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Peptide nucleic acid (PNA) binding-mediated gene regulation

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ABSTRACT

Peptide nucleic acids (PNAs) are synthetic oligonucleotides with chemically modified backbones. PNAs can bind to both DNA and RNA targets in a sequence-specific manner to form PNA/DNA and PNA/RNA duplex structures. When bound to double-stranded DNA (dsDNA) targets, the PNA molecule replaces one DNA strand in the duplex by strand invasion to form a PNA/DNA/PNA [or $(PNA)_2$/DNA] triplex structure and the displaced DNA strand exists as a single-stranded D-loop. PNA has been used in many studies as research tools for gene regulation and gene targeting. The D-loops generated from the PNA binding have also been demonstrated for its potential in initiating transcription and inducing gene expression. PNA provides a powerful tool to study the mechanism of transcription and an innovative strategy to regulate target gene expression. An understanding of the PNA-mediated gene regulation will have important clinical implications in treatment of many human diseases including genetic, cancerous, and age-related diseases.

Keywords: peptide nucleic acids (PNAs), PNA binding, single-stranded D-loop, transcription initiation.

Introduction

Peptide nucleic acids (PNAs) are synthetic oligonucleotides with modified backbones[1]. In PNAs, the sugar backbone is replaced with peptide-like backbones (Fig 1). PNAs can bind to both DNA and RNA targets in a sequence-specific manner to form a Watson-Crick type PNA/DNA and a PNA/RNA double helical structure. PNAs can also bind to double-stranded DNA (dsDNA) targets. In this case, the PNA molecule replaces one of the complimentary DNA strands by strand invasion and the displaced DNA strand then exists as a single-stranded D-loop at the PNA binding site[1-6]. When PNAs bind to homopurine/homopyrimidine sequences, the PNA/DNA/PNA [or $(PNA)_2$/DNA] triple helix structures can be formed (Fig 2A). Studies suggest that the first PNA molecule binds with the DNA strand to form a Watson-Crick type duplex structure and the second PNA molecule binds to the DNA strand of the PNA/DNA duplex by Hoogsteen hydrogen bonds [7, 8] to form the $(PNA)_2$/DNA triplex structure[1, 3, 9-11]. Most of the triplexes formed by PNAs are pyrimidine-motif triplex structures in which the pyrimidine-containing PNA molecule binds parallel to the purine-containing DNA strand of the PNA/DNA duplex to form the $(PNA)_2$/DNA triplex structure[1, 9, 12, 13]. The $(PNA)_2$/DNA triplex structure stabilizes both the bound PNAs and the single-stranded D-loop at the binding site[14].

PNAs have been used in a variety of research applications. One of the most common applications of the PNA technology is its antisense application in which the PNAs are designed to bind to messenger RNA (mRNA) targets to inhibit translation of the target genes[15-27]. PNAs are also used as probes for gene cloning and mutation detection[17, 28-30]. PNAs designed to bind to specific target gene sequences have been used in homologous recombination studies[31, 32]. In addition, PNAs designed as transcription factor decoy molecules have also been used for target gene induction[33].

The D-loops generated by PNA binding to dsDNA targets have been used to induce transcription and gene expression (Fig 2B). Early studies revealed that the PNA binding-generated D-loops could initiate transcriptions *in vitro* using T7 RNA polymerase[34]. The work of our laboratory demonstrated that the PNA binding-generated D-loops could initiate transcription in a HeLa nuclear extract *in vitro* transcription system and induce a GFP reporter gene expression in the CV-1 monkey kidney cells[35]. Most importantly, when PNAs designed to bind to the human γ-globin gene 5' untranslated region (5' UTR) sequence were used to treat the K562 human erythroleukemia cells, transcription of the endogenous γ-globin gene from the PNA binding sites was achieved[35]. These results suggest that PNA binding-mediated transcription may provide an innovative strategy to induce expression of specific target genes. It may also provide a novel approach to study gene modu-

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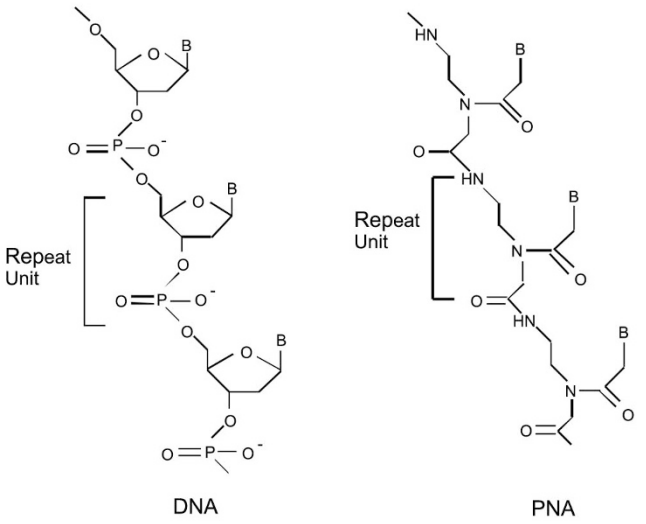

Fig 1. Structures of DNA and PNA oligonucleotides.

lating, transcription, and transcription regulation. Although the molecular mechanism of PNA binding-mediated transcription is relatively unclear, the results obtained from our recent studies provide some valuable information regarding the molecular basis of this process. We will discuss some of the progress that have been made in the PNA studies in the past few years and outline some of the difficulties that need to be overcome to bring the field forward.

PNA binding

PNAs can bind to both DNA and RNA targets in a sequence-specific manner to form duplex structures. Although the Watson-Crick duplex formation (anti-parallel duplex formation) is the preferred formation for the PNA bindings, parallel duplex structure can still be formed when PNAs bind to the target sequences[36].

The binding of PNAs to DNA and RNA targets is stronger than that of DNA/DNA or RNA/RNA bindings[1, 37]. This enhanced binding affinity is partially due to the uncharged property of the PNAs. Since PNAs are neutral in charge, the duplexes formed by PNA/DNA or PNA/RNA hybrids lack the electrostatic repulsion formed by DNA/DNA or RNA/DNA duplexes, resulting in a very stable duplex formation even at a relatively high temperature and a very high binding affinity. For example, the melting temperature (T_m) of a normal dA_{10}-dT_{10} DNA hybrid is 23ºC while the T_m of a similar dA_{10}-dT_{10} DNA/PNA hybrid is 86ºC[1].

As a result of this high binding affinity, the binding specificity of PNAs, in general, is much higher than that of DNA/DNA or RNA/RNA bindings. Compared with the

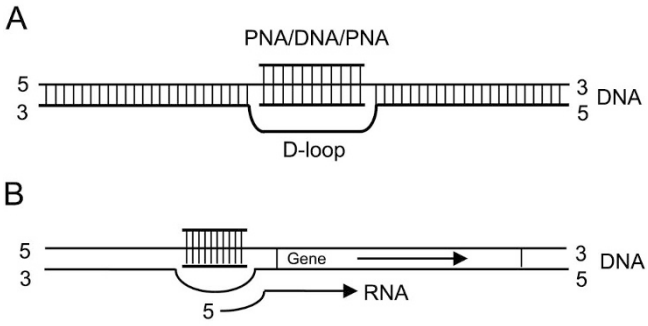

Fig 2. PNA binding-generated $(PNA)_2$/DNA triplex and single-stranded D-loop structure (**A**) and the transcription initiated from the D-loop site (**B**).

DNA/DNA or RNA/RNA bindings, any mismatches contained at the PNA binding sites will have greater impact on the stability of the duplexes formed by PNA/DNA and PNA/RNA hybrids[1, 38-42]. For example, binding kinetics studies reveal that the binding affinity of the T_5CT_4 PNA to the dA_{10} target is 100 times lower than that of the T_{10} PNA binding to the same target[42]. For this reason, PNAs are often used as probes for identifying desired target gene sequences and for detecting single base mutations with high accuracy[17, 28-30, 43]. The lengths of PNAs used in the hybridization studies are also relatively shorter than the normal DNA or RNA probes because of this high binding affinity. In most of the hybridization studies, PNAs with lengths of 8-12 mer are sufficient to form strong duplex formation and to distinguish single base mutations [17, 28-30]. When long PNA molecules are used as probes in the hybridization, however, reduced specificities may result since the stronger binding affinity between the PNAs and the homologous non-target sequences may lead to an increased false positive identification rate for the desired target sequences.

The effects of salts on PNA binding

Although the binding of PNA with DNA and RNA is stronger, this binding process of PNAs to dsDNA targets can be influenced by the presence of salts. This is because the binding of PNAs to dsDNA targets needs to replace one of the complimentary DNA strands in the duplex and the presence of positively charged ions, especially at high concentrations, can inhibit the strand invasion process by preventing the interaction of PNA molecule with the DNA targets, resulting in ineffective binding[6]. In comparison, the binding of PNAs to single-stranded targets such as mRNA or single-stranded DNA targets is not affected by the presence of salts. For example, in our previous studies the presence of 150 mM KCl, which is relevant to the physiological K^+ concentration, significantly inhibited the

binding of PNA-1 to the target[35].

To enhance the binding ability of PNAs to the dsDNA targets in the presence of salts, several strategies have been explored. Incorporation of positively charged groups such as lysine and arginine into the PNA molecules has been demonstrated to lead to an enhanced PNA binding in the presence of high concentrations of salts[10, 34, 44, 45]. Conjugating a positively charged peptide has also been determined to lead to an increase in PNA binding, especially the PNA binding to dsDNA targets[22]. This is likely due to the positive charges contained in the PNA that help the PNA molecule compete with other positively charged ions for an interaction with the negatively charged DNA targets, resulting in enhanced PNA bindings in the presence of salts. Increasing target gene transcription has also lead to an increase in the PNA binding efficiency in the presence of high concentrations of salts[46]. The sequence context of the PNA binding targets may also contribute to the PNA binding inhibition in the presence of high concentrations of salts. In our earlier studies, we found that two different PNAs of slightly different sequences but targeting to the regions of the genome separated by only 6bp had quite different salt dependence[35]. Therefore, if the salt inhibition effect is observed in the chosen PNA targets, changing PNA binding sites to other positions may help overcome this inhibition effect.

The stability of PNA

In comparison to normal DNA and RNA oligonucleotides, PNAs are much more stable inside cells[47]. PNAs are very resistance to nuclease digestion since the nucleases cannot digest their altered backbones[48]. PNAs are also very resistant to protease digestion. Regular DNA and RNA oligonucleotides are very sensitive to the nuclease digestion. Inside cells, for example, the half-life for most of the unmodified DNA and RNA oligonucleotides is approximately 15 min or shorter. In contrast, PNAs are very stable inside cells. Studies indicate that PNAs are stable inside cells for at least 48 h[49]. This extreme stability makes PNAs an ideal candidate for the antisense and antigene application.

PNA delivery

Although PNAs have many advantages in its application, some disadvantages have limited its application. One of the most important issues in the PNA application is its delivery efficiency. Unlike most of the DNA and RNA oligonucleotides, which can be easily delivered into cells through endocytosis, PNAs are poorly penetrated through the cell membrane[9, 50]. This is partially due to its uncharged property. Since the cell membrane carries negative charges, positively charged molecules such as normal DNA and RNA oligonucleotides can be attracted to the cell membrane, resulting in penetration of the oligonucleotides through the cell membrane by endocytosis. In contrast, uncharged PNA molecules cannot be attracted to the membrane, resulting in poor penetration through the membrane by this endocytotic process.

To enhance the efficiency in PNA delivery, many strategies have been explored. Since a positive charge will enhance the attraction of molecules to cell membrane, some studies have been attempted by incorporating positively charged residues such as lysine and arginine to the PNA molecules to enhance the PNA delivery efficiency[51]. Studies have also been done using ligands to enhance the attachment of PNAs to the cell membrane[15, 16, 18 ,20, 21, 52-59]. For example, PNAs have been conjugated with short peptide sequences, to enhance the PNA delivery efficiency[15, 53]. PNAs conjugated with other ligands such as antibodies or steroids have also been used to increase the PNA delivery efficiency[16, 52, 54, 60-64].

PNA binding-induced transcription

The possibility of using the PNA binding-generated D-loops to induce gene-specific transcription and gene expression has been studied both *in vitro* and *in vivo*[34, 35, 65]. The work reported by Mollegard et al indicated that a PNA T_{10} binding-generated D-loop was able to induce transcription from the D-loop site in both a T7 RNA polymerase -based *in vitro* transcription system and a rat nuclear extract *in vitro* transcription system[34]. In that work, the transcription activity of the T_{10} PNA-generated D-loop was determined by comparison with the transcription activity initiated from an *E. coli* lacUV5 promoter. The *in vitro* transcription results indicated that the transcription activity of the T_{10} PNA-generated D-loop is comparable to that of the lacUV5 promoter[34].

In our previous studies, we determined the transcription initiated from PNA binding-generated D-loops using two PNAs designed to bind to the human γ-globin gene −280 region, one with a 10-base target sequence and the second with a 12-base target sequence. The results obtained from our *in vitro* transcription studies indicated that both PNAs can induce transcription in a HeLa nuclear extract *in vitro* transcription system; the D-loop generated by the 12-mer PNA showed a much stronger transcription activity than that by the 10-mer PNA[35]. We also studied the abilities of these PNAs to induce gene expression using a plasmid construct that carries both PNA binding site sequences with a promoterless green fluorescent protein (GFP) gene[35]. When the PNA-bound plasmid was transfected into the CV-1 monkey kidney cells by microinjection, expression of the GFP protein was detected in

the transfected cells[35]. This result suggests that the PNA binding-generated D-loops could not only induce transcription *in vitro* but also induce expression of target gene *in vivo*, revealing a great potential of PNAs in inducing gene-specific expression in living cells.

The possibility of utilizing PNAs to induce expression of endogenous genes was also investigated in our previous studies using the PNAs designed to bind to the human γ-globin gene 5' UTR sequence[35]. When the K562 human erythroleukemia cells were treated with the PNAs for two days, specific transcripts initiated from the PNA binding sites were detected from the treated K562 cells; however, these transcripts were not detected in the untreated K562 cells. Interestingly, the results obtained from our studies indicate that the PNA treatment also enhances the transcription of the endogenous γ-globin gene from its natural promoter, suggesting that a transcription event occurring at the nearby region might result in an altered chromatin structure, leading to an increased transcription activity from its promoter. These results suggest that the PNA binding-generated D-loops could indeed provide an innovative strategy in inducing desired target gene expression, indicating the important clinical potentials of PNAs in inducing expression of some therapeutic-important genes.

The PNA length requirement for inducing transcription from the PNA binding sites

The results of our studies and the data published by others suggest that the D-loops generated by the PNA bindings could be used as artificial promoters to initiate transcription[34, 35, 66]. The lengths of the PNA required for the PNA binding-mediated transcription, however, were not determined in these studies. To determine the optimal PNA length requirement for inducing transcription from the PNA binding sites, we have designed and synthesized a series of PNAs (8 mer-20 mer) that bind to a 20 bp homopurine/homopyrimidine site in a GFP reporter gene-containing plasmid construct. The transcription activity initiated from the PNA binding-generated D-loops was then determined[65]. The results obtained from our *in vitro* transcription assay indicated that the transcription activity was detected with PNAs of 14 mer to 20 mer in length. The highest transcription activity, however, was detected when the PNAs of 16 mer and 18 mer PNA were pre-incubated with the plasmid DNA. To determine the PNA length requirement for inducing gene expression *in vivo*, various lengths of the PNA-bound plasmid DNA were transfected into human normal fibroblast (NF) cells. The GFP gene expression was then monitored with results indicating high levels of GFP expression by the 16 mer and 18 mer-PNA bound plasmid DNA. These results suggest that PNAs with lengths of 16-18 mer are more effective in inducing transcription and target gene expression.

The basal transcription components involved in the PNA binding-induced transcription

Although PNA binding-generated D-loops have been demonstrated for their abilities in inducing transcriptions [34, 35, 65], the molecular mechanism for PNA binding-induced transcription has not been established. Detection of PNA binding-induced GFP expression in mammalian cells suggests that a class II nuclear gene transcription may be involved in the PNA binding-induced transcription. To define the transcription factors involved in the PNA binding-mediated transcription, we have recently studied the PNA binding-mediated transcription *in vitro* using a depleted HeLa nuclear extract system (Wang, unpublished data). The basal transcription factors, including TFIIB, TFIID, TFIIE, and TFIIH, and the RNA polymerase II, were individually depleted in the HeLa nuclear extract by immuno-precipitation. The PNA binding-mediated transcription was then studied in both the immuno-depleted HeLa nuclear extracts and the extracts supplemented with purified individual basal transcription factors. The results obtained from our *in vitro* transcription experiments indicate that the basal transcription factors TFIID and TFIIH and the RNA polymerase II are essential for the PNA binding-induced transcription. The basal transcription factors TFIIB and TFIIE, however, are not essential for the PNA binding-mediated transcription. This result suggests that the class II gene transcription is indeed involved in the PNA binding-induced transcription process. Since the PNA binding-generated D-loops are structurally different from the class II gene promoters, it remains unclear as to how the basal transcription factors, such as the TFIID, can recognize the PNA binding-generated D-loops and initiate the transcription from the D-loop sites. Further studies are needed to determine the interactions between the PNA binding-generated D-loops and the basal transcription factors and the roles of these interactions in the initiation of transcription.

Conclusion

PNA binding-mediated transcription provides an innovative strategy to induce gene expression. This strategy will have both scientific importance and clinical relevance. The PNA binding-mediated transcription provides a powerful method to study the molecular mechanism of transcription initiation and gene regulation. The PNA binding mediated gene expression will also have important clinical implications in treatment of many human disease such as cancer and genetic diseases. However, some difficulty

exists in the technology that has limited its applications. The molecular mechanism of PNA binding-induced transcription needs to be established to provide a better understanding for the molecular basis of this important approach. Once these issues are resolved, the vast potential of this novel technology may be realized in the treatment of many human diseases and possible other applications.

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