Inducing and modulating anisotropic DNA bends by pseudocomplementary peptide nucleic acids

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DNA bending is significant for various DNA functions in the cell. Here, we demonstrate that pseudocomplementary peptide nucleic acids (pcPNAs) represent a class of versatile, sequence-specific DNA-bending agents. The occurrence of anisotropic DNA bends induced by pcPNAs is shown by gel electrophoretic phasing analysis. The magnitude of DNA bending is determined by circular permutation assay and by electron microscopy, with good agreement of calculated mean values between both methods. Binding of a pair of 10-meric pcPNAs to its target DNA sequence results in moderate DNA bending with a mean value of 40–45°, while binding of one self-pc 8-mer PNA to target DNA yields a somewhat larger average value of the induced DNA bend. Both bends are found to be in phase when the pcPNA target sites are separated by distances of half-integer numbers of helical turns of regular duplex DNA, resulting in an enhanced DNA bend with an average value in the range of 80–90°. The occurrence of such a sharp bend within the DNA double helix is confirmed and exploited through efficient formation of 170-bp-long DNA minicircles by means of dimerization of two bent DNA fragments. The pcPNAs offer two main advantages over previously designed classes of nonnatural DNA-bending agents: they have very mild sequence limitations while targeting duplex DNA and they can easily be designed for a chosen target sequence, because their binding obeys the principle of complementarity. We conclude that pcPNAs are promising tools for inducing bends in DNA at virtually any chosen site.

Intrinsic or induced DNA bending plays an important role in various biological processes such as transcription, replication, recombination, DNA packaging, and repair (1–6). It has therefore been recognized that biological functions relying on DNA bending may be influenced by nonnatural DNA-bending agents (7–11). So far, two classes of artificial DNA-bending agents have been investigated: tethered triple-helix-forming oligonucleotides (7–9) and six-zinc-finger peptides (10, 11). In both classes, two DNA-binding domains are connected by a linker and targeted to two separated sites in double-stranded DNA (dsDNA), thus inducing bending at the intervening region. Whereas tethered triplex-forming oligonucleotides can easily be designed, they suffer from severe sequence limitations because they require the presence of two ≥15-bp-long homopurine-homopyrimidine (hypr) tracts, located not far from each other in the target DNA. On the other hand, the design of artificial DNA-binding peptides such as the reported six-zinc-finger peptides is not straightforward and may require a selection process starting with a pool of peptides for any chosen DNA target sequence (12). Thus, a DNA-binding ligand capable of sequence-specific recognition and bending of DNA, which could easily be designed for a chosen DNA target sequence, would be of great interest.

Certain types of peptide nucleic acids (PNAs) are known to bind complementary target sites in dsDNA sequence-specifically through invasion of the double helix (13–23). Of those types, however, only hpyrPNAs and pseudocomplementary (pc)PNAs form stable PNA–dsDNA complexes and thus are of practical value as functional tools. Along with ordinary guanines and cytosines, pcPNAs carry 2,6-diaminopurines (D) and 2-thiouracils (^{s}U) instead of adenines and thymines, respectively (Fig. 1*A*). Lohse *et al.* (17) and Demidov *et al.* (20) showed that corresponding pairs of pcPNAs recognize their natural A-T or G-C counterpart, but form unstable duplexes with one another due to steric interference (see Fig. 1*B*). As a result, stable double-duplex invasion complexes (Fig. 1*C*) are formed when a pair of pcPNAs is added to dsDNA carrying the target sequence (17, 20).

For hpyrPNAs as well as pcPNAs, the corresponding invasion complexes with dsDNA show a reduced electrophoretic mobility compared with uncomplexed DNA, which may be caused by directional (anisotropic) or nondirectional (isotropic) deviations from the DNA helix axis. The former is usually referred to as DNA bending, whereas the latter is commonly designated as increased flexibility.¶ Earlier data with hpyrPNAs suggested that triplex invasion complexes do not cause anisotropic DNA bending (24), whereas recent results with pcPNAs have indicated that these ligands induce a directional DNA bend (25). However, a study analyzing the nature and degree of the DNA curvature induced by either type of PNA has been lacking. Because pcPNAs, unlike hpyrPNAs, provide with essentially sequence-unrestricted targeting of dsDNA, we have chosen to investigate DNA bending by this class of PNA.

By using gel electrophoretic methods in combination with electron microscopy (EM), we now provide clear evidences that pcPNAs induce bending of the DNA molecule in only one direction. We show that the degree of bending can be significantly increased by targeting two closely located sites of duplex DNA with pcPNAs. We therefore demonstrate that pcPNAs represent a very promising class of DNA-bending ligands. Because pcPNAs produce substantial directional bends in a highly sequence-specific manner and allow DNA targeting with very mild sequence restrictions, they are much superior to previously proposed DNA-bending ligands.

Materials and Methods

pcPNAs. The following pcPNAs carrying ^{s}U and D were used: I, H-K_2-^{s}U^{s}UGD^{s}UCDD-K-NH_2; II, H-K_2-^{s}UCDDDCD^{s}UGC-K-NH_2; III, H-GCD^{s}UG^{s}U^{s}U^{s}UGD-K-NH_2.

Like other types of PNAs (15, 22), pcPNAs are tagged by lysine residues (K) at their termini to increase solubility and affinity. PNAs were synthesized, purified, and characterized as described (17).

DNA. Plasmids pPA2-pPA24 used in our study are pUC19 derivatives with inserts cloned into the *Eco*RI/*Hin*dIII site of the

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Abbreviations: dsDNA, double-stranded DNA; hpyr, homopyrimidine; pc, pseudocomplementary; PNA, peptide nucleic acid; EM, electron microscopy; D, 2,6-diaminopurine; ^{s}U, 2-thiouracil; CPA, circular permutation assay.

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¶It should be noted that anisotropic DNA bending by increased local flexibility cannot entirely be ruled out. An anisotropic DNA bend can be static (or fixed), dynamic (or flexible), or can include both static and dynamic components.

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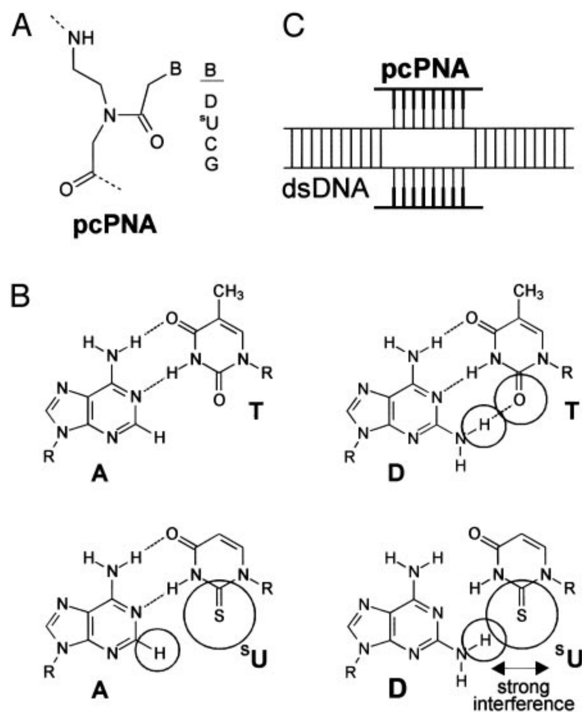


Fig. 1. (*A*) Chemical structure of pcPNA. In pcPNA, the nucleobases A and T are substituted by the modified nucleobases D and ${}^{s}U$, respectively. (*B*) The modified nucleobases prevent pcPNAs from forming a stable PNA–PNA duplex due to steric hindrance between D and ${}^{s}U$ bases, whereas they do not prevent pcPNAs from forming stable PNA–DNA heteroduplexes with complementary DNA strands (17, 20). (*C*) Double-duplex invasion complex formed at binding of a pair of pcPNAs to the target sequence in dsDNA.

polylinker. Recombinant plasmids were isolated from a dam^-, dcm^- strain of *Escherichia coli*, and sequences were verified by dideoxy sequencing. The plasmids were used to generate a set of blunt-end DNA fragments of 251 bp in length through PCR by using different primer pairs (sequences of plasmid inserts and primers are given in *Supporting Text*, which is published as supporting information on the PNAS web site). DNA samples that were obtained after PCR or other enzymatic reactions were isolated by a standard procedure, i.e., desalted by gel filtration (Sephadex G-50), purified by phenol and chloroform extraction, precipitated by the addition of ethanol and centrifugation, and suspended in buffer containing 10 mM Tris·HCl (pH 7.4) and 0.1 mM EDTA.

pcPNA–DNA Complex Formation. PCR-generated DNA fragments (≈300 ng) were incubated in a volume of 40 μl for 2 h at 37°C with the corresponding pcPNAs (1 μM final concentration) in the presence of 10 mM Tris·HCl (pH 7.4 at 25°C) and 0.1 mM EDTA. Surplus pcPNAs were removed by gel filtration (Sephadex G-50).

Gel Mobility-Shift Assays. Free and pcPNA-bound DNA fragments (≈30 ng each) were resolved by 5% nondenaturing PAGE (29:1 acrylamide/bis-acrylamide), run for 2–3 h (12.5 V/cm) in 1× TBE buffer (90 mM Tris-borate/2 mM EDTA/pH 8.0). Gels were stained with ethidium bromide, illuminated at 302 nm, and scanned with a charge-coupled device camera. Mobilities of free and bound DNA fragments were taken as the distance from the center of the electrophoresis well to the center of the corresponding band. The mobility of pcPNA–DNA complexes (bent DNA) was normalized to the mobility of the free (or unbent) DNA to give the relative mobility $R_{\mathrm{bound}}/R_{\mathrm{free}}$.

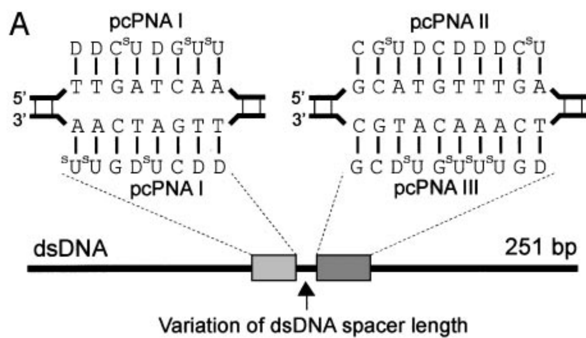
Preparation and Purification of DNA Minicircles. DNA monomer duplex, obtained after incubation of a precursor 99-bp DNA duplex with *Bbs*I (see *Supporting Text*), was bound by pcPNAs, followed by incubation with 20 units of T4 DNA ligase for 6 h at 16°C in a volume of 100 μl containing 1× ligation buffer (Fermentas, Hanover, MD). Samples were then heated for 1 h at 60°C, during which pcPNAs irreversibly dissociate from their targets (see Fig. 7, which is published as supporting information on the PNAS web site), and the DNA was isolated. An aliquot of the obtained product was incubated for 2 h at 37°C with 200 units of exonuclease III in a volume of 100 μl containing 1× reaction buffer (United States Biochemical), and the DNA was isolated. For EM analysis, aliquots of exonuclease-treated samples were subjected to gel filtration chromatography (see Fig. 8, which is published as supporting information on the PNAS web site).

EM and Data Analysis. Samples were diluted in a buffer containing 10 mM Na-Hepes (pH 7.5), 50 mM NaCl, and 0.1 mM EDTA to a final DNA concentration of ≈0.5 μg/ml, mounted on EM grids coated with a thin carbon film, and visualized as described (26). Angle measurements and determination of the mean value of the bending angle and its SD were based on described methods (26). Briefly, by using IMAGEJ software, we first measured the exact position of bends of the major part of molecules that had a clearly visible bend, thus verifying that bends were located at the center of the DNA fragments. We then measured the smallest angle, φ, between the tangents to the DNA arms emerging from the center of the fragment from which the bending angle, α, was calculated as $\alpha = 180° - \varphi$. Analyzing all DNA molecules for each pcPNA–DNA complex, a large number ($n > 500$) of bend angles was measured and converted to a cumulative distribution (normalized by n) in steps of 0.18°. Mean bending angle values and standard deviations were obtained by fitting the experimental cumulative distribution to the cumulative distribution of the folded Gaussian probability density function by minimizing the Kolmogorov–Smirnov statistic using MATHEMATICA 4.2 (26).

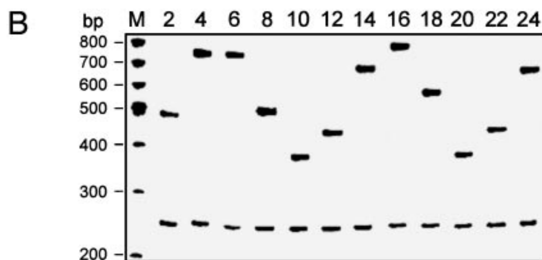
Results and Discussion

Phasing Analysis. Phasing analysis is widely used to detect and analyze DNA bending and allows, under certain conditions, discrimination between anisotropic (directional) bending and increase in DNA local flexibility (27–30). We prepared a set of 251-bp-long DNA fragments that varied in the length of regular duplex DNA between two target sites for pcPNAs (Fig. 2*A*). Because one target site contained a palindromic sequence, only one self-pcPNA (pcPNA I) was sufficient for binding in this case, whereas for the other, nonpalindromic target sequence, a pair of pcPNAs (pcPNAs II/III) was required. If binding of pcPNAs were to induce directional DNA bends at the target sites, the electrophoretic mobility of pcPNA-bound fragments would strongly depend on the spacer length that determines the relative angle between the bends. It would be slowest when the two bends are in the same plane and direction, i.e., in phase (cis orientation), and fastest when the two bends are 180° out of phase (trans orientation).

Indeed, when the DNA fragments were complexed with pcPNA I–III and resolved together with the initial free DNA by 5% nondenaturing PAGE, we observed significant sinusoidal variation in gel mobility of the pcPNA-bound DNA fragments as a function of phasing distance (Fig. 2*B*). The data were analyzed by plotting the relative mobility of pcPNA-bound DNA fragments as a function of spacer length between the target sites and fitting to a cosine function (Fig. 2*C*). Note that even for very short spacer lengths, the obtained data points lie perfectly on the cosine fit, indicating the occurrence of dsDNA throughout the spacer region. This result could be accredited to a duplex-stabilizing effect of the positively



Plasmid	Spacer sequence	Phasing distance
pPA2	5'-TT	2
pPA10	5'-TTGCAGTCCT	10
pPA16	5'-TTGCAGAGTCGATCCT	16
pPA24	5'-TTGCAGAGTCCATTCGACGATCCT	24



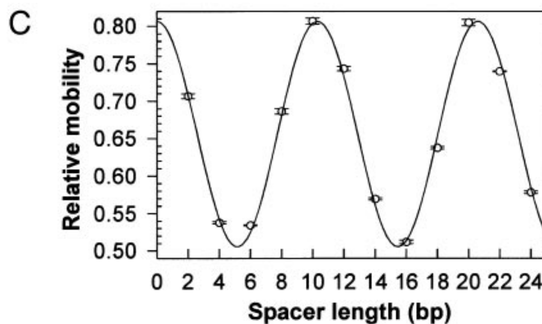


Fig. 2. (*A*) Constructs for phasing analysis. A set of 12 DNA fragments with variable spacer length (2–24 bp) of regular dsDNA between the target sites for pcPNAs I–III is used. Spacer sequences of four constructs are shown. (*B*) Phasing analysis monitored by nondenaturing PAGE. Upper and lower bands correspond to pcPNA-bound or free DNA fragments, respectively, with phasing distances indicated above each lane. (*C*) Plot of the relative mobility of DNA fragments bound with pcPNAs as a function of the spacer length. Data points are connected by the best fit of a cosine function. SD are plotted as vertical bars (in all cases they are smaller than the symbols used to plot the data).

charged lysine residues that are tagged to pcPNAs and to the extreme tightness of double–duplex structures (see below).

The considerable amplitude of phasing variation we observed for the pcPNA-bound DNA fragments can be interpreted as a direct evidence of anisotropic DNA bending. The bends were found to be in phase when the pcPNA-binding sites were separated by a half-integer number of helical turns.‖ The helical periodicity of the DNA between the bends was determined as 10.3 bp, which is very close to the canonical value for DNA in solution (31).

‖Typically, an A-tract reference bend is employed in phasing analysis to determine the absolute orientation of an unknown bend at binding of a ligand to dsDNA. Due to the nature of the duplex invasion complexes, such an approach is not feasible here.

Circular Permutation Assay (CPA). This method, originally developed by Wu and Crothers (32), is based on the principle that the electrophoretic mobility of a bent DNA fragment depends on the location of the bend within the DNA fragment. We prepared a set of DNA fragments differing in the locations of the target sites for pcPNAs (schematics in Fig. 3), and bound pcPNAs to one or both target sites. As expected, the formation of pcPNA–DNA complexes resulted in significant alteration of gel mobility with maximum values at central binding locations (Fig. 3 *A–D*). Bend angles were then derived from second-order polynomial or cosine fits to the plots of the relative mobility of pcPNA-bound DNA fragments against the bend location (Fig. 3 *E–H*), by using described algorithms (33, 34).

As shown in Table 1, binding of the self-pcPNA I to its DNA target resulted in a bend with a mean value of ≈70°, whereas the pcPNA pair II/III provided with a moderate bend of ≈45° on average when it was bound to the corresponding target sequence on duplex DNA. Binding of pcPNAs to both target sites resulted in an overall mean bend angle of ≈90° when the bends were in phase (16-bp spacer; complex I/II/III cis) and an overall mean bending angle of ≈50° when the bends were out of phase (10-bp spacer; complex I/II/III trans). The quantitation is based on the assumption that the electrophoretic mobilities of probes containing two closely juxtaposed in-phase or out-of-phase bends are comparable with those probes containing single DNA bends with the bend angle being the sum or difference of the two individual bend angles. This assumption has been previously found to be approximately valid for closely spaced bends with long flanking sequences (30). It is also worth mentioning that each pcPNA–DNA complex produced only a single band on polyacrylamide gels indicating the absence of structural isomers with different electrophoretic mobility that have been observed for complexes of duplex DNA with hpyrPNAs (16, 35).

The CPA has been found to overestimate bend angles because other factors such as increased DNA flexibility, direct size effects, or fractional occupancy can affect the electrophoretic migration (33, 36–38). Because pcPNAs have a low molecular weight as compared with the target DNA fragment and because pcPNA–DNA complexes are stable under the electrophoretic conditions used here, the latter two factors should not play a role in case of pcPNAs. However, an increase in DNA flexibility in pcPNA–DNA complexes cannot be ruled out. Therefore, we additionally determined bend angles by EM.

Determination of Bend Angles by EM. EM allows visualization of specific DNA complexes and measurement of the extent of DNA bending (39–41). In addition, a bending angle distribution is readily obtained providing useful information about the flexibility of a specific complex (42). Visualization and angle measurements of a large number of DNA fragment molecules bound centrally with pcPNA I, pcPNAs II/III, or with all three pcPNAs revealed significant DNA bending in the middle of the fragments, as shown in the histograms and microphotographs in Fig. 4. Mean values for the EM-measured bending angles and their SD, obtained from folded Gaussian fits to the experimental data, are given in Table 1. It can be seen that pcPNA I bends the DNA axis with a mean value of 57°. A lower magnitude of 43° is obtained at binding of pcPNA pair II/III to the DNA target fragment. The complex of all three pcPNAs with both DNA target sites in cis orientation gives rise to an enhanced bend with an average of 77°.

The mean bend angle values for pcPNA–DNA complexes I and I/II/III cis are 13–17° smaller in the EM measurements as compared with the values obtained by CPA, while good agreement in the magnitude of DNA bending induced by pcPNAs II/III was obtained by both methods. The tendency to slightly higher bending angle values by gel electrophoresis indicates that most probably a cumulative value was calculated by this method,

Fig. 3. CPA. A set of 251-bp-long PCR products is used (schematics), differing in the bend location *L*. The *L* value is defined as the distance of the pcPNA target site center (one target site) or the distance of the center between pcPNA-binding sites (two target sites) from one end of the DNA fragment divided by the total length of the DNA fragment. (*A–D*) Permutation analysis monitored by nondenaturing PAGE. Lanes a–i depict free (lower bands) and pcPNA-bound DNA fragments (upper bands) of the constructs shown above each gel. (*E–H*) Analysis of induced DNA bends. The relative mobility of pcPNA-bound DNA fragments was plotted against the bend location *L*. SD are indicated by vertical bars. Bending angles α were derived from the best fits of cosine functions (black lines) or of second-order polynomial functions (data not shown) to the experimental data (see *Supporting Text* for details).

consisting of a major contribution of directional DNA bending in pcPNA–DNA complexes and of a minor contribution due to increased flexibility.

An increased flexibility of a specific DNA complex can also be deduced from the analysis of SD, σ, of bending angles. A value for σ that significantly exceeds the measured value for free DNA can be explained by a higher flexibility of the complex (42). The mean value of σ for each pcPNA–DNA complex was found to be 1–3° higher than the corresponding value for free DNA (data not shown), indicating that the complexes may have a higher flexibility. It should be noted, however, that the possible gain in DNA flexibility at binding of pcPNAs is relatively small. Therefore, it appears that anisotropic DNA bends induced by pcPNAs are predominantly responsible for the kinks seen by EM and for the retarded migration observed in polyacrylamide gels, whereas the contribution of an increased isotropic flexibility is rather small.

The fact that we found the bend induced by the double-duplex invasion complex to be highly directional is hardly surprising. Indeed, two bulky DNA–PNA heteroduplexes, which include two negatively charged DNA backbones, two neutral PNA backbones, and positively charged oligolysine "tails," are situated in a side-by-side manner within the complex (see schematics in Fig. 2*A* where oligolysines are omitted), and must create a very tight and strained structure. Moreover, as such a complex constitutes a distortion of the DNA duplex at the target sequence, it should be bent. The apparent extreme tightness of the double-duplex complex is emphasized by our data in Fig. 2*C*, according to which two double-duplex complexes separated by a DNA duplex spacer do not open the spacer up, even when it consists of only 2 bp. These properties of the double-duplex invasion complex contrast to the properties of the triplex-invasion complex formed by hpyPNAs with dsDNA, which has been extensively studied before (14, 16, 22). In fact, two triplex-invasion complexes form an extended open region even if separated by as many as 10 bp (43). Thus, significant flexibility can be expected in a triplex-invasion complex. Data by Kim *et al.* (24) and D.I.C. (unpublished observations) seem to support this expectation.

Table 1. Quantitative estimation of DNA bends in pcPNA–DNA complexes by CPA and EM

Complexes	CPA bend angle α,*°		EM bend angle α,†°	σ,°
I	70	74	57	35
II/III	43	46	43	32
I/II/III trans	50	52	ND‡	ND‡
I/II/III cis	90	93	77	38

*Values represent means of triplicate determinations using the algorithms of Kerppola and Curran (ref. 33, left column) or of Ferrari *et al.* (ref. 34, right column). Errors are 1–5°.

†Mean values of bending angles, α, and SD, σ, were obtained from the folded Gaussian fit to the experimental data according to Cherny *et al.* (26). Numbers of complexes scored: 913 (I), 621 (II/III), and 504 (I/II/III cis). Errors for mean bend angles and SD are 2–4°.

‡ND, not determined.

DNA Minicircles. It is well known that the rate of ligase-mediated cyclization of "straight," linear DNA fragments into circular products decreases dramatically below 300 bp, reaching essentially zero somewhere between 126 and 200 bp, i.e., in the size

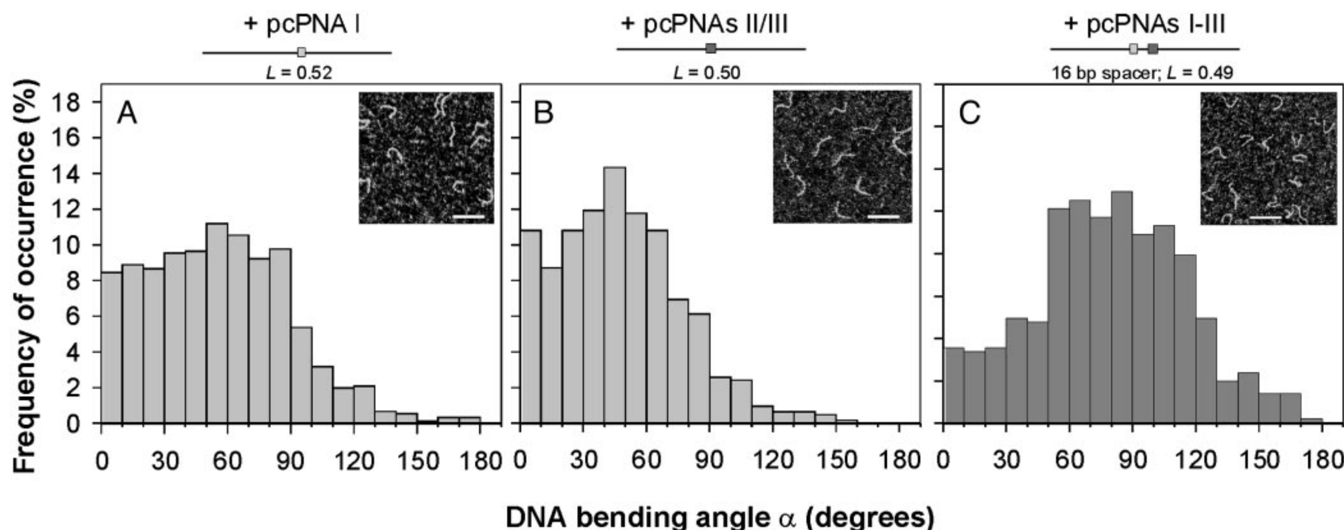


Fig. 4. Histograms showing frequencies of bending angles measured for DNA fragments 251 bp in length bound centrally by pcPNAs. Shown are results of binding to one target site (*A* and *B*) and binding to both target sites (*C*). Calculated mean values of bending angles and SD are given in Table 1. (*Insets*) Exemplary EM images of the pcPNA–DNA complexes. (Scale bars, 100 nm.)

range of the persistence length of duplex DNA (44, 45). Thus, to date only two methods exist where DNA minicircles below 200 bp can be obtained to a significant extent: (*i*) ligation of fragments containing multiple phased intrinsic bends such as A-tracts and (*ii*) ligation of fragments associated to a DNA-binding protein that induces strong DNA bending. Hence, the introduction of a strong bend in duplex DNA by small, synthetic ligands such as pcPNAs would present a new method for the ring closure of short duplex DNA fragments, which could be performed sequence-specifically on any DNA fragment.

To test this possibility, we designed a DNA fragment of 85-bp monomer length that carries two in-phase bends with an overall average value of 80–90° upon binding of pcPNAs I–III, and could be head-to-tail ligated due to complementary 5′-protruding termini (Fig. 5*A*). The choice of the fragment size was based on reported ring closure data of DNA duplexes containing phased A-tracts. With DNA fragments of 21-bp monomer length that contain two phased A-tracts a relatively narrow distribution of circle sizes in the range of 105–210 bp has been observed (46–48). Because those monomers have an intrinsic bend of ≈28–36°, the maximum circularization efficiency of such constructs at 147 bp corresponds to an overall bend angle of 200–250°. Therefore, we expected that the ligation reaction with the 85-bp monomer DNA fragment bound to pcPNAs I–III would lead to efficient cyclization of its dimer to 170-bp DNA minicircles, whereas the yield of circles originating from ligation of the monomer or of multimers with three or more subunits should be very limited.

The formation of DNA minicircles as major products of ligation with the DNA monomer complexed with pcPNAs was first verified by means of analysis of samples by nondenaturing PAGE before and after exonuclease treatment, as shown in Fig. 5*B*.** Because sizes of circular DNA cannot be directly established from gel electrophoresis, the assignment of specific bands in electrophoregrams as 170- or 255-bp DNA circles was based on visualization and analysis of samples by EM (Fig. 6).

The gel electrophoretic mobility of the major ligation products obtained with the pcPNA–DNA complexes were strongly dependent on the percentage of the used polyacrylamide gel, indicating already the presence of circular DNA before analysis of exonuclease-purified samples was performed. For instance, in

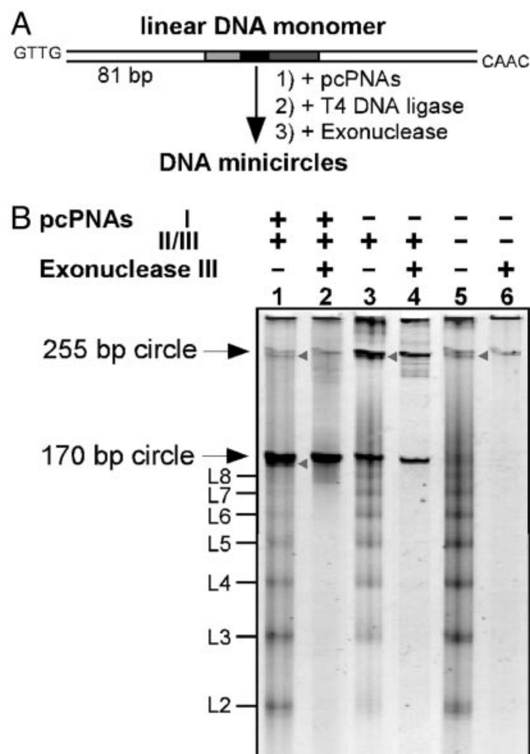


Fig. 5. Formation of DNA minicircles by means of pcPNA-induced DNA bending. (*A*) Schematics of the DNA monomer construct and outline of the experimental procedure. Binding sites for pcPNAs (gray areas) are separated by 5 bp. (*B*) Analysis of ligation products by 6% nondenaturing PAGE. Note that after ligation, the samples were heated to irreversibly dissociate pcPNAs. Black arrows show intact DNA minicircles, whereas gray arrowheads show nicked DNA minicircles. Linear DNA ligamers are assigned in the range of L2 (dimer) to L8 (octamer).

**Note that pcPNAs remained stably bound to DNA under the conditions employed for ligation, thereby causing different gel retardation effects with each ligamer (data not shown), so that they had to be irreversibly dissociated from their targets before analysis of ligation products could be performed.

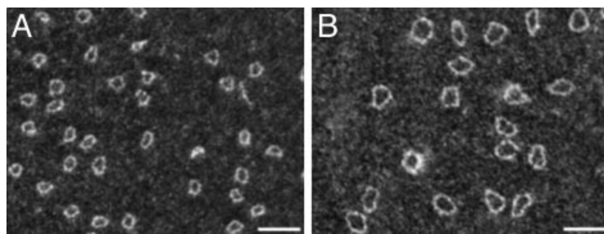


Fig. 6. EM images of DNA minicircles obtained from pcPNA–DNA complexes after ligation and purification. Shown are 170-bp (*A*) and 255-bp (*B*) DNA circles. (Scale bars, 50 nm.)

a 6% polyacrylamide gel, the major ligation product (60%) of the monomer complex with pcPNAs I–III comigrated with a linear 850-bp DNA fragment, whereas its mobility was reduced to the one of a linear 1,500-bp DNA size marker in a 7% polyacrylamide gel. To prove that this product was comprised of an intact DNA minicircle, we treated it with exonuclease III. As shown in Fig. 5*B*, it was resistant to cleavage by exonuclease III, whereas all other products with the exception of one minor byproduct (identified by EM as trimer circles) were completely digested by this enzyme (compare lanes 1 and 2). Based on their specific gel electrophoretic mobilities, the cleaved byproducts were identified as linear ligamers L or nicked DNA circles, respectively. The exonuclease-treated sample was then further analyzed by EM. As expected, it consisted almost exclusively (>96%) of covalently closed dimer circles. The minute fraction of larger circles present in this sample was subsequently removed by gel filtration chromatography (Fig. 8*A*), thus yielding a homogeneous sample of intact 170-bp DNA circles (Fig. 6*A*).

As expected, moderate bending of the DNA monomer induced by binding of pcPNAs II and III led to a wide distribution of DNA minicircles upon ligation with circular dimers and trimers as main products (Fig. 5*B*, lanes 3 and 4). We were able to separate the mixture of DNA circles in the exonuclease-treated sample by gel filtration chromatography into fractions containing either cyclic multimers (≥4-mers), trimers, or dimers (Fig. 8*B*), enabling accurate quantitation of yields of the small DNA circles and in detail analysis of ligation products by EM. A representative EM image of the fraction comprised of trimer DNA circles is shown in Fig. 6*B*.

Control ligation reactions, performed in the absence of pcPNAs, led predominantly to linear multimers of the initial duplex (Fig. 5*B*, lane 5). The small quantity of DNA circles obtained after exonuclease III treatment of this sample contained mostly circles in the range between tri- and pentamers, as identified by EM (data not shown).

It should be noted that the formation of DNA minicircles competes with the formation of linear ligamers at the used input concentration of monomer in the ligation reactions (49). Thus, the obtained high yield (60%) of the 170-bp DNA minicircle for the monomer bound to pcPNAs I–III is quite remarkable and confirms the presence of highly bent DNA in this complex.

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