3050–3055 Nucleic Acids Research, 1995, Vol. 23, No. 15

Enhanced PCR amplification of VNTR locus D1S80 using peptide nucleic acid (PNA)

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Received January 9, 1995; Revised and Accepted June 19, 1995

ABSTRACT

Use of the polymerase chain reaction (PCR) to amplify variable numbers of tandem repeat (VNTR) loci has become widely used in genetic typing. Unfortunately, preferential amplification of small allelic products relative to large allelic products may result in incorrect or ambiguous typing in a heterozygous sample. The mechanism for preferential amplification has not been elucidated. Recently, PNA oligomers (peptide nucleic acids) have been used to detect single base mutations through PCR clamping. PNA is a DNA mimic that exhibits several unique hybridization characteristics. In this report we present a new application of PNA which exploits its unique properties to provide enhanced amplification. Rather than clamping the PCR, PNA is used to block the template making it unavailable for interstrand and intrastrand interactions while allowing polymerase to displace the PNA molecules and extend the primer to completion. Preferential amplification is reduced and overall efficiency is enhanced.

INTRODUCTION

Use of the polymerase chain reaction (PCR) to amplify variable numbers of tandem repeat (VNTR) loci has been widely used in gene mapping, linkage studies, diagnostics, forensics and paternity testing (1–5). Unfortunately, there is an inverse relationship between the size of the VNTR allele and the efficiency of amplification, such that small allelic products may be preferentially amplified when present in a heterozygous state with a large allele. In some circumstances, differential amplification may result in dropout of the larger allelic product and misclassification of a heterozygous individual as homozygous for the over amplified smaller allele. The mechanism for preferential amplification has not been elucidated. Walsh *et al.* (6) suggested that preferential amplification, in some cases, is the result of incomplete denaturation, differential priming, limiting enzyme or small sample size. However, recent work with 'long PCR' may suggest that it results from premature termination of primer extension, due either to template nicking, incorporation of mismatched base pairs or premature dissociation of polymerase (7–9). The results of our work suggest that, at least in some systems, another mechanism may play a role in premature termination: reannealing of complementary template (complete or truncated) and subsequent clamping of the PCR.

Recently, PNA oligomers (peptide nucleic acids) have been used to detect single base mutations through PCR clamping (10). PNA is a DNA mimic in which the deoxyribose-phosphate backbone has been replaced by an oligoamide consisting of *N*-(2-aminoethyl)glycine units. PNA mimics DNA in terms of its ability to recognize and bind to complementary nucleic acid sequences but does so with higher thermal stability and specificity than corresponding oligodeoxynucleotides. However, a single base mismatch in a PNA–DNA duplex is much more destabilizing than in the corresponding DNA–DNA duplex. Furthermore, PNA cannot function as a primer for DNA polymerase (11,12).

In this report we present a new application of PNA which exploits these unique properties to provide enhanced amplification of VNTR locus D1S80 and renders genotypic patterns which are more readily interpretable. We demonstrate that PNA can sufficiently block template to make it unavailable for reassociation but at the same time allow primer extension. The D1S80 locus was selected as a model to test the system and, although its amplification is fairly robust, it can demonstrate preferential amplification when alleles are widely disparate in length. The VNTR locus, D1S80, consists of a 16 bp repeat with at least 29 alleles ranging in size from 369 to 801 bp corresponding to 14 and 41 repeats, respectively (13,14).

MATERIALS AND METHODS

The composite PNA H-CTT(G/T)CCGGTGGTC(C/T)TC-NH_2 was synthesized by PerSeptive Biosystems (Framingham, MA). PNA sequences were synthesized on a modified Expedite (PerSeptive Biosystems) automated instrument using Boc/Z-protected monomers and Boc-BHA-PEG polystyrene based solid supports (PerSeptive Biosystems) with an optimized solid phase methodology (15). Mixed sites were introduced by alternating the pulses of the monomers to be incorporated at the mixed site. The oligomers were cleaved from the resin, and protecting groups removed using TFMSA/TFA/m-cresol (2:7:1) for 2 h at room temperature in an Ultrafree-MC with a PTFE membrane (Millipore). The support was retained by the Ultrafree-MC device, and the filtrate containing the PNA was precipitated with

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anhydrous ether placed in dry ice for 5 min. The precipitate was then collected by centrifugation at 2 000 *g* for 2–3 min and washed twice with ether. The crude PNA samples were purified by reverse phase chromatography on a Deltapak C18, 3.9 × 150 mm column and their mass confirmed on a MALDI-TOF mass spectrometer. Oligodeoxynucleotide primers used in the PCR reactions were synthesized by Research Genetics (Huntsville, AL). The sequence of the primers according to Kasai *et al.* (13) were 5′-GAAACTGGCCTCCAAACACTCCCCGCCG-3′ (forward primer) and 5′-GTCTTGTTGGAGATGCACGTGCCCC-TTGC-3′ (reverse primer). The forward primer was end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP.

DNA was extracted from EDTA anticoagulated whole blood using a Puregene DNA Isolation Kit (Gentra Systems, Inc., Minneapolis, MN). Purified DNA from cell line K562 was purchased from Promega Corporation (Madison, WI).

Amplification of the D1S80 locus was carried out in a total volume of 12.5 µl using 1.75 ng of genomic DNA. Each reaction contained 0.75 µM of each primer, 250 µM each of dCTP, dATP, dGTP and dTTP (Promega Corporation), and 1× Stratagene buffer (10 mM Tris–HCl, pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin and other stabilizers not specified). The PCR reactions were overlaid with 1 drop of mineral oil. Prior to addition of 0.5 U *Taq* DNA polymerase (Stratagene, La Jolla, CA), the reaction mixtures were heated to 95°C for 4–10 min in a GTC-2 Genetic Thermal Cycler (Precision Scientific, Chicago, IL). Subsequently, 5 cycles were carried out each consisting of 95°C for 1.25 min for denaturation, 67°C for 30 s to allow primer annealing, and 4 min at 72–76°C (exact extension temperatures are indicated in the figure legends) for primer extension, followed by 25 cycles each consisting of 95°C for 1.25 min, 66°C for 30 s, and 72–76°C for 4 min. The final extension was carried out for an additional 5 min. The concentration of PNA was as indicated in the figure legends.

Electrophoresis of the amplified DNA was carried out on a 5% Long Ranger denaturing gel (J. T. Baker, Inc., Phillipsburg, NJ) at 1000 V for 3.5 h. PCR product (5 µl) was mixed with 5 µl of tracking dye (95% v/v formamide, 20 mM EDTA, 0.5% w/v bromophenol blue, 0.5% w/v xylene cyanol) and heated to 95°C for 5 min prior to loading. Following electrophoresis, the polyacrylamide gels were dried using a Savant Slab Gel Dryer (ATR, Inc., Laurel, MD). The amplification products were visualized by autoradiography using Kodak XAR5 imaging film and Biotech L-Plus intensifying screens (Fisher Scientific, Pittsburgh, PA). Quantitative analysis of relative band intensities was performed using a Beckman Appraise Densitometer (Beckman Instruments, Inc., Brea, CA). The instrument was used in the absorbance mode with an interference filter with a peak transmittance at 600 nm and a 2 mm slit length.

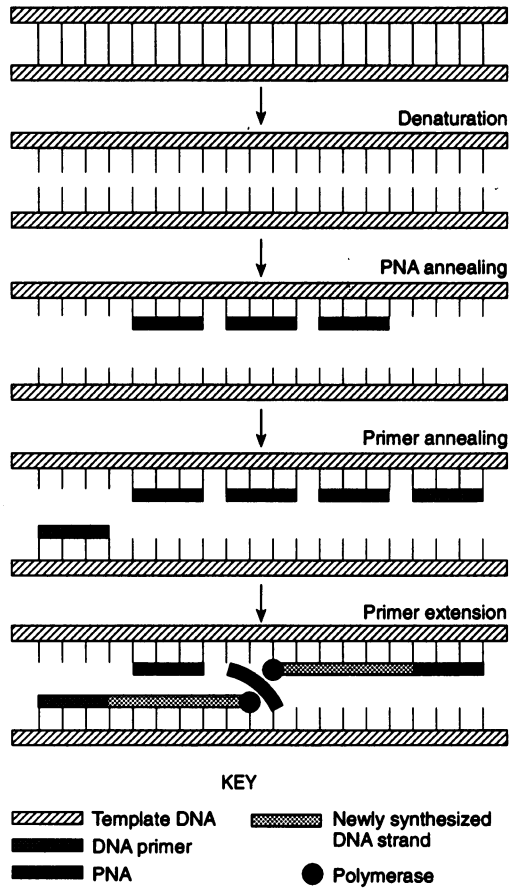

Figure 1. Schematic diagram of the modified PCR reaction in the presence of PNA. Following template denaturation, PNA molecules anneal to and block the DNA template. A separate PNA annealing step (at ~78°C) may be used, or PNA annealing may occur while cooling to the primer annealing temperature. Once DNA primer molecules have annealed to the template, then the temperature is raised for primer extension. During primer extension, PNA molecules are displaced from the template allowing primer extension to proceed to completion.

RESULTS

Optimization of PNA concentration

The PNA molecules were designed to block the repeat sequence at the D1S80 locus during amplification, but at the same time not prevent the polymerase from displacing them and extending the primers to completion (Fig. 1). To accomplish this the PNA had to anneal to its target sequence at a relatively high temperature and yet be destabilized enough at extension temperatures to be displaced by the polymerase. It would also require a concentration high enough to effectively compete with the build up of product during later rounds of the PCR but not clamp the PCR as it has been previously shown to do so effectively (10). Figure 2 shows the result of a PCR experiment using K562 DNA and increasing amounts of PNA. Amplification of K562 DNA generates two fragments of 433 and 609 bp corresponding to 18 and 29 repeats, respectively. Differential amplification is readily apparent when PNA is absent as evidenced by the less intense upper band (allele with 29 repeats). As the concentration of PNA increases the intensity of the larger fragment increases up to a maximum at ~1.2–1.8 µM PNA. At PNA concentrations >1.8 µM the efficiency of the amplification process appears to decline. Furthermore, the smaller fragment also appears to benefit from the presence of PNA as its amplification is enhanced, although to a lesser extent. K562 DNA was also amplified at the D1S80 locus in the presence of an unrelated 10mer PNA ($[CGG]_3C$, data not shown). The results were as expected with no enhancement in the reactions containing no PNA or CGG repeat PNA, and enhancement in both reactions containing D1S80 PNA with or without CGG repeat PNA. Thus, enhancement appears to be locus

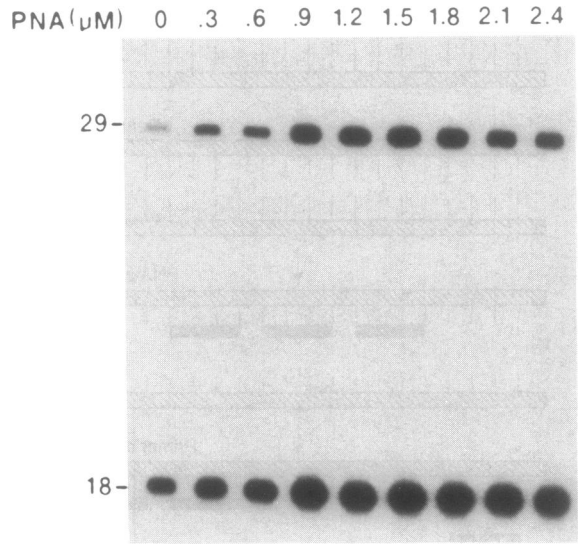

Figure 2. Optimization of the PNA concentration required for enhanced amplification of K562 DNA. K562 DNA was amplified without (lane 1) and with increasing amounts of PNA (lanes 2–9). The allele size (in repeats) is given in the left. Primer extension was carried out at 72°C.

specific, requiring PNA oligomers that are complementary to the sequence of interest. As previously shown, the presence of unrelated PNA molecules has no effect on the PCR (10).

Scanning lanes 1 (no PNA) and 6 (1.5 μM PNA) of the autoradiograph (Fig. 2) with a densitometer revealed a 2.6-fold reduction in the degree of preferential amplification when PNA was included in the PCR (data not shown). The percentage OD contribution of each band in lane 1 (no PNA) were 19.1 and 80.9% for the larger (29 repeats) and smaller (18 repeats) alleles, respectively. That corresponds to 4.2 times greater efficiency for the PCR amplification of the smaller allele. In contrast, when PNA (1.5 μM) was included in the PCR, the large and small allele represent 38.9 and 61.1% of the profile, respectively. The smaller allele was still preferentially amplified but with only a 1.6-fold greater efficiency.

Evaluation of enhancement at elevated primer extension temperatures

Historically, primer extension has been carried out at 72°C when amplifying the D1S80 locus using the PCR (13,16). Since the T_m of the PNA–DNA duplexes are significantly higher than the T_m for DNA–DNA duplexes (11) we were concerned that the PNA oligomer would, to a small degree, be clamping the PCR at that extension temperature. If so, elevating the extension temperature might further enhance the amplification. Preliminary experiments (data not shown) had indicated that an extension temperature of 76–77°C exhibited a slight increase in the amplification enhancement. Presumably, a primer extension temperature closer to the T_m of the PNA, allows the polymerase to more easily displace the PNA molecules that are bound to the template. To determine the optimum temperature for primer extension in the presence of PNA, K562 DNA was amplified with PNA (1.5 μM) and without PNA at temperatures ranging from 72 to 78°C. Figure 3 and Table 1 demonstrate that without PNA the efficiency of amplification is unaffected at elevated extension temperatures. However, the enhancing effects of PNA are significantly diminished at an extension temperature of >77°C.

Analysis of random DNA specimens

To evaluate the general application of PNA for enhancing PCR at the D1S80 locus, nine DNA specimens from routine paternity casework were randomly selected. Each was amplified with (1.5 μM) and without PNA. All samples were prepared together from the same master mix of reagents and cycled together in the same thermal cycler. Primer extension was carried out at 76°C. The results are shown in Figure 4 and Table 2. Fragment sizes for the specimens tested ranged from 433 to 657 bp as determined by comparison to a D1S80 allelic ladder (Perkin-Elmer Cetus, Norwalk, CT, data not shown). Each DNA specimen demonstrated enhanced amplification, particularly of the larger allele, when PNA was present. It was most apparent in DNA samples demonstrating >6 repeats difference in allele size (samples 2, 4, 5 and 8). However, amplification of close heterozygotes (samples 1, 3 and 6) and apparent homozygotes (samples 7 and 9), also appeared to benefit from the presence of PNA as evidenced by the increased product yield.

Table 1. Densitometric analysis of band intensities (expressed in % relative absorbance) demonstrating the enhancing effects of PNA when amplification is carried out at elevated primer extension temperatures (Fig. 3)

	Relative Absorbance (%)					
	–PNA			+ PNA		
Temp. °C	Upper Band	Lower Band	Ratio Lower/Upper	Upper Band	Lower Band	Ratio Lower/Upper
72	24.0	76.0	3.2	31.1	68.9	2.2
74	23.5	76.5	3.3	28.7	71.3	2.5
76	25.6	74.4	2.9	32.5	67.5	2.1
77	24.5	75.5	3.1	34.2	65.8	1.9
78	16.2	83.8	5.2	16.4	83.6	5.1

Upper and lower bands correspond to the 29 and 18 repeat alleles, respectively.

Table 2. Densitometric analysis of band intensities (expressed in % relative absorbance) demonstrating the enhancing effects of PNA on random DNA specimens (Fig. 4)

	Relative Absorbance (%)					
	– PNA			+ PNA		
Sample	Upper Band	Lower Band	Ratio Lower/Upper	Upper Band	Lower Band	Ratio Lower/Upper
1	47.2	52.8	1.1	47.9	52.1	1.1
2	13.2	86.8	6.6	30.4	69.6	2.3
3	47.6	52.4	1.1	45.2	54.8	1.2
4	28.7	71.3	2.5	37.5	61.9	1.6
5	21.3	78.7	3.7	33.4	66.6	2.0
6	46.8	53.2	1.1	50.0	50.0	1.0
7	100	–	–	100	–	–
8	8.7	91.3	10.5	21.9	78.1	3.6
9	100	–	–	100	–	–
10	17.4	82.6	4.7	22.9	77.1	3.4

Upper and lower bands correspond to the larger and smaller allele respectively. Homozygous samples (7 and 9) have 100% of their profile represented by one band.

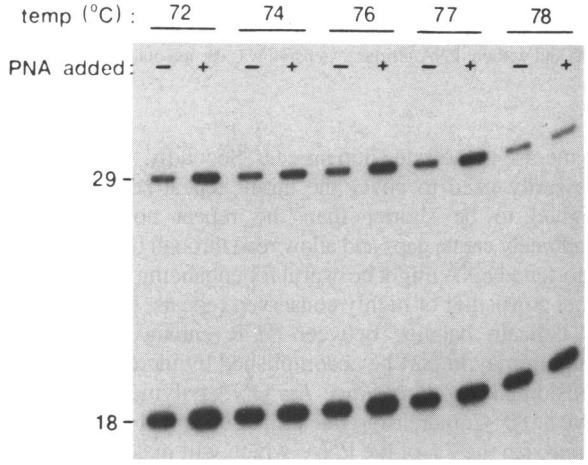

Figure 3. Evaluation of the enhancing effects of PNA when amplification is carried out at elevated primer extension temperatures. K562 DNA was amplified with (1.5 μM) and without PNA at primer extension temperatures of 72, 74, 76, 77 and 78°C. –, no PNA added; +, PNA added. The allele size (in repeats) is given to the left.

PNA enhancement occurs in later rounds of amplification

Since PNAs are not consumed in the amplification process, the ratio of PNA to template decreases with each cycle as more template is produced. Therefore, the kinetics of PNA annealing are constantly changing in the reactions. To evaluate these changes and determine where in the PCR process PNA exerts its effect, K562 DNA was amplified without PNA and with 0.9, 1.2, 1.5 and 1.8 μM of PNA for 10, 20 and 30 cycles. The result is shown in Figure 5. After 10 cycles of the PCR no detectable product was observed. With 20 cycles of PCR amplification, product can be detected in each lane. No improvement in signal was observed when PNA was present and may have actually reduced the efficiency of amplification, presumably through clamping. However, after 30 rounds of the PCR and a theoretical 10^9-fold increase in product, enhancement is readily apparent even at suboptimal PNA concentrations.

DISCUSSION

We have presented a method using PNA to enhance the efficiency of the PCR amplification of VNTR alleles. If properly designed and present during PCR amplification at the appropriate concentration, PNA allows fragments of different size to be more effectively and more evenly amplified. Differential amplification is less apparent and as a result, the risk of misclassification is greatly reduced. In addition, fragments of the same or similar size are also amplified more effectively when PNA is included in the PCR.

Design of the PNA is critical if blocking of PCR template is to occur without clamping the reaction. The PNA molecule must anneal to the template before the complementary DNA strand. However, the PNA–DNA duplex must also be sufficiently destabilized at the primer extension temperature to allow the polymerase to displace it and extend through to the end of the template. There are several ways to accomplish this objective. First, the normal sequence variation present within most VNTR repeats may provide some assistance in attaining the delicate balance between blocking and clamping. In fact, it may not be possible to design a PNA mixture that will be exactly complementary to all repeats of all alleles. In the case of the D1S80 locus, the published sequence revealed length and sequence variation. The most common repeat consisted of a 16 bp sequence with an adenine or guanine at position 3 of the repeat, and a cytosine or adenine at position 13. Therefore, we constructed a composite PNA mixture representing all four possible sequences. However, the composite PNA did not include a PNA complementary to other less common variants of the consensus sequence, not to mention incomplete repeats. And, as previously shown (10), even a single base mismatch has significant destabilizing effects on the

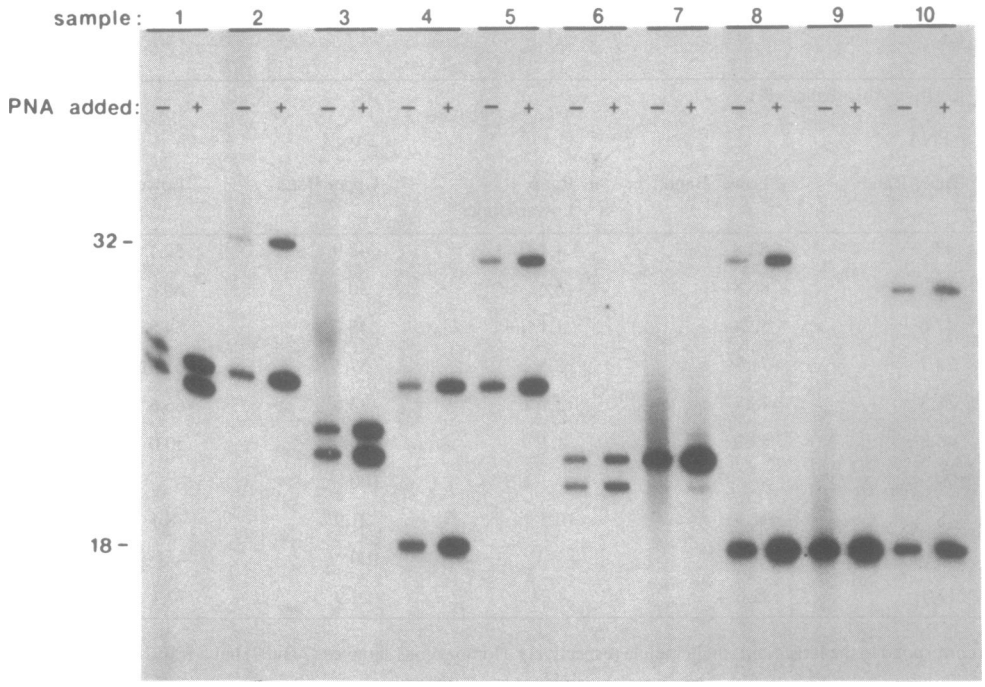

Figure 4. A direct comparison of the efficiency of the PCR when random DNA specimens are amplified with and without PNA. Nine randomly selected DNA samples (samples 1–9) and K562 DNA (sample 10) were amplified by the PCR with PNA (1.5 μM) and without PNA. Primer extension was carried out at 76°C. Allele size ranged from 18 to 32 repeats. –, no PNA added; +, PNA added.

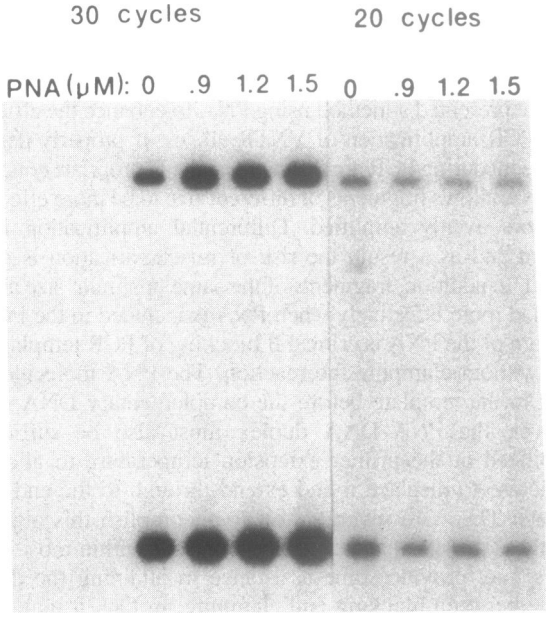

Figure 5. A comparison of PNA enhancement of the PCR at 20 and 30 cycles of amplification. K562 DNA was amplified with PNA (0.9, 1.2 and 1.5 μM) and without PNA for 10 (data not shown), 20 and 30 cycles. Primer extension was carried out at 76°C.

PNA–DNA duplex. Thus, blocking of most, if not all alleles is probably incomplete. But, incomplete blocking is still sufficient to prevent complementary template strands from reannealing and the gaps between adjacent PNAs may actually facilitate the polymerase in the extension process. Secondly, the PNA does not necessarily need to cover the entire repeat sequence. A PNA designed to be shorter than the repeat could be used to intentionally create gaps and allow read through by the polymerase. A shortened PNA might be useful for enhancing the amplification of loci consisting of highly conserved repeats. Finally, obtaining that delicate balance between PCR enhancement and PCR clamping may in part be accomplished by increasing the primer extension temperature. Since *Taq* DNA polymerase is optimally active at 80°C, increasing the temperature will allow the system to approach the T_m of the PNA, which will make it easier for the polymerase to dislodge it, while still allowing primer extension to proceed.

The degree of enhancement obtained when randomly selected samples were amplified with PNA (Fig. 4) was variable. While some samples demonstrated greatly enhanced amplification in the presence of PNA, the enhancement of other samples was less significant. Such variation may reflect the sequence and length variation present within the D1S80 repeat. If the tested alleles contain polymorphisms not represented by the composite PNA molecules, then blocking of those alleles may have been incomplete, resulting in reduced enhancement. Sequencing additional alleles should reveal additional variations of the repeat unit.

Peptide nucleic acid enhancement of the PCR process appears to occur during the later rounds of amplification. Reduced amplification efficiency after 20 cycles in the presence of PNA suggests that excess PNA molecules are initially, to some extent, clamping the PCR. This suggests that the accumulation of product in the later rounds of the PCR may be the cause, at least in part, for preferential amplification of VNTR loci. It is in later cycles that the PCR components are depleted (primer is depleted while the ratio of polymerase to template is reduced) while product is

greatly increased. Complementary strands of the template may be reannealing before extension takes place thereby blocking primer extension and possibly primer binding. Since larger alleles contain more repeat units, they reanneal at a faster rate, thereby reducing the efficiency of the PCR amplification of those fragments. Although most polymerases exhibit 5′ to 3′ nuclease activity, they are not likely to effectively cope with the extensive interstrand interaction occurring in later rounds of the PCR. Blocking of the repeat units by PNA molecules prevents reannealing of complementary template strands, allowing primer extension to occur without impediment.

Walsh *et al.* (6) proposed differential priming, differential denaturation, limiting *Taq* DNA polymerase and stochastic fluctuation of input allele copy number as mechanisms for preferential amplification. While they presented evidence for each, none are likely to be the primary mechanism in preferential amplification of VNTR loci. Differential priming due to sequence polymorphism under the primers is likely to affect most systems to some degree. However, polymorphisms should be randomly distributed and affect the amplification of small as well as large alleles. And, since most thermal stable polymerases tolerate a T_{den} of 94°C, differential denaturation should not be a problem, providing, as the authors stated, that the PCR parameters are optimized and the wells of the thermal cycler heat block are properly controlled. The amount of *Taq* DNA polymerase while certainly an important parameter, is usually not a limiting reagent. Even when enzyme is titrated to find the lowest amount that still gives good amplification, most regimens still provide the system with an excess. In our experience it has not been necessary to use >1 U enzyme in the PCR and usually 0.5 U in a 50 µl reaction is sufficient. However, in the present application in which PNA molecules must be displaced by the polymerase, a further increase (0.5 U enzyme in a 12.5 µl reaction was used) in enzyme may provide further enhancement. Finally, stochastic fluctuation can occur when low copy number of target DNA is used, but as stated by the authors, utilizing nanogram amounts of input DNA should alleviate that problem.

Premature termination of primer extension due to template nicking, incorporation of mismatched base pairs, or dissociation of the polymerase due to limited processivity are thought to be responsible for the length limitations of PCR amplification (7–9). They may also, in part, be responsible for the preferential amplification of small allelic products when present in a heterozygous state with a large allele. However, premature termination of primer extension due to clamping by complementary template (complete or truncated) may also be a significant factor. Further enhancement of the PCR might be attained by combining 'long PCR' methods with PNA.

PNA may also be useful in reducing the anomalous products, or ladder-like pattern, so often generated during the PCR amplification of repeat sequences, particularly short tandem repeats (STRs). Mechanisms such as slipped strand mispairing (17–19), template independent addition of a single nucleotide to the 3′ end (20) and recombination involving 'out of register' annealing of truncated PCR product (21,22) or 'out of register' template switching (23–25) have been invoked to explain this phenomenon. In the case of slipped strand mispairing in which a region of the primer–template complex may dissociate during primer extension thereby allowing slippage, PNA may inhibit the slippage process. Since PNA binds to the repeat sequences, it is not likely to prevent the addition of non-template directed extra nucleotides at the 3′ end of DNA. However, 'out of register' annealing of truncated products (or complete product) could be significantly reduced if PNA molecules were present to block interstrand interaction. Similarly, bound PNA might also interfere with template switching by making secondary template less available for polymerase to jump to.

The mechanisms involved in preferential amplification, the production of anomalous products (or ladder-like products), and the length limitations for PCR amplification are complex. Usually these phenomena are discussed in isolation without consideration of the others. However, it is feasible that they are interrelated and that the complex milieu of a PCR simultaneously sustains several (depending on the conditions; buffer, primers, enzyme and locus) interstrand and intrastrand interactions. If so, PNA technology may prove useful, not only for reducing the effects of preferential amplification, but for reducing the production of the ladder-like patterns associated with the amplification of STRs and for improving the size of the template span successfully traversed by polymerase in the PCR.

ACKNOWLEDGEMENTS

We are grateful to David P. Bick and Shannon J. Odelberg for critical review of the manuscript and their helpful suggestions. We thank Geoff Swift for the computer graphics.

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