized DNAs that might be suited for many different purposes in disciplines such as drug discovery, drug delivery, nanoelectronics, and nanocomputing.

Materials and Methods

Polymerases and PCR Additives. *Tth*, *Pwo*, and *Tgo* DNA polymerase were purchased from Roche Diagnostics (Mannheim, Germany). *Bst*, Vent (exo-) DNA polymerase, and Klenow Fragment (exo-) were from New England Biolabs (Frankfurt, Germany). *Taq* DNA polymerase was from Promega (Madison, WI). *Pfu* (exo-) DNA polymerase was purchased from Stratagene (Heidelberg, Germany). Sequenase V. 2.0 was from Amersham Biosciences (Freiburg, Germany).

The PCR additives Betaine (BioChemika, anhydrous), formamide (MicroSelect for molecular biology), tetramethylammonium chloride (Ultra for molecular biology), dimethyl sulfoxide (Ultra for molecular biology) were purchased from Fluka.

Primer Extension Reactions. 5′-[^{32}P]-labeled primer **P3′** (2 pmol) was annealed to the appropriate template (6 pmol) in 1X polymerase reaction buffer (provided by the supplier of the DNA polymerase) by heating the mixture to 95 °C for 5 min and subsequently allowing the solution to cool over 1 h to room temperature. Thermostable inorganic pyrophosphatase (0.2 U), DNA polymerase (1 U), and dNTPs (final concentration 50 μM) were added to a final volume of 20 μL in 1X polymerase reaction buffer. The reactions were immediately performed by heating the mixture to the optimal temperature of the enzyme in a thermocycler for 30 min. Reactions were stopped by addition of 60 μL of stop-solution (80% formamide solution containing 20 mM EDTA) and heating at 99 °C for 10 min. Reaction products were separated by 8% denaturing PAGE (8 M urea, 50−60 °C) and visualized and quantified by autoradiography or phosphorimaging.

Preparation of Single-Stranded Functionalized Templates. For separation of single-stranded fDNA, 10 pmol of a 5′-[^{32}P]-labeled primer **P3′** was extended on 30 pmol of the appropriate 5′-biotinylated template with nucleotides **dA2**, **dC1**, **dG1**, **dT3** (final concentration 50 μM each), 0.4 U thermostable inorganic pyrophosphatase, and 2 U Vent (exo-) DNA polymerase in a final reaction volume of 40 μL of 1X polymerase reaction buffer. The mixture was heated for 2 h to 72 °C. After the reaction, 10 μL of 5X buffer (750 mm NaCl, 0.5 mm EDTA, 250 mm HEPES, pH 7.0) was added, and the mixtures were immobilized on 50 μL of streptavidin-agarose (Ultra-Link Plus, Pierce, Rockford, IL), prewashed with five 100 μL volumes of wash buffer (150 mM NaCl, 0.1 mm EDTA, 50 mM HEPES, pH 7.0). The column was washed with 15 × 100 μL wash buffer at 25 °C. The fDNA was eluted with two 50 μL volumes of ice-cooled elution buffer (0.1 M NaOH, 150 mM NaCl). Reactions were neutralized with 7 μL of 5% acetic acid. The concentration of the fDNA templates was determined by scintillation counting.

PCR Reactions with Functionalized DNA Templates and Natural dNTPs. PCR experiments were performed on an Eppendorf Mastercycler gradient. The reactions were done in an overall reaction volume of 50 μL in 1X GC-rich reaction buffer (GC-rich PCR System, Roche) containing 1 M GC-rich resolution solution (GC-rich PCR System, Roche) using natural dNTPs (final concentration 200 μM each), 2.5 U *Pwo* DNA polymerase, and the modified template (about 0.1 pmol per reaction). Initially, 10 PCR cycles were performed with only 5′-[^{32}P]-labeled primer **P5′** (50 pmol) with the following conditions: initial denaturing at 99 °C for 3 min, denaturing at 99 °C for 3 min, primer annealing at 50 °C for 2 min, and extension at 72 °C for 5 min. Subsequently primer **P3′** (50 pmol) was added to the reaction mixture and an additional 8 cycles of PCR were performed with conditions: denaturing 99 °C for 1 min, annealing 50 °C for 1 min, extension 72 °C for 1 min, and a final extension step of 5 min at 72 °C. The PCR reactions were analyzed on a 2.5% agarose gel stained with ethidium bromide (5 μL sample). Additionally, 5 μL of the PCR reaction mixture was mixed with 15 μL of stop solution, denatured for 5 min at 99 °C, and the products separated on an 8% denaturing PAGE (8 M urea, 50−60 °C) and quantified by phosphorimaging.

PCR Reactions with Natural DNA Templates and Modified dNTPs. The reactions were done in an overall volume of 25 μL in *Pwo* reaction buffer (1X) containing 10% DMSO, 5% formamide, 0.75 M betaine, and 50 mM TMAC using modified nucleotides **dA2**, **dC1**, **dG1**, **dT2** (final concentration 200 μM each), 25 pmol of each primer **P3′** and 5′-[^{32}P]-labeled primer **P5′**, 0.5 pmol of the respective template, *Pwo* DNA polymerase (1.25 U), and thermostable inorganic pyrophosphatase (0.2 U). As a control, PCRs were performed under the same conditions using 200 μM natural dNTPs instead of the modified nucleotides. Amplification was performed through an initial denaturing at 99 °C for 3 min, followed by 18 cycles of denaturing at 99 °C for 2 min, primer annealing at 50 °C for 2 min, and extension at 72 °C for 2 min. A negative control without template was also carried out. 5′-[^{32}P]-labeled and denatured 100 bp DNA ladder (peqLab) served as a length standard. An aliquot of 10 μL of the PCR reaction mixture was mixed with 30 μL of formamide/water (4:1), containing 20 mM EDTA, and denatured at 99 °C for 10 min. 5 μL of each reaction mixture was analyzed by 10% denaturing PAGE (8 M urea, 50−60 °C), and visualized and quantified by phosphorimaging.

Circular Dichroism. CD experiments were performed with a Jasco 810 spectrophotometer. CD spectra of the duplex solutions (0.4 OD base concentration of duplexes **A**, **C**, **D**, **E**, 0.6 OD of duplex **B**, and 0.22 OD of duplex **G**, in 10 mM phosphate buffer and 1 M NaCl, pH 7.0 at 25 °C) and were recorded using a 0.2 mm quartz cell. A spectrum of the buffer was measured separately and subtracted from the spectra resulting from the samples. On average, seven spectra were recorded for each experiment. The samples for CD (**ds fM59** (duplexes **A**−**E**,

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G)) were generated by large-scale PCR in an overall volume of 5 mL, using the same conditions and modified nucleotides as described in the earlier paragraph but with **M59** template (primers **P3′** and **P5′**). Following the PCR, the duplexes were precipitated with cold EtOH and purified by anion exchange chromatography or by native PAGE (15%). The full details can be found in the Supporting Information.

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Supporting Information Available: Details on synthetic procedures, spectroscopic characterization, primer extension experiments, melting behavior of fDNA, MS-characterization and seuencing data of modified DNA primer extension products, and sequencing data of modified DNA PCR products. This material is available free of charge via the Internet at http://pubs.acs.org.

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