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Peptide nucleic acid (PNA) binding-mediated induction of human γ-globin gene expression

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ABSTRACT

Peptide nucleic acids (PNAs) can bind to homopurine/homopyrimidine sequences of double-stranded DNA targets in a sequence-specific manner and form $[PNA]_2$/DNA triplexes with single-stranded DNA D-loop structures at the PNA binding sites. These D-loop structures have been found to have a capacity to initiate transcription *in vitro*. If this strategy can be used to induce transcription of endogenous genes, it may provide a novel approach for gene therapy of many human diseases. Human β globin disorders such as sickle cell anemia and β-thalassemia are very common genetic diseases that are caused by mutations in the β-globin gene. When γ-globin genes are highly expressed in sickle cell patients, the presence of high levels of fetal hemoglobin (HbF, α2γ2) can compensate for the defective β-globin gene product and such patients have much improved symptoms or are free of disease. However, the γ-globin genes are developmentally regulated and normally expressed at very low levels (>1%) in adult blood cells. We have investigated the possibility of inducing γ-globin gene expression with PNAs. Using PNAs designed to bind to the 5' flanking region of the γ-globin gene, induction of expression of a reporter gene construct was demonstrated both *in vitro* and *in vivo*. More importantly, PNA-mediated induction of endogenous γ-globin gene expression was also demonstrated in K562 human erythroleukemia cells. This result suggests that induction of γ-globin gene expression with PNAs might provide a new approach for the treatment of sickle cell disease. PNA-induced gene expression strategy also may have implications in gene therapy of other diseases such as genetic diseases, cancer and infectious diseases.

INTRODUCTION

Peptide nucleic acids (PNAs) are synthetic oligonucleotides with modified polyamide backbones that bind sequence-specifically to single- and double-stranded DNA targets and form Watson–Crick double helix structures (1). When bound to double-stranded DNA targets, the PNA molecule replaces one DNA strand by strand displacement to form a PNA/DNA duplex structure, and the displaced DNA strand forms a D-loop structure at the PNA binding site. A $[PNA]_2$/DNA triplex structure can be formed when PNAs bind to a homopurine/homopyrimidine DNA target (2–6). The D-loops generated by PNA triplex structures are very stable since the triplex formation at the PNA binding sites stabilizes the bound PNAs. Interestingly, the D-loop structures have been found to be able to initiate gene transcription *in vitro* (7). This result and other *in vitro* gene transcription studies (8,9) suggest that it might be possible to use the PNA strategy to induce gene-specific expression. However, no *in vivo* studies have been completed to test this hypothesis.

Human β-globin disorders such as sickle cell anemia and β-thalassemia are very common genetic diseases. More than one million people worldwide are affected with the disease. The molecular basis of the disease is well characterized: an A to T point mutation in the sixth codon of the β-globin gene leads to a replacement of glutamic acid with valine and results in sickle cell anemia; deletions in the β-globin gene lead to imbalanced synthesis of the α-chain and β-chain of hemoglobin in erythrocytes and cause β-thalassemia. Interestingly, when γ-globin genes are highly expressed in sickle cell patients, the presence of high levels of fetal hemoglobin (HbF, α2γ2) in erythrocytes (~20–30% total hemoglobin) can compensate for the defective β-globin and such patients have much improved symptoms or are free of disease (10–12). Unfortunately, the γ-globin genes are developmentally regulated and normally expressed at high levels only during the fetal stage of human development (from 8 weeks in the embryo to birth). In adults, the β-globin gene is predominantly expressed (98%) while the γ-globin gene is expressed at very low levels (>1%).

To increase the levels of HbF in sickle cell anemia patients, many drugs have been developed. Butyric acid and its analogs have been found to increase the levels of HbF (13,14). Hydroxyurea, another drug that has been used in the treatment of sickle cell disease, has also been found to increase HbF levels in patients (15–17). However, many patients could not achieve increased HbF with this treatment. With hydroxyurea treatment, for example, only ~60% of patients were found to have

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increased HbF in their erythrocytes (18). Therefore, a novel approach to activate γ-globin gene expression would be beneficial.

We have investigated the possibility of inducing γ-globin gene expression with PNAs. Using PNAs designed to bind to a homopurine/homopyrimidine sequence of γ-globin gene –280 region, we demonstrate that binding of the PNAs can induce expression of a reporter gene in a reporter gene construct both *in vitro* and *in vivo*. More importantly, induction of endogenous γ-globin gene expression was also achieved when K562 human erythroleukemia cells were treated with PNAs. The strategy of inducing endogenous γ-globin gene expression with PNAs may provide a novel approach for gene therapy of sickle cell diseases. This strategy may also have implications in gene therapy of other genetic diseases, cancer and infectious diseases. PNA-induced gene expression may provide a new approach for studying gene transcription and regulation.

A. Sequence of the Gγ-globin gene -300 region.

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  -324                                                        -265
5'-CTTCTATGGTGGGAGAAGAAAACTAGCTAAAGGGAAGAATAAATTAGAGAAAAATTGGAA-3'
3'-GAAGATACCACCCTCTTCTTTTGATCGATTTCCCTTCTTATTTAATCTCTTTTTAACCTT-5'
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B. Sequences of PNA oligonucleotides

PNA-1 NH2-T CTTCCCTTT-OOO-*TTTJJJTTJT*-COOH

PNA-2 NH2-TTTTCTTCTCCC-OOO-*JJJTJTTJTTTT*-COOH

CP-1 NH2-TTCCTTCCTT-OOO-TTJJTTJJTT-COOH

C. Structure of pUSAG3

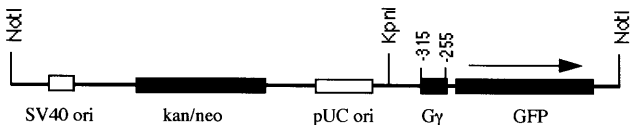

Figure 1. The sequences of the –300 region of Gγ-globin gene and the PNAs. (**A**) The DNA sequences around the PNA binding sites. The numbers correspond to the nucleotide positions in the human Gγ-globin gene. The underlined nucleotide sequences show the PNA binding sites. (**B**) The sequences of PNA oligonucleotides. Cytosines were replaced by pseudoisocytosines (J) for third strand binding; three linkers (O) were incorporated to link the DNA binding domain and the triplex binding domain. The italic sequences in the PNA oligonucleotides were designed for triplex binding.

MATERIALS AND METHODS

Cell lines, oligonucleotides and vectors

The CV1 monkey kidney cells were obtained from the American Type Culture Collection (ATCC CCL70, Rockville, MD) and were maintained in DMEM medium supplemented with 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD). The K562 human erythroleukemia cells were obtained from the American Type Culture Collection (ATCC CCL243, Rockville, MD) and were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum.

Oligonucleotides used in this study were synthesized by either the Keck Oligo Synthesis Laboratory at the Yale University School of Medicine or the Biopolymer Laboratory at the University of South Alabama College of Medicine. The PNAs were synthesized by PerSeptive Biosystems (Framingham, MA).

The pUSAG3 plasmid was constructed by standard molecular cloning procedures (19). A 60 bp DNA fragment containing the –315 to –255 region sequence of the human Gγ-globin gene (Fig. 1A) was inserted into the *Sma*I site of the pEGFP-1 plasmid, a green fluorescent protein (GFP) promoter reporter vector (Clontech Laboratories, Palo Alto, CA), to obtain the pUSAG3 plasmid (Fig. 1C).

PNA binding assay

One 60mer oligonucleotide that contains the sequence from –315 to –255 of the human Gγ-globin gene was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. This labeled 60mer oligonucleotide was annealed with a complementary 60mer oligonucleotide to form a 60 bp DNA duplex. A fixed concentration of the 60 bp duplex DNA (5×10^{-9} M) was incubated with increasing concentrations of PNA in TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA) at 37°C for 4 h. The samples were analyzed by polyacrylamide gel electrophoresis in a 20% gel, followed by autoradiography. A GS-250 PhosphorImager (BioRad, Hercules, CA) was used for quantitation of the reaction products. The concentration at which PNA binding was half-maximal was taken as the equilibrium dissociation constant (K_d) (20).

In vitro transcription assay

The *in vitro* transcription assays were performed in a HeLa Nuclear Extract *in vitro* Transcription System under conditions recommended by the manufacturer (Promega, Madison, WI). Briefly, pUSAG3 was digested with *Not*I to linearize the plasmid. The linearized plasmid DNA (1 μg) was incubated with PNA-1 (10 μM) and/or PNA-2 (2 μM) in TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA) at 37°C for 4 h to induce the $[PNA]_2$/DNA-loop formation. The $[PNA]_2$/DNA triplex complex was incubated in a 25 μl reaction containing 200 μM each of ATP, CTP and UTP, 4 μM GTP, 10 μCi [α-^{32}P]GTP (Amersham, Arlington Heights, IL) and 8 U of HeLa nuclear extract at 30°C for 60 min. Termination of the reaction was performed by addition of 175 μl of Stop Mix. The products were extracted with 200 μl of phenol:chloroform and were precipitated with 500 μl of ethanol. The pellets were resuspended in 10 μl of nuclease-free water and mixed with 10 μl of loading dye. The samples were analyzed by polyacrylamide gel electrophoresis in a 5% gel containing 7 M urea, followed by autoradiography.

Reverse transcription primer extension assay

RNA was prepared by an *in vitro* transcription reaction or was isolated from living cells. The reverse transcription reaction was performed in a 25 μl volume containing 1× reverse transcription buffer (50 mM Tris, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT), total RNA (2 μg) or mRNA (0.1 μg), 1 pmol γ-^{32}P-labeled primer, 400 μM of each dATP, dCTP, dGTP and dTTP, and 400 U of M-MLV reverse transcriptase (Promega, Madison, WI). The reactants were incubated at 37°C for 90 min. The newly synthesized DNAs were precipitated with ethanol, resuspended in 10 μl of formamide solution, analyzed by 7% polyacrylamide sequencing gel, and visualized by autoradiography.

In vivo green fluorescent protein expression assay

pUSAG3 (5 μg) was incubated with PNA-1 (10 μM) or PNA-2 (2 μM) in 20 μl of TE buffer (pH 7.5) at 37°C for 4 h. The PNA-bound plasmid DNA was separated from free PNAs by centrifugation through Centricon 100 microcentrators (Amicon, Beverly, MA). The plasmid DNA was adjusted to a concentration of 0.1 μg/μl and mixed with rhodamine-labeled BSA at a concentration of 0.2 μg/μl. About 0.1 pl of the plasmid DNA mixture was microinjected into the nuclei of individual CV1 cells grown on etched glass coverslips using borosilicate needles and a WPI picopump (WPI, Sarasota, FL) as described by Dean (21). Microinjections were performed using a Narishige micromanipulator mounted on an Olympus IMT2 microscope and about 50 individual cells were microinjected for each DNA substrate. The cells were incubated at 37°C for 20 h and then fixed in 3% paraformaldehyde in phosphate-buffered saline. Expression of GFP was detected by epifluorescence microscopy using an Olympus BMAX50 microscope equipped with a fluorescein filter (BP470–490 nm/BA515 nm) and a rhodamine filter (BP530-550 nm/BA590 nm) and was photographed with Kodak TriX Pan 400 film. False color images of digitized negatives were recreated using Adobe Photoshop 4.0.

Introducing PNAs into human cells

The cultured K562 cells were harvested from the flask and resuspended into fresh medium. Approximately 4×10^6 cells were mixed with PNA-1 (5 μM) and/or PNA-2 (2 μM) in 300 μl of medium and incubated on ice for 10 min. The cells were electroporated in a 0.4-cm cuvette with a setting of 950 μF/250 V (BioRad Gene Pulser Plus, BioRad, Hercules, CA). Following electroporation, the cells were incubated on ice for 30 min, diluted into T25 flasks and incubated at 37°C for 2 days in a tissue culture incubator supplemented with 5% CO_2. Total RNA was isolated from the cells using the RNeasy isolation kit (Qiagen Inc., Santa Clarita, CA).

RNase protection assay

A 170 bp human γ-globin gene probe was generated in an *in vitro* transcription reaction using the *Bst*EII-linearized pT7Aγ plasmid DNA as a template (22). A 316 bp glyceraldehyde phosphate dehydrogenase (GAPD) gene probe was prepared in an *in vitro* transcription reaction using human pTRI-GAPD plasmid DNA (Ambion; Austin, TX) as a template. Three micrograms of total RNA were incubated with 10^6 c.p.m. of radiolabeled probe at 45°C overnight. After digestion with RNase A, the protected fragments were analyzed on a 6% denaturing polyacrylamide gel containing 8 M urea followed by autoradiography. The levels of human γ-globin gene and GAPD gene mRNA were quantitated using a GS-250 PhosphorImager. The amount of human γ-globin mRNA production was estimated relative to the amount of GAPD gene expression.

RESULTS

Designing and testing of PNAs that bind to the γ-globin gene 5' flanking region

To efficiently induce gene expression by PNA binding, stable D-loop structures need to be maintained in the target genes. The $[PNA]_2$/DNA triplex structures at the PNA binding sites have been found to greatly stabilize such D-loop structure (1–3). For transcription to initiate, the D-loop structures need to be formed on the DNA template strand since the RNA transcript is synthesized from this strand. The –315 to –255 region of the human Gγ-globin gene contains several homopurine/homopyrimidine stretches with the homopurine strand located on the template strand (23). Therefore, this region was a suitable target for this study and was chosen for studying PNA-induced γ-globin gene expression (Fig. 1A). Two complementary 60mer oligonucleotides that contain the –315 to –255 region of the Gγ-globin gene were synthesized. Two PNAs, one designed to bind to position –286 to –277 of the Gγ-globin gene (PNA-1), and the other designed to bind to position –304 to –293 of the Gγ-globin gene (PNA-2), were also synthesized (Fig. 1B). Both PNAs were synthesized to contain two binding domains: the DNA binding domain that binds to the homopurine strand of the target DNA forming a Watson–Crick duplex structure, and the triplex binding domain that binds to the PNA/DNA duplex to form a triple helix. These two PNA binding domains were linked by three hydrophilic spacer units.

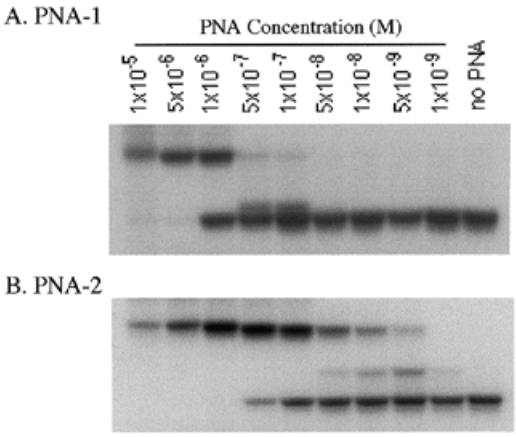

Figure 2. Comparison of the binding of PNA-1 and PNA-2. A fixed concentration of the ^{32}P-labeled 60 bp DNA fragment (5×10^{-9} M) was incubated with increasing concentrations of PNA in TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA) at 37°C for 4 h. The reactants were analyzed by polyacrylamide gel electrophoresis in a 20% gel followed by autoradiography. An equilibrium dissociation constant (K_d) was determined to estimate the binding affinity of the PNA oligonucleotides. (**A**) Binding of PNA-1 to DNA target. (**B**) Binding of PNA-2 to DNA target.

We tested the binding of these PNAs to their target sites. One of the 60mer oligonucleotides was end-labeled with [γ-^{32}P]ATP. This oligonucleotide was mixed with the complementary 60mer oligonucleotide at a 1:1 ratio to form a 60 bp DNA duplex. A fixed concentration of the 60 bp DNA fragment was incubated with increasing concentrations of PNA in TE buffer at 37°C for 4 h to allow the PNA to bind to the target. A gel mobility shift assay was performed to analyze the binding of the PNA and the binding capacity (which was designated as the concentration of PNAs at which the shifted DNA probe/total DNA probe = 50%) was used to determine the affinities of PNAs binding to their target sites (Fig. 2). The binding capacities of PNA-1 and PNA-2 were estimated to be 1×10^{-6} M and 7×10^{-8} M, respectively.

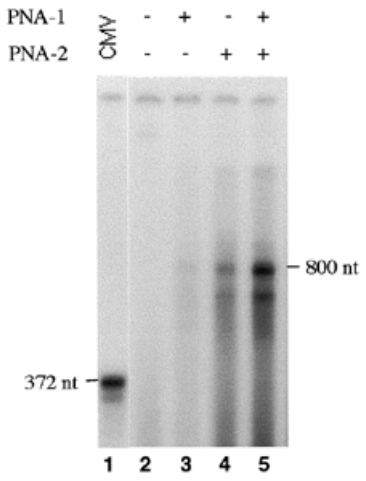

Figure 3. PNA-mediated GFP gene transcription in HeLa nuclear extract. The pUSAG3 plasmid DNA was linearized by digestion with *Not*I. The linearized pUSAG3 plasmid DNA was used as a template for *in vitro* transcription in a HeLa nuclear extract transcription system. Lane 1, CMV promoter-driven 372 nt RNA transcript as a positive control; lane 2, pUSAG3 plasmid DNA alone; lane 3, pUSAG3 plasmid DNA in the presence of PNA-1; lane 4, pUSAG3 plasmid DNA in the presence of PNA-2; lane 5, pUSAG3 plasmid DNA in the presence of both PNA-1 and PNA-2.

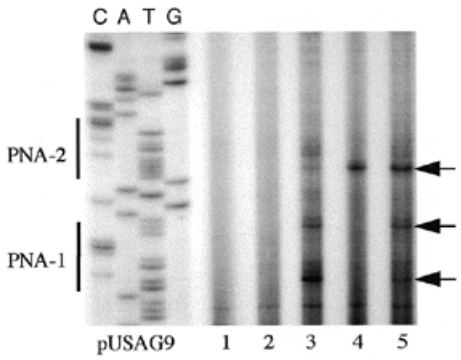

Figure 4. Detection of PNA binding-induced gene transcription initiation site. The pUSAG3 plasmid DNA sequencing reaction was performed using a primer binding to the 5' coding region of the GFP gene. The same primer was used as a primer for the reverse transcription. Lane 1, pUSAG3 plasmid DNA alone; lane 2, pUSAG3 plasmid DNA treated with control PNA (CP-1); lane 3, pUSAG3 plasmid DNA treated with PNA-1; lane 4, pUSAG3 plasmid DNA treated with PNA-2; lane 5, pUSAG3 plasmid DNA treated with PNA-1 and PNA-2. The sequences show both the PNA-1 (lower) and the PNA-2 (upper) binding site in the plasmid pUSAG3.

PNA-mediated gene transcription in a HeLa nuclear extract *in vitro* transcription system

To investigate if binding of the PNAs to the target can induce gene-specific transcription *in vitro*, a HeLa nuclear extract *in vitro* transcription assay was performed using a GFP expression plasmid (pUSAG3) that contains the PNA target sites upstream of the GFP gene instead of a promoter. The restriction enzyme *Not*I linearized pUSAG3 plasmid DNA was used as a template for the *in vitro* transcription assay in the presence or the absence of PNAs (Fig. 3). No specific RNA transcripts were synthesized from the pUSAG3 plasmid alone due to the lack of a functional mammalian promoter within the plasmid (Fig. 3, lane 2). However, in the presence of both PNA-1 and PNA-2, an ~800 nt RNA transcript was synthesized in the HeLa nuclear extract. This size of the RNA transcript corresponded to transcription initiating from the PNA binding sites since the PNA binding sequences of the Gγ-globin gene are located ~800 bp upstream of the *Not*I site in the plasmid (Fig. 3, lane 5). The ~800 nt RNA transcript was also detected in the presence of either PNA-1 or PNA-2 alone (Fig. 3, lanes 3 and 4). However, the expression levels of the GFP in these reactions were lower than that observed in the presence of both PNAs. This result indicated that both PNA-1 and PNA-2 bind sequence-specifically to the DNA target sites and that binding of these PNAs to their target sites initiates GFP transcription from these PNA binding sites.

Detection of the transcription initiation site in PNA binding-induced transcription

To determine the PNA binding-induced transcription initiation site, a reverse transcription primer extension assay was performed using RNA prepared from the *in vitro* transcription reaction and a γ-^{32}P-labeled primer that binds to the 5' coding region of the GFP mRNA with a sequence of 5'-CGTCCAGCTCGAC-CAGGATG-3' (Fig. 4). A sequencing ladder of pUSAG3 using the same primer was loaded on the gel as a size marker. pUSAG3 plasmid DNA alone or pUSAG3 plasmid DNA pre-incubated with a control PNA (CP-1) which does not bind to the plasmid did not induce any specific RNA transcripts from the PNA-1 or PNA-2 binding sites (Fig. 4, lanes 1 and 2). In contrast, when PNA-bound pUSAG3 plasmid DNA was used as a template, specific RNA transcripts were detected. When the PNA-1-bound pUSAG3 plasmid DNA was used as a template, two specific RNA transcripts, one starting at the position one base inside the 3' end and the other one starting at the position one base inside the 5' end of PNA-1 binding sequence, were detected (Fig. 4, lane 3). When the PNA-2-bound pUSAG3 plasmid was used as a template, one RNA transcript starting at the position one base inside the 3' PNA-2 binding sequence was generated (Fig. 4, lane 4). Interestingly, when the pUSAG3 plasmid DNA treated with both PNA-1 and PNA-2 was used as a template, three RNA transcripts, corresponding to the two PNA-1 and one PNA-2-specific transcripts, were produced (Fig. 4, lane 5). This result suggested that binding of either PNA-1 or PNA-2 alone to the target site was able to initiate RNA transcription; however, the binding of both PNA-1 and PNA-2 enhanced the transcription activity at the PNA binding targets.

PNA-induced green fluorescent protein gene expression *in vivo*

To investigate whether binding of PNAs to the DNA target sites could induce gene expression in living cells, PNA-induced gene expression was studied in CV1 monkey kidney fibroblast cells using the GFP gene as a reporter. The pUSAG3 plasmid DNA was first incubated with either PNA-1 or PNA-2 *in vitro* to form $[PNA]_2$/DNA triplex structures. Rhodamine-labeled BSA was mixed with the PNA/DNA complexes and co-microinjected into the nuclei of CV1 cells as an inert marker to determine which cells had been microinjected.

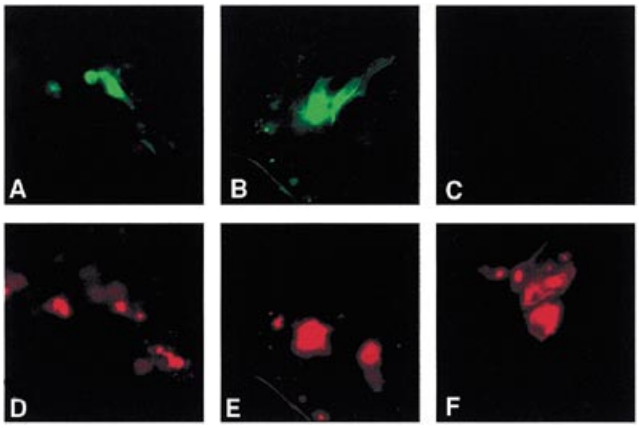

Figure 5. PNA binding-mediated GFP gene expression in CV1 cells. Twenty hours post-injection of plasmid or plasmid–PNA complexes, GFP expression was determined. GFP fluorescence is shown in the top panels and the rhodamine-labeled BSA signal is shown in the bottom panels. (**A**) and (**D**) CV1 cells injected with pUSAG3.PNA-1; (**B**) and (**E**) CV1 cells injected with pUSAG3.PNA2; (**C**) and (**F**) CV1 cells injected with pUSAG3 plasmid DNA alone. In all cases, rhodamine-labeled BSA was co-injected with the DNAs to the cells.

Expression of the GFP gene was monitored 20 h after micro-injection by fluorescence microscopy (Fig. 5, top panels) in relation to the number of injected cells (Fig. 5, bottom panels). GFP expression was not detected in any CV1 cells that were microinjected with pUSAG3 alone (Fig. 5C). In contrast, when either PNA-1 or PNA-2 bound pUSAG3 plasmid DNA complexes were injected into CV1 cells, ~30% of the injected cells displayed intense expression of GFP (Fig. 5A and B).

PNA-induced endogenous γ-globin gene expression

To test the possibility of inducing endogenous γ-globin gene expression in human cells, PNA-mediated γ-globin gene expression was investigated in K562 human erythroleukemia cells which have low levels of endogenous γ-globin gene expression (22). The K562 cells were treated with either PNA-1 and/or PNA-2 and were incubated in cell growth medium for 2 days. As a control, some K562 cells were also treated with control PNA in the same condition. Total RNA was isolated from the cells and an RNase protection assay (RPA) was performed to determine the level of γ-globin gene mRNA production (Fig. 6). The amount of γ-globin mRNA was increased when the K562 cells were treated with PNAs for 2 days. The expression level of the γ-globin gene was returned to normal 7 days after the PNA treatment (data not shown). The level of γ-globin mRNA synthesis was increased as much as 2.8-fold in the K562 cells that were treated with PNAs for 2 days compared to the untreated K562 cells (Fig. 6, lane 2 versus lane 1). In a similar experiment in which the K562 cells were treated for 3 days with 75 μM hemin, a γ-globin gene-specific inducer, the level of γ-globin gene expression was increased 4.6-fold (Fig. 6, lane 5). Expression of the GAPD gene was used as an internal control and did not change in any of the experiments. This result suggests that the PNAs designed to bind to the γ-globin gene 5' flanking region could induce endogenous γ-globin expression in K562 cells.

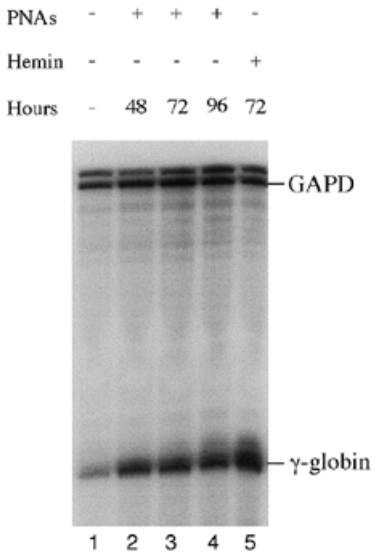

Figure 6. RNase protection analysis to detect PNA-induced γ-globin gene expression in K562 cells. Autoradiograph of the RNase protection analysis in a 6% polyacrylamide gel containing 8 M urea. Lane 1, K562 cells; lanes 2–4, K562 cells treated with PNAs for 2, 3 and 4 days, respectively; lane 5, K562 cells treated with hemin (75 μM) for 3 days.

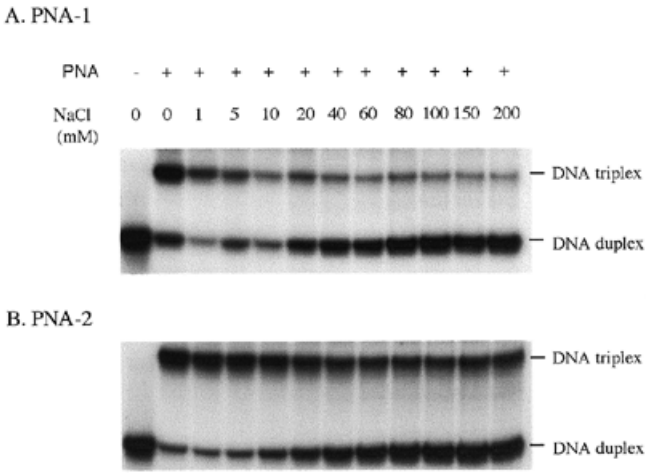

Figure 7. NaCl-mediated inhibition of PNA binding. (**A**) PNA-1 and (**B**) PNA-2 binding inhibition by NaCl. The PNA-1 used in the reaction is 5×10^{-6} M and the PNA-2 used in the reaction is 1×10^{-6} M. The target DNA substrate used in the reaction is 5×10^{-9} M.

The ability of the PNAs to induce gene expression when electroporated into cells indicated that the PNAs are capable of binding to their target sites under physiological conditions. This is somewhat surprising since it has been shown that PNA to DNA binding is inhibited as the concentration of salt increases. To confirm that our PNAs indeed are binding to their targets under these salt conditions, we performed an *in vitro* binding reaction with varying concentrations of NaCl (Fig. 7). As can be seen, the binding of PNA-1 and PNA-2 showed very different sensitivity to the presence of salt. The binding of PNA-1 is very sensitive to the presence of NaCl. In

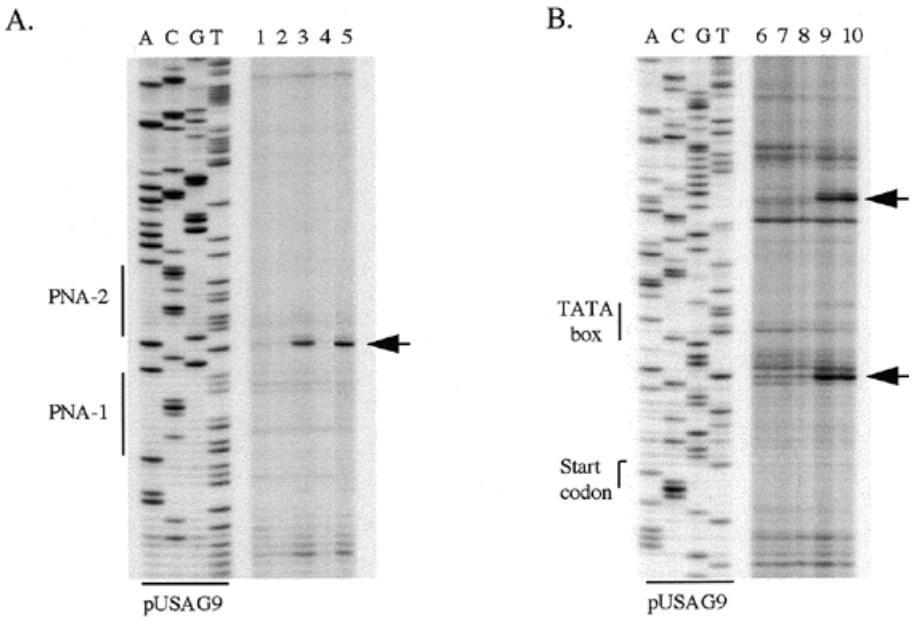

Figure 8. Determination of PNA-induced endogenous γ-globin gene transcription using reverse transcription primer extension. (**A**) Detection of γ-globin gene transcription initiation sites around the PNA binding sites. A primer that binds to the γ-globin gene at the position –207 to –225 (5'-CCAAGAGGATACTGCTGCT-3') was used for both reverse transcription primer extension and DNA sequencing reactions. pUSAG9 plasmid DNA was used as a template for the DNA sequencing reaction. (**B**) Detection of γ-globin gene transcription initiated from the native γ-globin gene promoter. A primer that binds to the γ-globin gene coding region at the position 80 to 61 (5'-TCTCCTCCAGCATCTTCCAC-3') was used for both reverse transcription primer extension and DNA sequencing reactions. pUSAG9 plasmid DNA was used as a template for the DNA sequencing reaction. Lanes 1 and 6, total RNA isolated from K562 cells; lanes 2 and 7, total RNA isolated from control PNA-treated K562 cells; lanes 3 and 9, total RNA isolated from PNA-2 treated K562 cells; lanes 4 and 8, total RNA isolated from PNA-1 treated K562 cells; lanes 5 and 10, total RNA isolated from PNA-1 plus PNA-2 treated K562 cells.

the presence of 40 mM of NaCl, for example, only ~15% DNA substrate was bound by PNA-1 (Fig. 7A). The binding of PNA-2, however, is more resistant to the presence of salt. At 150 mM NaCl conditions, ~30% of DNA substrate was still bound to PNA-2 (Fig. 7B).

To determine if the increased γ-globin mRNA in PNA-treated K562 cells was due to transcription initiated from the PNA binding sites, a reverse transcription primer extension assay was performed using total RNA isolated from K562 cells and a primer binding to the γ-globin gene –207 to –225 region (5'-CCAAGAGGATACTGCTGCT-3') (Fig. 8A). A specific RNA transcript starting at the position two bases 3' to the PNA-2 binding sequence was detected from the K562 cells that were treated with either PNA-2 alone or PNA-1 and PNA-2 together (Fig. 8A, lanes 3 and 5). In contrast, no specific RNA transcripts synthesized around the PNA binding sites were detected from untreated K562 cells or the K562 cells treated with control PNA (Fig. 8A, lanes 1 and 2). Treatment of K562 cells with PNA-1 alone was not sufficient to induce specific RNA transcripts around the PNA binding sites (Fig. 8A, lane 4).

We have also determined levels of γ-globin mRNA production from its native promoter in these PNA-treated K562 cells using a primer that binds to the γ-globin gene coding region position 80–61 (5'-TCTCCTCCAGCATCTTCCAC-3') (Fig. 8B). Interestingly, it was found that the K562 cells that had induced γ-globin transcription from the PNA binding site due to PNA treatment also showed increased γ-globin transcription from the γ-globin gene native promoter as well (Fig. 8B, lanes 9 and 10 versus lanes 6 and 7).

DISCUSSION

In this study we have shown that PNAs designed to bind to the human γ-globin gene 5' flanking region can be used as gene-specific activators to induce gene expression. Using a GFP promoter reporter construct, we have demonstrated that binding of the PNAs induced GFP reporter gene transcription in an *in vitro* transcription system in the absence of mammalian promoters. When the same vector construct containing the *in vitro*-bound PNAs was microinjected into CV1 monkey kidney cells, significant GFP expression was detected by fluorescence microscopy. This result suggests that binding of PNAs to the target sites not only initiates RNA transcription *in vitro* but also induces gene expression in living cells. More importantly, PNA-induced endogenous γ-globin gene expression was demonstrated in K562 human erythroleukemia cells using RNA protection assays and our reverse transcription primer extension assays confirmed a specific RNA transcript synthesized from the PNA binding site. This result indicates that PNAs cannot only bind to naked DNA targets *in vitro* and induce gene expression, but also bind to endogenous gene targets under physiological conditions and induce gene expression in living cells. This result provides strong evidence for a possible future gene therapy via PNA binding-induced endogenous gene expression.

The initiation sites of PNA binding-induced transcription were determined from both the *in vitro* and the *in vivo* studies using reverse transcription primer extension assays. In the PNA binding-induced *in vitro* transcription studies, the initiation sites of PNA-1 binding mediated RNA transcription

were located to positions one to two bases inside 3' and the 5' ends of the PNA-1 binding sequence and the initiation site of PNA-2 binding-induced transcription was located to one base inside the 3' end of PNA-2 binding sequence (Fig. 4). In our *in vivo* study, the initiation site of PNA-induced γ-globin gene transcription was located to the position two bases 3' to the PNA-2 binding site (Fig. 8A). Mechanisms for the observed difference in transcription initiation sites between the *in vitro* and the *in vivo* studies are unknown. One possibility is that this may be due to different RNA template used in the assay: the RNA template used in our *in vitro* study was pre-mRNA synthesized by the HeLa nuclear extract *in vitro* transcription system without any post-transcriptional modification; while the RNA template used in our *in vivo* study was isolated from K562 cells that post-transcriptional modification already occurred in RNA templates. However, this result may also reveal differences in environment for PNA binding and/or transcription initiation used the *in vitro* and the *in vivo* system. Further studies need to be done to determine the molecular mechanisms of different transcription initiation sites observed in these *in vitro* and *in vivo* studies.

One of the interesting observations obtained from the *in vivo* studies is that it seems that PNAs not only induced endogenous γ-globin gene transcription from the PNA binding site but also increased the γ-globin gene transcription from its native promoter (Fig. 8B). Since the PNA binding site is located at a far distance 5' to the γ-globin gene native promoter region (~250 bp from the PNA binding site to the γ-globin gene TATA box), this increased γ-globin transcription could not have directly resulted from the D-loops generated by the PNA binding. It is possible that transcription of the γ-globin gene from upstream sequences makes the native promoter more accessible for RNA polymerase and/or other transcription factors and, therefore, leads to increased γ-globin transcription from its native promoter.

Both the human Gγ and the Aγ-globin genes contain similar DNA sequences at the 5' flanking region. The target sequences of PNA-1 are identical in both genes while the target sequences of PNA-2 are different for only 1 bp in both genes. Whether this single base difference at the PNA-2 binding site could discriminate the Gγ and the Aγ gene in PNA binding and therefore lead to different levels of Gγ- and Aγ-gene expression is unknown.

The PNAs used in this study were designed to bind to homopurine target sites of either 10 or 12 bases in the human Gγ globin gene. The binding affinities of these PNAs are much stronger than would be expected for normal triplex-forming oligonucleotides including phosphodiester and phosphorothioate oligonucleotides. The apparent binding capacity of PNAs used in this study is in a range of 1×10^{-6} to 8×10^{-8} M, which is at least 30-fold higher than phosphodiester triplex-forming oligonucleotides with similar lengths (24). The strong binding affinity of PNAs is contributed to the modified polyamide backbones which eliminate the charge in the PNA strand and stabilize both the duplex and the triplex structures. This result is consistent with other published data (1–3,6). The strong binding affinity of PNAs may help overcome the target sequences limitations of applying triplex technology in gene therapy of many human diseases in the future.

PNAs can bind to both single- and double-stranded DNA targets. However, the process for binding to both targets is very different in the presence of high concentrations of salts. Binding of PNAs to single-stranded DNA (ssDNA) targets is not affected by the presence of salt since the process of binding PNA to ssDNA targets is independent of salt concentration. When bound to double-stranded DNA (dsDNA) targets, however, one DNA strand must be replaced with a PNA molecule in order to form a PNA/DNA duplex and this process is partially inhibited in the presence of high concentrations of salts (4,25) In our *in vitro* PNA binding studies, different PNA binding inhibition patterns were observed between PNA-1 and PNA-2. In the presence of 150 mM NaCl, ~30% of PNA-2 can still bind to duplex DNA target, however, only ~15% of PNA-1 can bind to DNA target in the presence of 40 mM of NaCl in the *in vitro* PNA binding assay (Fig. 7). The different binding inhibition profiles of PNA-1 and PNA-2 in the presence of high concentrations of salt may contribute to the *in vivo* result: treatment of PNA-2 alone was able to induce the endogenous γ-globin gene transcription at the PNA-2 binding site in K562 cells, while no transcription was observed at the PNA-1 binding site when the K562 cells were treated with PNA-1. To improve the binding of PNA at high salt concentrations, several methods have been studied (25,26). Modifications of PNAs such as incorporation of positively charged amino acids (lysine) to the ends of PNAs, have been found to increase the rate of PNA binding in high salt conditions (25). Altering target DNA dynamics by increasing negative supercoiling within the DNA targets has also been reported to enhance the rate of PNA binding (26).

Gene transcription has been reported to enhance the binding of PNA to dsDNA targets (26). Transcription might lead to local DNA conformational changes (such as temporarily increased single-strand loops and disrupted histones bound at the target sites) and hence favor PNA binding conditions. In our studies, PNA-induced endogenous γ-globin gene expression was achieved in K562 cells. We have noticed that low levels of γ-globin gene expression are present in K562 cells: whether this low level of γ-globin gene expression increased PNA binding and led to γ-globin expression induction is unclear. Experiments are in progress to test whether the PNAs can induce expression of the γ-globin gene in other human cells that carry silenced γ-globin genes in their chromosome.

Our results provide direct evidence to show that binding of PNAs to DNA targets can induce endogenous gene expression in human cells. This may provide a novel approach to study fundamental mechanisms of gene expression and regulation. For example, the established gene transcription model suggests that transcription in both prokaryotic and eukaryotic cells is initiated from the binding of RNA polymerase and other transcription initiation factors (such as sigma factor in *Escherichia coli* and TFIID in mammalian cells) to the promoter region. This results in the unwinding of an ~12 bp region, and at the same time, the core polymerase undergoes a major conformational change yielding a stable and highly processive elongation complex that completes synthesis of the nascent transcript (27). However, in PNA binding-induced gene transcription, binding of the PNAs to DNA targets opens single-stranded loops at the target sites in the absence of polymerase and accessory factors. Whether a similar transcription initiation procedure takes place for PNA binding-induced transcription remains to be seen.

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