Peptide Nucleic Acid. A Molecule with Two Identities

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Received September 29, 1998

Introduction

Since the elucidation of the double helical structure of DNA (deoxyribonucleic acid), our genetic material, the properties of this remarkable molecule have fascinated chemists, and tremendous efforts have been devoted to understanding its structure and function in biology as well as from a chemical and physicochemical standpoint. Yet very simple, just built from four heterocyclic nucleobases (adenine (A), cytosine (C), guanine (G), and thymine (T)) assembled via a polymer backbone composed of deoxyribose phosphodiesters (Figure 1), this molecule holds the key to life on earth and biological evolution. Part of the secret is nucleobase molecular complementarity (Figure 2), which together with the structural features of the deoxyribose phosphate backbone allows two sequence complementary, antiparallel DNA strands to form a double helix. This also ensures high-fidelity information transfer—as written by the linear sequence of the nucleobases—during synthesis of a new DNA copy (replication), and during transcription of the DNA into the cell's messenger molecule, RNA (ribonucleic acid) (which is finally translated to the work molecules of the cell, proteins). Some very simple and very fundamental questions still need ato be answered. Why has Nature "chosen"/settled on DNA as the central genetic material of life? Is DNA the only possibility or could other chemical structures fulfill the requirements and support (another form of) life? What makes DNA such a good candidate in terms of structure, stability, and molecular recognition? We will most probably never get the final answers to all of these questions, but studying DNA will of course bring us closer. Also, studies of DNA analogues and mimics may help to shed light on these questions. Furthermore, oligomers of both synthetic DNA and DNA analogues are finding widespread applications within molecular (biology), genetic diagnostics, and gene therapeutic medicine. Recently the properties of a novel DNA mimic, peptide nucleic acid (PNA), has forced us to modify our view of the uniqueness of DNA. In the present Account, I will try to give a brief overview of the properties and (possible) applications of PNA, pointing out the implications and future directions of the research including PNA chemistry, PNA structure, recognition of double-stranded DNA, applications, origin of life, and molecular recognition. More comprehensive reviews on PNA can be found in refs 1−4.

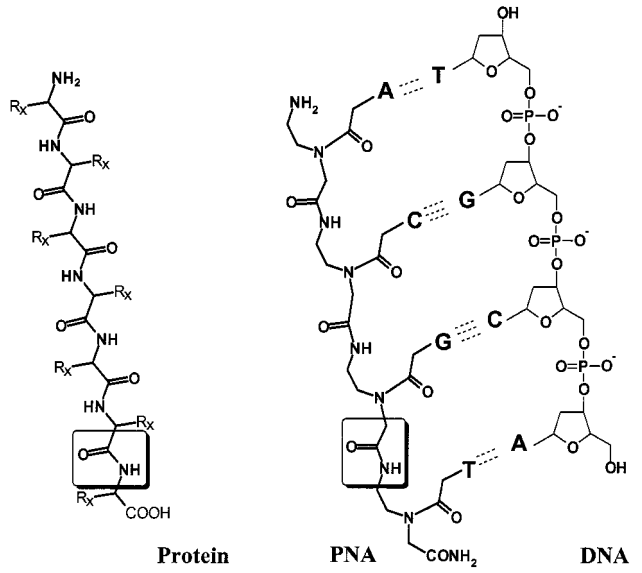

FIGURE 1. Chemical structures of a protein (peptide) (where Rx is an amino acid side chain), a PNA molecule, and a DNA molecule. The amide (peptide) bond characteristic for both PNA and protein is boxed in.

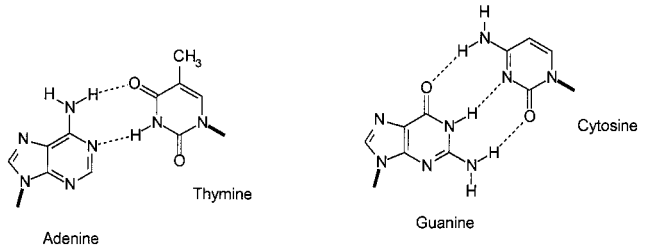

FIGURE 2. Nucleobase complementarity by the adenine−thymine and guanine−cytosine base pairs.

Peptide Nucleic Acid

PNA was originally designed and developed as a mimic of a DNA-recognizing, major-groove-binding, triplex-forming oligonucleotide.[5−7] However, the pseudopeptide (polyamide) backbone of PNA (Figure 1) has proven to be a surprisingly good structural mimic of the ribose−phosphate backbone of nucleic acids. Therefore, PNA has attracted wide attention in medicinal chemistry for development of gene therapeutic (antisense and antigene) drugs, and in genetic diagnostics. However, while PNA is conceptually a DNA mimic, it is chemically a pseudopeptide (polyamide), and this fact makes PNA of interest for

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10.1021/ar980010t CCC: $18.00
Published on Web 04/01/1999

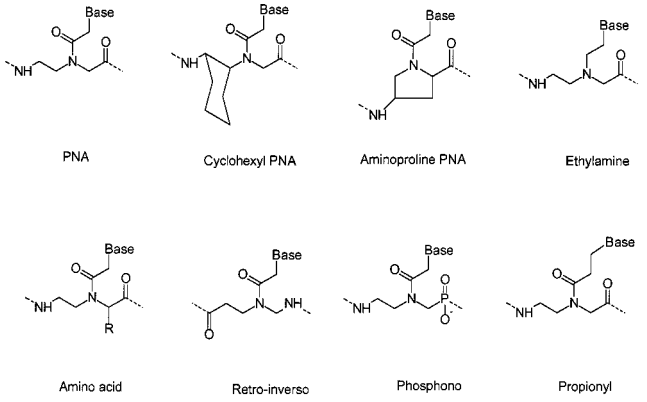

FIGURE 3. Chemical structures of a selection of PNA monomer units. Cf. refs 16 (cyclohexyl), 17 (aminoproline), 68 (ethylamine), 69 and 70 (amino acids), 71 (retro-inverso), 72 (phosphono), and 73 (propionyl).

more basic questions regarding DNA structure, evolution, and function. This functional duality of PNA is central for understanding the properties of PNA. In a sense it gives the molecule—and the researcher—an identity problem: is it a "peptide" or a "nucleic acid"?

PNA Chemistry

The success with PNA has revived the interest in "nucleobase amino acids" and their oligomers which dates back to the 1970s when De Koning and Pandit prepared homouracil "nucleopeptides" derived from lysine,[8] and Buttrey et al. constructed polymers of thyminyl-β-alanine.[9] However, these oligomers do not hybridize efficiently to polyA, and subsequent work on DNA analogues primarily related to antisense technology has to a great extent followed a strategy of "minimal chemical deviation" from the (deoxy)ribose phosphodiester backbone of natural DNA.[10−12] The morpholino derivatives introduced by Summerton et al.[13] probably represent the first glimpse of a successful DNA mimic with a non-phosphodiester/non-ribose backbone, although these are clearly not optimal DNA mimics and have later been replaced by morpholinophosphoamidates[14] which are promising antisense candidates.

Nonetheless, the extraordinary and quite surprising properties of "aminoethyl glycine PNAs" have since their presentation in 1991[5] given new inspiration and momentum to explore "peptide nucleic acids" in general, as defined as oligomers of "nucleobase amino acids". The simple and straightforward chemistry of PNA basically being constructed of three parts, an α-amino acid (glycine), a nucleobase attached via an amide linker (acetyl), and a spacer (ethyl) (Figure 3), is also an open invitation to chemical modification.

The "field of peptide nucleic acids" has followed two main directions. One is to explore the structure−activity relationship of PNA in terms of nucleic acid hybridation properties, also with the aim of being able to design analogues of the original "aminoethyl glycine" PNA having improved hybridization potency. The other direction springs from the demonstration with PNA that extremely good structural mimics of DNA not relying on a DNA-related backbone are possible, and therefore mimics based on other types of chemical structures when designed properly can most certainly also be found.

Why is PNA such a good DNA mimic? Most probably the answer resides in a favorable geometry of the backbone combined with a structure of constrained flexibility. This allows the oligomer to adapt to the helical structure preferred by DNA (B-form helix) or RNA (A-form helix) without losing the entire gain in binding enthalpy in decreased entropy upon formation of the much more rigid duplex (or triplex) structures (even though these structures are distinctly different from the P-form helix structure preferred by the PNA itself (vide infra)).

In support of this notion of constrained flexibility, reduction of the "nucleobase linker amide" (thereby removing the rigidity imposed by the planar amide) is detrimental to DNA binding.[15] Conversely, it should be possible to rigidify the PNA structure in such a way as to favor the structure found in duplexes and thereby increase the duplex stability owing to a smaller loss of entropy. A couple of partially successful attempts in this direction have been reported. In one case the ethylene part of the linker was locked in a cyclohexyl structure[16] (Figure 3). Although improved hybridization was not attained by this modification, PNAs containing cyclohexyl units (the SS isomer) in the backbone formed PNA−DNA duplexes with a smaller loss in entropy as compared to the unmodified PNA. However, the binding enthalpy was correspondingly reduced, indicating that the cyclohexyl backbone does indeed reduce the number of energetically favorable conformations for the single-stranded PNA, but unfortunately, these seem not to be the optimal for PNA−DNA duplex formation.

Cyclic structures within the backbone have also been described, and in one case—by using a few proline-derived backbone units in a PNA (Figure 3)[17]—improved stability of PNA_2−DNA triplexes was reported.[17] There seems little doubt that rigidifying the backbone structure using cyclization will eventually lead to stronger hybridization as also the recent results with conformationally locked DNA analogues have demonstrated.[18]

PNA Structure

Three-dimensional structures have been determined for the major families of PNA complexes. Two duplex structures, a PNA−RNA[19] and a PNA−DNA[20] duplex, were solved by NMR, and a PNA_2−DNA triplex[21] and a PNA−PNA duplex[22] were solved by X-ray crystallography (Figure 4). These structures clearly demonstrate that the PNA does indeed have the flexibility enabling adaption to the A- and B-form helices preferred by RNA or DNA, but the results also clearly show that the structure preferred by PNA is a much wider (28 Å) and more slowly winding helix (18 base pair pitch), now referred to as the P-form.[21,22] These results therefore also stress that PNA is *not* the perfect structural mimic of DNA or RNA, thereby leaving ample room for chemical improvement. It is also noteworthy that

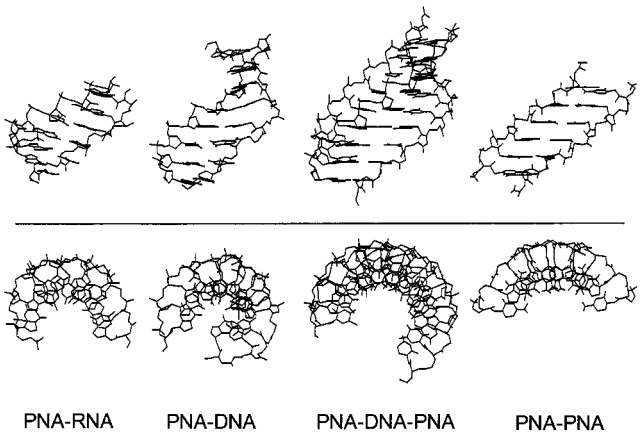

FIGURE 4. Structures of various PNA complexes. Adapted from ref 3.

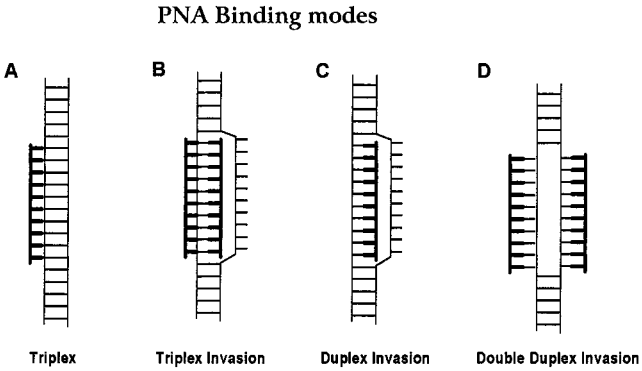

FIGURE 5. PNA binding modes (schematics) for targeting double-stranded DNA. Thick structures signify PNA.

the ethylene linker is in the gauche conformation, and thus the "gauche effect" may be a stabilizing factor.

The conformation of the single-stranded oligonucleotides is of utmost interest when nucleic acid hybridization is discussed. The hybridization process is enthalpically driven and is usually accompanied by a large entropy loss. Preformation of a hybridization-competent single-strand conformation would minimize this entropy loss (but could, of course, also reduce the enthalpic gain from base stacking) and should therefore increase duplex stability. The preferred single-strand conformation(s) of PNA is not known, but the very complex ^{1}H NMR of single-stranded PNAs caused by multiple amide cis/trans rotamers which are resolved upon hybridization to DNA or RNA[20,23] clearly shows that oligomers exist in multiple conformations. By proper chemical modification it should be possible to (energetically) lock the structure in the correct rotamer.

Recognition of Double-Stranded DNA

It came as a great surprise that targeting of homopyrimidine PNA to double-stranded DNA resulted in strand displacement complexes rather than the traditional and expected triple helix structure (Figure 5B). Due to the very high stability of PNA_2−DNA triplexes, it is thermodynamically favorable to form such a structure and thereby open the DNA helix (Figure 5B). This quite special binding mode

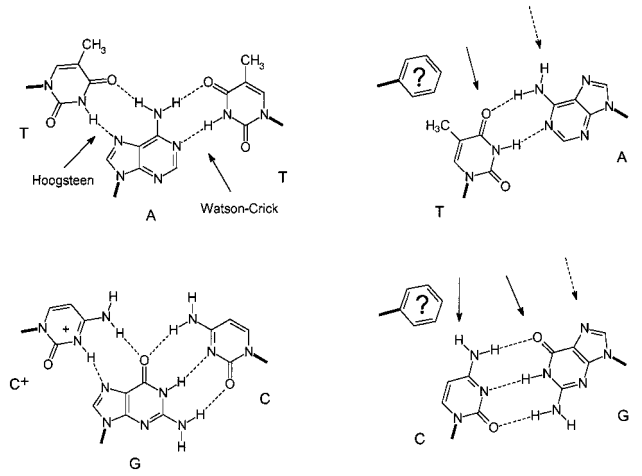

FIGURE 6. The "triplex recognition problem". While thymine and N3-protonated cytosine recognize the A−T and C−G base pairs, respectively, no natural nucleobases that specifically recognize the T−A or C−G base pairs have been found. Because of the asymmetry of triple helices, the hydrogen donor (arrows) and hydrogen acceptor (broken arrows) recognition sites are positioned further out in the major groove than the incoming third strand (simple) nucleobase (?).

also imposes unusual characteristics on the binding process. Most results have been obtained with 10-mer PNAs, and in this case the complex lifetime (>24 h) usually greatly exceeds the time of the experiment.[24] Therefore, equilibrium is not reached, and the binding in terms of both efficiency and sequence specificity is kinetically controlled.[25] Furthermore, the binding rate is greatly inhibited by increasing the ionic strength of the medium,[26−28] which stabilizes the DNA duplex, and dramatically increased by permanent or transient opening (unwinding) of the duplex at or near the PNA binding site. Such opening can be caused by a passing RNA polymerase (transient),[29] by negative DNA supercoiling,[30] or by an adjacent strand displacement complex.[28]

Triplex formation with PNA as with natural oligonucleotides is essentially restricted to homopurine targets. Although many attempts—and some of them at least partly successful[31,32]—have been made to develop the "two missing nucleobases" that should be able to recognize thymine and cytosine, respectively, in the DNA target, no general solution to the "triplex recognition problem" has yet been found (Figure 6).

One may argue that this problem should be more easily addressed using PNA as compared to conventional triple helix formation (with oligonucleotides) since part of the recognition—and binding energy—is supplied by the Watson−Crick bound PNA strand. Therefore, a more "relaxed" recognition could be tolerated in the Hoogsteen strand. This contention has yet to be experimentally supported. As a small step toward this goal, we have designed a novel nucleobase that is able to recognize thymine (Figure 7).

However, PNA shows a much greater binding mode repertoire than do oligonucleotides. In a few cases which appear to be associated with high cytosine content of the PNA, a traditional PNA−DNA triplex (Figure 5A) has been observed.[34,35] These have lower stability than the corre-

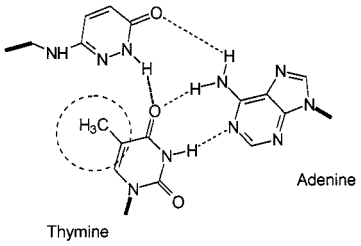

FIGURE 7. Proposed recognition mode of the T−A base pair recognizing E-base. This nucleobase was built with an extended linker to the backbone in order to reach the O4 hydrogen acceptor of thymine and also to avoid steric clashing with the 5-methyl group.

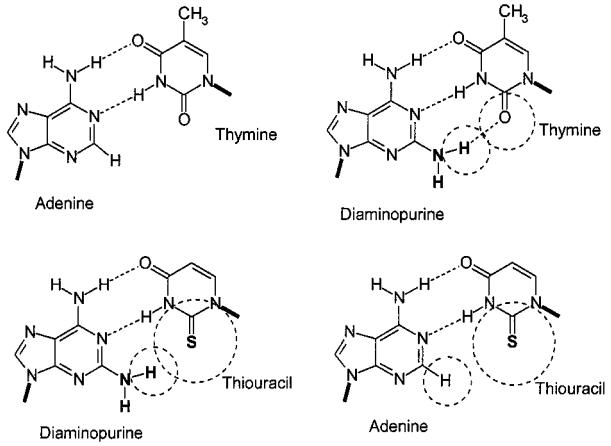

FIGURE 8. Chemical structures showing how adenine−thymine, diaminopurine−thymine, and adenine−thiouracil can be formed, whereas steric hindrance interferes with the formation of the diaminopurine−thiouracil base pair (cf. refs 37 and 38).

sponding strand invasion complexes but are formed at a faster rate. Furthermore, invasion of double-stranded DNA has been demonstrated using a homopurine PNA decamer, which forms only a duplex with the complementary oligonucleotide (Figure 5C).[36] Finally, very recent results using the sterically compromised base pair, diaminopurine−thiouracil (Figure 8)[37,38] have demonstrated that the double duplex invasion principle (Figure 5D) is quite effective in a PNA context[38] as opposed to a DNA context.[37]

Therefore, general principles for designing PNAs (or other DNA mimics or analogues) targeted to any desired sequence (10−15 bases in length) in double-stranded DNA is absolutely within close reach.

Applications

The very favorable RNA and DNA hybridization properties of PNA have, not surprisingly, led to the development of a large variety of biomolecular and potential medical applications of PNA. These include hybridization techniques for genetic detection such as in situ hybridization,[39] "pre-Southern" blotting,[40] array hybridization,[41] hybridization detection by mass spectrometry,[42] nucleic acid biosensors,[43] modulation of PCR analyses,[44] and tools for genome mapping[45,46] as well as antisense[47−50] and antigene[47,51−52] drug approaches. Exploitation of the antisense potential of PNA has been slowed by inefficient uptake of PNA in most eukaryotic cells. However, this area may rapidly be regaining momentum owing to three recent discoveries. Several cell penetrating peptides have been identified within the past few years. These are typically relatively short (∼15 amino acids), polycationic peptides which are believed to physically destabilize the predominantly cationic surface of the cellular membrane.[53] Upon conjugation to, e.g., a PNA oligomer, such peptides have been found to quite efficiently deliver PNAs to cells,[54−56] and down-regulation of target gene expression compatible with a PNA antisense mechanism was reported both for nerve cells in culture[55,56] and upon injection of such PNA−peptide conjugates into the brain of rats.[56] Furthermore, it was also reported that "naked" PNAs targeted to neural receptors when injected directly into the brain of rats are capable of exerting behavioral effects that are compatible with an antisense down-regulation of the receptor.[57] Finally, it was quite surprisingly found that the prokaryote *E. coli* is slightly "leaky" toward PNA.[58] Using a mutant form (AS19) of *E. coli* which is around 10-fold more sensitive to antibiotics in general, it was demonstrated that a bis-PNA targeted to the α-sarcin loop of the 23S ribosomal RNA inhibits growth of these cells ($C_{50} \approx 2\ \mu$M) and indeed also to wild-type *E. coli* (K12). Furthermore, full antisense down-regulation of the enzymes β-galactosidase and β-lactamase was demonstrated in *E. coli* AS19 using PNA, and most interestingly, formerly penicillin-resistant bacteria treated with such an anti-β-lactamase PNA (β-lactamase confers penicillin resistance) now became sensitive to ampicillin.[59] Thus, the resistant phenotype was reverted by the antisense treatment. These results open possibilities for development of novel bacteriostatics based on genetic (antisense) principles. Furthermore, the antisense technology should find wide applications within "functional genomics" and medicinal target validation for studying the phenotypic effects of down-regulating or extinguishing specific genes.

Origin of Life

It is generally believed that our present day DNA−RNA−protein world was preceded by an "RNA world" in which RNA molecules served both the purpose of genetic storage and as functional enzymes.[60] From a prebiotic point of view, it is, however, much easier to create (and imagine) conditions under which amino acids and nucleobases could have been formed on the prebiotic earth than conditions that would favor formation of ribose sugar and nucleosides and -tides. Therefore, the duality of PNA, in essence being a "peptide" that carries genetic information, presents the possibility of a "peptide nucleic acid world".[61] In support of this idea, it has been shown that "chemical transfer" of genetic information from PNA to another PNA and to RNA (or DNA) (and vice versa) is in principle feasible, since homocytosine PNA oligomers can direct template-dependent synthesis of a complementary homoguanine PNA oligomer or RNA oligonucleotide.[62−64] Furthermore, a peptide nucleic acid—like PNA—can be

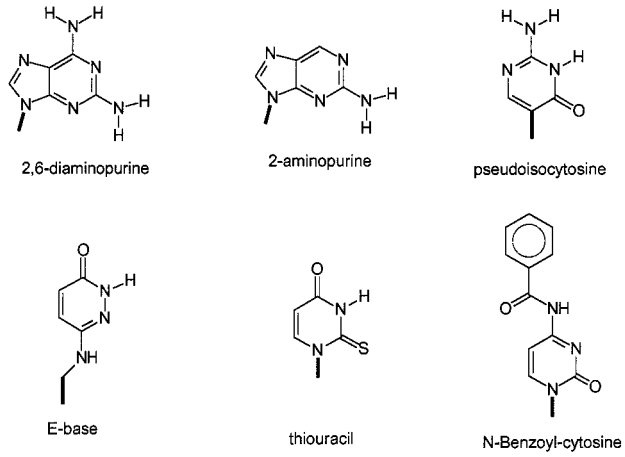

FIGURE 9. Examples of nonstandard nucleobases that have been in PNA oligomers. Cf. refs 74 (2,6-diaminoadenine), 75 (2-aminopurine), 76 (pseudoisocytosine), 33 (E-base), 38 (thiouracil), and 77 (*N*-benzoyl cytosine).

achiral, thereby "postponing" Nature's choice of chirality to a later stage in evolution. Admittedly, these experiments and ideas are very crude, but nonetheless the properties of PNA should inspire us to also consider these types of molecules in our speculations and models of the origin of life on Earth.

Molecular Recognition

Molecular recognition between nucleic acids—via nucleobase complementarity—is probably the most elegant molecular recognition system in Nature. It is beautifully simple, yet very powerful. Also it is very appealing to chemists because it can be modified and even expanded by using nonnatural nucleobases.[65] It has even been possible to use DNA oligonucleotides as designer building blocks for the formation of nanostructures, such as cubes and two- or three-dimensional networks.[66] Furthermore, the inclusion of nonnucleobase ligands instead of the natural nucleobases in DNA is providing tools to understand the recognition process in terms of contributions from hydrogen bonding and internucleobase stacking.[67] Using PNA in analogue approaches could add further dimensions to these studies by examining the effect of change in backbone structure(s), and should also allow construction of more sophisticated and chemically functionalized nanostructures. A large variety of backbone structures are already available (Figure 3), whereas only a few alternative nucleobases have so far been reported in a PNA context (Figure 9).

The Dual Identity

I believe that the best use of PNA both scientifically and technically comes from always realizing that PNA is not an oligonucleotide *analogue* but rather a DNA *mimic* with chemical and physical properties of its own. Therefore, the optimal exploitation of PNA is to take advantage of its unique properties, the ones that distinguish it from DNA, instead of just trying to use it as a substitute for DNA. I also think that the molecule is very attractive to chemists because of its (only partly explored) possibilities for chemical modification. In a way PNA combines the recognition and structural power of DNA with the robustness, flexibility, and opportunities of peptide (amide) chemistry.

I thank all the students, postdoctoral fellows, and colleagues in the laboratory at the University of Copenhagen as well as collaborators abroad who during the past 7 years through their interest in and hard work with the PNA molecule have unveiled its properties and with ingenuity developed novel applications. Finally I am indebted to the co-inventors of PNA, Professor Ole Buchardt (deceased) and Drs. Michael Egholm and Rolf Berg.

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AR980010T