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Antisense properties of duplex- and triplex-forming PNAs

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ABSTRACT

The potential of peptide nucleic acids (PNAs) as specific inhibitors of translation has been studied. PNAs with a mixed purine/pyrimidine sequence form duplexes, while homopyrimidine PNAs form $(PNA)_2$/RNA triplexes with complementary sequences on RNA. We show here that neither of these PNA/RNA structures are substrates for RNase H. Translation experiments performed in cell-free extracts showed that a 15mer duplex-forming PNA blocked translation in a dose-dependent manner when the target was 5′-proximal to the AUG start codon on the RNA, whereas similar 10-, 15- or 20mer PNAs had no effect when targeted towards sequences in the coding region. Triplex-forming 10mer PNAs were efficient and specific antisense agents with a target overlapping the AUG start codon and caused arrest of ribosome elongation with a target positioned in the coding region of the mRNA. Furthermore, translation could be blocked with a 6mer bisPNA or with a clamp PNA, forming partly a triplex, partly a duplex, with its target sequence in the coding region of the mRNA.

INTRODUCTION

The use of oligodeoxynucleotides (ODNs) and their analogs as sequence-specific inhibitors of translation (antisense reagents) has attracted great attention due to the potential of these as therapeutic agents against genetic and virus-mediated diseases. A number of ODN analogs with modified backbone structures have been developed in an attempt to improve their antisense potency in terms of nuclease resistance, RNA affinity and cellular uptake. Although many of these analogs, including phosphorothioates, methylphosphonates, α-anomeric ODNs and 2′-*O*-alkyloligoribonucleotides are nuclease resistant, several problems, such as non-specific effects and poor cellular uptake, are still unsolved (for reviews see 1–5).

The mechanism of antisense effects by ODNs is believed to be either a ribonuclease H (RNase H)-mediated cleavage of the RNA strand in ODN–RNA hybrids or a physical blocking of the translation machinery at the ODN–RNA complex. ODN analogs that activate RNase H, such as phosphorothioates, have until now proven to be the most efficient antisense agents, due to the irreversibility of the mRNA cleavage (6–8). However, phosphorothioates have also been shown to exhibit non-specfic effects, probably due to RNase H cleavage of imperfectly matched target sites (9) and interactions with cellular proteins (10–12).

Peptide nucleic acids (PNAs) are DNA mimics with a pseudopeptide backbone composed of achiral and uncharged *N*-(2-aminoethyl)glycine units (13–17). PNAs have been shown to hybridize sequence-selectively and with high affinity to complementary sequences in single-stranded DNA or RNA, forming Watson–Crick double helices (13,18–19). Notably however, homopyrimidine PNAs form thermally highly stable $(PNA)_2$/RNA triplexes with complementary RNA targets, having T_m values >70°C for decamers (15–16,20). Furthermore, PNAs have been found to be stable in serum as well as in cell extracts (21). Previous reports have shown that a 10mer pyrimidine-rich PNA complementary to a sequence in the coding region of SV40 T antigen mRNA could block translation *in vitro* and that 15- and 20mer pyrimidine-rich PNAs targeted towards sequences in the same region inhibited T antigen expression when microinjected into Tsa 8 cells (22). Other experiments using microinjection showed that a 15mer homopyrimidine PNA targeted towards a sequence in the 5′-untranslated region of SV40 T antigen mRNA was able to inhibit T antigen expression in CV-1 cells by 99%, while a 15mer non-homopyrimidine PNA targeted towards the same region caused 51% inhibition of T antigen expression at estimated intracellular concentrations of 2 μM (8,23).

In the present paper we have undertaken studies in order to differentiate between the antisense potential of duplex-forming and triplex-forming PNAs respectively by performing *in vitro* translation in rabbit reticulocyte lysates. We show that both duplex- and triplex-forming PNAs can inhibit translation at targets overlapping the AUG start codon. Triplex-forming PNAs are able to block the translation machinery at targets in the coding region of mRNA, while duplex-forming PNAs are not. Furthermore, clamp PNAs, potentially forming partly triplex and partly duplex with its target on mRNA, have antisense properties similar to the full triplex-forming PNAs.

MATERIALS AND METHODS

PNA and ODN synthesis

PNAs were synthetized as described (24,25). The PNAs were purified by HPLC and their identity confirmed by mass

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spectrometry. ODNs were synthetized on a Biosearch 7500 DNA synthesizer by standard phosphoamidite chemistry.

In vitro transcripts

A plasmid containing the chloramphenicol acetyltransferase (CAT) gene plus 43 bp of the 5′-untranslated region for CAT was obtained by cloning the *Cla*I–*Bam*HI fragment of plasmid pCM7 (Pharmacia) into the polylinker of pBluescriptKS+ (Stratagene). A polypurine sequence was inserted 514 bp downstream from the ATG translation start sequence by cloning oligonucleotides 5′-CATGAAAAGAAGAAT-3′ (non-template strand) and 5′-CATGATTCTTCTTTT-3′ (template strand) into the *Nco*I site. The resulting plasmid, pKSCAT, was linearized with *Eco*RV and 1 μg used for transcription in a reaction containing 40 mM Tris–HCl, pH 8.0, 25 mM NaCl, 8 mM $MgCl_2$, 2 mM spermidine, 5 mM DTT, 10 mM each ATP, CTP and UTP, 2 mM GTP, 0.5 mM cap analog m^7(5′)Gppp(5′)G (Boehringer Mannheim) and 50 U T3 RNA polymerase (Gibco BRL) in a total volume of 20 μl. Transcription was carried out for 30 min at 37°C. The *in vitro* transcript was used without further purification as template in the translation reactions.

^{32}P-Labeled transcripts were made by addition of 0.1 μCi [α-^{32}P]UTP to the transcription reaction with a UTP concentration of 1 mM; m^7(5′)Gppp(5′)G was omitted and the GTP concentration adjusted to 10 mM. The transcript was ethanol precipitated in order to remove unincorporated [α-^{32}P]UTP.

RNase H assay

The ^{32}P-labeled pKSCAT transcript was incubated with variable concentrations of PNA or ODN in 10 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 25 mM NaCl, 1 mM DTT in a total volume of 20 μl for 1 h at room temperature. Then 1 U *Escherichia coli* RNase H (Boehringer Mannheim) was added and the incubation continued for 1 h at 37°C. The reaction was stopped by cooling the samples on ice. Formamide buffer was added and the RNA was analyzed on a 7 M urea–8% polyacrylamide gel followed by autoradiography.

Translation reactions

Translation was performed using a rabbit reticulocyte lysate kit according to the manufacture's recommandations (Boehringer Mannheim). pKSCAT *in vitro* transcript (1 μl) and rabbit globin mRNA (0.25 μg) (Gibco BRL) were used. PNA was added without preincubation to the reactions at the desired concentrations. Translation was carried out in the presence of [^{35}S]Met at 30°C for 1 h and terminated by boiling the samples for 2 min in 60 mM Tris–HCl, pH 6.8, 10% (v/v) glycerol, 2% SDS, 5% (v/v) mercaptoethanol. The samples were then analyzed by 18% (w/v) SDS–PAGE and protein bands were visualized by autoradiography.

RESULTS

PNA–RNA complexes are not substrates for RNase H

RNase H selectively cleaves the RNA moiety of DNA/RNA heteroduplexes and is believed to be responsible for the majority of the effects of antisense ODNs in intact cells (26). It has been reported (22), but not actually shown, that PNA–RNA complexes which we would predict to form triplexes are not substrates for

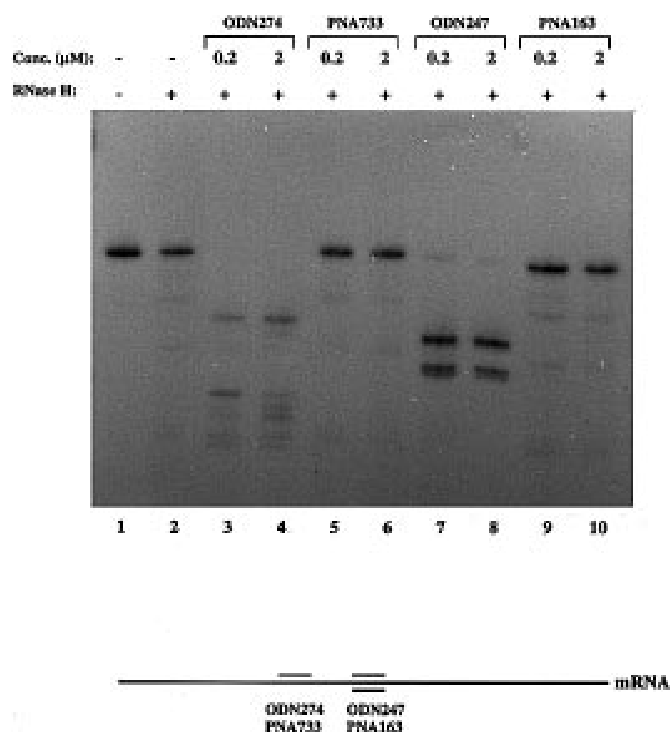

Figure 1. RNase H cleavage of a 200 nt ^{32}P-labled transcript alone or hybridized with a PNA or ODN in different concentrations as indicated above each lane. The positions of the PNA and ODN targets on the RNA are shown in the model below.

RNase H. We therefore performed experiments to determine whether $(PNA)_2$/RNA triplexes and PNA/RNA duplexes respectively were cleaved by RNase H. For this purpose a 200 nt [^{32}P]UTP-labeled transcript was incubated with either a 15mer PNA containing both purines and pyrimidines (here refered to as a mixed sequence PNA) or a 10mer homopyrimidine sequence PNA. Binding of the PNAs to their targets on the RNA was confirmed by gel shift of the ^{32}P-labeled RNA on native gel electrophoresis (data not shown). As controls, parallel samples were prepared with two phosphodiester ODNs having the same sequences as the PNAs. After incubation for 1 h in the presence of RNase H the cleavage pattern was examined by denaturing PAGE. As seen in Figure 1, cleavage products of the predicted sizes were obtained with both ODNs (lanes 3–4 and 7–8), whereas no RNA cleavage was observed with either of the PNAs (lanes 5–6 and 9–10). Thus we conclude that neither of the PNAs activate RNase H when hybridized to RNA.

Antisense effects of duplex-forming PNAs

In order to examine the antisense characteristics of PNAs an *in vitro* translation rabbit reticulocyte lysate model system was chosen. CAT mRNA, which encodes a protein of 25 kDa, was used as reporter mRNA and globin mRNA, encoding a protein of 17 kDa, was used as an internal control for translation efficiency. PNAs complementary to target sequences in different regions of the CAT mRNA were employed (listed in Table 1).

First, we tested PNAs with mixed purine/pyrimidine sequences with the propensity to form only duplexes with complementary

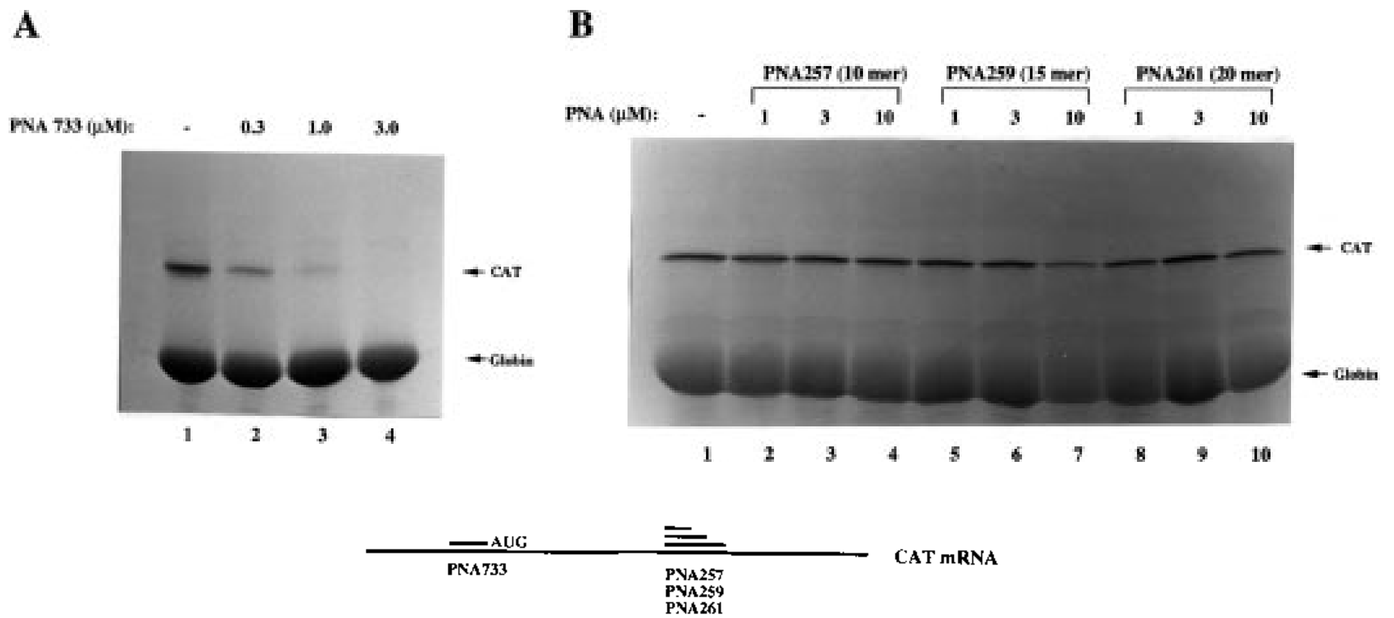

Figure 2. Translation of CAT mRNA in the presence of either a 15mer PNA with mixed purine/pyrimidine sequence hybridized to a region immediately 5′ of the AUG start codon (**A**) or 10mer, 15mer or 20mer mixed purine/pyrimidine PNAs targeted towards sequences in the coding region (**B**) as shown in the model below. PNA identity and concentration is indicated above the lanes. Rabbit globin mRNA was used as an internal control. Positions of CAT protein and globin are shown by arrows.

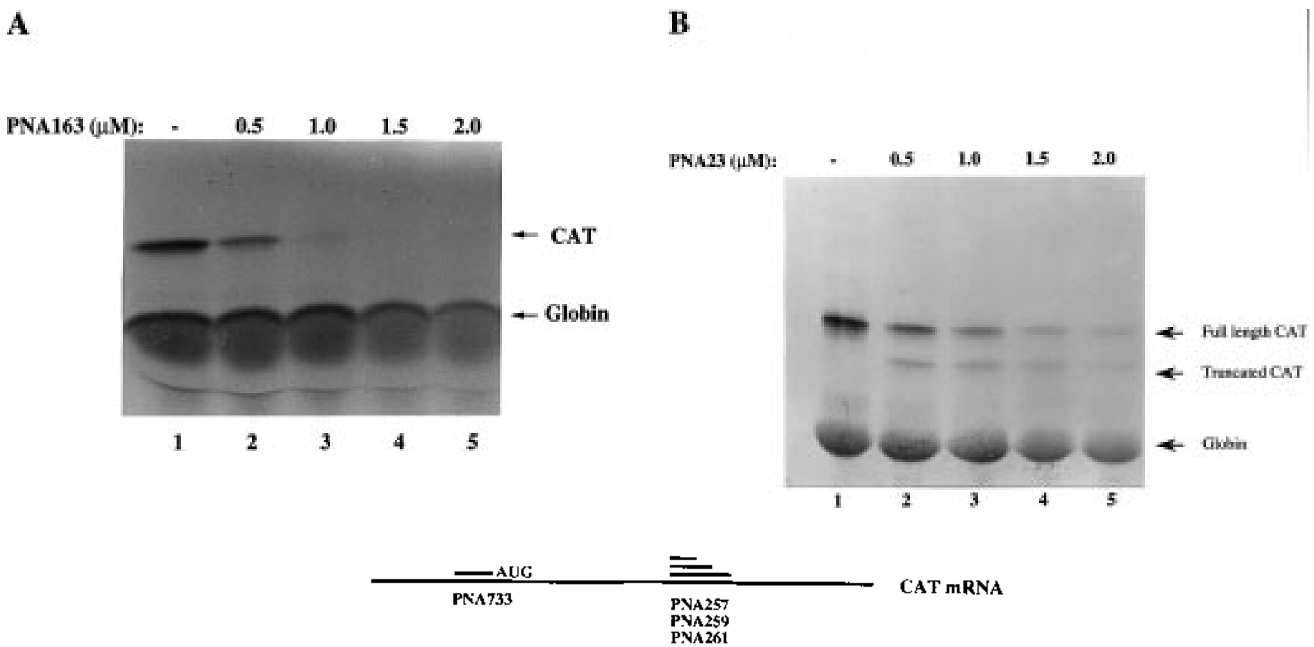

Figure 3. Antisense effect of polypyrimidine PNAs targeted to sequences adjacent to the AUG start codon (**A**) or in the coding region of CAT mRNA (**B**) as shown in the model below. The concentration of PNA is indicated above each lane.

RNA targets. When the target was positioned immediately 5′ of the AUG start codon the 15mer PNA 733 efficiently blocked translation of CAT mRNA in a PNA concentration-dependent manner, while translation of globin mRNA was largely unaffected (Fig. 2A). However, when 10mer, 15mer or 20mer PNAs were targeted to a sequence in the coding region of CAT mRNA no inhibition of translation was observed (Fig. 2B), indicating that duplex-forming PNAs are not able to arrest the elongating ribosome.

Table 1. Sequences of PNAs and ODNs used in the study

Name	Sequence[a]	Target	Effect
PNA 23	H-TTC TTC TTT T-Lys-NH_2	Coding region	+++
PNA 163	H-CTC TTT TTT T-Lys-NH_2	AUG region	+++
PNA 257	H-ACA TCT TGC G-Lys-NH_2	Coding region	–
PNA 259	H-ACG CCA CAT CTT GCG-Lys-NH_2	Coding region	–
PNA 261	H-GTA ACA CGC CAC ATC TTG CG-LysNH_2	Coding region	–
PNA 311	H-JTT TT-$(eg1)_3$-TTT TCA CCG T-NH_2	Coding region (clamp)	?
PNA 733	H-TTT AGC TTC CTT AGC-Lys-NH_2	AUG region	+++
PNA 817	H-TCT CAA TAA ACC CTT T-$(eg1)_3$-TTT JJJ-Lys-NH_2	Coding region (clamp)	++
PNA 818	H-TCT CAA TAA ACC CTT-$(eg1)_3$-TTJ JJ-Lys-NH_2	Coding region (clamp)	–
PNA 928	H-TCT CAA TAA ACC TTT-NH_2	Coding region	–
PNA 952	H-CCC TTT-(eg1)-TTT JJJ-Lys NH_2	Coding region (clamp)	+
ODN 247	5′-TTT TTT TCT CCA-3′	AUG region	–
ODN 274	5′-TTT AGC TTC CTT AGC-3′	AUG region	–

The position of their target in CAT mRNA is listed together with their relative effect as inhibitors of translation in rabbit reticulocyte lysate.
[a]eg1, 8-amino-3,6-dioxaoctanoic acid; J, pseudoisocytosine.

Triplex-forming PNAs prevent translation at initiation codon targets and inhibit ribosome elongation at coding region targets

The CAT mRNA contains a polypurine sequence, GAGAAAAAAA, immediately downstream of the AUG start codon. When a PNA targeting this region, presumably forming a $(PNA)_2$/RNA triplex, was used translation of CAT mRNA was efficiently and specifically inhibited (Fig. 3A). Inhibition of CAT mRNA translation by PNA 163 was in the same dose range as observed for the mixed sequence PNA 733.

When a triplex-forming PNA (PNA 23), targeted towards an AAAAGAAGAA sequence 514 nt downstream of the AUG start codon was used translation of CAT mRNA was specifically repressed and a truncated product appeared (Fig. 3B, lanes 2–4). Increasing the PNA concentration led to a decrease in both the full-length and the truncated CAT products, while the globin translation level was less affected.

Translation inhibition by clamp PNAs

On the basis of these results we found it interesting to investigate whether PNAs which would form partly a triplex and partly a duplex with their target (here refered to as clamp PNAs) were able to inhibit translation. In this way the triplex half of the complex could be shortened and specific recognition of the target might be assured by the duplex-forming half. Restrictions on target sequences would be less stringent if five to six purines were needed, rather than 10 or more. For this purpose PNAs were designed in which the Hoogsteen recognizing strand was linked to the Watson–Crick recognizing strand by a flexible ethylene glycol linker. The cytosine residues in the Hoogsteen strand were substituted with pseudoisocytosine, in order to obtain optimal hybridization at neutral pH (27). A target for these PNAs positioned in the middle of the coding region of the CAT mRNA was chosen and the PNAs were designed to form the triplex at the 5′-end and the duplex at the 3′-end of the target. As seen in Figure 4B, PNA 817, having 6 + 6 pyrimidines and 10 mixed bases, inhibited CAT mRNA translation in a specific manner, but the concentration needed for inhibition was rather high (>3 µM). PNA 818, which differs from PNA 817 in having one less T on each of the triplex strands, did not inhibit CAT mRNA translation even at 10 µM (Fig. 4A). PNA 928, which was lacking the Hoogsteen strand but otherwise had the same sequence as PNA 817, thus forming a duplex with the target mRNA, also failed to have any effect on CAT mRNA translation (data not shown). In contrast, a PNA consisting of only the triplex-forming part of PNA 817 (= PNA 925) did exhibit an inhibitory effect on CAT mRNA translation at concentrations >3 µM. However, at 10 µM a CAT mRNA translation product was still visible.

PNA 311 was designed to form a triplex at the 3′-end and duplex at the 5′-end of the target. This PNA reduced translation of both CAT and globin mRNA (Fig. 4C), which indicates a non-specific effect.

Comparison of PNA and ODN antisense effects

In order to compare the antisense effects of PNA and a phosphodiester ODN parallel translation experiments were carried out with duplex-forming PNA 733 and a phosphodiester ODN having the same sequence. RNase H activity in rabbit reticulocyte lysate is known to be low under the conditions used for *in vitro* translation (28), therefore exogenous RNase H (*E.coli*) was added to the translation reactions. As seen in Figure 5, inhibition of CAT translation by PNA takes place in a dose-dependent manner (lanes 2–5) and is almost complete at 1 µM, whereas oligonucleotide concentrations up to 10 µM fail to inhibit CAT translation (lanes 6–11).

The antisense effects of the different PNAs and ODNs are summarized in Table 1.

DISCUSSION

The results presented here have implications for fundamental aspects of the antisense potential of PNAs. First, it is clearly demonstrated that neither PNA/RNA duplexes nor $(PNA)_2$/RNA

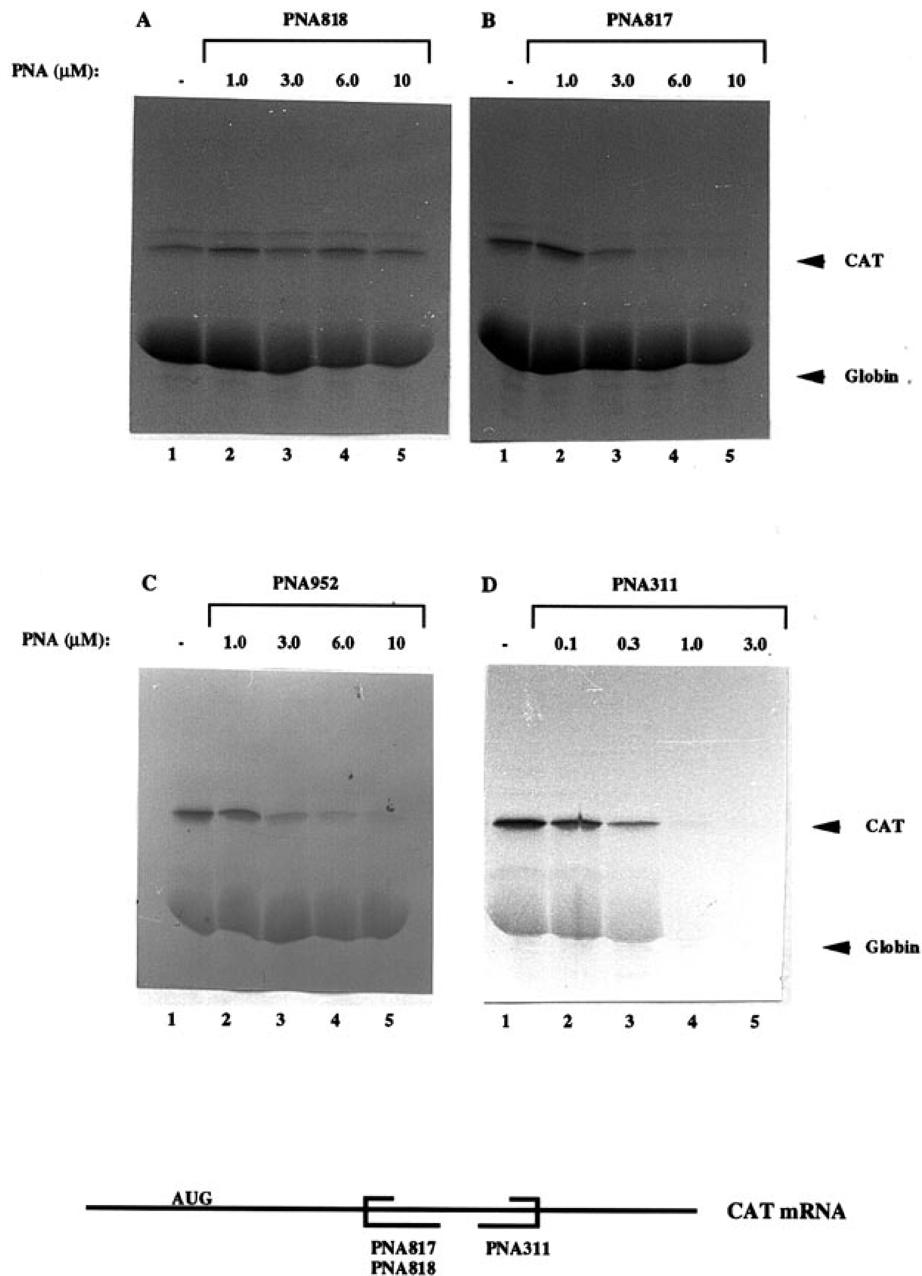

Figure 4. Translation of CAT and globin mRNA in the presence of (**A**) 5 + 15, (**B**) 6 + 16 or (**C**) 6 + 6 clamp PNAs with the triplex part facing the 5′-end of the target or (**D**) a 5 + 10 clamp PNA with the triplex part facing the 3′-end of the target as shown in the model below.

triplexes are substrates for RNase H. Considering the very different structure of the PNA backbone compared with DNA this is not unexpected and is in accordance with previous claims regarding $(PNA)_2$/RNA triplexes (22). Likewise, other ODN analogs with modified backbones, such as methylphosphonates (29), α-ODNs (30) and 2′-*O*-alkyloligoribonucleotides (31), are not RNase H activators.

Inhibition of translation initiation with antisense reagents hybridizing to targets overlapping the AUG start codon can take place by an RNase H-independent mechanism (32). Similarily, duplex- as well as triplex-forming PNAs targeted towards sequences adjacent to the AUG start codon efficiently blocked translation of CAT mRNA, presumably by physically blocking assembly of the 80S ribosome initiation complex. A phosphodiester ODN targeted to the same region as the duplex-forming PNA adjacent to the AUG start codon did not inhibit translation, even when *E.coli* RNase H was added to the translation reaction.

Antisense effects of phosphodiester and phosphorothioate ODNs targeted to sequences in the coding region of a mRNA are RNase H dependent (7,26,32–33). ODN analogs that do not activate RNase H (as mentioned above) are capable of blocking translation at targets in the coding region only when conjugated to crosslinkers, such as psoralen (34–36), or to alkylating reagents (37–38). In agreement with previous findings (8,22–23), we here show that a PNA forming a $(PNA)_2$/RNA triplex in the coding region of CAT mRNA causes arrest of the elongating ribosome,

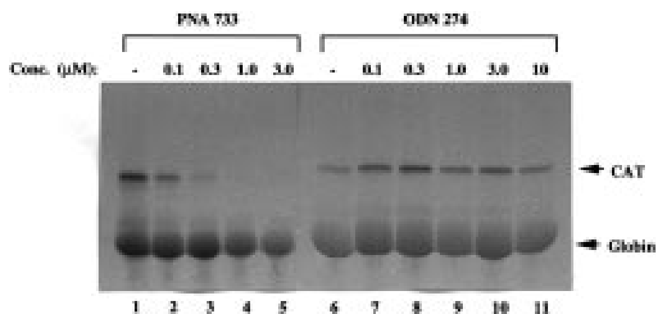

Figure 5. Comparison of the antisense effects of duplex-forming PNA 733 (lanes 2–5) and an ODN (lanes 6–10) with similar sequence, targeted adjacent to the AUG start codon on CAT mRNA. Translations were performed in the presence of 3 U *E.coli* RNase H. Concentrations of PNA and ODN are indicated above the lanes.

giving rise to a truncated protein product. A total conversion from full-length to truncated CAT protein is, however, not obtained when raising the PNA concentration. Rather, a general inhibition of CAT translation takes place. A plausible explanation for this observation could be that the elongating ribosome is arrested when encountering the $(PNA)_2$/RNA structure and remains associated with the mRNA. Subsequent ribosomes would consequently stack up on the CAT mRNA and thereby prevent re-initiation of translation of these mRNAs.

Duplex-forming PNAs binding to complementary sequences in the coding region of CAT mRNA do not seem able to arrest the translation machinery. Compared with the $(PNA)_2$/RNA triplex, which is thermally more stable for a similar length (15–16) and presumably more 'tight', the PNA/RNA duplex may allow 'breathing' at the ends, making it easier for the elongating ribosome to unwind and remove the PNA. Alternatively, the $(PNA)_2$/RNA triplex, being a more bulky structure, acts as a block to the translation machinery. Clearly it is not simply the stability of the complex that determines the translation elongation blockage, since the T_m of the oligonucleotide–PNA 261 complex is 81°C, whereas that of the oligonucleotide–PNA 23 complex is only 69°C.

The requirement for triplex formation in order to attain efficient translation arrest with targets downstream of the AUG limits the possibilities of finding appropriate targets in a given gene. We found that a clamp PNA forming a 6mer triplex and a 10mer duplex with a target sequence in the coding region of CAT mRNA inhibited its translation, while a control PNA forming just the duplex part did not. However, a bisPNA forming just the 6 + 6 triplex part also had an inhibitory effect on CAT translation, albeit slighly less efficient than the 6 + 16 clamp. Notably, a clamp PNA forming a 5mer triplex and a 10mer duplex failed to inhibit translation, indicating that a 6mer triplex in this case was necessary to arrest the ribosome. These results open interesting possibilities that deserve further exploration. Clamp ODNs forming full triplexes with polypurine targets on either single-stranded DNA or RNA have previously been found to be able to arrest replication (39) and reverse transcription (40) respectively. It remains, however, to be seen if such ODN clamps can work as blocks to the elongating ribome.

It is generally observed that the position of the target on the mRNA drastically influences the efficiency of antisense ODNs, so that targets shifted just a few nucleotides can have varied effects on translation. The design of a PNA therefore most likely has to be optimized for each target with respect to length and, in the case of clamp PNAs, pyrimidine content. Furthermore, it cannot be ruled out that other coding regions of (other) mRNA could be sensitive to translation inhibition by duplex-forming PNAs by a mechanism that does not involve ribosome elongation arrest.

In conclusion, triplex-forming PNAs as well as duplex-forming PNAs are potent antisense reagents. Fully or partly triplex-forming PNAs differ in their antisense potential from duplex-forming PNAs and other ODN analogs in being able to arrest translation at coding region targets. Whether these PNAs will have specific antisense effects in intact cells remains to be seen.

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