Electron microscopy mapping of oligopurine tracts in duplex DNA by peptide nucleic acid targeting

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Received September 24, 1994; Revised and Accepted November 9, 1994

ABSTRACT

Biotinylated homopyrimidine decamer peptide nucleic acids (PNAs) are shown to form sequence-specific and stable complexes with complementary oligopurine targets in linear double-stranded DNA. The non-covalent complexes are visualized by electron microscopy (EM) without chemical fixation using streptavidin as an EM marker. The triplex stoichiometry of the PNA-DNA complexes (two PNA molecules presumably binding by Watson–Crick and Hoogsteen pairing with one of the strands of the duplex DNA) is indicated by the appearance of two streptavidin ‘beads’ per target site in some micrographs, and is also supported by the formation of two retardation bands in a gel shift assay. Quantitative analysis of the positions of the streptavidin ‘beads’ revealed that under optimized conditions PNA–DNA complexes are preferably formed with the fully complementary target. An increase in either the PNA concentration or the incubation time leads to binding at sites containing one or two mismatches. Our results demonstrate that biotinylated PNAs can be used for EM mapping of short targets in duplex DNA.

INTRODUCTION

Recently, new approaches have been developed for physical mapping of specific sequences of different length in double-stranded DNA by electron microscopy (EM). It was demonstrated that a methyltransferase tetranucleotide recognition sequence could be mapped with the enzyme per se as an EM marker (1). 12–17 bp long homopurine/homopyrimidine tracts in DNA can be detected with biotinylated purine or pyrimidine deoxyligoids by means of triplex formation using streptavidin as an EM marker (2). Accurate EM mapping of long DNA sequences (hundreds of bp) is possible by homologous DNA targeting with RecA-coated single-stranded DNA probes (3). A novel DNA targeting reagent, peptide nucleic acid (PNA), a DNA analogue in which the nucleobases are attached via a linker to an N-(2-aminoethyl)glycine backbone, has been demonstrated to bind strongly and sequence-specifically to double-stranded DNA (4–10). It was shown that the complexes formed between PNA H-T10-LysNH2 molecules and an extended double-stranded DNA insert, A98/T98, could be observed directly by electron microscopy as an ‘eye’ structure (6). In the present study we examine the interaction of biotinylated decaprimidine PNAs with linear duplex DNA and show that these PNA probes exhibit sequence-specific binding and form stable complexes with their complementary decapurine DNA targets. These complexes can be unambiguously visualized with streptavidin as an EM marker and used for physical mapping of DNA molecules.

MATERIALS AND METHODS

PNA H-T10-LysNH2 (abbreviated to PNA-T10) was synthesized as described by Egholm et al. (12,13). The biotinylated derivatives, bio-PNA-T10 and bio-PNA-T2CT2CT4 (Figure 1) were prepared by active ester coupling: 1 mg PNA (H-T10-LysNH2 or H2N-(CH2)5-CO-T5CT5CT5CT5-NH2) was dissolved in 4 ml of a 1:1:2 mixture of 0.2 M NaH2CO3/0.2 M Na2HCO3/CH3CN. Subsequently 40 μl of a solution of biotin-N-hydroxy-succinimide ester (20 mg/ml in dimethyl formamide)

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was added and the mixture was incubated at room temperature until complete conversion of the PNA (the reaction was followed by HPLC). The PNAs were purified by reversed-phase HPLC on a C18 column using a 40 min linear 0-40% acetonitrile gradient in 0.1% trifluoroacetic acid/water. Verification of the chemical compositions of the bio-PNAs was done by fast atom bombardment mass spectrometry (MS:FAB*).

Plasmids containing the target sequences were obtained by cloning of the appropriate oligonucleotide into the vector pUC19 plasmid as described by Nielsen et al. (7): for bio-PNA-T₁₀ the target was cloned into the BamHI site (pT10 plasmid), for bio-PNA-T₂CT₂CT₄ the target was cloned into the PstI site (pA8G2 plasmid).

Electron microscopy experiments were performed with plasmids linearized with restriction enzyme Scal. Biotinylated complexes were prepared by preincubation of plasmid DNA with bio-PNAs at 37°C for the desired time in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5). After formation of the complex, the mixture was passed through a Sephacryl S-200 column equilibrated with 10 mM Tris–HCl, 50 mM NaCl, pH 7.5, to remove non-bound PNA molecules. After addition of streptavidin to a final concentration of 10 µg/ml and 5 min incubation at room temperature, the gel filtration step was repeated. Then the mixture was diluted to a final DNA concentration of 1 µg/ml and adsorbed to carbon films activated by glow discharge in tripropylamine vapours according to Dubochet et al. (14). The samples were stained with a 0.5–1% aqueous solution of uranyl acetate and shadowed with Pt/C (95/5). Micrographs were digitized and analyzed with the aid of an

![Image](image-url)

**Figure 1.** Chemical structures of the biotinylated PNAs. Since PNA H-T₁₀-LysNH₂ contains two primary amino groups which both react with the biotinylated active ester, two mono-biotinylated and one bis-biotinylated product are possible. Two products were observed: a mono- and a bis-biotinylated PNA. It is our experience that the amino group of the lysine is more reactive than the intrinsic N-terminal amino group of the PNA, so we have purified and assigned the mono-biotinylated PNA-T₁₀ to the structure shown. The PNAs showed the expected molecular weights by fast atom bombardment mass spectrometry and their purity was >90% as analyzed by reversed-phase high performance liquid chromatography.
RESULTS AND DISCUSSION

It has been established that 10mer PNA molecules bind sequence-specifically to the target sites in duplex DNA by formation of a stable triplex comprising two PNA molecules and the complementary DNA strand of the target, while the other DNA strand is displaced (4,6-11). To detect the complexes formed by biotinylated PNA (Figure 1) with the 10mer DNA targets we used the EM technique and applied an approach previously used for EM detection of intermolecular oligonucleotide–DNA triplexes using biotinylated probes and streptavidin as an EM marker (2).

We first checked the binding of bio-PNAs to duplex DNA and the possibility of detecting the complexes with streptavidin using a gel retardation assay. These experiments clearly showed that binding of PNA-T₁₀ alone leads to a noticeable retardation of a labeled DNA fragment containing the target sequence (7,15) (Figure 2, lanes 1 and 2). Incubation with bio-PNA-T₁₀ also leads to retardation, but with somewhat decreased efficiency of the binding (Figure 2, lane 4). Inclusion of streptavidin leads to a pronounced further retardation of the band corresponding to the bio-PNA-DNA complex (Figure 2, lane 5), while the complex with the non-biotinylated PNA is not affected by the presence of streptavidin (Figure 2, lane 3). These results, and analogous data for bio-PNA-T₂C₃T₄C₅–plasmid pA8G2 complexes (not shown), clearly indicate that the interaction between duplex DNA and bio-PNAs results in stable complexes, which could be detected via streptavidin coupling. Note also the appearance of two streptavidin retarded bands. This probably reflects the stoichiometry of the (bio-PNA)₂–DNA complexes to which two streptavidin molecules per target site may bind.
Complexes involving two streptavidin molecules were also detected by EM (see below).

Electron microscopy was employed to visualize the bio-PNA–DNA complexes using streptavidin as an EM marker. Figure 3A shows a micrograph of Scal-linearized DNA–bio-PNA-T10–streptavidin complexes. The computer-assisted analysis of the micrographs was done as described by Kurakin et al. (1). It is observed that the streptavidin ‘beads’ are clearly located at the oligoadenine tract targeted by bio-PNA-T10. The major peak on the resulting histogram (Figure 3B) is positioned 924 bp from the closest end of the pT10/Scal plasmid and coincides, within experimental error (~20 bp), with the position of the A10/T10 insert (932–941 bp). The position of a small additional peak near 1950 bp correlates with an intrinsic target site of pUC19 (T7-GT7/A3-CA7, positioned at 1932–1942 bp) which contains a mismatch. This peak becomes more pronounced upon longer incubation. Increasing the PNA concentration (from 10 to 30 μM) yielded several other PNA–DNA complexes correlating with intrinsic pUC19 sites having two mismatches (data not shown).

Similar results were obtained with bio-PNA-T2CT2CT4 targeted to the pA8G2 plasmid. Nielsen et al. (11) have shown that PNA-T2CT2CT4 binds strongly to its target on the pA8G2 plasmid by strand displacement via PNA-DNA triplex formation. The complex is stabilized by lowering the pH of the medium. We believe that a similar complex is formed by bio-PNA-T2CT2CT4. To facilitate complex formation, incubation of DNA with this bio-PNA was performed at pH 5.0, as is usually done for cytosine-containing pyrimidine oligonucleotides which form triplexes involving protonated cytosines in the Hoogsteen strand (2). It was found that the bio-PNA-T2CT2CT4–DNA complexes did not dissociate upon shifting pH from 5.0 at the incubation step to 7.5 at the step of streptavidin coupling and EM analysis. This is important for EM mapping of long genomic DNAs. In acidic solution, these usually give poor micrographs with tangled DNA balls, instead of extended DNA threads as seen at neutral pH. Figure 4 shows the micrograph of these complexes and the histogram of the distribution of streptavidin ‘beads’ along DNA molecules. The major peak in the histogram positioned at 934 bp (Figure 4B) coincides within experimental error (~ 30 bp) with the position (950–959 bp) of the PNA-targeted oligopurine tract. Minor PNA binding sites, which manifest themselves in the form of the two small peaks on the histogram, are ascribed to intrinsic pUC19 targets with one (A7-G7AGA7/GT7CTC7/GT7, site at 724–733 bp) or two (three neighboring sites at 1932–1941, 1965–1974 and 1990–1999 bp) mismatches.

It is interesting that careful inspection of the micrographs revealed the appearance of double streptavidin ‘beads’ in some molecules (Figure 5). Such structures require the proper orientation and shadowing for their EM visualization and were most frequently observed when the bio-PNA–streptavidin complex was formed prior to binding to the DNA. We believe that this directly reflects the stoichiometry of the bio-PNA–DNA complexes involving two PNA molecules, presumably binding by Watson–Crick and Hoogsteen base pairing with the complementary strand of the DNA duplex. This stoichiometry is consistent with an observed quadratic dependence on the PNA
concentration of the rate of PNA–DNA complex formation (16) and the triplex (PNA)$_2$–DNA structure indicated by DMS probing (11).

We have shown that binding of PNA to double-stranded DNA takes place by strand displacement (4,6,9,11). The permanganate probing experiments presented in Figure 6 show that this is also the case for the bio-PNA (although with somewhat decreased binding affinity, which is primarily due to the difference in charge between the two PNAs), as evidenced by the pronounced increase in sensitivity to permanganate oxidation of the thymines within the PNA target. The sequence specificity of the interaction of bio-PNA-T$_{10}$ with DNA by strand displacement was further examined by S$_1$ probing (9). Incubation of bio-PNA-T$_{10}$ with plasmid pT10 linearized with Scal and subsequent digestion with S$_1$ endonuclease should yield two fragments of the plasmid if the bio-PNA bound specifically at the complementary target at the BamHI site. The results presented in Figure 7 shows that this is the case. When the DNA was preincubated in TE buffer for 5 min with 10 µM PNA, two distinct bands of the expected sizes were seen in the gel (Figure 7, lane 4; compare with lane 7 of Scal/BamHI double digest of the pT10 plasmid). Thus, following this protocol we target biotinylated PNA strictly to the specific site. Longer incubation time (lanes 5 and 6) or higher PNA concentration (lanes 8 and 9) led to additional bands. We attribute these bands as originating from complexes formed between PNA and intrinsic plasmid sequences with one or more mismatches, which show lower affinity as compared with the fully matched site. Similar sequence discrimination was also observed for bio-PNA-T$_3$CT$_2$CT$_4$ binding to plasmid pA8G2 (data not shown).

CONCLUSION

Our results show that biotinylated PNAs may be used as sequence-specific markers for electron microscopy location of short oligopurine tracts in DNA. This could be useful for physical mapping of DNA. A high efficiency of detection of the target sequences can be reached: the occupancy of the true target sites for both the bio-PNAs studied is between 50 and 90% without compromising the sequence specificity. By obtaining a set of histograms at different incubation times or PNA:DNA ratios, it should be possible to map sites with one and two mismatches in addition to the fully matched ones. As compared to the EM mapping of DNA with RecA-coated single-stranded DNA probes (3) or with intermolecular oligonucleotide triplexes (2), the PNA–DNA complexes are perfectly stable to be used with shorter targets.

ACKNOWLEDGEMENTS

This work was supported by ISIS Pharmaceuticals as well as by grants M41000 from the International Science Foundation and 94-04-11424 from the Russian Foundation for Fundamental Research.

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