Peptide Nucleic Acid (PNA) Facilitates Multistranded Hybrid Formation between Linear Double-Stranded DNA Targets and RecA Protein-Coated Complementary Single-Stranded DNA Probes†

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ABSTRACT: RecA protein-coated single-stranded DNA probes, known as RecA nucleoprotein filaments, bind specifically to homologous DNA sequences within double-stranded DNA targets, forming multistranded probe−target DNA hybrids. This DNA hybridization reaction can be used for sequence-specific gene capture, gene modification, and gene regulation. Thus, factors that enhance the efficiency of the hybridization reaction are of significant practical importance. We show here that the hybridization of a peptide nucleic acid (PNA) within or adjacent to the probe−target homology region significantly enhances the yield of hybrid DNA formed in the reaction between linear double-stranded DNA targets and RecA protein-coated complementary single-stranded (css)DNA probes. The possible mechanisms and the advantages of using RecA nucleoprotein filaments in combination with PNA for genomic DNA cloning and mutagenesis are presented.

RecA protein-coated single-stranded DNA, known as RecA nucleoprotein filaments, are a key to the homologous recombination process. These nucleoprotein filaments bind homologous, or partially homologous, sequences within double-stranded DNA targets (for review see *1*). This hybridization process is used for sequence-specific gene capture, gene modification, and gene regulation (for review see *2*, *3*). In these applications, the RecA protein-coated nucleoprotein filaments are referred to as "probes", double-stranded DNA molecules are referred to as "targets", and the products of their binding ("targeting") are referred to as "hybrids".

An important special case of the DNA targeting reaction occurs when two complementary single-stranded (css)DNA probes are used in the reaction. In this case, four-stranded probe−target hybrids, also known as double D-loops, are formed (*4*, *5*). Double D-loops are kinetically stable within linear and open-circular DNA targets, even after removal of RecA protein (*4*, *5*). In contrast, protein-free single D-loop hybrids are stable only in negatively supercoiled DNA targets (*6*). The kinetic stability of double D-loop DNA hybrids is probably due to the slow reversible initiation step in their dissociation process (*7*, *8*). The superior stability of double D-loop DNA hybrids is important for many practical applications, including DNA cloning (for review see *3*). Another advantage of using the cssDNA[1] probes is that they can be conveniently obtained directly from the PCR product amplified from the target DNA (*4*).

Because of numerous applications for manipulation of native double-stranded DNA, the methods to increase the efficiency of the homologous DNA targeting are of interest. One possible way to increase the efficiency of homologous DNA targeting is to tether the homologous probe to a triplex-forming oligonucleotide (TFO). This approach is possible when the target DNA sequence is flanked by a homopurine-homopyrimidine DNA sequence motive, which can efficiently bind a TFO (for review see *9*). Usually TFO-binding reactions are robust and efficient because they do not require an energetically "costly" target DNA-opening process, which is necessary for DNA strand invasion into the duplex target. The "anchoring" of the homologous DNA probe to the target via TFO binding dramatically increases the "local concentration" of the homologous probe in the vicinity of the target and thus facilitates double-stranded DNA target invasion. This approach was successfully used for negatively supercoiled DNA targets in vitro (*10*, *11*). Similar mechanisms may apply to corrections of mutant DNA targets in vivo (*12*).

A second approach is based on the fact that the RecA protein-coated nucleoprotein filament binding eventually unwinds the double-stranded DNA target (for review see *1*); therefore, one can hypothesize that preformed stable local openings in the target DNA within (or adjacent to) the region of the probe−target homology would stimulate RecA-mediated nucleoprotein filament hybridization, thus increasing the yield of the probe−target hybrids.

Acceleration of the DNA probe's hybridization because of the local unwinding of the target DNA was demonstrated for negatively supercoiled target DNA, where the local

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[1] Abbreviations: PNA, peptide nucleic acid; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; cssDNA probes, complementary single-stranded DNA probes; bp, base pair; TFO, triplex-forming oligonucleotide.

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unwinding was provided by cruciform DNA formation (*13*). However, in the absence of negative superhelical stress (i.e., for linear or open-circular double-stranded DNA targets), this mechanism of local unwinding is not possible.

Likely candidates for facilitating local DNA openings are peptide nucleic acids (PNAs). PNAs are DNA analogues that contain peptide-like backbones instead of phosphodiester DNA backbones (*14*). Under the appropriate conditions, homologous PNA oligomers hybridize to specific sequences within double-stranded DNA targets with very high efficiencies. Because of their superior DNA (and RNA) binding properties, PNAs have numerous significant biological applications (for review see *9*, *15*, *16*).

PNAs hybridize to double-stranded DNA targets, forming various types of D-loop-like structures, which are summarized in (*17*). Remarkably, at least some of these PNA−DNA hybrid structures are stable under the positive superhelical stress that destabilizes D-loop-like structures (*18*).

Within these D-loop-like structures, except for the special case of pseudocomplementary PNAs (*17*), one of the target DNA strands is not base-paired. In the case of pseudocomplementary PNAs, both target DNA strands are base-paired; however, transient target DNA opening would occur at the junctions of the PNA−DNA hybrid because of thermal fluctuations.

It has already been demonstrated that PNA-induced double-stranded DNA opening facilitates the invasion of a second PNA into the neighboring site on the DNA target (*19*) and enables the hybridization of single-stranded DNA oligonucleotides to the complementary strand of the double-stranded DNA target (PD-loop formation (*20*)).

In the work presented here, we investigated PNA facilitation of the reaction between RecA protein-coated complementary single-stranded DNA probes and the double-stranded DNA.

MATERIALS AND METHODS

Target DNAs. As a specific target, the following 530 bp fragment from the human HPRT gene was used:

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1   ATCACAGTTC ACTCCAGCCT CAACATCCTG CACTAAAGTG ATTTTCCCAC
    CTCACCTCTC AAGTAGCTGG GACTACAGGT ACATGCTACC ATGCCTGGCT 100
101 AATTTTTTTT TTTTTGCAGG CATGGGGTCT CACTATATTG CCCAGGTTGG
    TGTGGAAGTT TAATGACTAA GAGGTGTTTG TTATAAAGTT TAATGTATGA 200
201 AACTTTCTAT TAAATTCCTG ATTTTATTTC TGTAGGACTG AACGTCTTGC
    TCGAGATGTG ATGAAGGAGA TGGGAGGCCA TCACATTGTA GCCCTCTGTG 300
301 TGCTCAAGGG GGGCTATAAA TTCTTTGCTG ACCTGCTGGA TTACATCAAA
    GCACTGAATA GAAATAGTGA TAGATCCATT CCTATGACTG TAGATTTTAT 400
401 CAGACTGAAG AGCTATTGTG TGAGTATATT TAATATATGA TTCTTTTTAG
    TGGCAACAGT AGGTTTTCTT ATATTTTCTT TGAATCTCTG CAAACCATAC 500
501 TTGCTTTCAT TTCACTTGGT TACAGTGAGA
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This fragment was obtained by PCR from genomic DNA and cloned into the EcoRI−HindIII sites of either pBluescript II SC(+) vector (producing plasmid pHP3A) or pUC18 vector (producing plasmid pHP3ApUC). These plasmids were constructed by Drs. S. Pati, A. Vallerga, and K. Lentine. Both plasmids were sequenced, and the inserts in both cases were identical to the sequence shown above. This fragment contains the sequence $(dT)_{13}/(dA)_{13}$ (at positions 103−115), which provides a specific binding site for PNA Lys−T_{10}−Lys. Superhelical plasmids were purified using a large-scale plasmid purification kit (QIAGEN), with additional ethanol precipitation and 70% ethanol washing steps. The samples were air-dried and dissolved in TE buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA). Linear plasmids were obtained by digestion of the superhelical plasmid with the restriction enzyme Sca I (Gibco BRL), which has a single recognition site distant from the HPRT gene insert. To avoid contamination with residual superhelical plasmid DNA, the linear plasmids were purified from agarose gels using a QIAquick gel-purification kit (QIAGEN), followed by additional ethanol precipitation, a rinse with 70% ethanol, air-drying, and resuspension in TE buffer. Experiments performed with non-gel-purified linear plasmids produced results similar to those with the gel-purified linear plasmids (data not shown).

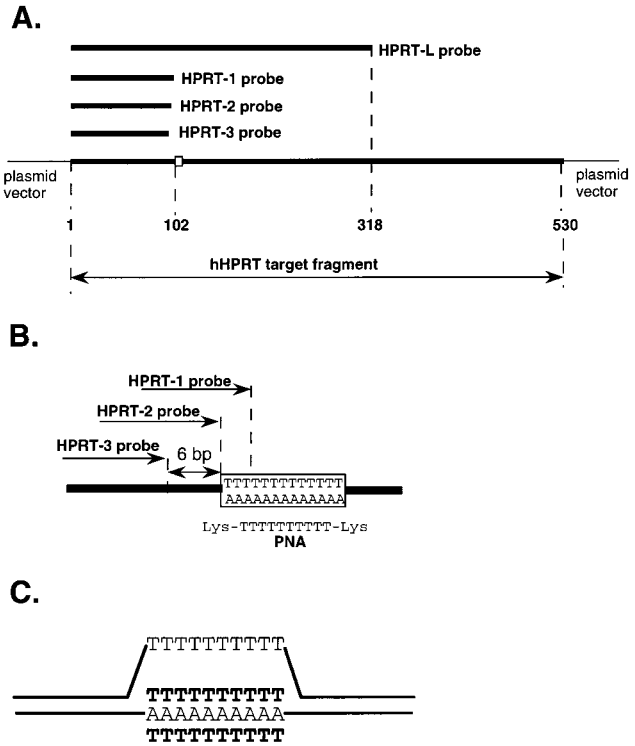

FIGURE 1: DNA substrates and PNA. (A) Scheme of probes. The PNA-binding site $(dA/dT)_{13}$ is shown by a small open rectangle. (B) Detailed scheme of the probe locations in the vicinity of the PNA binding site. Note that because the PNA is 10 bases long, it could be "shifted" relative to the edge of the $(dA/dT)_{13}$ insert on up to three bases. We used the shorter PNA because the oligoT-PNA solubility strongly decreases with increasing length. (C) Complex formed by oligo T-PNA and the target. Two PNA strands form a triplex with the dA strand of the target, while the dT strand of the target remains unpaired.

Complementary Single-Stranded (css)DNA Probes. The double-stranded DNA fragments for producing (css)DNA probes were obtained by PCR from the target plasmid using 5′-biotinylated primers, which allows monitoring of probe−target hybrid formation using chemiluminiscent assays for biotin-labeled DNA (see below). The position of the DNA probes relative to that of the PNA-binding site is shown in Figure 1A, B. In the case of the probes HPRT-1, HPRT-2, and HPRT-3, only one (left) primer, which is distal from PNA-binding sequence T_{13}, was biotinylated. In the case of the long HPRT-L probe, we used either one or both biotinylated primers (indicated in captions to Figures) and obtained similar results.

The precise positions of the PCR primers within the human HPRT gene fragment were as follows (positions are defined relative to the human HPRT gene fragment sequence shown above): For all DNA probes, the left primer was the same

and occupied the position 1→21. The right primer position was at 105→85 in the case of the HPRT-1 probe, at 102→82 in the case of the HPRT-2 probe, at 96→76 in the case of the HPRT-3 probe, and at 318→296 in the case of HPRT-L probe.

PCR products were purified from agarose gels using a QIAquick gel-purification kit (QIAGEN), followed by additional ethanol precipitation, rinsing with 70% ethanol, air-drying, and resuspention in TE buffer.

For homologous DNA targeting of pBluescript II SK(+) plasmid, a 537 bp-long PCR fragment was used, corresponding to the pBluescript II SK(+) sequence between positions 513 and 1049.

To obtain RecA protein-coated cssDNA probes, 50 ng of the PCR fragment in 13.4 μL was denatured by incubation in a boiling water bath for 3 min and then chilled in ice for 2 min. Next, 9.7 μL of a freshly prepared mixture containing 6 μL of buffer (50 mM Tris acetate (pH 7.5), 250 mM sodium acetate (pH 7), 10 mM magnesium acetate, and 5 mM DTT) and 3.7 μL of 16.2 mM ATPγS was added, immediately followed by 0.7 μL of RecA protein (3 mg/mL). The mixture was incubated for 17 min at 37 °C.

PNA. The sequence $(dT)_{13}/(dA)_{13}$ within the human HPRT gene fragment provides a specific binding site for PNA $(H_2N)Lys(T_{10})Lys(NH_2)$, which was used in this work (Figure 1B). This PNA binds to its homologous DNA sequence forming a structure where the A-strand of the double-stranded target DNA forms a triplex with two PNA molecules and the displaced T-strand of the target DNA is unpaired (Figure 1C) (for review see *9*, *15*, *16*).

PNA $(H_2N)Lys(T_{10})Lys(NH_2)$ was purchased from PE Biosystems (MA) and dissolved in ddH_2O at a final concentration of 100 μM, following the instructions provided by the supplier. The stock solution was kept at 4 °C. Before use, PNA was heated at 50 °C for 10 min and then cooled to room temperature.

Preincubation of Target Plasmid with PNA and the Homologous Targeting Reaction. In most experiments, 500 ng of the target plasmid DNA was incubated with PNA (final concentration of 8 μM) for 3.5 h at 37 °C in 8 mM Tris HCl, pH 8.0, and 0.8 mM EDTA. The total volume of the mixture was 5 μL. Next, 1.2 μL of 200 mM magnesium acetate was added, followed by the addition of the mixture containing the RecA protein-coated cssDNA probe.

The targeting reaction mixture was incubated for 2 h at 37 °C. Next, 4 μL of 10% SDS (w/v) and 3.8 mL of the loading buffer (30% glycerol (v/v), 0.1% bromphenol blue in TAE buffer) were added, and then samples were incubated for 5 min at 20 °C and loaded onto the gel. All modifications of this standard procedure are indicated in the captions to the corresponding figures.

Gel Electrophoresis and Visualization of Probe−Target DNA Hybrids. Gel electrophoresis was performed in 1.2% agarose gels in TAE buffer at 1 V/cm for 20 h. Next, DNA in the gels was stained with ethidium bromide solution (1μg/mL) in TAE buffer and photographed using a CCD camera. The gels were then soaked in 10 × SSC buffer (*21*), and DNA was transferred from the gels onto a positively charged nylon membrane (TROPIX) by capillary transfer (*21*) during at least 20 h. Ethidium bromide staining of DNA in the gels after the transfer indicated that most of the target DNA was transferred to the membrane. After the transfer, DNA was cross-linked to the membrane using a UV Stratalinker (Stratagene). Next, the membrane was processed using a kit for detection of biotinylated DNA (TROPIX) and monitored by exposure to X-ray film.

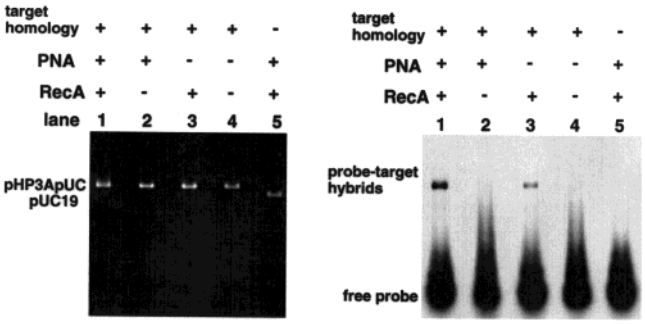

FIGURE 2: Targeting reactions with the HPRT-L probe. Reactions were performed under standard conditions with plasmid pHP3ApUC linearized by Sca I. Both probe strands were 5′-biotinylated. The left panel shows DNA in an ethidium bromide-stained gel. The right panel shows DNA transferred from the gel to a membrane, processed using a kit for detection of biotinylated DNA, and monitored by X-ray film, where only biotinylated DNA (i.e., probe−target DNA hybrids and free probes) can be observed (see Materials and Methods).

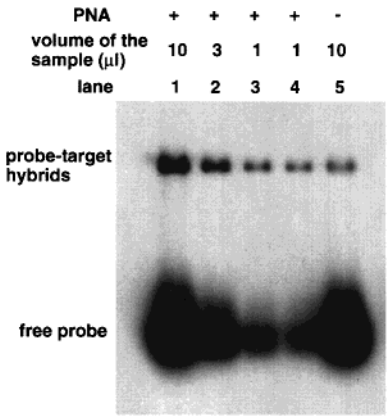

FIGURE 3: Estimation of the effect of PNA on hybrid yield. Reactions were performed under standard conditions with the probe HPRT-L (both probe strands were 5′-biotinylated), and the plasmid pHP3ApUC was linearized by Sca I. After the targeting reaction was complete, 10, 3, and 1 μL of the PNA(+) sample and 10 μL of the PNA(−) sample were loaded onto gel (as shown). The load of 1 μL of the PNA(+) sample was duplicated. The intensity of 1 μL of the PNA(+) sample is close to the intensity of 10 μL of the PNA(−) sample. Thus, PNA increases the yield of the hybrids by about 10-fold.

RESULTS AND DISCUSSION

Effects of PNA on Targeting Linear DNA. Figure 2 shows a DNA targeting experiment using the probe HPRT-L, where the PNA-binding site is localized within the region of the probe−target DNA homology. Hybrid formation was detectable only with a homologous DNA target in the presence of RecA protein (right panel, lanes 1, 3), and the presence of PNA strongly increased the yield of probe−target DNA hybrids (lane 1 versus lane 3). Figure 3 shows that the addition of PNA increased the yield of the probe−target DNA hybrids by about 10-fold. A similar enhancing effect of PNA was observed for probe HPRT-1, which has a three-base overlap with the PNA binding site, and also for probe HPRT-2, which is adjacent to the PNA-binding site (Figure 4, lanes 1−4).

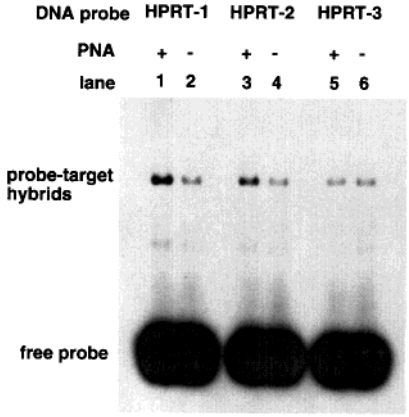

FIGURE 4: Effect of PNA on hybrid yields for HPRT-1, HPRT-2, and HPRT-3 probes. The reactions were performed under standard conditions, with the plasmid pHP3ApUC linearized by Sca I. PNA increases the yield of the hybrids for HPRT-1 and HPRT-2 probes but does not noticeably affect the yield of the hybrids for the HPRT-3 probe.

In the case of the probe HPRT-3, which is separated from the PNA-binding site by six bp, the yield of the hybrids was not affected by PNA (Figure 4, lanes 5, 6). Also, PNA did not improve the targeting of the linear double-stranded pBluescript plasmid, which does not have a specific binding site for this PNA (data not shown). Thus, to improve the RecA protein-mediated targeting reaction, the PNA binding must occur within or adjacent to the area of the probe−target homology.

Possible Mechanisms for PNA-Enhanced Probe−Target DNA Hybrid Formation. In the experiments described above, we tested the linear DNA targets and linear probes, which are homologous to the sequence that is distant from the end of the target DNA. In this case, only double D-loop DNA hybrids are stable following deproteinization, as detected by gel-shift assay (*4*, *5*, *7*). Thus, the hybrids in Figures 2−4 probably are double D-loop DNA hybrids. During double D-loop formation, both cssDNA probes hybridize to one target DNA molecule (Figure 5). In accordance with our initial hypothesis, PNA binding can facilitate the first probe strand's invasion by providing the target DNA opening. However, in the case of double D-loops, another explanation is also possible. According to the experimental data, within the three-stranded complex formed by the first RecA protein-coated probe strand and the target, the displaced target strand (which is complementary to the second probe strand) is localized close to two other strands and could interact with two other strands and with the RecA protein within the complex (*22*−*26*). Thus, the displaced strand would not be readily accessible to a bulky RecA protein-coated nucleoprotein filament; consequently, the second RecA protein-coated probe strand would not readily hybridize with it.

However, we hypothesize that occasionally, because of thermal fluctuations, the three-stranded complex may adopt a rare "open" conformation where the displaced target strand has enough room for hybridization with the RecA protein-coated second probe strand. This energetically unfavorable open conformation can be achieved by the opening of several additional base pairs of the target DNA at the flanks of the complex or by "conformational stretching" at the flanks of the displaced strand. Though the binding of the second probe strand to the displaced strand is energetically favorable and

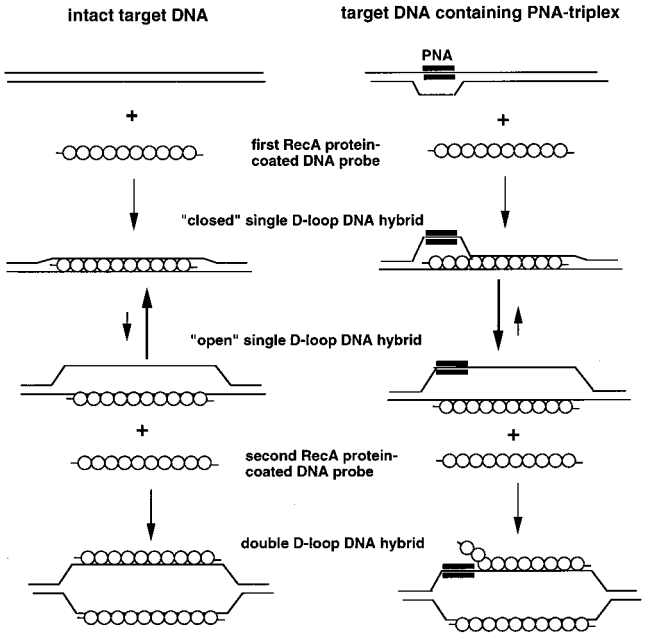

FIGURE 5: Scheme for double D-loop DNA hybrid formation using intact target DNA (left) and a complex of the target DNA with PNA (right). Small circles symbolize the RecA protein. PNA binding interferes with the close contact of the displaced target strand with two other strands and with RecA protein, thus facilitating the "open conformation" of the complex. If the PNA binding site is localized at the flank of the probe−target complex, then PNA binding provides stable unwinding at the flank, which also facilitates the open conformation.

would compensate for the energetically unfavorable distortions at the junctions of the complex, the requirement for these distortions creates a kinetic barrier for the hybridization of the second probe strand. As shown in Figure 5, PNA shifts the equilibrium toward the open complex by disrupting the contact between the displaced strand and two other strands and the RecA protein within the complex and by opening the flanks. Consequently, PNA accelerates double D-loop formation. Our experiments with the supercoiled target DNA (see below) suggest that this mechanism is at least partially responsible for the effect of PNA on RecA-mediated targeting.

Effect of PNA on Targeting Negatively Supercoiled DNA. We investigated hybrid formation with negatively supercoiled DNA targets (Figure 6). The yields of the hybrids are significantly higher with negatively supercoiled DNA (Figure 6, right panel, lanes 3, 4, 9, 10) than with linear DNA (Figure 6, right panel, lanes 1, 2, 7, 8) because negative supercoiling facilitates the formation of structures (including D-loops) in which the target DNA strands are unwound (for review see *27*). Because of this fact, (i) strand invasion into superhelical DNA occurs much faster, and (ii) both single and double D-loops are kinetically stable within negatively supercoiled DNA. D-loop formation partially relaxes negative superhelical stress within the target DNA, which decreases the electrophoretic mobility of the hybrids (Figure 6, right panel, lanes 3, 4, 9, 10) in comparison with that of the untargeted superhelical DNA (Figure 6, left panel, lanes 3, 4, 9, 10).

Figure 6 shows that, in contrast to the linear DNA targeting (right panel, lanes 1, 2), PNA decreases the yields of the hybrids formed by RecA protein-coated cssDNA probes with negatively supercoiled DNA (right panel, lanes 3, 4). In the

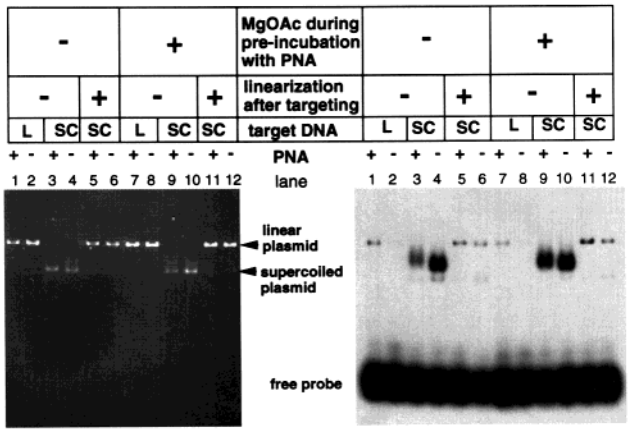

FIGURE 6: Comparison of the effect of PNA on double-stranded linear DNA targets and negatively supercoiled DNA targets. The left panel shows DNA in an ethidium bromide stained gel. The right panel shows DNA transferred from the gel to a membrane, processed using a kit for detection of biotinylated DNA, and monitored by X-ray film where only biotinylated fractions (i.e., probe−target hybrids and free probes) can be observed (see Materials and Methods). Probe HPRT-1 and the target plasmid pHP3ApUC (either superhelical (SC) or linearized (L) by Sca I) were used in the targeting reaction. For lanes 1−6, all PNA preincubation and targeting reaction conditions were standard. In lanes 7−12, the only modification from the standard protocol was that 1.2 μL of 200 mM magnesium acetate was added to the target DNA before PNA addition rather than after preincubation with PNA. After the targeting reaction was completed, 3 μL of each sample (which contain formed probe−target DNA hybrids) was mixed with 12 μL of buffer REact6 (50 mM Tris HCl, 50 mM NaCl, 50 mM KCl, 6 mM $MgCl_2$, pH 7.4) (GibcoBRL). Next, to samples 5, 6, 11, and 12, restriction enzyme Sca I (1 μL, 10 units/μL) was added, which linearizes the supercoiled target DNA by cutting in the site distant from the HPRT insert. Thus, for samples 5, 6, 11, and 12, the hybrids were formed with supercoiled targets and then were linearized. All samples were incubated at 37 °C for 1.5 h, deproteinized with SDS, and analyzed by gel electrophoresis.

case of preincubation with PNA, hybrids with supercoiled targets have a slower electrophoretic mobility, as monitored in agarose gels (compare lanes 3 and 4 in the right panel). Because the hybrids with linear DNA are not retarded in their electrophoretic mobility in gels, the retardation of the migration of hybrids with supercoiled targets is due to a larger relaxation of the superhelical stress in the presence of PNA. This finding suggests that under conditions of low ionic strength and negative superhelical stress, both of which strongly facilitate PNA invasion (*14*, *18*, *28*), PNA invades short and mismatched "semispecific" (dT/dA) stretches, producing local DNA unwinding. Under high negative superhelical stress, target DNA unwinding can expand into the flanking sequences (especially if they are AT-rich), causing further relaxation of negative superhelical stress within negatively supercoiled DNA targets. Because negative superhelical stress strongly facilitates RecA-filament invasion, this relaxation most likely explains the PNA inhibition of RecA-mediated targeting in superhelical target plasmids. We observed similar inhibition by PNA for the targeting of superhelical pBluescript plasmid (data not shown). Thus, this effect does not require the presence of an HPRT insert in the target. However, the magnitude of this effect might depend on the plasmid sequence.

When PNA invasion was performed in the presence of Mg^{2+}, the inhibitory effect of PNA on superhelical DNA targeting (as well as additional target relaxation induced by PNA) strongly decreased (Figure 6, right panel, lanes 9 and 10). This decrease is most likely caused by additional salt that inhibits PNA invasion into semispecific sites.

Remarkably, when the superhelical hybrids were linearized by digestion with the restriction enzyme Sca I (Figure 6, lanes 5, 6, 11, 12), the amounts of linear hybrids obtained were noticeably higher in the presence of PNA, despite the fact that the initial amounts of superhelical hybrids were lower in the presence of PNA (Figure 6, right panel, lanes 3, 4, 9, 10). Because only double D-loop DNA hybrids are stable following target linearization (*4*, *5*, *7*), these results show that the ratio of double D-loops to single D-loops is higher in the presence of PNA, which strongly suggests that the facilitation of probe−target hybrid formation by PNA for linear DNA targets is at least partially due to facilitated binding of the second probe strand to the three-stranded probe−target complex rather than to the intact target molecule.

The answer to the question of whether PNA facilitates the first probe strand invasion into the linear DNA target would require investigating single D-loop formation in linear DNA in both the presence and absence of PNA. However, single D-loops are not stable enough to be monitored by gel-shift assay, which we used in this work. Thus, more complex methods such as chemical or enzymatic probing must be used to establish the mechanism of PNA-facilitated DNA targeting directly.

PNA Enhances RecA Protein-Mediated Probe−Target Hybrid Formation at Physiological-like Concentrations of Mg^{2+} Ions. The rate of PNA invasion into double-stranded DNA strongly decreases with increasing ionic strength. However, when the PNA has already invaded the double-stranded DNA target, the PNA−DNA hybrid complex is stable at high ionic strength at physiological temperatures (*18*). Thus, PNA invasion can be performed at low ionic strength, and when it is required for further manipulations, the ionic strength can be increased. However, a slower PNA invasion was observed at physiological ionic conditions (*29*).

According to our standard protocol (see Materials and Methods), PNA and the target DNA were preincubated at low ionic strength (8 mM Tris HCl, pH 8, 0.8 mM EDTA) for 3.5 h at 37 °C, and then magnesium acetate was added to a final concentration of 39 mM. Next, RecA protein-coated cssDNA probes were added, and the targeting reaction was incubated for 2 h at 37 °C. The final concentration of metal ions during the targeting was 10 mM Mg^{2+} and 50 mM Na^+ (see Materials and Methods for details). However, we found that the addition of magnesium acetate into the target DNA-containing solution before the addition of PNA does not decrease the PNA-induced enhancement of targeting to linear DNA (Figure 6, right panel, lanes 7, 8) and decreases the PNA-induced inhibition of targeting to negatively supercoiled DNA (Figure 6, right panel, lanes 9, 10), presumably by inhibition of semispecific PNA invasion into the superhelical target.

Further experiments showed that the preincubation of the target DNA with PNA before addition of RecA protein-coated cssDNA probes can be omitted without a noticeable effect on the yield of the hybrids (Figure 7). Thus, both PNA and cssDNA probes can react with the target simultaneously at physiological-like ionic conditions. These observations

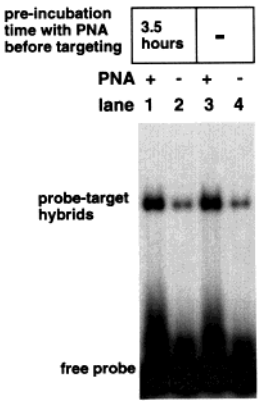

FIGURE 7: Preincubation with PNA before the targeting reaction is not required for PNA-enhanced targeting. Probe HPRT-L, 5′-biotinylated at the left end, and the target plasmid pHP3A, linearized by Sca I, were used in these experiments. The target DNA in samples 1 and 2 was incubated with and without PNA, respectively, for 3.5 h at 37 °C in the presence of magnesium acetate, as was done for samples 7−12 in Figure 6. For samples 3 and 4, PNA and an equivalent amount of H_2O, respectively, were added to the reaction mixture containing the target plasmid and magnesium acetate immediately before the addition of the RecA protein-coated cssDNA probes.

become important in the potential biological application of PNA-improved RecA-mediated DNA targeting.

Potential Applications of PNA-Enhanced Homologous DNA Targeting. In this work, we demonstrated that PNA binding within or adjacent to the area of the probe−target homologous sequence significantly increases the yield of the hybridization reaction between linear DNA targets and the RecA protein-coated cssDNA probes.

An important application would be in sequence-specific physical isolation of double-stranded DNA from complex mixtures. This procedure is often referred to as DNA capture. In DNA capture protocols, various modes of the target DNA recognition can be utilized, including homopurine−homopyrimidine triplexes (*30*), DNA displacement loops (D-loops) (*31*), RecA nucleoprotein filaments−DNA hybrids (*32*−*34*), and composite PNA−DNA hybrid structures ("PD-loops") (*20*, *35*).

One DNA capture protocol, which is commonly used for rapid DNA cloning from plasmid DNA libraries, utilizes RecA protein-mediated recognition of homologous DNA by cssDNA probes (*4*). In this method, the biotinylated RecA protein-coated cssDNA probes hybridize to the homologous target DNA or to the family of target DNAs that share significant homology with the probe. Next, the probe−target DNA hybrids are captured and retained on streptavidin-coated magnetic beads, while nonbound DNA is washed out. The DNA population is thus enriched with the DNA of interest and then is eluted. This method can be applied to the capture of linear DNA targets, including sheared genomic DNA (*4*). The data presented in this work shows that PNAs can improve the linear target DNA capture by increasing the yield of probe−target hybrids.

Other potential applications of PNA with cssDNA probes include modification of target DNA sequences in vivo, using cssDNA probes containing heterologous sequence insertions or deletions. This process probably involves probe invasion into the target DNA (reviewed in *2*, *3*), and recently it was hypothesized that DNA mismatch correction by RNA−DNA chimeras may proceed via probe invasion into the target DNA, followed by formation of double D-loop structures (*36*). In addition to facilitating the probe invasion, as demonstrated in the present work, PNA−DNA hybrids may create "hot spots" for DNA recombination and DNA repair by creating structural distortions within the target DNA or by interfering with target DNA copying. Our observations (Figure 7) that PNA and RecA protein-coated cssDNA probes invade the target DNA in the same reaction mixture under near-physiological conditions support potential in vivo gene modification applications of PNA, together with RecA protein-coated cssDNA probes.

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