Pyrophosphorolysis-Activated Polymerization (PAP): Application to Allele-Specific Amplification

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ABSTRACT

To measure mutation load or to detect minimal residual disease, a robust method for identifying one mutant allele in the range of 10^6 – 10^9 wild-type alleles would be advantageous. Herein, we present evidence that pyrophosphorolysis-activated polymerization (PAP) has the potential to provide a highly specific and robust method of allele-specific amplification if DNA polymerases with higher pyrophosphorolysis activity can be found or engineered. In PAP, pyrophosphorolysis and polymerization by DNA polymerase are coupled serially by utilizing a pyrophosphorolysis-activatable oligonucleotide (P^*). P^* , which is an allele-specific oligonucleotide with a dideoxynucleotide at the 3' terminus, can be activated by pyrophosphorolysis to remove the 3' terminal dideoxynucleotide in the presence of pyrophosphate (PP_i) and the complementary strand of the allelic template; then the activated P^* can be extended by DNA polymerization. Specificity results from both pyrophosphorolysis and polymerization because significant nonspecific amplification requires the combination of mismatch pyrophosphorolysis and misincorporation by the DNA polymerase, which is an extremely rare event. Proof of principle has been achieved with a polymorphic site within the human D_1 dopamine receptor gene. The effects of the dideoxyligonucleotide sequences, DNA polymerases, PP_i concentrations, allele-specific templates, pH and dNTP concentrations were examined.

INTRODUCTION

A method of detecting one mutant allele in 10^6 – 10^9 wild-type alleles would be advantageous for many applications including detecting minimal residual disease (rare remaining cancer cells during remission, especially mutations in the *p53* gene or other tumor suppressor genes previously identified within the tumors) and measurement of mutation load (the frequency of specific somatic mutations present in normal tissues such as blood or urine). Individuals with a high mutation load may be at increased risk for cancer due to either environmental exposure or endogenous defects in any of the hundreds of genes necessary to maintain the integrity of the genome. For those individuals found to have a high mutation load, clues to etiology can be obtained by defining the mutation pattern.

Multiple methods for detecting mutations present in less than 10% of cells (i.e., rare alleles) have been developed including PCR amplification of specific alleles (PASA), peptide nucleic acid (PNA) clamping blocker PCR, allele-specific competitive blocker PCR, mismatch amplification mutation assay (MAMA) and restriction fragment-length polymorphism (RFLP)/PCR (16). These methods (i) amplify the rare allele selectively, (ii) destroy the abundant wild-type allele or (iii) spatially separate the rare allele from the wild-type allele. RFLP/PCR has been reported to have the highest specificity of 10^{-8} (17), but in our hands the specificity has been 10^{-3} – 10^{-4} (8). Methods that selectively amplify the rare allele include PASA, which routinely has a specificity of less than or equal to 1 part in 40 (21).

DNA polymerases, which are critical to nucleic acid amplification, catalyze some or all of the following reactions: (i) polymerization of deoxynucleotide triphosphates; (ii) pyrophosphorolysis of duplexed DNA in the presence of pyrophosphate (PP_i), $[dNMP]_n + x[PP_i] \rightarrow [dNMP]_{n-x} + x[dNTP]$; (iii) 3'→5' exonuclease activity; and (iv) 5'→3' exonuclease activity (5,9). For *Taq* and *Tfl* DNA polymerases, the polymerization and 5'→3' exonuclease activity have been reported (4,7,13). For T7 Sequenase® and Thermo Sequenase™ DNA polymerases (both from Amersham Pharmacia Biotech, Piscataway, NJ, USA), pyrophosphorolysis can lead to the degradation of specific dideoxynucleotide-terminated segments in Sanger sequencing reaction (23,25). Herein, we describe pyrophosphorolysis-activated polymerization (PAP), an approach that has the potential to enhance dramatically the specificity of PASA.

MATERIALS AND METHODS

Preparation of Template by PCR

A 640-bp region of the human D_1 dopamine receptor gene was amplified by PCR with two primers (T = 5'-GAC-CTGCAGCAAGGGAGTCAGAAG-3' and U = 5'-TCATAC CGGAAAGGG CTGGAGATA-3') (Figure 1A). The TU:UT duplexed product spans nucleotides 33–672 in GenBank® accession no. X55760 and the GC content is 55.3%. A common A to G polymorphism is located at nucleotide 229, resulting in three genotypes of GG, AA and GA (11). The PCR mixture contained a volume of 50 μ L: 50 mM KCl,

10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 200 μM each of the four dNTPs (Roche Molecular Biochemicals, Indianapolis, IN, USA), 0.1 μM each primer, 2% dimethyl sulfoxide (DMSO), 1 U *Taq* DNA polymerase (Roche Molecular Biochemicals) and 250 ng genomic DNA from GG homozygote, AA homozygote or GA heterozygotes. Cycling conditions includ-

ed denaturation at 94°C for 15 s, annealing at 55°C for 30 s and elongation at 72°C for 1 min, for a total of 35 cycles (GeneAmp® PCR System 9600; Applied Biosystems, Foster City, CA, USA). The PCR product was purified from primers and other small molecules by approximately 10 000-fold by three times of retention on an Amicon® Centricon® 100 microconcentrator (Milli-

pore, Bedford, MA, USA). The amount of recovered PCR product was determined by UV absorbance at 260 nm.

Synthesis of a Pyrophosphorolysis-Activatable Oligonucleotide (P*) by Adding a 3' Dideoxynucleotide

The dideoxynucleotide was synthesized by a Model 8909 Synthesizer

Figure 1. Schematic of PAP. (A) A duplexed DNA template TU:UT is amplified with two oligonucleotides, P* and U, *Taq* DNA polymerase, dNTPs, pyrophosphate and [α-³²P]dCTP. P* = pyrophosphorolysis-activatable oligonucleotide. In this example, P* is D₃G*, and TU:UT is a 640-bp segment of the dopamine D1 receptor gene. (B) D₃G* has a G dideoxynucleotide at the 3' terminus, and it is specific to the complementary strand of the G allele but mismatches the A allele at the 3' terminus (Table 1). Removal of the dideoxy G by pyrophosphorolysis is followed by polymerization for each amplification. (C) Autoradiogram of PAP from the GG, AA and GA genotypes. When the G allele is present, the radioactively labeled specific products of 469 bases (duplex PU:UP and excess antisense strand UP) are produced because the low rate of pyrophosphorolysis by *Taq* polymerase implies that oligonucleotide U has a much higher efficiency than oligonucleotide P*. Electrophoresis for a longer period separates PU:UP from UP. Other products of UT and TU:UT are indicated. Note that TU:UT derives from annealing of excess radioactively labeled UT with nonradioactively labeled TU original template.

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Table 1. Oligonucleotides Used in PAP

Template		5'...AATCTGACTGACCCCTATTCCTGCTT- ^G _A GGAAC...3' ^a						
Name	Oligonucleotide Sequence 5'→3'					Purpose		
D ₁	ACTGACCCCTATTCCTGCTT					Control		
D ₁ G [*]	ACTGACCCCTATTCCTGCTT G *					3' ddG and G allele specificity co-localized		
D ₂ G [*]	ACTGACCCCTATTCCTGCTT GG *					G allele specificity 5' to ddG		
D ₃ G [*]	ACTGACCCCTATTCCTGCTT GGG *					G allele specificity 5' to ddG		
D ₄ G [*]	ACTGACCCCTATTCCTGCTT GGGG *					3' ddG mismatches template		
D ₅ G [*]	TCTGACTGACCCCTATTCCTGCTT G *					D1G*, with 5' extended bases		
D ₆ A [*]	TGACTGACCCCTATTCCTGCTT A *					3' ddA and A allele-specificity co-localized		
U	TCATACCGGAAAGGGCTGGAGATA					Upstream oligonucleotide		
Name	3' Terminal Nucleotide ^c		Allele-Specific Nucleotide ^d		Size (base)	T _m (°C) ^e	Amplification ^f	
	Type	Match	Type	From 3' Terminus (bp)			G allele	A allele
D ₁	dT	Yes	—	+1	21	64	Yes	Yes
D ₁ G [*]	ddG	Yes	G	0	22	68	No	No
D ₂ G [*]	ddG	Yes	G	-1	23	72	No	No
D ₃ G [*]	ddG	Yes	G	-2	24	76	Yes	No
D ₄ G [*]	ddG	No	G	-3	25	80	No	No
D ₅ G [*]	ddG	Yes	G	0	26	80	Yes	No
D ₆ A [*]	ddA	Yes	A	0	24	72	No	No
U	dA	Yes	—	—	24	72	Yes	Yes

^aThe bold **G** and **A** correspond to the G and A alleles, respectively.

^bD₁ G^{*} = D₁ with ddG to the 3' terminus; * denotes a dideoxynucleotide at the 3' terminus. The first base at the 5' terminus corresponds to nucleotide 208 in GenBank accession no. X55760.

^cThe 3' terminal base is a deoxynucleotide or dideoxynucleotide and creates a match (Yes) or a mismatch (No) with the corresponding base on the complementary strand of the template.

^dThe allele-specific nucleotide is G or A, and its distance to the 3' terminus is assigned: 0 = at the 3' terminus; +1 = 1 base downstream from the 3' terminus; -1 = 1 base upstream from the 3' terminus; -2 = 2 bases upstream from the 3' terminus and -3 = 3 bases upstream from the 3' terminus.

^eThe T_m for oligonucleotides was estimated to be 4°C × (G + C) + 2°C × (T + A) at 1 M NaCl (15).

^fAmplification with U and one P* or with only one P*.

(PerSeptive Biosystems, Framingham, MA, USA) and purified by oligopure cartridges (Hamilton, Reno, NV, USA) in the City of Hope DNA/RNA Chemistry Laboratory. The 3' terminal dideoxynucleotide was added by terminal transferase. The mixture contained a total volume of 40 µL: 200 mM potassium cacodylate, 25 mM Tris-HCl (pH 6.6 at 25°C), 2.5 mM CoCl₂, 0.25 mg/mL BSA, 4000 pM of the oligonucleotide, 2.5 mM 2',3'-ddNTP (the molar ratio of

the 3' OH terminus to ddNTP was 1:25), 125 U terminal transferase (the last two items are both from Roche Molecular Biochemicals). The reaction was incubated at 37°C for 1 h and then stopped by adding EDTA at 5 mM final concentration. After desalting by using butanol, the dideoxyoligonucleotide was purified by preparative 7 M urea/20% polyacrylamide gel electrophoresis (PAGE) in TBE buffer (90 mM Tris-borate, 1 mM EDTA, pH 8.3) (14). The amount of the

recovered P* was determined by UV absorbance at 260 nm.

Since small amounts of unterminat-ed oligonucleotide would result in non-specificity of pyrophosphorolysis, each dideoxyoligonucleotide was ³²P-labeled at the 5' terminus by T4 polynucleotide kinase and then was electrophoresed through a 7-M urea/20% polyacrylamide gel. Only P* products were visible even when the gel was overexposed (data not shown). It is

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estimated that more than 99.99% of P* contained a dideoxynucleotide at the 3' terminus. The purity of P* was supported by the absence of PCR product or PAP product at pH 8.1 (Table 2).

PAP

A 469-bp region within the TU:UT duplexed template was amplified by PAP with oligonucleotides P* and U or with only P* (Table 1 and Figure 1A). The PU:UP duplexed product corresponds to nucleotides 204–672 in GenBank accession no. X55760, and the GC content is 55.6%. Unless stated, the PAP reaction mixture contained a total volume of 25 μ L for *Tfl* DNA polymerase: 75 mM KCl, 20 mM Tris-HCl (pH 7.4), 1.5 mM MgCl₂, 40 μ M each of the four dNTPs (dATP, dTTP, dGTP and dCTP), 0.2 μ M P*, 0.05 μ M U oligonucleotide, 300 μ M Na₄PP_i (the 20 mM stock solution was adjusted by HCl to pH 8.0), 1 μ Ci [α -³²P]dCTP (3000 Ci/mmol; Amersham Pharmacia Biotech), 1 U *Tfl* DNA polymerase

(Promega, Madison, WI, USA) and 2 ng TU:UT. For *Taq* DNA polymerase, the reaction mixture was the same except for 50 mM KCl, 10 mM Tris-HCl (pH 7.4), 2.0 mM MgCl₂ and 1 U *Taq* DNA polymerase. The mixtures of PCR and other controls were the same except for the primers added. Cycling conditions included: 94°C for 15 s, 55°C for 1 min, ramping to 72°C for 1 min and 72°C for 2 min, for a total of 15 cycles.

The reaction was electrophoresed through a standard 2% agarose gel. The gel was stained with ethidium bromide for UV photography by a charge-coupled device (CCD) camera (Gel Doc 1000; Bio-Rad Laboratories, Hercules, CA, USA), dried and subjected to X-OMATTM AR film (Eastman Kodak, Rochester, NY, USA) for autoradiography.

Restriction Digestion

Each of three restriction endonucleases of *Aci*I (5'-C↓CGC-3'/3'-GGC↑G-5'), *Eae*I (5'-Py↓GGCCPu-3'/3'-PuCCGG↑Py-5') and *Eco*0109I (5'-PuG↓

GNCCPy-3'/3'-PyCCNG↑GPu-5') has a restriction site within the PU:UP duplex. The GG alleles were amplified by PAP with D₃G* and U; PCR amplification with D₁ and U was used as the control. Forty microliters of the PAP reaction and 2 μ L PCR were purified and concentrated with a Centricon 100 microconcentrator, and the products were digested by the restriction endonuclease: 2.5 U *Aci*I in 1 $\times$ NE buffer 3, or 3 U *Eae*I in 1 $\times$ NE buffer 1 or 30 U *Eco*0109I in NE buffer 4 with BSA (all the above enzymes and buffers are from New England Biolabs, Beverly, MA, USA). The reaction (10 μ L) was incubated at 37°C for 2 h, and the digestion reaction was electrophoresed through a standard 2% agarose gel as described above.

RESULTS

Principle of PAP

Tfl and *Taq* DNA polymerases were shown to contain pyrophosphorolysis activity (data not shown). *Tfl* DNA polymerase was utilized to detect the G allele at nucleotide 229 of the D₁ dopamine receptor gene (11) (Figure 1A). P* was synthesized with either ddG or ddA at the 3' terminus (see Table 1). The 3' terminal dideoxynucleotide inhibits direct extension by polymerization but can be removed by pyrophosphorolysis in the presence of PP_i when the P* is specifically hybridized with the complementary strand of the G allele. The degraded oligonucleotide can be extended by polymerization in the 5'→3' direction (Figure 1, B and C).

The enhanced specificity of PAP relative to PASA is provided by serially coupling pyrophosphorolysis and polymerization. Significant nonspecific amplification requires mismatch pyrophosphorolysis and misincorporation by DNA polymerase, which is an extremely rare event (Figure 2).

Specific Amplification with D₃G* and D₃G*

PAP was performed with two oligonucleotides (P* and U), *Tfl* DNA polymerase and DNA template of the

Figure 2. Enhanced specificity of PAP with D₃G*. The specificity of PAP is compared with that of PASA to exponentially amplify a template pool of G and A alleles. (A) The specific amplification of PASA derives from the high efficiency of primer extension when the primer matches the G allele. The nonspecific amplification results from mismatch extension from the A allele. When this occurs, it results in an efficient substrate for further amplification. The thickness and position of the arrow represent the amplification efficiency in each cycle. (B) The specific amplification of PAP from the G allele occurs at high efficiency. Two types of nonspecific amplifications originate from the A allele: (i) nonspecific amplification can occur at low efficiency by mismatch pyrophosphorolysis resulting in a A:T homoduplex PU:UP product, which is not an efficient template for subsequent amplification; and (ii) nonspecific amplification can occur at extremely low efficiency by both mismatch pyrophosphorolysis and misincorporation to produce a G:T heteroduplex PU:UP product, but once it occurs, it provides an efficient template for subsequent amplification. A similar tendency of nonspecific amplifications is suggested for linear amplification by PAP with only D₃G*. It should be noted that the allele-specific nucleotide of P*, such as D₃G*, may be near but not at the 3' terminus. In that case, nonspecific amplification of PAP requires mismatch pyrophosphorolysis and mismatch extension. While both variations of PAP should have higher specificity than PASA, the highest specificity is predicted when the 3' terminal dideoxynucleotide is also the allele-specific nucleotide.

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GG and AA alleles. Multiple P* were tested (Table 1). The allele-specific nucleotide and dideoxynucleotide co-localized to the 3' terminus (D₃G*) and the allele-specific nucleotide two bases from the 3' terminus (D₃G*) specifically amplified the G allele in the presence of PP_i (Figure 3A). Without added PP_i, no specific product was observed with D₃G*, indicating that added PP_i was an essential component for PAP (Figure 3B, lanes 6 and 15). Faint products with D₃G* in lane 4 and with D₄G* in lane 5 were observed (Figure 3B) (see Discussion).

Effects of pH, Concentration of PP_i and Concentration of dNTP and Enzyme

Each of above parameters was examined. PAP was most efficient at pH

between 7.4 and 7.7, at PP_i concentration between 200 and 400 μM and at dNTPs concentrations between 25 and 50 μM (Table 2). *Taq* DNA polymerase can substitute for *Tfl* with similar efficiencies (Table 2).

Identity of Specific Products

To confirm the identity of the specific products, restriction endonuclease digestion was performed (Figure 4). Each of the three restriction endonucleases of *AclI*, *EaeI* and *Eco0109I* has a restriction site within the PU:UP duplex. The expected restriction fragments were found. Similar results were observed with D₃G* and U (data not shown).

The specific products of PAP with D₃G* and U revealed two specific bands on the agarose gel (i.e., PU:UP

and UP), because U was more efficient than D₃G* under our amplification conditions. To confirm this, the GG alleles were amplified by PAP using *Tfl* DNA polymerase with D₃G* and U as was done previously. The products were denatured and electrophoresed through a denaturing polyacrylamide gel. Only one specific band in single-stranded form was observed, indicating that the specific PAP products contain the duplexed and single-stranded segments. The same result was observed with D₃G* and U (data not shown).

Linear PAP

PAP was performed for linear amplification with only one P* from the GG and AA alleles in the presence of PP_i. The specific products of PAP were obtained with D₃G* and with D₃G*, but not with the other P* (Figure 5, lanes 4 and 6). The efficiency of P* was affected by the oligonucleotide size, the 3' terminal dideoxynucleotide and the position of the allele-specific nucleotide.

DISCUSSION

Enhanced Specificity of PAP with D₃G*

PAP relies on the serial coupling of the two reactions, each with high specificity. A mutation-specific P* is activated by pyrophosphorolysis on the mutated template; the extended fidelity product contains the mutation. In the presence of wild-type template, pyrophosphorolysis occurs inefficiently because the 3' terminus of P* does not match; the extended fidelity product will contain the wild type sequence. For the extended product to contain the specific mutation, a specific incorporation error is also required.

Evidence is presented that pyrophosphorolysis followed by polymerization may be used to increase the specificity of PASA. Significant non-specific amplification requires the serial coupling of the two types of errors (Figure 2). The mismatch pyrophosphorolysis rate to remove a mismatch dideoxynucleotide at the 3' terminus, expressed as the removal rate of an incorrect versus a correct dNMP, was reported

Figure 3. Specific amplification with D₃G* and D₃G*. PAP was performed in the presence (A) or the absence (B) of added PP_i with two oligonucleotides for exponential amplification. The oligonucleotides are listed in Table 1. Extension controls with only U identify the positions of TU:UT and UT. Extension controls with D₁ identify the position of PU. PCR controls of D₁ and U identify the positions of PU:UP and PU:UT. Only 20% of the extension product with D₁ and the PCR product with D₁ and U was loaded relative to other lanes.

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Table 2. Parameters Affecting PAP

Parameter		PAP Efficiency ^a	
		D ₅ G*-U	D ₃ G*-U
pH ^b	8.1, 7.9	-	-
	7.7, 7.5, 7.4	++	+++
	7.15	+	+
PP _i ^b (μM)	1000	-	-
	800	-	±
	600	-	++
	400, 200	++	+++
	0	-	±
All dNTPs changed ^b (μM)	200	-	±
	100	-	++
	50	++	+++
	25	++	++++
dGTP changed ^{b,c}	100, 50, 25	±	++
dATP changed ^{b,c}	100, 50	-	+
	25	-	++
<i>Taq</i> DNA polymerase	G allele and PP _i	++	+++
	A allele and PP _i	-	-
	G allele and no PP _i	-	±

^aThe PAP efficiency is indicated as: -, no specific product(s); ±, very weak specific product(s); +, weak specific product(s); ++, moderate specific product (s); +++, strong specific product(s); +++++, very strong specific product(s).

^b*T7* DNA polymerase was used to amplify the GG alleles under the conditions in Materials and Methods, except for the factors indicated.

^cThe indicated concentration was changed, but the others were kept at 200 μM.

ed at less than 10⁻⁵ for *T7* DNA polymerase (9,26). The misincorporation rate to create a substitution mutation by polymerization, expressed as the incorporation rate of an incorrect versus a correct dNMP, was reported as to be 10⁻⁵ for *T7* DNA polymerase and 10⁻⁴ for *E. coli* DNA polymerase I (3,9,26). Similar results were reported for *Taq* DNA polymerase and for 3'→5' exonuclease-deficient mutants of *T7* DNA polymerase and *E. coli* DNA polymerase I (3,6,9,26). The specificity due to the nonspecific amplification, in one instance, in PAP with D₃G* is estimated to be 10⁻⁵ per cycle if the mismatch pyrophosphorolysis rate of a ddNMP is the same as dNMP. The specificity due to the nonspecific amplification, in another instance, is estimated to be 3.3 × 10⁻¹¹ if the mismatch pyrophosphorolysis and the misincorporation are serially coupled by *T7* DNA polymerase. The product of the first example of

nonspecific amplification can be differentiated from that of the second example of nonspecific amplification or that of the specific amplification, such as by a single-strand conformation polymorphism (SSCP) nondenaturing gel electrophoresis because their sequences are different in a single base.

Essential Components of PAP

Each P* was tested by utilizing *T7l* or *Taq* DNA polymerases to amplify the GG and AA alleles. The specific amplification requires the presence of PP_i and allele-specific template. In addition, the amplification efficiency is affected by the oligonucleotide size, the 3' terminal dideoxynucleotide and the position of the allele-specific nucleotide relative to the 3' terminus of P*.

It is not clear why D₁G* and D₂G* did not generate the specific signals, but it may be related to a threshold sta-

bility of duplex between P* and the template. D₆A*, which contains an A dideoxynucleotide at the 3' terminus, did not generate the specific signal. It is possible that the efficiency of PAP will vary in response to the terminal ddNMP. In fact, ddNTPs are known to have different incorporation efficiencies by polymerization. Klenow fragment of *E. coli* DNA polymerase I, *Taq* DNA polymerase and Δ*Taq* DNA polymerase incorporate ddGTP more efficiently than other ddNTPs (20,24,25). The rate of ddNTP incorporation also varies depending on the template sequence and can be tenfold higher at some bases relative to others (20). Another possibility is that D₆A* is shorter in size with a lower melting temperature (T_m).

In PAP without added PP_i, very faint false signals were generated with D₃G* and with D₄G* (Figure 3B). One possibility is that oligonucleotide dimers can

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form and trigger nonspecific pyrophosphorolysis of P^* in later cycles, with PP_i released from the by-polymerization or with PP_i contaminated in dNTPs. 3' terminal degraded D_3G^* and D_4G^* can be hybridized and extended as false signal. Oligonucleotide dimers were observed with D_3G^* and D_4G^* (data not shown). Another possibility with D_3G^* is that the specific pyrophosphorolysis can occur in later cycles with PP_i released or with PP_i contaminated. A third possibility is that D_3G^* and D_4G^* were contaminated by minimal D_3 and D_4 , which did not have G dideoxynucleotide at 3' termini.

Comparison with Other Technologies

A number of methods for enzymatic nucleic acid amplification in vitro have been developed and can be adapted to detect known sequence variants. These include PCR (18,19), ligase chain reaction (LCR) (2,10) and rolling circle amplification (RCA) (1,12). PAP is different in many ways: (i) pyrophosphorolysis and polymerization are serially coupled for each amplification; (ii) there is at least one dideoxynucleotide for PAP. Other chemically modified nucleotides lacking the 3' hydroxyl group at the 3' terminus can serve the same function; (iii) one format is for linear amplification and the other is for exponential amplification; (iv) PP_i is necessary for the amplification; (v) significant nonspecific amplification requires both mismatch pyrophosphorolysis and misincorporation; and (vi) PAP can detect known point mutations and may greatly increase the specificity to detect an extremely rare mutant allele from the wild-type allele.

The mechanistic basis is that two or more reactions are serially coupled for amplification with increased specificity. The key component of PAP is P^* . The blocked 3' terminus in these experiments is a dideoxynucleotide, but any nonextendable nucleotide susceptible to pyrophosphorolysis could, in principle, be substituted. Indeed, any enzyme that cleaves an oligonucleotide 5' to a mismatch could serve the same function as pyrophosphorolysis activation. For example, a blocked oligonucleotide including the methylated recognition

sequence (such as G^mATC) is annealed to its target with the unmethylated recognition sequence, then restriction endonuclease (such as *DpnI*) can only cleave the methylated site and so activate the oligonucleotide for extension.

If a mismatch is located 5' to the cleavage site, significant nonspecific amplification requires the serial coupling of mismatch cleavage and a misincorporation, which is a rare event. Activatable oligonucleotides may also be combined

Figure 4. Restriction endonuclease digestion. To show specificity of PAP, samples from the experiment shown in Figure 3 were digested with *Acil*, *EaeI* and *Eco0109I* restriction endonucleases. Each enzyme has a restriction site within PU:UP. PAP amplified the GG alleles with D_3G^* and U. Five percent of PCR product with D_1 and U were taken as control. *Acil* produces a 236- and a 233-bp fragment from PU:UP and a 407- and a 233-bp fragment from TU:UT. *EaeI* produces a 289- and a 180-bp fragment from PU:UP and a 460- and a 180-bp fragment from TU:UT. *Eco0109I* produces a 348- and a 121-bp fragment from PU:UP and a 107-, a 412- and a 121-bp fragment from TU:UT. The arrows indicate the digestion products expected from PU:UP.

Figure 5. Linear PAP. PAP was performed with only one P^* in the presence of added PP_i . Twenty percent of the extension product with D_1 was loaded relative to other lanes (lanes 1 and 10). No = no oligonucleotide added.

with "minisequencing" primer extension. This may provide a more specific assay for detection of single-base changes that might be particularly amenable to chip technology in which specificity can be a problem (22). Demonstration that PAP can occur in the linear format (Figure 5) supports the feasibility of this approach.

In summary, a novel method of PAP has been developed. In PAP, pyrophosphorolysis and polymerization by DNA polymerase are coupled serially for each amplification by using P*. PAP can be applied for exponential amplification or for linear amplification. Various parameters, including allelic template, P* sequence, DNA polymerase, pH, PP_i and dNTP concentration, were examined. PAP, adapted for detection of the polymorphism in the D₁ dopamine receptor gene, is a promising approach for detection of rare mutations.

REFERENCES

- Banér, J., M. Nilsson, M. Mendel-Hartvig and U. Landegren. 1998. Signal amplification of padlock probes by rolling circle replication. *Nucleic Acids Res.* 26:5073-5078.
- Barany, F. 1991. Genetic disease detection and DNA amplification using cloned thermostable ligase. *Proc. Natl. Acad. Sci. USA* 88:189-193.
- Bebenek, K., C.M. Joyce, M.P. Fitzgerald and T.A. Kunkel. 1990. The fidelity of DNA synthesis catalyzed by derivatives of *Escherichia coli* DNA polymerase I. *J. Biol. Chem.* 265:13878-13887.
- Chien, A., D.B. Edgar and J.M. Trela. 1976. Deoxyribonucleic acid polymerase from the extreme thermophile *Thermus aquaticus*. *J. Bacteriol.* 127:1550-1557.
- Duetcher, M.P. and A. Kornberg. 1969. Enzymatic synthesis of deoxyribonucleic acid. 28. The pyrophosphate exchange and pyrophosphorolysis reactions of deoxyribonucleic acid polymerase. *J. Biol. Chem.* 244:3019-3028.
- Eckert, K.A. and T.A. Kunkel. 1990. High fidelity DNA synthesis by the *Thermus aquaticus* DNA polymerase. *Nucleic Acids Res.* 18:3739-3744.
- Kaledin, A.S., A.G. Sliushenko and S.I. Gorodetskii. 1981. Isolation and properties of DNA-polymerase from the extreme thermophilic bacterium *Thermus flavus*. *Biokhimiia* 46:1576-1584.
- Knoll, A., R.P. Ketterling and S.S. Sommer. 1996. Absence of somatic mosaicism in 17 families with hemophilia B: an analysis with a sensitivity 10- to 1000-fold greater than that of sequencing gels. *Hum. Genet.* 98:539-545.
- Kornberg, A. and T.A. Baker. 1992. DNA Replication, 2nd ed., p.113-226. W.H. Freeman and Company, New York.
- Landegren, U., R. Kaiser, J. Sanders and L. Hood. 1988. A ligase-mediated gene detection technique. *Science* 241:1077-1080.
- Liu, Q., J.L. Sobell and S.S. Sommer. 1995. Screening the dopamine D1 receptor gene in 131 schizophrenics and eight alcoholics: identification of polymorphisms but lack of functionally significant sequence changes. *Am. J. Med. Genet. (Neuropsych. Genet.)* 60:165-171.
- Lizardi, P.M., X. Huang, Z. Zhu, P. Bray-Ward, D.C. Thomas and D.C. Ward. 1998. Mutation detection and single-molecule counting using isothermal rolling-circle amplification. *Nat. Genet.* 19:225-232.
- Longley, M.J., S.E. Bennett and D.W. Mosbaugh. 1990. Characterization of the 5' to 3' exonuclease associated with *Thermus aquaticus* DNA polymerase. *Nucleic Acids Res.* 18:7317-7322.
- Maniatis, T., E.F. Fritsch and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. CSH Laboratory Press. Cold Spring Harbor, NY.
- Miyada, C.G. and R.B. Wallace. 1987. Oligonucleotide hybridization techniques. *Methods Enzymol.* 154:94-107.
- Parsons, B.L. and R.H. Heflich. 1997. Genotypic selection methods for the direct analysis of point mutations. *Mutat. Res.* 387:97-121.
- Pourzand, C. and P. Cerutti. 1993. Genotypic mutation analysis by RFLP/PCR. *Mutat. Res.* 288:113-121.
- Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis and H.A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239:487-491.
- Saiki, R.K., S. Scharf, F. Faloona, K.B. Mullis, G.T. Horn, H.A. Erlich and N. Arnheim. 1985. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230:1350-1354.
- Sanger, F., S. Nicklen and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitor. *Proc. Natl. Acad. Sci. USA* 75:5463-5467.
- Sarkar, G., J. Cassady, C.D.K. Bottema and S.S. Sommer. 1990. Characterization of polymerase chain reaction amplification of specific alleles. *Anal. Biochem.* 186:64-68.
- Syvanen, A.C. 1999. From gel to chips: "minisequencing" primer extension for analysis of point mutations and single nucleotide polymorphisms. *Hum. Mutat.* 13:1-10.
- Tabor, S. and C.C. Richardson. 1990. DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. *J. Biol. Chem.* 265:8322-8328.
- Tabor, S. and C.C. Richardson. 1995. A single residue in DNA polymerases of the *Escherichia coli* DNA polymerase I family is critical for distinguishing between deoxy- and dideoxyribonucleotides. *Proc. Natl. Acad. Sci. USA* 92:6339-6343.
- Vander Horn, P.B., M.C. Davis, J.J. Cunniff, C. Ruan, B.F. McArdle, S.B. Samols, J. Szaasz, G. Hu et al. 1997. Thermo Sequenase™ DNA polymerase and *T. acidophilum* pyrophosphatase: new thermostable enzymes for DNA sequencing. *BioTechniques* 22:758-762.
- Wong, I., S.S. Patel and K.A. Johnson. 1991. An induced-fit kinetic mechanism for DNA replication fidelity: direct measurement by single-turnover kinetics. *Biochemistry* 30:526-537.

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