Peptide nucleic acids (PNAs) complementary to the 15 bases around the fusion point of both genomic DNA and cDNA of the promyelocytic leukemia/retinoic acid receptor α (PML/RARα) gene was synthesized and shown by gel retardation experiments to specifically bind oligonucleotides corresponding to the fusion region of the P/R molecule. PNA was also shown to specifically compete with anti-P/R DNA for duplex formation with P/R DNA and to displace the anti-P/R DNA from dsDNA. In vitro transcribed P/R RNA from two inserts of ~350 and ~700 bp were tested in gel retardation experiments with fluorescein-conjugated PNA and showed stable binding (resistant to denaturing conditions) of PNA to the newly transcribed RNA. Control RNA or transcripts from the noncoding strand did not bind PNA. However, this PNA, although able to specifically clamp polymerase chain reaction, was incapable of inhibiting in vitro translation of the PML/RARα mRNA, even when a bis-PNA was used. Therefore, a PNA was targeted against the start region of the P/R cDNA and against poly-purine regions of the gene. Specific inhibition in vitro translation and transcription was shown, starting at concentrations as low as 100 nM. When oligonucleotides presenting the same sequence were compared, PNA proved to be approximately 40 times more active. In conclusion, in vitro inhibition of translation and transcription of the P/R cDNA can be obtained with PNA; however, it is still necessary to target the start region of the gene.

95% of PNAs were synthesized as described. Fluorescein-conjugated PNA was obtained by reacting the PNA with fluorescein isothiocyanate. PNA sequences are as follows: 137 (complementary to the genomic P/R cDNA, accession number AF001574), no. 205, H-Gly-CTCAATGGCTGCCTC-NH₂, and no. 946, H-Gly-CTCAATGGCTGCCTC-CCT-NH₂. In conclusion, in vitro inhibition of translation and transcription of the P/R gene can be obtained with PNA. Further studies are needed to target the ATG start region of the gene.

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Submitted November 28, 1995; accepted April 8, 1996.

Supported in part by the Italian Association for Cancer Research (AIRC), the Italian Research Council (CNR No. 95.00842.CT04), BIOMED-2 Grant No. BMH4-CT96-0848, CEVA, and the Danish National Research Foundation.

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T4 polynucleotide kinase) were incubated for 30 minutes at room temperature (step 1) in TE buffer and then (step 2) at either room temperature or 37°C for an additional 30 minutes alone or with either the complementary ODN or PNA. The samples were then run on a 15% polyacrylamide gel and visualized by autoradiography. A competition assay was accomplished by incubating in the same tube both DNA and PNA. To verify the strand displacement ability of PNA, P/R ODNs were incubated with their antiserum ODNs during step 1 (30 minutes at room temperature). Subsequently, PNA was added and samples incubated for 30 additional minutes (step 2) at either room temperature or 37°C.

PNA binding to P/R RNA (gel acceleration experiments). Fluorescein-conjugated PNA (500 to 1,000 ng) was incubated in TE buffer with cold ODN (1 to 10 µg), with the transcription products obtained from three reactions (see below) from the LAAB and MOL plasmids or with control RNA (30 minutes at 37°C). Samples were then loaded on a 15% (ODNs) or 4% (transcripts) polyacrylamide gel and run at 200 V. Gels were then mounted on 3M paper (Whatman, Maidstone, UK) and dried, and bands were visualized (as green light) by direct UV illumination. Size markers were obtained by the migration of RNA standards (RNA ladder; Gibco, Gaithersburg, MD) and detected on the wet gel by ethidium bromide staining and UV transillumination. In some experiments, samples were mixed one-third with formamide and loaded on 10 mol/L urea gels.

In vitro transcription experiments. Plasmids were first linearized by digestion with HindIII (used for MOL and anti-LAAB) or EcoRI (used for LAAB and anti-MOL). Template DNA was purified by phenol/chloroform extraction and used for in vitro transcription at 1 µg/reaction (SP6/T7 transcription kit; Promega, Madison, WI) in the presence of 20 pCi of [cU-32P]UTP for 10 to 30 minutes at 37°C, as described.17 For gel acceleration experiments, cold UTP was used. Transcription was initiated from the SP6 (LAAB and anti-MOL) or T7 (MOL and anti-LAAB) site. PNA was incubated with purified DNA at 37°C for 30 minutes before starting transcription. Unincorporated UTP was removed with NUC-TRAP mini columns (Stratagene, La Jolla, CA), and the samples were run on 4% to 5% polyacrylamide gel electrophoresis (PAGE), dried, and visualized by autoradiography. The intensity of different bands was compared using the 620 CCD densitometer (Bio-Rad, Richmond, CA) and the I-D Analyst II data analysis software.

In vitro translation experiments. Plasmids BCR1 or pGF-1 were directly translated into poly-epitides using the TNT coupled reticulocyte lysate system (Promega, La Jolla, CA). Briefly, 1 µg DNA, 77 RNA polymerase, TNT buffer, aminoacid mixture, L-[35S]-methionine (20 µCi), RNasin (20 U; Promega), TNT rabbit reticulocyte lysate, and PNA were mixed and incubated at 37°C for 30 minutes, according to TNT protocol. As internal controls, 0.5 µg of the pGF-1 plasmid was incubated in the same tube. Samples were run in 6% to 7% polyacrylamide gel electrophoresis (PAGE), dried, and visualized by autoradiography. Molecular weight markers (Bio-Rad) were run in parallel.

Polymerase chain reaction (PCR) clamping. Anti-P/R PNA no. 205 and the bis-PNA no. 496 were used to target the fusion point of P/R in the BCR1 plasmid. Two plasmids carrying the β-glucocerebrosidase gene and the factor V gene were used as specificity controls.17 The following primers were used for amplification: 5'-GGA GCC AGC CAT TGA GAC 3' (forward) and 5'-CAT AGT GGT AGC CTA AGG ACT T-3' (reverse) for P/R, 5'-GAA TGT CCC AAG CTT TTG A-3' (forward) and 5'-AAG CTG AAG CAA GAG AAT CG-3' (reverse) for β-glucocerebrosidase, and 5'-GGA ACA ACA CCA TGA TCA GAG CA-3' (forward) and 5'-TAG CCA GGA GAC CTA ACA TGT TC-3' (reverse) for factor V. The amplified fragments were, respectively, 131, 357, and 287 bp in length. PCR was performed in a 50 µL reaction containing 3 ng of the plasmid, 200 µmol/L of each dNTP, 0.5 µmol/L of each primer, 50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.3, 2.6 mmol/L MgCl₂, and 1.25 U Taq polymerase on the GenAmp PCR System 9600 machine (Perkin Elmer, Branchburg, NJ) using a 4-step cycle: 94°C for 15 seconds, 62°C for 1 minute, 54°C for 20 seconds, 72°C for 90 seconds for 25 cycles. PNA was either preincubated overnight with the plasmid or added in the tube at the beginning of the reaction.

RESULTS

Anti-P/R PNA is able to bind specifically to P/R DNA and to successfully compete with anti-P/R DNA (gel retardation experiments). To investigate the ability of PNA to bind P/R DNA, 15 mer ODNs corresponding to the fusion region of the P/R gene were synthesized and used in gel retardation experiments.

Two types of P/R fusions were studied. Initially, the sequence corresponding to genomic fusion (from the patient identified as no. 8) was targeted. In this patient, the fusion region (AGAGTTTACGAGGGA) has an approximate A+G content of 75%. Gel retardation experiments are shown in Fig 1A. Both anti-P/R DNA and PNA can bind to P/R DNA. Anti-P/R DNA slows the migration of P/R DNA (lanes 2 and 3, consistent with the formation of dimers). PNA forms two bands (lanes 4 and 5) that migrate more slowly than the DNA/DNA dimers because of the lack of net charges on the PNA molecule. These two bands are compatible with the formation of a PNA/DNA duplex (lower band) and of a nonperfect PNA/DNA triplex. As expected, the triplex predominates when working in an excess of PNA (lane 4). If DNA/DNA dimers are allowed to form and then are challenged with PNA (lane 6), then about 48% of the DNA/DNA complex is displaced, with the formation of PNA/DNA dimers (and triplexes, upper band). However, if the second incubation is performed at 37°C instead of at room temperature, then a near complete displacement of the DNA/DNA dimers is observed (Fig 1B, lanes 8 through 11), showing the superior binding efficiency of PNA. Anti-P/R PNA does not bind to PML or RARα ODNs (mismatch of 5 and 7 bases, respectively) or to anti-P/R ODN. PNA also does not interfere with the binding of PML (Fig 1A, lanes 9 through 11) and RARα (data not shown) ODNs to their complementary ODNs.

Subsequently, ODNs corresponding to the BCR1 type cDNA (GAGGCAGCCATTGAG) were used (Fig 1C). This represents a mixed sequence with only 66% A+G content, thus, only PNA/DNA duplexes are observed (lanes 5 through 7). PNA successfully competed with DNA (lane 8, 77% PNA/DNA χ 23% DNA/DNA). Anti-P/R PNA is able to successfully displace anti-P/R DNA from DNA/DNA dimers and to bind specifically to P/R DNA when incubated with the DNA/DNA dimers for at least 60 minutes at 37°C (lanes 10 through 12).

These experiments show that PNA is able to specifically bind to ODNs representing both the genomic and the cDNA P/R fusion region and to successfully compete with anti-P/R DNA for binding to P/R DNA.

Ability of PNA to bind transcripts of the P/R gene (gel acceleration experiments). P/R RNA was transcribed in vitro from the two plasmids LAAB and MOL that contain ~700- and ~350-bp inserts of the P/R cDNA, including

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Fig 1. Ability of anti-P/R PNA to bind specifically to both single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) representing fusion regions containing poly-purine or mixed sequences. (A) A 15-mer ODN representing a genomic P/R fusion region (AGAGTTACAGAGGA, high purine [75%] content) named P/R was labeled and used. Lane 1, P/R alone (10 pmol); lanes 2 and 3, P/R + anti-P/R ODN (100 and 10 pmol, respectively); lanes 4 and 5, P/R + anti-P/R PNA (100 and 10 pmol, respectively); lane 6, P/R + anti-P/R ODN (100 pmol, step 1) followed by PNA (100 pmol, step 2 [room temperature]); lanes 7 and 8, P/R + anti-PML or anti-RARe ODN; lane 9, labeled PML ODN alone; lane 10, PML + anti-PML ODN; and lane 11, P/R + anti-P/R ODN (100 pmol, step 1) followed by anti-P/R PNA. (B) Lane 1, P/R alone (10 pmol); lanes 2 through 4, P/R + anti-P/R ODN (100, 10, and 5 pmol, respectively); lanes 5 through 7, P/R + anti-P/R PNA (100, 10, and 5 pmol, respectively); lanes 8 and 9, P/R + anti-P/R ODN (10 pmol, step 1) followed by anti-P/R PNA (step 2, 37°C; 100 and 10 pmol, respectively); lanes 10 and 11, same as lanes 8 and 9 but containing anti-P/R ODN at 5 pmol/L. (C) Ability of anti-P/R PNA to bind an ODN representing the fusion region of the P/R cDNA, a non-poly-purine DNA sequence. A 15-mer ODN representing the BCR1-type fusion region (GAGGGACCCATTGAG, mixed sequence), named 004, was labeled and used. Lane 1, 004 alone (10 pmol); lanes 2 through 4, 004 + anti-P/R ODNs (10, 30, and 100 pmol, lanes 2 through 4, respectively) or PNA (lanes 5 through 7); lane 8, 004 + anti-P/R ODN and PNA; lanes 9 through 12, 004 + anti-P/R ODN (100 pmol, step 1) followed by PNA (100 pmol, step 2 [37°C]) for 30 minutes (lane 9) or for 60, 120, or 240 minutes (lanes 10 through 12, respectively). All samples were run in 12% PAGE. The bands corresponding to ssDNA, dsDNA, PNA/DNA, and PNA/DNA are indicated.

the fusion point. Because PNA does not contain phosphate molecules, conventional binding techniques could not be used. Therefore, a fluorescein-conjugated PNA-based technique was used. PNA is not charged and therefore is expected to have little migration when electric current is applied. The binding to RNA would confer mobility to PNA, which can be visualized as a green signal by direct UV illumination of the dried gel. An example using ODNs is presented in Fig 2A. PNA no. 205 (500 ng [≈100 pmol]), which binds specifically to P/R ODN, can migrate into the gel and forms a discrete band. Down to 40 pmol of PNA can be visualized (data not shown). Transcripts from the MOL and LAAB plasmids (containing partial P/R inserts of 326 and 738 bases, respectively) were then incubated with PNA and run on PAGE (Fig 2B through D). Unbound PNA show little shift, as expected. When incubated with P/R transcripts, PNA migrates (gel acceleration) at positions compatible with the length of the transcripts. Incubation of PNA
Fig 2. Ability of anti-P/R fluorescein-conjugated PNA to bind to P/R mRNA transcribed by plasmids LAAB (738 bases) and MOL (326 bases) or to P/R ODNs. (A) PNA (100 pmol) was incubated for 30 minutes at room temperature with anti-P/R ODN (lanes 1 and 2, 100 and 1,000 pmol, respectively) or with anti-PML or anti-RARα ODNs (lanes 3 and 4, 1,000 pmol). Lane 5, PNA alone. Samples were run on 10% PAGE. PNA was visualized as described under the Materials and Methods. (B) PNA (lane 5) was incubated for 30 minutes at 37°C with transcripts from MOL (lane 1), LAAB (lane 2), or their antisense transcript (lanes 3 and 4). (C) PNA (lane 5) was incubated (as in [B]) with control RNA (lanes 1 and 2, 10 and 20 μg, respectively) and with transcripts from LAAB (sense [lane 3] and antisense [lane 4]). (D) Lane 1, PNA alone; lane 2, PNA + anti-MOL RNA; lane 3, PNA + MOL RNA. Samples were mixed with formamide and run in denaturing (10 mol/L urea) PAGE. Molecular markers are indicated.

with antisense transcripts or with unrelated control RNAs did not induce any shift of the PNA (Fig 2C, lanes 1 and 2). Whereas control PNAs enter the gel in Fig 2C, they apparently fail to form a band in Fig 2B. Although a clear explanation is not available, it is possible that, in the experiment depicted in Fig 2B, a partial precipitation of PNA occurred that prevented control samples from forming a distinct band.

The binding of PNA to its target is very stable, because, once formed, it is resistant to denaturing (10 mol/L urea) conditions (Fig 2D). In conclusion, PNA is able to bind specifically P/R RNA and forms a stable complex.

PNA-mediated inhibition of PCR amplification of P/R.

To establish a possible functional role for the observed binding of PNA no. 205 (and of its corresponding bis PNA no. 496) to nucleic acids, we performed a PCR clamping test. In this assay, the specific arrest of Taq polymerase-mediated amplification of a P/R sequence by PNA is assessed. PNA no. 205 failed to affect the amplification of a 131-bp fragment containing the P/R fusion point, whereas PNA no. 496 showed significant (≥ 50%) inhibition in the μmol/L range. The block was significantly increased when PNA no. 496 was preincubated overnight with template DNA, reaching a significant inhibition at 300 nmol/L and a total block at 2
PNA inhibits PML/RARα gene translation

whereas the lower band (76 kD) refers to the product of pGF-I (internal control). PNA concentrations were 0, 50, 100, 300, and 500 nmol/L (lanes 1 through 5, respectively).

In vitro translation experiments. The ability of anti-P/R PNA to inhibit the in vitro translation of the P/R fusion protein from the expression plasmid plasmid BCR1 was evaluated. PNA no. 205, which showed a stable binding to the P/R fusion region, was added to the in vitro translation assay, but failed to inhibit the translation of the P/R polypeptide from plasmid BCR1 (data not shown). A second PNA (no. 496), synthesized as a bis-PNA against the fusion region, inhibited translation at high (>2 μmol/L) concentrations, but in a nonspecific way (data not shown). Similarly, both PNA 116-496 and against a poly-purine region (no. 783, residues 122-136) of PML, which is retained in the fusion gene, to inhibit translation.

Figure 3 shows the results using PNA 752 to inhibit in vitro translation of the P/R protein from plasmid BCR1. Specific inhibition starts at 50 to 100 nmol/L and complete inhibition is achieved at 300 nmol/L. Translation of the control plasmid pGF-I (lower band) was unaffected. The activity of PNA 752 was also compared with an ODN with the same sequence (Fig 4A and B). The ODN achieved a significant translation inhibition at 8 μmol/L. The concentrations of 200 nmol/L (PNA) and 8 μmol/L (ODN) achieved similar (~80%) translation inhibition. The activity of PNA 783 was also evaluated (Fig 4C). At 300 nmol/L, a 60% inhibition was obtained (compared with 100% for PNA 752); no accumulation of a truncated product was evident. Further increases in concentration led to nonspecific inhibition.

In vitro transcription experiments. A bis PNA (no. 753) targeted against a homopurine stretch in the PML gene was used to inhibit in vitro transcription from the LAAB plasmid (Fig 5). This PNA would be expected to block transcription approximately 100 bases after the T7 promoter site. A truncated ~100-base-long transcript became evident at 500 nmol/L, progressively increased in intensity at 1 and 2 μmol/L, and reached 30% of the total transcript (as assessed by densitometric analysis). A proportional decrease of the full-length transcript was also observed. Further increases in PNA concentration resulted in the nonspecific inhibition of both the full-length and the truncated products.

These experiments show that PNAs directed against the start codon or poly-purine regions can specifically inhibit both in vitro transcription and translation of the P/R gene. PNA can achieve a degree of translation inhibition similar to that obtained using 40 times higher concentrations of ODN. Inhibition of transcription can be achieved by targeting a poly-purine region in the coding DNA strand.
DISCUSSION

The hybrid protein P/R epitomizes one of the growing list of fusion molecules generated by chromosomal translocations associated with human cancer. The causal role of the P/R protein and of most other fusion proteins in the pathogenesis of human cancer is now well established, although the molecular mechanism by which each protein transforms cells is still obscure in many cases. Our ability to modulate the molecular mechanism by which each protein transforms cells is still obscure in many cases. Our ability to modulate the expression and/or the function of fusion proteins is even more limited at present.

PNA represents a promising tool in this direction. PNA could potentially be used as both an antisense and an antigen molecule. We initially verified the DNA-binding ability of PNA using labeled ODN representing the fusion region of the P/R molecule. Subsequent experiments confirmed that anti-P/R PNA could bind to transcripts from the P/R gene.

Unfortunately, even a stable binding to the fusion region of the mRNA was not sufficient to inhibit P/R protein synthesis, although specific termination of PCR amplification of a fragment containing the P/R fusion was attained. It is possible that the ribosomes could displace that bound PNA and continue synthesis downstream. These data also show the difficulties present when trying to precisely target the fusion region of hybrid genes, mainly due to the limited variability and number of sequences that can be generated. However, this problem is not restricted to the targeting of DNA or RNA, but is also present when different strategies (e.g., the elicitation of an immune response against the fusion region of the P/R protein\(^{21,22}\)) are followed.

Subsequently, the potential target region was extended to the entire PML part of the fusion molecule. Because it is known that non-RNase H-activating ODN analogues can have better translation inhibition when targeted to the initiation codon, a new 15-mer PNA was prepared. With this PNA, a specific and sensitive translation inhibition was obtained at concentrations as low as 100 nmol/L. This represents the first description of translation inhibition obtained by PNA-mediated targeting of a mixed (non-poly-purine) sequence corresponding to a naturally occurring gene. Translation inhibition by PNA has been shown so far only when targeting homopurine sequences\(^{13}\) artificially introduced in nonbiologically relevant genes.

When the same sequence ODN was evaluated, it appeared to be several times less effective than PNA on a molar basis. A different PNA (no. 783), directed against a homopurine region of the cDNA, also exhibited some activity at 300 nmol/L; however, this became nonspecific at higher concentrations. This observation requires further investigation. It is important to note that, when specific translation inhibition was obtained with PNA no. 783, no accumulation of a truncated product was found, which is in variance with in vitro transcription (see below). This fact could be explained with the successive block of several translation complexes at different positions and the ensuing release of polypeptides of distinct molecular weight that, therefore, do not form a single band. The absence, or nonstoichiometrical amount, of a truncated translation product has been observed in other systems too.\(^{23}\)

A bis-PNA directed against a homopurine region of the coding strand was used to evaluate transcription inhibition. In this case, a progressive accumulation of a truncated product (with decrease in the full-length transcript) was observed, although at high PNA concentrations, close to the micromoles per liter mark, and without reaching a complete suppression. In this case, higher concentrations produced non-specific inhibition of both the full-length and truncated transcript.

Although additional important information are not yet available, including the ability of PNA to enter cells,\(^{24}\) these data support the possible use of PNA as a specific tool to modulate gene expression. Although results in intact cells are an essential prerequisite for clinical development, these data show that an oncogene can be targeted at both the translation and transcription level and epitomize potentials and limitations of targeting different regions of the gene. In this context, it is worth saying that, although the targeting of the PML start (ATG) region would not be absolutely tumor-specific, the function of PML in normal cells could be easily vicariated, as data in PML knock-out mice seem to suggest (P.P. Pandolfi, unpublished observations).

The observed inhibition of transcription, although not complete, is particularly promising, because it is known that only few molecules (2 in theory) need to be blocked inside a cell to completely inhibit transcription. PNA could thus be used as an antigen molecule, with the aim of selectively blocking gene transcription.

ACKNOWLEDGMENT

The authors thank Drs G. Parmiani for discussion, G. Manenti for helpful criticism and support, and J. Barrett and N. Young for reviewing the manuscript.

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In vitro transcription and translation inhibition by anti-promyelocytic leukemia (PML)/retinoic acid receptor alpha and anti-PML peptide nucleic acid

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