Pergamon

Bioorganic & Medicinal Chemistry 9 (2001) 2429–2434

BIOORGANIC & MEDICINAL CHEMISTRY

Strand Displacement Recognition of Mixed Adenine–Cytosine Sequences in Double Stranded DNA by Thymine–Guanine PNA (Peptide Nucleic Acid)

Peter E. Nielsen* and Michael Egholm†

Center for Biomolecular Recognition, Department for Biochemistry and Genetics, Biochemistry Laboratory B, The Panum Institute, Blegdamsvej 3c, DK-2200 N Copenhagen, Denmark

Dedicated to the late Professor Ole Buchardt.

Received 11 December 2000; accepted 18 May 2001

Abstract—Mixed pyrimidine–purine peptide nucleic acids (PNAs) composed of thymines and guanines are shown to form a PNA_2–DNA triplex with Watson–Crick complementary adenine–cytosine oligonucleotides and to bind complementary adenine–cytosine targets in double stranded DNA by helix invasion. These results for the first time demonstrate binding of an unmodified PNA oligomer to a mixed pyrimidine–purine target in double stranded DNA and illustrate a novel binding mode of PNA. © 2001 Published by Elsevier Science Ltd.

Introduction

Peptide nucleic acids (PNA) are DNA mimics with a pseudopeptide backbone consisting of *N*(2-aminoethyl)glycine units.[1–5] Due to the chemical and physico-chemical properties of PNA, for instance with regard to binding to single and double stranded DNA and RNA, PNA is of significant current interest in molecular biology, bioorganic and medicinal chemistry.[6–11]

Sequence specific recognition of and binding to duplex DNA by homopyrimidine PNA takes places via P-loop strand displacement[12–14] and is ascribed to the extraordinarily high stability of an internally formed PNA_2/DNA triplex.[1,13,14] Interestingly, the formation of a conventional (PNA/DNA_2) triplex was not observed under these conditions and appears to be of lower stability.[13] However, for very C-rich PNAs evidence for the existence of traditional PNA–DNA_2 triplexes have been obtained by DNaseI footprinting,[15] and likewise the complex between PNA $(TC)_5$ and poly d(AG)-poly d(TC) was found to be a traditional PNA–DNA_2 triplex.[16] In general, mixed purine-pyrimidine, duplex forming PNAs do not invade double stranded DNA, although such complexes were implicitly postulated to account for some recent chromatin capture and in situ transcription inhibitory results obtained using PNA.[17,18] However, these reports presented no data demonstrating strand invasive duplex binding by the PNAs and thus alternative explanations for the results could be possible. Nonetheless, a homopurine PNA (A_4G_2AGAG) which forms a very stable duplex ($T_m = 70\,^{\circ}C$) with the antiparallel, complementary deoxyoligonucleotide, was shown to bind this target in double stranded DNA forming a strand displacement complex.[19] Furthermore, PNA oligomers conjugated to cationic peptides were shown to invade targets in supercoiled DNA.[20,21] Finally, the use of pseudo complementary PNA oligomers containing 2,6-diaminopurine and 2-thiouracil instead of adenine and thymine, respectively, allows targeting of A/T-rich (>50%) mixed purine/pyrimidine sequences by double duplex invasion.[22,23]

In DNA triple helices, guanine/thymine oligonucleotides bind specifically and efficiently to homopurine guanine-adenine targets in an antiparallel configuration via G·G–C and T·A–T triplets.[24] In order to study this

*Corresponding author. Tel.: +45-35327762; fax: +45-35396042; e-mail: pen@imbg.ku.dk

†Present address: Molecular Staging, Inc., 300 George Street, New Haven, CT 06511, USA.

0968-0896/01/$ - see front matter © 2001 Published by Elsevier Science Ltd.
PII: S0968-0896(01)00244-9

triplex motif in a PNA context, we synthesized a guanine-thymine PNA (H-T_4G_2TGTG-LysNH$_2$) and analyzed the binding of this to single and double stranded DNA targets.

Surprisingly, we find that such GT–PNAs form triplexes with complementary AC-oligonucleotides. Furthermore, we could detect no binding to a 'triplex complementary' double stranded AG–DNA target, whereas the PNA bound to a 'Watson–Crick complementary' double stranded AC–DNA target by strand displacement. These results demonstrate a novel binding mode for duplex invading PNAs and show specific binding of an oligonucleotide analogue to a mixed purine(A)–pyrimidine(C) duplex DNA target.

Materials and Methods

PNA monomers and oligomers were synthesized by the tBoc-solid-support method as described.[3,4] Construction and isolation of plasmid DNA, labeling of restriction fragments and $KMnO_4$ probing experiments were performed as previously described.[13,25] Briefly, plasmids containing the 5′-A_4C_2ACAC target were constructed by cloning of oligonucleotides 5′-GATCA_4C_2ACAC and 5′-GATCGTGTG_2T_4G into the *Bam*HI site of *pUC*19, while plasmids containing the 5′-A_4C_2GCAC target were constructed by cloning of oligonucleotides 5′-GATCA_4C_2GCAC and 5′-GATCGTGCG_2T_4G into the *Bam*HI site of *pUC*19.[25] The plasmids were digested with restriction enzymes *Eco*RI and *Pvu*II (or *Hind*III and *Pvu*II) and 3′-^{32}P-end-labelled at the *Eco*RI (or *Hind*III) site using α-^{32}P-ATP and the Klenow fragment of E. coli DNA polymerase. The large (246 base pair) *Eco*RI–*Pvu*II DNA fragment (or the *Hind*III-*Pvu*II DNA fragment) was isolated by polyacrylamide gel electrophoresis and used for the probing experiments.

Probing experiments with $KMnO_4$ or dimethyl sulphate (DMS) were performed in 100 μL buffer (10 mM Na-cacodylate, 1 mM EDTA, pH 7.0 or as indicated) containing approximately 200 cps ^{32}P-labeled DNA fragment, 0.5 μg calf thymus DNA and the desired amount of PNA. Following a preincubation of DNA with the PNA for 60 min at 37 °C, the probing reagent was added, the incubation was continued at room temperature and the reactions were finally terminated by the addition of a stop-buffer. Probing conditions were for $KMnO_4$: 1 mM $KMnO_4$, 15 s, stopped with 50 μL 1 M β-mercaptoethanol, 1.5 M NaOAc, pH 7.0; and for DMS: 1% DMS, 15 s, stopped as for $KMnO_4$ probing. The samples probed were subsequently treated with piperidine (0.5 M, 90 °C, 20 min) prior to gel analysis. The DNA was precipitated by addition of 200 μL 2% KOAc in 96% EtOH and was analyzed by electrophoresis in 10% polyacrylamide sequencing gels. Radioactive DNA bands were visualized by autoradiography using intensifying screens and Agfa curix RP1 X-ray films exposed at −70 °C.

Job plots were made by mixing varying ratios of PNA and oligonucleotide keeping the total concentration constant (10 μM). The samples were heated to 90 °C, slowly cooled to 20 °C and the absorbance at 260 nm was measured.

Gel shift experiments were performed by mixing varying concentrations of PNA with a constant amount of oligonucleotide (5 μM) containing a small amount of the same 5′-^{32}P labeled oligonucleotide in 10 μL TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.0). The samples were heated to 90 °C, slowly cooled to 20 °C. After addition of 2 μL gel loading buffer (450 mM Tris–Borate, 5 mM EDTA, pH 8.3, 50% glycerol), the samples were analyzed on 15% polyacrylamide gels run in TBE (90 mM Tris–Borate, 1 mM EDTA, pH 8.3) buffer. Radioactive DNA bands were visualized by autoradiography using intensifying screens and Agfa curix RP1 X-ray films exposed at −70 °C.

Thermal denaturation experiments were performed in 10 mm cells using a Gilford Response spectrophotometer. T_m values are reported as the maximum of the 1st derivative of the T_m curve.

Results and Discussion

Binding of the H-T_4G_2TGTG-LysNH$_2$ PNA to complementary A_4C_2ACAC oligonucleotides resulted in complexes that both Job-plot (Fig. 1a) and a gel-shift analysis (Fig. 1b) strongly indicated to be PNA_2–DNA triplexes. However, as opposed to the Py·Pu–Py PNA_2–DNA triplexes (for which the Watson–Crick PNA strand should preferentially be antiparallel and the Hoosteen PNA strand preferentially parallel to the DNA target[26]), in this case the anti-parallel configuration (which is favored by PNA–DNA Watson–Crick duplexes) for both PNA strands resulted in the most stable triplexes (Table 1, Fig. 1b). Furthermore, thermal denaturation of the complexes showed only minimal hysteresis (Fig. 2); also in sharp contrast to the behaviour of homopyrimidine PNA_2–DNA triplexes.

These results prompted us to study the binding of T_4G_2TGTG PNAs to a 5′-A_4C_2ACAC-3′ duplex DNA target. Although we were not able to detect stable complex formation using a gel-shift assay, $KMnO_4$ probing unequivocally showed (Fig. 3a) that strand displacement complexes having the non-complementary TG–DNA strand in a single stranded conformation were formed using either the antiparallel PNA 1 or the parallel PNA 2, whereas virtually no binding was observed with the one-mismatch PNAs 3 or 4. We also note that binding of the parallel PNA 1 results in a probing pattern that produces only faint signals at the two G*T*G*T*-sites and extends outside the putative binding site. This indicates the possibility of a different binding mode for this PNA; e.g., by antiparallel binding to the sequence TTTTGG).

Formally, the binding of PNA 2 could be the result of at least two sets of nucleobase triplets: T·T–A, G·G–C (I) or T·A–T, G·C–G (II), i.e., either a PNA·PNA–DNA (I) or a PNA·DNA–PNA (II) triplex. Both sets involve

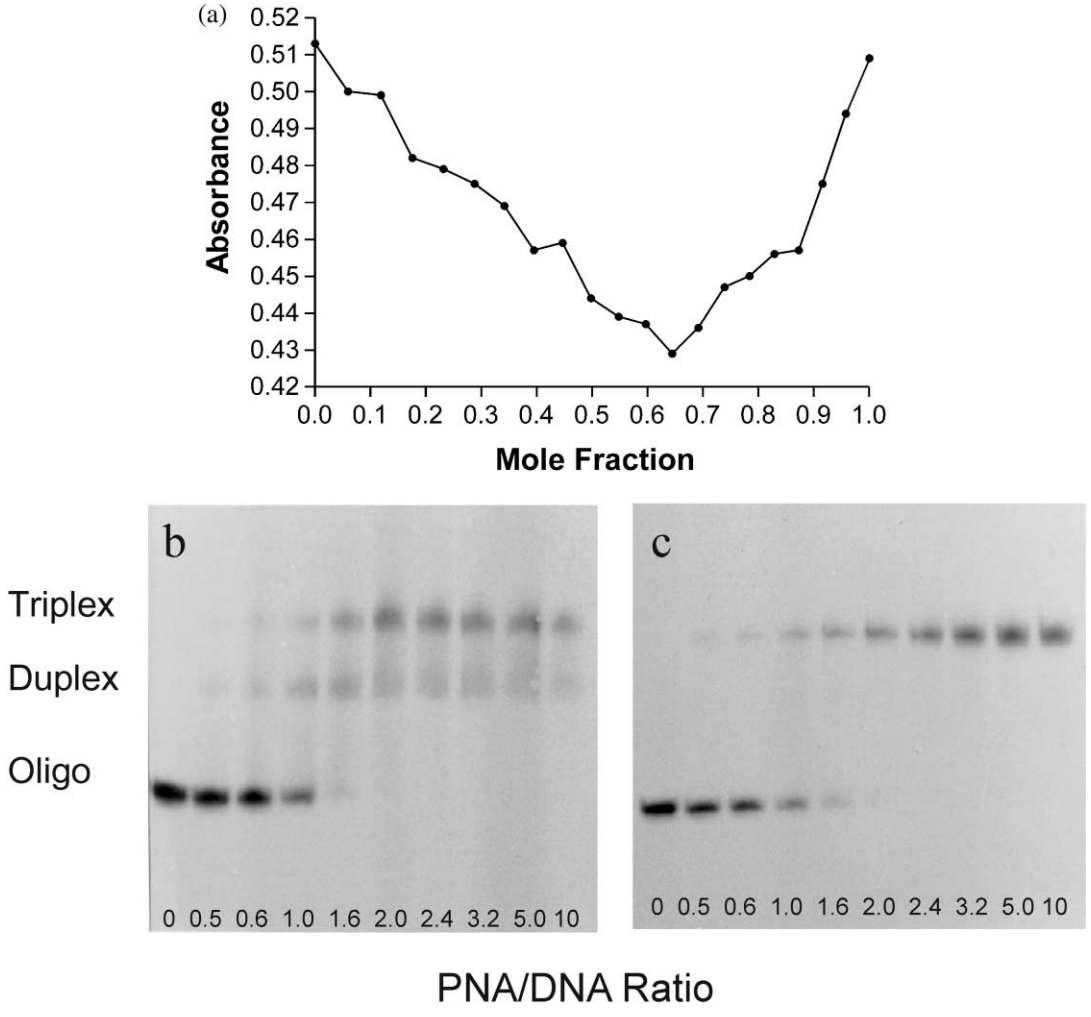

Figure 1. Binding stoichiometry of PNA H-T_4G_2TGTG-LysNH_2 to complementary oligonucleotides: (a) Job plot analysis using oligonucleotide 5′-d($CACAC_2A_4$); (b and c) Gel shift assay using oligonucleotides 5′-d(A_4C_2ACAC) (b) and 5′-d($CACAC_2A_4$) (c). The PNA concentrations were: 0, 2.5, 3, 5, 7,10, 12, 16, 25 and 50 μM while the concentrtion of the 5′-^{32}P labeled oligonucleotide (5′-GATCC*AAAACCACACC*G (parallel) or 5′-GATCC*CACACCAAAA*G (antiparallel)) was 5 μM. The samples (10 °C mu;L) were incubated in 10mM Tris–HCl, 1 mM EDTA, pH 7.4 for 5 min at 90 °C, cooled slowly to 20 °C, and finally put on ice before 2 μL 50% glycerol in TBE (90 mM Tris-borate, 1 mM EDTA, pH 8.5) buffer was added and the samples analyzed by gel electrophoresis in 20% polyacrylamide/urea gels run at 4 °C in TBE buffer. Radioactive DNA bands were visualized by autoradiography using intensifying screens.

Table 1. Thermal stabilities[a] (T_m, °C) of T/G motif PNA–DNA complexes

	Complementary DNA oligonucleotide		
	Antiparallel[b]	Parallel	Mismatch[c]
PNA			
H-T_4G_2TGTG-LysNH_2 (1)	52.5 (53.8)	41.5	45.0
H-GTGTG_2T_4-LysNH_2 (2)	50.0 (53.7)	45.5	35.0
(1) + (2)	48.5	49.5	nm
H-T_4G_2CGTG-LysNH_2 (3)	63.0 (58.7)	46.5	42.5
H-GTGCG_2T_4-LysNH_2 (4)	58.0 (58.6)	nm	33.0
H-TG_2TG_2TGTG-LysNH_2 (5)	71.0 (66.0)	65.0	nm
(5′-d(T_4G_2TGTG) (DNA reference)	37.0	nd	nm

[a]Thermal stabilities (T_m) were measured in 10 mM Na-phosphate, 100mM NaCl, 0.1 mM EDTA, pH 7.0 at a heating rate of 0.5 °C/min (5–90 °C).
[b]5′-3′-direction for the oligonucleotide versus carboxy-amino direction for the PNA is termed the antiparallel complex. The DNA oligomers are d($CACAC_2A_4$) for 1, 2 and d(T_4G_2TGTG); d($CACAC_2AC_2A$) for 3 and 4, and d($CACGC_2A_4$) for 5;. nd: no complex detected, nm: not measured. The values in parentheses are calculated according to Giesen et al..[27]
[c]T_m for the antiparallel complex is reported: DNA oligomer d($CACGC_2A_4$) was used for 1 & 2, and oligomer d($CACAC_2A_4$) was used for 3 & 4.

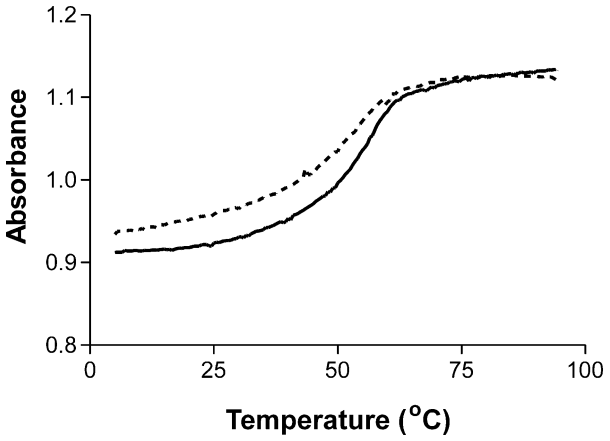

Figure 2. Thermal denaturation (T_m) profile of the complex between PNA 1 and oligonucleotide 5′-d($CACAC_2A_4$). The heating rate was 0.5 °C/min. The heating curve is represented by the solid line while the cooling curve is represented by the broken line. The hysteresis was 2 °C.

an unconventional (and presumably not optimal) base pairing (T·T and G·C). Two types of experiments were designed in an attempt to discriminate between these two models. Increasing the G/T ratio in the PNA should influence the stability of the complex differently according to the models: Model one would predict a stabilization owing to the decrease of the presumably less favorable T·T–A triplets, whereas model II would predict a destabilization owing to the decrease in the likewise presumably less favorable G·C-G triplets. The results (Table 1) show a significantly increased thermal stabililty for PNA 5 (H-TG_2TG_2TGTG-LysNH_2) and thus support model I. However, this complex was found by Job-plot analysis to be a duplex and the increased thermal stability is therefore ascribed to the higher purine (G) content of this PNA.[27] Nonetheless, PNA 5 also bind weakly to double stranded DNA by strand displacement (Fig. 3b). The strand invading property of this PNA is ascribed to the high thermal stability of the PNA–DNA duplex as previously observed with a homopurine PNA.[19]

In another binding experiment, we incorporated a guanine instead of one of the adenines into the target (5′-$CACGC_2A_4$). Using the complementary PNA (T_4G_2CGTG), we rationalized that in model II this would create a conventional C^+·G–C triplet, which should not compromise the complex stability and, furthermore, the C^+·G Hoogsteen base pairing should be detectable by dimethyl sulfate probing of guanine-N7. This substitution increased the T_m of the PNA–DNA complex by approximately 10 °C (Table 1) (which is ascribed to stabilization by the A–T to G–C transversion[27]). However, no protection of the guanine in the double stranded DNA target against DMS modification as evidence for Hoogsteen PNA–DNA interaction could be detected in the presence of PNA 4 (Fig. 3d) despite the fact that this PNA by $KMnO_4$ probing was shown to invade the target in the antiparallel configuration (Fig. 3c, lanes 15–17). Furthermore, the parallel PNA 3 did not bind (Fig. 3c, lanes 11–14). (We also note that PNA 1 exhibits binding at the mismatched parallel site (Fig. 3b, lanes 2–5), but as observed with the fully matched target (Fig. 3a, lanes 2–5), this binding is somewhat off the target, and thus most likely reflects alternative target binding). Therefore these results also support model I, although they by no means prove it. A possible base pairing Scheme is depicted in Fig. 4. It is noteworthy that precedence AC/TG triplex structures have previously been proposed to occur in DNA telomers, although the authors did not explicitly consider a possible T·T interaction.[28]

The present results have demonstrated a novel mode of PNA strand invasion. The results also show that recognition of double stranded DNA by PNA can occur by mechanisms that are more complex than straight forward invasion via PNA–DNA–PNA triplexes or PNA–DNA duplexes. However, the observation that PNA 5 did not form a stable PNA_2DNA triplex indicate that this novel putative PNA–PNA–DNA recognition motif may have not yet resolved unrecognized sequence context constraints, and therefore further studies are required to assess the potential of exploiting it for sequence specific recognition of double stranded DNA using PNA or other oligonucleotide analogues. However, it could well be worth exploring the triplet motifs suggested by Trapane et al.[29] in a PNA·PNA–DNA triplex concept. These motifs involve bidentate nucleobases having a Watson–Crick face for binding the targeting and a 'triplex face' to which a second designed oligomer can bind.

Finally, the present results on the duplex DNA binding of GT–PNA oligomers also invite to speculations on the factors that determine the helix invasive power of a PNA. Limiting the discussion to decamer PNAs, it is well established that triplex forming homopyrimidine PNAs form very stable strand displacement complexes.[1,12–15,25,26,30–33] It also seems clear that PNAs, such as A_4G_2 AGAG [19] or TG_2TG_2TGTG (PNA 5) that form PNA–DNA duplexes of comparable thermal stability (ca. 70 °C for a decamer), are also capable of invading the duplex although the resulting complexes are significantly less stable than the triplex invasion complexes. (It is noteworthy, however, that a further stabilization is possible using modified nucleobases, such as diaminopurine.[34]) The results obtained with PNA *1* are most interesting in this context. The PNA–DNA complex with this PNA has a thermal stability (T_m = 52.5 °C, ~5.3 °C/base pair) that in our experience should not allow strand displacement binding. For instance, we have not been able to detect strand displacement binding with PNA GTAGATCACT (T_m = 51 °C ~5.1 °C/base pair) or PNA TGTACGTCACAACTA (T_m = 69 °C ~4.6 °C/base pair). Nonetheless, the present results clearly demonstrate that PNA 1 does indeed invade the DNA double helix, and we can only ascribe this to its propensity to form a triplex structure which by some means—most likely kinetic in nature—is able to stabilize the strand displacement complex. Thus by further unraveling the various mechanistic aspect of PNA strand displacement binding, one should be able to exploit this knowledge to

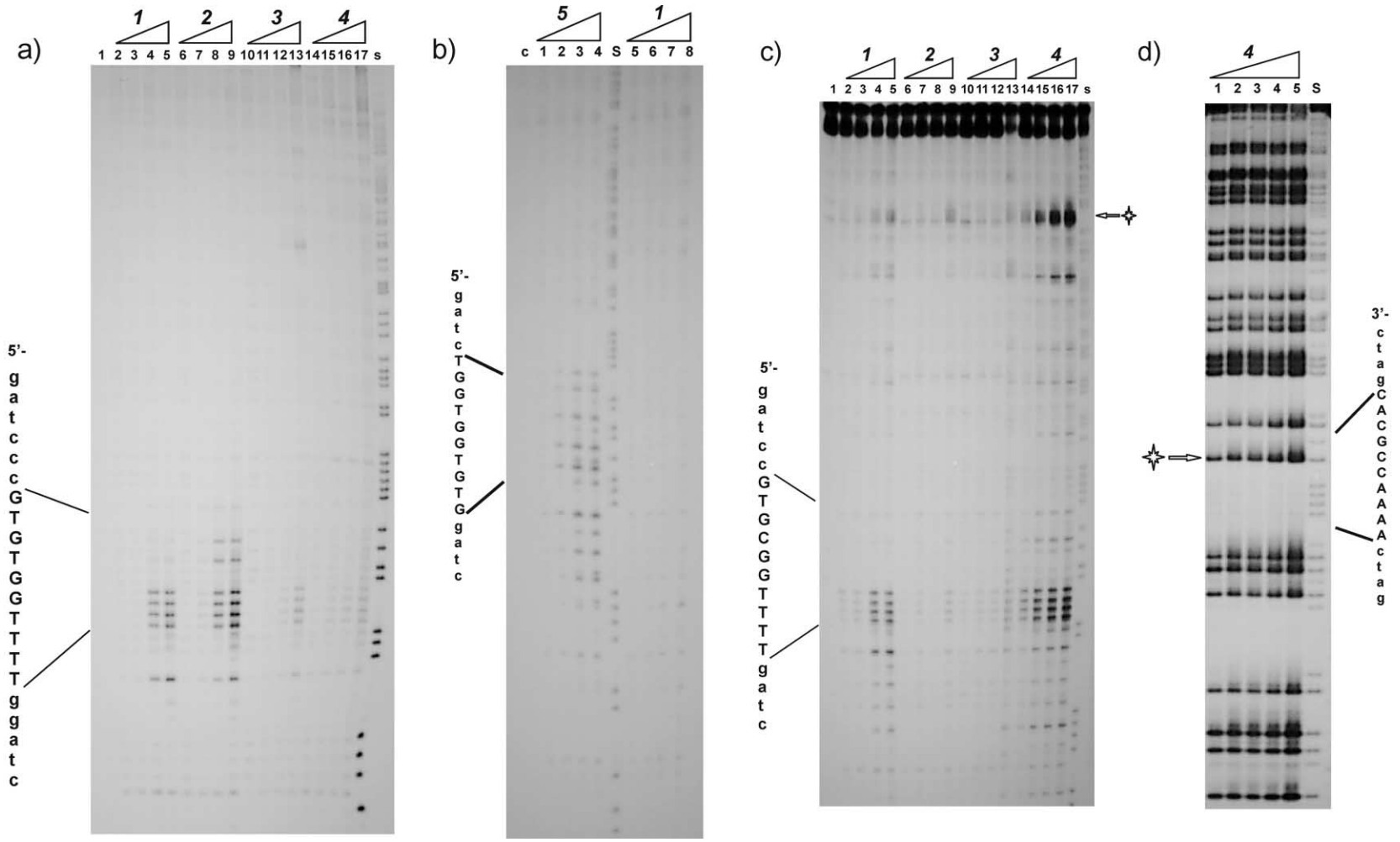

Figure 3. Binding of PNAs 1, 2, 3 and 4 (panels a & c) to a double stranded DNA target as probed by $KMnO_4$. Lanes 2–5: PNA 1, lanes 6–9: PNA 2, lanes 10–13: PNA 3, lanes 14–17: PNA 4, lane 1: control without PNA, lane S: A + G sequence reaction. Panel b: Lanes 2–4: PNA 5, lanes 6–9: PNA 1. Panel D: PNA 4 as probed with dimethyl sulfate. The PNA concentrations were: 0.5 μM (lanes 2,6,10,14), 1.5 μM (lanes 3,7,11,15), 5 μM (lanes 4,8,12,16), 15 μM (lanes 5,9,13,17). The DNA targets were: 5′-A_4C_2ACAC (panel a), 5′-CACACCACCA (panel b) and 5′-A_4C_2GCAC (panel c and d). The PNA targets are indicated. The asterix in panel c) indicates a secondary target for the PNA having the sequence: 5′-gggAAAACCCTGG, but it is not clear how much of this target is actually bound by the PNA. The asterix in panel d) points to the guanine which should be selectively protected.

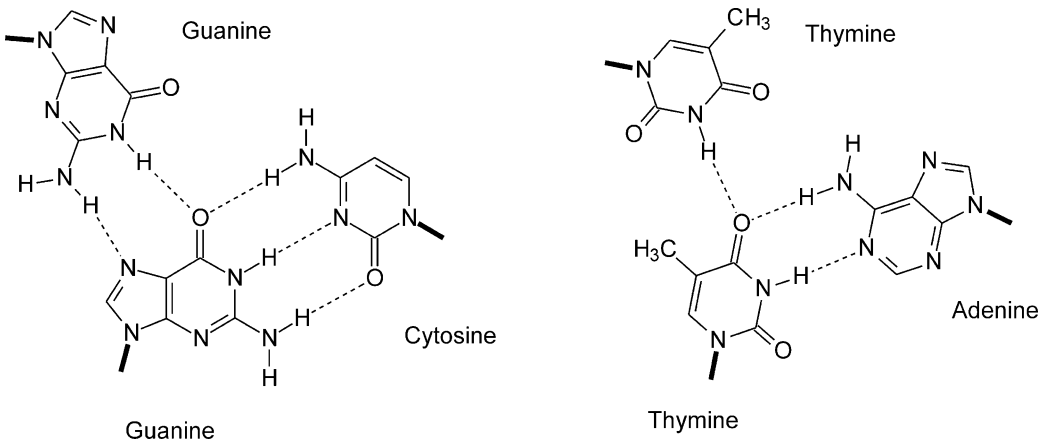

Figure 4. Possible base pairing for the T·T-A and G·G-C triplets.

design PNAs (and other DNA binding ligands) with greatly improved binding characteristics.

Acknowledgements

The expert technical assistance of Ms. Karin Frederiksen and Ms. Annette W. Jørgensen as well as the financial support of The Danish National Research Foundation and The Lundbeck Foundation is gratefully acknowledged.

References and Notes

1. Nielsen, P. E.; Egholm, M.; Berg, R. H.; Buchardt, O. *Science* **1991**, *254*, 1497.
2. Egholm, M.; Buchardt, O.; Nielsen, P. E.; Berg, R. H. *J. Am. Chem. Soc.* **1992**, *114*, 1895.
3. Dueholm, K. L.; Egholm, M.; Behrens, C.; Christensen, L.; Hansen, H. F.; Vulpius, T.; Petersen, K.; Berg, R. H.; Nielsen, P. E.; Buchardt, O. *J. Org. Chem.* **1994**, *59*, 5767.
4. Christensen, L.; Fitzpatrick, R.; Gildea, B.; Petersen, K. H.; Hansen, H. F.; Koch, T.; Egholm, M.; Buchardt, O.; Nielsen, P. E.; Coull, J.; Berg, R. H. *J. Peptide Sci.* **1995**, *3*, 175.
5. Egholm, M.; Buchardt, O.; Christensen, L.; Behrens, C.; Freier, S. M.; Driver, D. A.; Berg, R. H.; Kim, S. K.; Norden, B.; Nielsen, P. E. *Nature* **1993**, *365*, 556.
6. Nielsen, P.E.; Egholm, M. eds (1999) *Peptide Nucleic Acid (PNA). Protocols and Applications*. Norfolk; Horizon Scientific Press
7. Ganesh, K. N.; Nielsen, P. E. *Current Organic Chemistry* **2000**, *4*, 916.
8. Demidov, V.; Bukanov, N. O.; Frank-Kamenetskii, M. D. *Curr. Issues Mol. Biol.* **2000**, *2*, 31.
9. Ray, A.; Nordén, B. *FASEB J.* **2000**, *14*, 1041.
10. Nielsen, P. E. *Curr. Opin. Biotech.* **2001**, *12*, 16.
11. Nielsen, P. E. *Exp. Opin. Invest. Drugs* **2001**, *10*, 331.
12. Cherny, D. Y.; Belotserkovskii, B. P.; Frank-Kamenetskii, M. D.; Egholm, M.; Buchardt, O.; Berg, R. H.; Nielsen, P. E. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 1667.
13. Nielsen, P. E.; Egholm, M.; Buchardt, O. *J. Mol. Recognit.* **1994**, *7*, 165.
14. Demidov, V. V.; Yavnilovich, M. V.; Belotserkovskii, B. P.; Frank-Kamenetskii, M. D.; Nielsen, P. E. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 2637.
15. Praseuth, D.; Grigoriev, M.; Guieysse, A. L.; Pritchard, L. L.; Harel-Bellan, A.; Nielsen, P. E.; Hélène, C. *Biochim. Biophys. Acta* **1997**, *1309*, 226.
16. Wittung, P.; Nielsen, P. E.; Norden, B. *Biochemistry* **1997**, *36*, 7973.
17. Boffa, L. C.; Carpaneto, E. M.; Allfrey, V. G. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 1901.
18. Boffa, L. C.; Morris, P. L.; Carpaneto, E. M.; Louissaunt, M.; Allfrey, V. G. *J. Biol. Chem.* **1996**, *271*, 13228.
19. Nielsen, P. E.; Christensen, L. *J. Am. Chem. Soc.* **1996**, *118*, 2287.
20. Ishihara, T.; Corey, D. R. *J. Am. Chem. Soc.* **1999**, *121*, 2012.
21. Zhang, X.; Ishihara, T.; Corey, D. R. *Nucleic Acids Res.* **2000**, *28*, 3332.
22. Lohse, J.; Dahl, O.; Nielsen, P. E. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 11804.
23. Izvolsky, K. I.; Demidov, V. V.; Nielsen, P. E.; Frank-Kamenetskii, M. D. *Biochemistry* **2000**, *39*, 10908.
24. Frank-Kamenetskii, M. D.; Mirkin, S. M. *Ann. Rev. Biochem.* **1996**, *64*, 65.
25. Nielsen, P. E.; Egholm, M.; Berg, R. H.; Buchardt, O. *Nucleic Acids Res.* **1993**, *21*, 197.
26. Egholm, M.; Christensen, L.; Dueholm, K.; Buchardt, O.; Coull, J.; Nielsen, P. E. *Nucleic Acids Res.* **1995**, *23*, 217.
27. Giesen, U.; Kleider, W.; Berding, C.; Geiger, A.; Ørum, H.; Nielsen, P. E. *Nucleic Acids Res.* **1998**, *26*, 5004.
28. Veselkov, A. G.; Malkov, V. A.; Frank-Kamenetskii, M. D.; Dobrynin, V. N. *Nature* **1993**, *364*, 496.
29. Trapane, T. L.; Ts'o, P. O. P. *J. Am. Chem. Soc.* **1994**, *116*, 10437.
30. Kuhn, H.; Demidov, V.; Frank-Kamenetskii, M. D.; Nielsen, P. E. *Nucleic Acids Res.* **1998**, *26*, 582.
31. Bentin, T.; Nielsen, P. E. *Biochemistry* **1996**, *35*, 8863.
32. Bukanov, N. O.; Demidov, V. V.; Nielsen, P. E.; Frank-Kamenetskii, M. D. *Proc. Natl. Acad. Sci. U.S.A* **1998**, *95*, 5516.
33. Kuhn, H.; Demidov, V. V.; Nielsen, P. E.; Frank-Kamenetskii, M. D. *J. Mol. Biol.* **1999**, *286*, 1337.
34. Haaima, G.; Hansen, H. F.; Christensen, L.; Dahl, O.; Nielsen, P. E. *Nucleic Acids Res.* **1997**, *25*, 4639.