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Single base pair mutation analysis by PNA directed PCR clamping

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ABSTRACT

A novel method that allows direct analysis of single base mutation by the polymerase chain reaction (PCR) is described. The method utilizes the finding that PNAs (peptide nucleic acids) recognize and bind to their complementary nucleic acid sequences with higher thermal stability and specificity than the corresponding deoxyribooligonucleotides and that they cannot function as primers for DNA polymerases. We show that a PNA/DNA complex can effectively block the formation of a PCR product when the PNA is targeted against one of the PCR primer sites. Furthermore, we demonstrate that this blockage allows selective amplification/suppression of target sequences that differ by only one base pair. Finally we show that PNAs can be designed in such a way that blockage can be accomplished when the PNA target sequence is located between the PCR primers.

INTRODUCTION

A multitude of human genetic diseases result from single base mutations in specific genes (1). To facilitate the *in vitro* analysis of such mutations several techniques have been devised. These include enzymatic (2) or chemical (3–4) probing of mismatch complexes, gradient gel electrophoresis (5), use of nucleotide analogues (6) hybridization with allele specific oligonucleotide probes (7) and the oligonucleotide ligation assay (8). To enhance the sensitivity of these methods the target nucleic acid is normally amplified to detectable quantities by the polymerase chain reaction (PCR) (9).

PCR itself has also been used to analyse directly single base mutations by using allele specific oligonucleotides as amplification primers (10–12). Unfortunately, the general applicability of this approach is limited by the fact that the majority of primer–template mismatches have no significant effect on the amplification process (13).

We recently found that PNA (Peptide Nucleic Acid) is a potent DNA mimic in terms of sequence specific hybridization, and obtained results showing that at physiological ionic strength PNA/DNA duplexes are generally 1°C per base pair more stable thermally than the corresponding DNA/DNA duplexes (14–17). Furthermore, our results indicated that the base pair mismatch discrimination is greater for PNA/DNA than for the corresponding DNA/DNA duplexes. In the special case of homopyrimidine PNA, $(PNA)_2$/DNA triplexes are formed of unprecedented thermal stability and sequence discrimination with complementary oligonucleotides. For example, the complex of PNA T_{10} with dA_{10} exhibits a T_m of 76°C, with ΔT_m's for base mismatches ranging between 10–13°C (18). Similarly, T_m for a PNA $(T_4CT_5)_2/dA_4GA_5$ complex is 79°C, with ΔT_m's for base mismatches ranging between 30–35°C (18).

Taking advantage of these unique properties of PNA, and the fact that PNA cannot function as a primer for DNA polymerase, we now report that PNA can be used to block a PCR amplification process in a sequence specific manner. Furthermore, we show that the specificity of this approach, termed 'PCR clamping', is such that two alleles which differ by only one base pair can be discriminated. Thus this technique allows for direct analysis of single base mutations by PCR.

MATERIALS AND METHODS

The PNAs H-T_{10}-LysNH$_2$, H-T_5CT_4-LysNH$_2$, PNA62 (H-TG-TACGTCACAACTA-NH$_2$) and PNA176 H-GATCCTGTAC-GTCACAACTA-NH$_2$ were synthesized as described (15–17). The plasmid pT10KS was constructed by cloning the complementary oligonucleotides 5'-GATCCT_{10}G and 5'-GATCCA_{10}G into the *Bam*HI site of the Bluescript KS$^+$ plasmid (Stratagene). The plasmid pT9C was constructed by cloning the complementary oligonucleotides 5'-TCGACT_5CT_4G and 5'-TCGACA_4GA_5G into the *Sal*I site of pUC19. The plasmid p62-1 was constructed by first cloning the complementary oligonucleotides 5'-GATC-

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CTGTACGTCACAACTA-3′ and 5′-GATCTAGTTGTGACG-TACAG-3′ into the *Bam*HI site of pUC19 to obtain p62, followed by cloning of a 556bp *Pst*I/*Hind*III fragment from the phage λ genome into the *Pst*I/*Hind*III site of p62. The plasmids p62-A-KS, p62-T-KS and p62-C-KS were isolated from a mini-library constructed by cloning the degenerate, complementary oligonucleotides 5′-TCGACTCTAGAGGATCTAGTTGTGANGT-ACAG-3′ and 5′-GATCCTGTACNTCACAACTAGATCCT-CTAGAG-3′ into the *Sal*I/*Bam*HI site of bluescript KS^+ (Stratagene). The control plasmids pCKS and PCKS-1 were Bluescript KS^+ derivatives which do not contain a target sequence for any of the PNAs used in this study. Using standard techniques (19) plasmids were isolated from selected clones of recombinant *E.coli* JM103, purified by buoyant density centrifugation in CsCl gradients and sequenced by the dideoxy method.

The following oligonucleotide primers were used in the PCR reactions: reverse primer (5′-GAAACAGCTATGAC-3′), reverse-1 primer (5′-CACACAGGAAACAGCTATGAC), forward primer (5′-GTAAAACGACGGC-3′), forward-1 primer (5′-GTAAAACGACGGCCAGT), proximal primer (5′-TACC-CGGGGATC-3′) and primers specific for each of the p62 plasmids: p62-1 primer (5′-TGTACGTCACAACTA-3′), p62-A-1 primer (5′-TGTACATCACAACTA-3′), p62-A-2 primer (5′-CCTGTACATCACAACTA-3′), p62-A-3 primer (5′-ATCCTGTACATCACAACTA-3′), p62-A-4 primer (5′-G-GATCCTGTACATCACAACTA-3′), p62-A-5 primer (5′-GT-GGATCCTGTACATCACAACTA-3′), p62-T primer (5′-GGA-TCCTGTACTTCACAACTA-3′) and p62-C primer (5′-GGA-TCCTGTACCTCACAACTA-3′).

PCR amplifications were carried out in a 50μl volume containing 0.1ng of each plasmid, 0.2μM of each primer, 200μM dNTP and buffer (10mM Tris−HCl, pH 8.3 (at 25°C), 10mM KCl, and 3mM $MgCl_2$). The PCR reactions were overlaid with 2 drops of paraffin oil and incubated at 96°C for 2 minutes before the amplification process was initiated by the addition of 3U of the Stoffel polymerase (Perkin Elmer Cetus) or 1U of the supertaq polymerase (AH Diagnostics). When using the supertaq polymerase the buffer was changed to (50mM Tris−HCl, pH 9.0 (25°C), 50mM KCl, 7mM $MgCl_2$, 16mM $(NH_4)_2SO_4$ and 0.2mg/ml BSA). Experiments were carried out using either a Minicycler™ (MJ Research) amplifier machine or a LEP amplifier machine (IgG Biotech). Comparative results were obtained independent of the machine and polymerase used. PCR cycle profiles and concentrations of PNAs were as indicated in the figure legends.

T_m values for PNA/DNA DNA/DNA duplexes were determined spectrofotometrically at 260nm in 10mM Na-phosphate, 150mM NaCl and 1mM $MgCl_2$.

RESULTS

PNAs can effectively block the formation of a PCR product containing a complementary target sequence

Given the higher thermal stability of a PNA/DNA duplex compared to the corresponding DNA/DNA duplex we speculated that PNA might be able to block PCR in a sequence specific manner if targeted against one of the PCR primer sites. Clearly, for such a blocking mechanism to work the PNA must compete effectively against its cognate PCR primer in binding to their common recognition site. To facilitate this requirement, the normal 3 step PCR cycle was expanded with a distinct PNA annealing step which 1) precedes the PCR primer annealing step and 2) is set at a temperature that allows only the PNA to bind to its target sequence (Figure 1).

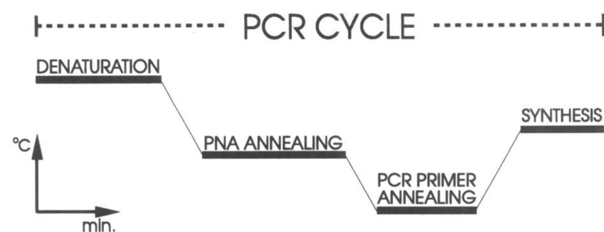

Figure 1. Schematic representation of the PCR cycle profile used in PNA directed clamping.

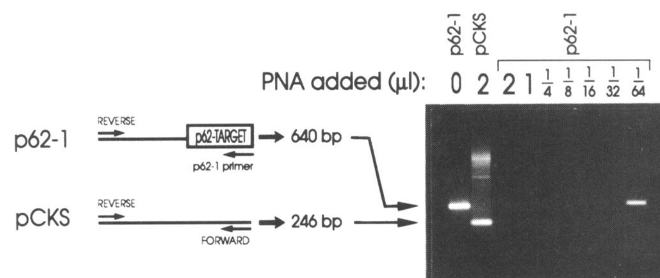

Figure 2. Experimental setup and result of a PCR clamping experiment in the presence of increasing concentrations of PNA62. Lane 1: amplification of the p62-1 plasmid in the absence of PNA62. Lane 2: amplification of the pCKS control plasmid (plasmid containing no PNA62 target) in the presence of 17.8μM PNA62. Lanes 3−9: amplification of the p62-1 plasmid in the presence of 17.8μM (3), 8.9μM (4), 2.2μM (5), 1.1μM (6), 0.6μM (7), 0.3μM (8) and 0.15μM (9) PNA62. PCR cycle conditions were 96°C, 2min−65°C, 1min−40°C, 30sec−60°C, 2min−30 cycles.

Figure 2 shows the experimental setup and result of a PCR clamping experiment in the presence of increasing amounts of a 15mer PNA, PNA62 (H-TGTACGTCACAACTA-NH_2). Two plasmid templates were used: the p62-1 plasmid which directs the amplification of a 640bp fragment containing a PNA62 target site and the control plasmid, pCKS, which directs the amplification of a 246bp non-target fragment. When PNA62 is either absent (lane 1) or present at a concentration of 0.15 μM (lane 9) the p62-1 plasmid directs the synthesis of the expected 640bp PCR fragment. At concentrations at or above 0.3μM PNA62, however, no product is produced (lanes 3 to 8). The absence of product is not due to a non-specific inhibitory effect of PNA62 on PCR, since even at the highest concentration used (17.8μM) PNA62 will not inhibit the amplification of the expected 246bp fragment from the pCKS control plasmid (lane 2). Furthermore, the ability to clamp PCR is not the result of some unique property of PNA62 since similar results could be obtained with other mixed sequence PNAs (data not shown).

Clamping can be accomplished when the PNA target site is located between the two PCR primers

We next analysed whether the PNA would be able to clamp PCR independent of the relative position of the PNA and PCR primer target sites. We compared the overlap of PNA and PCR primer target sites to the situations where the PNA target site is either 1) located adjacent to a PCR primer site or 2) located in the

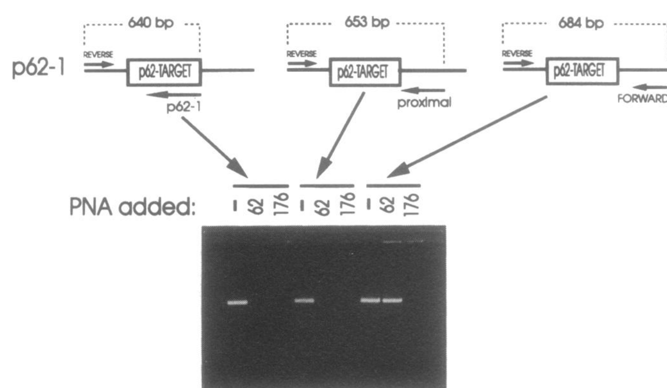

Figure 3. Experimental setup and effect on PNA62 directed clamping of changing the relative position of the PNA and PCR primer target sites. The 3'-end of the forward primer is located 31bp downstream of the PNA62 target site and 26bp downstream of the PNA176 target site. The 3'-end of the proximal primer is located 1bp downstream of the PNA62 target and overlaps the PNA176 by 4bp. The p62-1 primer exactly overlaps the PNA62 target site. Lanes 1−3: amplification of the p62-1 plasmid with reverse and p62-1 primers in the absence (1) or presence of 17.8μM of PNA62 (2) or 17.8μM of PNA176 . Lanes 4−6: amplification of the p62-1 plasmid with reverse and proximal primers in the absence (4) or presence of 17.8μM of PNA62 (5) or 17.8μM PNA176 (6). Lanes 7−9: amplification of the p62-1 plasmid with reverse and forward primers in the absence (7) or presence of either 17.8μM PNA62 (8) or 17.8μM PNA176 (9). PCR cycle conditions were 96°C, 2min−65°C, 1min−40°C, 30sec−60°C, 2min−30 cycles.

middle of the PCR region. There are fundamental differences in the underlying mechanism of clamping in these three cases. When the PNA and PCR primer target sites overlap, clamping operates by 'primer exclusion'. Conversely, when the target site is located at a distance from the PCR primer sites, clamping is expected to operate by preventing read-through by the Taq polymerase ('elongation arrest'). Finally, when the PNA target is located adjacent to the PCR primer site, clamping is likely to operate by either preventing polymerase access to the PCR primer and/or by preventing initiation of primer elongation.

Figure 3 shows the experimental setup and the result of changing the relative position of the PNA and PCR primer target sites. Using PNA62, clamping can be accomplished efficiently when the PNA target site either overlaps (lane 2) or is located adjacent to a PCR primer site (lane 4). However, when the PNA target site is located at a distance from the PCR primer site no clamping is observed after 30 cycles (lane 8) suggesting that this PNA/DNA complex is unable to prevent read-through by the polymerase. To test whether an extented PNA62, with an increased T_m for its complementary DNA target, was capable of clamping we synthesized PNA176 (H-GATCCTGTACGTC-ACAACTA-NH_2) which is complementary to the PNA62 target plus the first 5 flanking base pairs in the plasmid. As shown in Figure 3, PNA176 efficiently clamps the PCR process independent of the position of the PNA target site; lane 3: overlapping PNA and PCR primer target sites, lane 6: proximal PNA and PCR primer target sites and lane 9: widely spaced PNA and PCR primer target sites. In experiments with another DNA target sequence we have found that a shorter PNA than PNA62, with a correspondingly lower T_m, is succesful in clamping its cognate PCR when its target site is located at a distance from the PCR primers (data not shown). Thus, it would appear that this clamping ability may be a complex function of affinity and kinitics of dissociation.

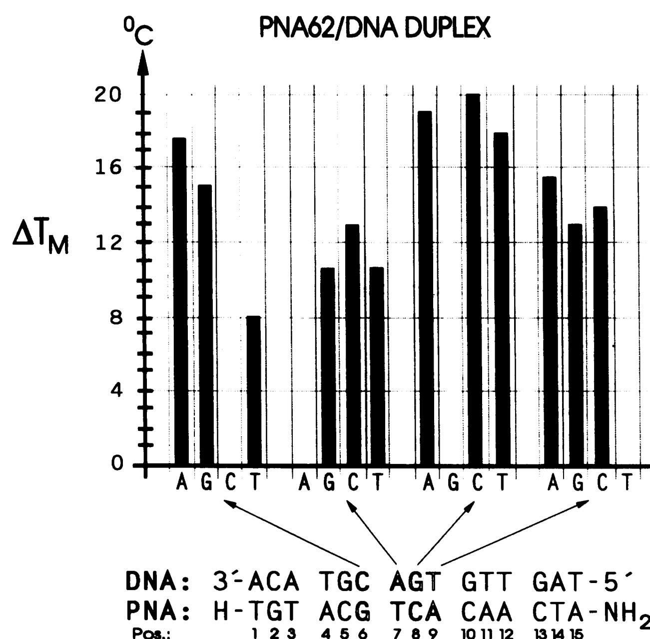

Figure 4. Schematic representation of the effect on T_m of introducing single base mismatches in a PNA62/DNA complex. A series of anti-parallel DNA oligonucleotides were synthesized which contained different single base mismatches to the PNA62 at position 6−9. The T_m value of the fully complementary PNA/DNA duplex is 69°C. The ΔT_m values shown in the Figure indicate the reduction in thermostability that results from the introduction of single base mismatches in the helix.

PNA directed clamping can be used to analyse single base mutations

To explore the potential of the method we tested whether PNA62 would be able to discriminate between fully complementary and single base mismatch targets in a mixed target PCR (i.e. where both targets are present in the PCR reaction mix). As shown in Figure 4 single base mismatches in the PNA62/DNA duplex lower the thermostability of the complex by 8−20°C depending on the type of mismatch and its position in the duplex. Based on these data we chose to analyse mutations at position 6 since these mutations span the largest temperature interval and also include the mutation that exhibits the least helix destabilizing effect, i.e. the PNA **G/T** DNA mutation (ΔT_m of 8°C).

The configuration chosen for the point mutation analysis was primer exclusion. The PCR reaction mix contained the p62-1 wildtype plasmid, the appropriate mutant p62-plasmid, primers specific for the wildtype and the mutant plasmids and the common reverse primer. Figure 5 shows the experimental setup and the result of the PNA **G/T** DNA mutation analysis. In the absence of PNA62 two PCR products of sizes corresponding to amplification of the p62-1 and p62-A-KS are produced (lanes 1, 3, 5, 7 and 9). In the presence of PNA62, however, the synthesis of a PCR product is dependent on the size of the mutant primer. When the mutant primer has a size similar to PNA62, addition of the PNA oligomer will suppress the amplification from both wildtype and mutant plasmid (lane 2). This is because the duplex between the mutant primer and its complementary target is less thermostable (T_m=52°C) than the mismatched PNA62/mutant duplex (T_m=61°C, Fig. 4). However, as the size of the mutant primer is increased its ability to compete with PNA62 for binding to its target sequence increases. Thus, at a

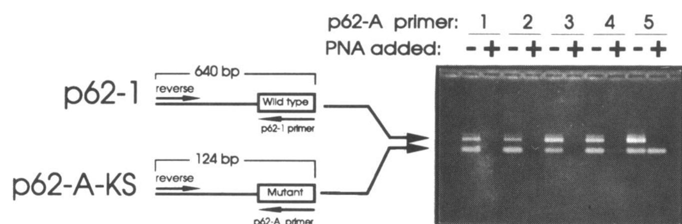

Figure 5. Optimization of the size of the mutant primer required to carry out selective PNA62 directed clamping of the wildtype p62-1 plasmid in the presence of the p62-A-KS single base mutated plasmid. Each reaction contains the p62-1 and p62-A-KS plasmid, the p62-1 primer, one of the p62-A-1 to 5 primers, the common reverse primer and 8.9μM PNA62. Lanes 1–2: amplification using the p62-A-1 primer in the absence (1) or presence (2) of PNA62. Lanes 3–4: amplification using the p62-A-2 primer in the absence (3) or presence (4) of PNA62. Lanes 5–6: amplification using the p62-A-3 primer in the absence (5) or presence (6) of PNA62. Lanes 7–8: amplification using the p62-A-4 primer in the absence (7) or presence (8) of PNA62. Lanes 9–10: amplification using the p62-A-5 primer in the absence (9) or presence (10) of PNA62. PCR cycle conditions were 96°C, 2min–65°C, 1min–40°C, 30sec–60°C, 2min–30 cycles.

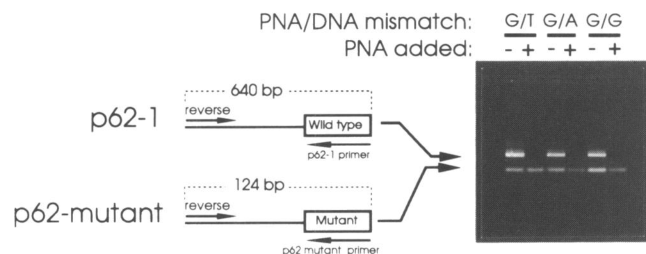

Figure 6. Point mutation analysis with PNA62. Each PCR reaction contains the p62-1 plasmid and one of the single base mutated plasmids p62-A-KS, p62-T-KS or p62-C-KS, the common reverse primer, the appropriate allele specific primers (p62-1 primer, p62-A-5 primer, p62-T-4 primer, p62-C-4 primer) and 8.9μM PNA62. Lanes 1–2: co-amplifications of the p62-1 and p62-A-KS plasmids in the absence (1) and presence (2) of PNA62. Lanes 3–4: co-amplifications of the p62-1 and p62-T-KS plasmids in the absence (3) and presence (4) of PNA62. Lanes 5–6: co-amplifications of the p62-1 and p62-C-KS plasmids in the absence (5) and presence (6) of PNA62. PCR cycle conditions were 96°C, 2min–65°C, 1min–40°C, 30sec–60°C, 2min–30 cycles.

mutant primer size of +8 nucleotides (relative to the 15mer PNA62) the p62-A-KS plasmid directs the amplification of a small amount of PCR product (lane 8) and at a mutant primer size of +10 this amplification product is readily visible in the gel (lane 10). Even at a size of +10, however, the mutant primer will not prevent PNA62 from clamping its wildtype target as shown by the lack of the 640bp band in lane 10.

Using a similar experimental setup we then determined the optimal size for the T-mutant and C-mutant primers. These data are compiled in Figure 6 which shows that PNA62 is able to carry out selective suppression of its fully complementary sequence in the presence of all 3 possible point mutations at the position analysed; lane 2: PNA **G/T** DNA mismatch, lane 4: PNA **G/A** DNA mismatch and lane 6: PNA **G/G** DNA mismatch.

Clamping with homopyrimidine PNAs.

In the examples described above the PNAs contained both purine and pyrimidine nucleobases. Such mixed sequence PNAs form highly thermostable duplexes in a preferred anti-parallel

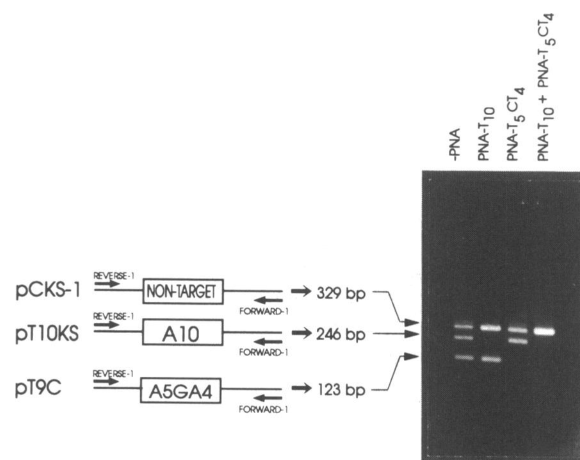

Figure 7. Single base mismatch analysis with two homopyrimidine PNAs complementary to a target sequence located at a distance from the PCR primers. Each PCR reaction contains the pT10KS, pT9C and pCKS-1 (control) plasmids and PNAs as indicated. Lane 1: co-amplifications in the absence of PNAs. Lane 2: co-amplifications in the presence of 3.3μM PNA H-T_{10}-LysNH_2. Lane 3: co-amplifications in the presence of 13.2μM PNA H-T_4CT_5-LysNH_2. Lane 4: co-amplification in the presence of 3.3μM H-PNA-T_{10}-LysNH_2 and 13.2μM PNA H-T_4CT_5-LysNH_2. PCR cycle conditions were 96°C, 2min–62°C, 3min–40°C, 1min–65°C, 2min–35 cycles.

orientation with their target DNA sequence (17). In contrast to this binding mode, homopyrimidine PNAs form extremely thermostable $(PNA)_2$/DNA triplexes with a preference for parallel orientation with their target DNA sequence (15–16). We therefore also wished to study the PCR clamp technique with such triplex forming PNAs. To obtain thermal stabilities comparable to the previously described PNA62/DNA duplex we chose two 10mer PNAs (PNA H-T_{10}-LysNH_2, T_m=75°C and H-T_5CT_4-LysNH_2, T_m=79°C) which differ from each other at a single base position. Both of these PNAs acted as efficient and sequence specific clamps in a PCR amplification process and both PNAs were very efficient in blocking their cognate PCR process independent of the location of the PNA and PCR primer sites (data not shown). Furthermore, as shown in Figure 7 both homopyrimidine PNAs were able to discriminate between their fully complementary and single base mismatch targets when the PNA target site is located at a distance from the PCR primer sites. In the absence of either of the PNAs three PCR products of sizes corresponding to amplification of the pT10KS, pT9C and pCKS-1 control plasmids are visible in the gel (Figure 4, lane 1). If PNA H-T_{10}-LysNH_2 is included in the PCR reaction alone the products corresponding to amplification of the pT9C and pCKS-1 control plasmids are seen (lane 2). Similarly, PNA H-T_4CT_5LysNH_2 suppresses the amplification of its cognate target fragment, whilst leaving amplification of the pT10KS and pCKS-1 control plasmids unaffected (lane 3). In the presence of both PNAs only the PCR product corresponding to the pCKS-1 control plasmid is seen.

DISCUSSION

We have used PNA to develop a method that converts a PCR amplification process into an efficient analytical tool for the direct detection of single base mutations. In our hands the method is very robust. The two different modes of PCR blocking by PNA

(primer exclusion and elongation arrest) and the two different ways in which PNA target recognition can occur (duplex vs. triplex), further provide great versatility and flexibility to the PNA/PCR clamp system.

It is interesting to note that clamping can operate efficiently even with incomplete binding of PNA to its DNA targets. For example, our calculations show that if 1% of all target sequences escape clamping in *each* cycle the maximum amplification factor after 30 cycles is only 9-fold, which will not generally produce a detectable signal on a gel. Indeed, in PCR amplifications of genomic material we predict that as much as 10% of the target sequences can escape clamping without generating a detectable signal (equivalent to a maximum amplification factor of 2500 in 30 cycles).

In a PNA clamping protocol with mixed sequence PNAs we prefer to use the primer exclusion principle for the following reasons. First, this clamping mode places the least physical demands on the PNA, i.e. clamping does not require that the PNA, once bound to its target, is able to prevent read-through by the polymerase as is the case in the elongation arrest clamping mode. Second, the only variables in the primer exclusion clamping mode are the T_m of the PNA and the PCR primers and these can be tuned to precision simply by changing either the sizes of the PNA and PCR primers, or by altering their exact position on the target DNA. Third, when using the primer exclusion principle there is the further advantage that, in addition to blocking its cognate target site, the PNA will compete with the PCR primer for any cryptic primer sites in the genome, thereby suppressing any occurrence of non-specific background in the PCR process directed by this primer.

In order to target unique sequences in the human genome a primer of at least 17bp is usually required, the T_m's of which typically range between 50−60°C. Thus, for the successful projection of our PNA clamping approach to the analysis of point mutations in the human genome, PNAs with T_m's above 60°C must be able to effectively discriminate between their fully complementary and single base mismatched target DNA. Using a mixed sequence 15mer PNA with a T_m of 69°C we have shown, in a model system, that three different point mutations at a single position can be discriminated, suggesting that PNA clamping can be used as an effective diagnostic tool for the analysis of mutations in complex genomes. We acknowledge that only three out of twelve possible mismatches have been analysed and only in a single sequence context. However, we have shown in the present study that the PCR clamp system can efficiently discriminate the most difficult case in our system (PNA G/T DNA mismatch with a ΔT_m of only 8°C). We are therefore confident that, given the great flexibility of the method, conditions can be found to discriminate any single point mutation. When such mutations are present at low frequency it is interesting to speculate that the PNA clamp may instead be directed against the large excess of non-mutated genes the presence of which leads to unwanted background in diagnostic procedures. We now intend to apply the PCR clamp technique to the analysis of point mutations in human, animal and plant genetic material.

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