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## Polymorphic forms of the protein L-isoaspartate (D-aspartate) O-methyltransferase involved in the repair of age-damaged proteins

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**Abstract** The protein L-isoaspartate (D-aspartate) O-methyltransferase (PCMT1) can initiate the repair of age-damaged aspartyl and asparaginyl residues of intracellular proteins. The human gene *PCMT1* encoding this enzyme has at least four polymorphic sites, one of which results in two major isoforms with either an Ile residue or a Val residue at amino acid position 119. The frequencies of the alleles encoding the Ile<sub>119</sub> and Val<sub>119</sub> variants are similar in Caucasian populations, but a predominance of the Ile<sub>119</sub> allele exists in Asian and African populations. Analyses of the enzymatic activities of the Ile<sub>119</sub> and Val<sub>119</sub> variants in red blood cell lysates show that the higher specific activity and thermostability of the Ile<sub>119</sub> isoform is balanced by the potentially compensating higher substrate affinity of the Val<sub>119</sub> isoform. In a preliminary attempt to find an association between genotype frequency at the *PCMT1* locus and healthy aging, we compared the distribution of genotypes in a healthy older population of Ashkenazi Jewish individuals with that in a younger ethnically matched control group. We found that 65% of the healthy older population had the heterozygous genotype, greater than the 50% expected by Hardy-Weinberg equilibrium, suggesting a possible selection for having both alleles of the repair methyltransferase in successful aging. Three additional polymorphisms in non-coding regions of the methyltransferase gene were found to be biallelic and demonstrated nonrandom association in a specific haplotype with the codon 119 polymorphism. Finally, we also detected a heterozygous mutation in the splicing branch site of intron 2 that did not appear to affect activity. This study will help define the normal physiological range of activity for this repair methyltransferase and give

us a better understanding of its role in the processes of aging and disease.

**Key words** Polymorphism · Isoaspartyl damage · Protein methylation · Protein repair · Aging

### Introduction

Intracellular proteins are subject to a variety of spontaneous chemical degradation reactions (Stadtman 1988; Harding et al. 1989). The resulting altered proteins can have compromised functions and may represent a manifestation of aging at the molecular level. The ability of cells to recognize and repair or degrade such damaged proteins would thus represent an evolutionary adaptation for long-term survival of the organism. Although protein degradation pathways are essential to cellular homeostasis, there is a substantial energy cost to completely degrade and resynthesize a protein. There are also situations where new protein synthesis does not occur or is severely curtailed, such as in mature red blood cells or in long-lived proteins such as the crystallins in the eye lens. These circumstances would favor the repair of damaged proteins as opposed to degradation. The protein L-isoaspartate (D-aspartate) O-methyltransferase pathway represents one means of at least partially repairing damaged aspartyl and asparaginyl residues that are susceptible to deamidation, isomerization, and racemization reactions (Fig. 1) (Geiger and Clarke 1987; Patel and Borchardt 1990; Clarke 1999).

The abundance of aspartyl and asparaginyl residues makes most proteins subject to these types of damage, and the nearly ubiquitous expression of this methyltransferase in both prokaryotes and eukaryotes suggests a housekeeping role in preventing the accumulation of damaged proteins (Kagan et al. 1997). In vitro experiments have shown that isoaspartyl damage can decrease the activity of specific proteins such as calmodulin (Potter et al. 1993) and the bacterial HPr phosphocarrier protein (Brennan et al. 1994), and that incubation with the repair methyltransferase and

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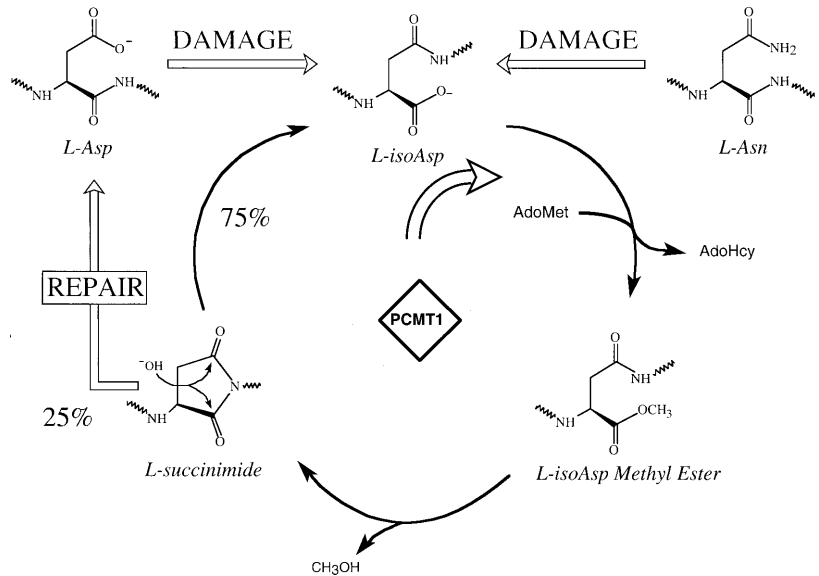
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**Fig. 1.** Repair mechanism of the protein L-isoaspartate (D-aspartate) methyltransferase (PCMT1) for the conversion of abnormal L-isoaspartyl residues to L-aspartyl residues. The formation of an isoaspartyl residue occurs from the nucleophilic attack of the peptide-bond nitrogen on the carboxyl group of the aspartyl or asparaginyl side chain via a succinimide ring intermediate. This reaction causes a redirection of the peptide backbone through the side chain and results in a kink in the protein (Lowenson and Clarke 1995). Such damage can result in a structural and subsequent functional change in the protein. The PCMT1 methyltransferase specifically recognizes the damaged form of the residue and initiates the repair cycle by transferring a methyl group from *S*-adenosyl methionine to the  $\alpha$ -carboxyl group of the L-isoaspartyl residue. The methyl ester forms spontaneously demethylate to give the cyclic L-succinimide residue that can regenerate the normal L-aspartyl residue by hydrolysis. Although the succinimide ring can also open to the L-isoaspartyl form, the constitutive presence of the methyltransferase leads to the net reconversion to the L-aspartyl residue in additional cycles of the reaction (Johnson et al. 1987b; McFadden and Clarke 1987). *AdoMet*, *S*-adenosyl methionine; *AdoHcy*, *S*-adenosyl homocysteine



the methyl-donating substrate *S*-adenosylmethionine can restore activity (Johnson et al. 1987a; Brennan et al. 1994). This type of damage is also found in the  $\beta$ -amyloid plaques of Alzheimer's Disease (Roher et al. 1993) and in cataractous eye lens tissue (McFadden and Clarke 1986), two common biomarkers of aging. The importance of repairing these damaged residues is most apparent in a mouse knockout model of the repair methyltransferase which shows substantially elevated levels of isoaspartyl damage in tissue proteins, coupled with the death of the mice within 40–50 days (Kim et al. 1997).

We have also been interested in the role of the human protein L-isoaspartate (D-aspartate) methyltransferase in the pathophysiology of the aging process. This enzyme is encoded by a single gene, *PCMT1*, that has been mapped to the q24–25 region of chromosome 6 (MacLaren et al. 1992b; DeVry and Clarke 1999) and is comprised of eight exons spanning approximately 60kb of genomic DNA (DeVry et al. 1996). Interestingly, the initial characterization of the human cDNA clones and peptide sequencing revealed allelic variations that suggested it was polymorphic (Ingrosso et al. 1989; MacLaren et al. 1992a; Tsai and Clarke 1994). We have subsequently begun to characterize the extent and complexity of these variations and have examined their effects on enzymatic activity. Recent studies have shown that the specific activity of the protein L-isoaspartate (D-aspartate) methyltransferase is influenced by the identity of the amino acid residue at position 119 (David et al. 1997). This polymorphic site has been characterized by a transition base substitution in the third position of codon 119 that results in either an Ile or Val residue (Tsai and Clarke 1994). Using red blood cell cytosolic fractions from a population of individuals with known genotype, higher specific activity and thermostability was seen for the Ile<sub>119</sub> homozy-

gotes compared with Ile<sub>119</sub>/Val<sub>119</sub> and with the Val<sub>119</sub> homozygotes (David et al. 1997).

In this paper, we expand the study of the position 119 polymorphism and establish its allele frequencies in different ethnic populations. Moreover, we show that the thermostability of the Ile<sub>119</sub> variant is balanced by an increased affinity of substrate recognition by the Val<sub>119</sub> variant. We also find an intriguing association when allele frequencies are compared between successfully aged individuals and younger ethnically matched controls. Finally, we perform a comprehensive screen of the *PCMT1* coding and flanking intron sequence to identify additional polymorphisms in the repair methyltransferase gene.

## Materials and methods

### Subjects

The population study of the polymorphism at amino acid position 119 in the *PCMT1* gene product included 395 unrelated individuals from the Los Angeles area, and 25 Norwegian and 20 Korean individuals collected by Dr. Richard Weinshilboum at the Mayo Clinic in Rochester, Minnesota. To ask if a correlation could be made between *PCMT1* genotype and healthy aging in human populations, 40 Ashkenazi Jewish individuals (31 women and 9 men; 75–104 years old; average age, 87.2 years) were studied as representatives of a successfully aged population that were communicative, ambulatory, self-sufficient, and living independently in a retirement community. Clinical histories were not available to establish any additional exclusion criteria. In order to compare this group with an ethnically matched younger popula-

tion, 40 Ashkenazi Jewish individuals (14 women and 26 men, 21–74 years old; average age, 36.2 years) were studied from the same metropolitan community. Human studies were approved by the Human Subject Protection Committee at the University of California, Los Angeles (UCLA) and at the Jewish Home for the Aging in Reseda, California.

## Genotyping

For the 395 individuals that were studied, genomic DNA was isolated from buccal samples that were obtained with informed consent from healthy volunteer subjects. Cells were collected on a sterile histology brush (Puritan Medical Products, Guilford, Maine, USA) by gently brushing the inner cheek for approximately 5–10s. Genomic DNA was prepared from the cheek cells as described by Richards et al. (1993) with the exception that the brush was immediately immersed in 1 ml of phosphate-buffered saline solution (pH 7.4) contained in a polypropylene microcentrifuge tube and vigorously twirled to release the cells. The brush was then removed and the cells were spun down at 14,000g for 1 min. The pellet was then resuspended in 600 µl of 50mM NaOH. After a 5-min incubation at 95°C, 60 µl 1 M Tris (pH 7.4) was added to neutralize the solution and the buccal cell lysate

containing genomic DNA was stored at –20°C. For the Norwegian and Korean samples, purified DNA was provided by Dr. Richard Weinsilbom.

Single-stranded conformational polymorphism (SSCP) screens were carried out by PCR amplification of each *PCMT1* exon (DeVry et al. 1996) using the primers listed in Table 1, followed by mutation detection enhanced polyacrylamide gel electrophoresis (Bailey 1995). Oligonucleotide primer synthesis and purification was performed by GIBCO BRL (Life Technologies, Grand Island, NY, USA). PCR reaction mixtures (25 µl) contained 20 pmol of each primer, 20 µM of a dNTP mix containing all four deoxyribonucleotide triphosphates (Pharmacia Biotech, Piscataway, NJ, USA), 1× reaction buffer (Promega, Madison, WI, USA), 3 mM MgCl<sub>2</sub>, 1 U *Taq* DNA polymerase (Promega), 5 µl buccal lysate containing genomic DNA, and 0.5 µl of 3000 Ci/mmol [ $\alpha$ -<sup>32</sup>P] dCTP (ICN Radiochemicals, Irvine, CA, USA). The MgCl<sub>2</sub> was added as a HotWax Mg<sup>2+</sup> bead (Invitrogen, San Diego, CA USA) to initiate a hot start PCR. The PCR cycling conditions for each amplification were 95°C for 2 min as an initial denaturation step and then 30 cycles of 95°C for 30s, 55°C for 30s, and 72°C for 30s, followed by a final extension step at 72°C for 5 min. A 2-µl aliquot of the reaction mixture was added to 9 µl of loading buffer containing 95% formamide, 10 mM NaOH,

**Table 1.** Sequences of primers used for PCR amplification and DNA sequencing

| Primer      | Exon | Sequence                               |
|-------------|------|--|
| CDI1F2      | 1    | 5'-CTAAGCCGCGGCGTCACAGAGC-3'           |
| EIF' (BT1F) |      | 5'-TGTGCTTAGCGATGGCCTGGAATC-3'         |
| CDI1F       |      | 5'-GGTGACGGTGTGGGAGGTGG-3'             |
| CD1R        |      | 5'-GGTGGCACTTACTGCGGAGATTGTGG-3'       |
| CDI1R4      |      | 5'-GCAACTCTGCGCCGAACCTCC-3'            |
| E2R (BT2R)  | 2    | 5'-CTGTAGCCAGCATCACTTCAAATAC-3'        |
| 2INF5'      |      | 5'-CAGTCTCTATAGCACAGCTGGTGTA-3'        |
| VB22LEUF    |      | 5'-CTTGTTTCAGAAAATGGAATCCTCAAG-3'      |
| VBE2F       |      | 5'-CTTGTTTCAGAAAATGGAATC-3'            |
| 2INF3'      |      | 5'-GTGTCCCAGGCTTTTTTCT-3'              |
| I3F         | 3    | 5'-TATTGTGAAAAATAACGTATGGA-3'          |
| CDI3R       |      | 5'-TGTAACCAAGTGAACAAAC-3'              |
| CDI4F2      | 4    | 5'-TGGCAGATAGCAAGATAAAGTGG-3'          |
| CDI4F       |      | 5'-CCATGATGTGTGTACTTTAGTC-3'           |
| CDE4F2      |      | 5'-CATGCATATGCGCTAGAAGTTC-3'           |
| CDI4R       |      | 5'-CATATAATATTCTTCTGTGGGGCC-3'         |
| E4R (BT4R)  |      | 5'-GCTTAGCTCCTTCATGCAACTG-3'           |
| E5F (DM5)   | 5    | 5'-GTTGGATGTACTGGAAAAGTCATAGG-3'       |
| EX5F        |      | 5'-GATAAACTGAGTGTTTAGCAATC-3'          |
| E5R (BT5R)  |      | 5'-CTGAGTCATCTACTAGCTCTTTAATG-3'       |
| E5R' (DM6)  |      | 5'-CAAGCTGACTCTCCCTGAAGACAG-3'         |
| I5R         |      | 5'-CTCTGAGTATTATTAGCTTGCATTGG-3'       |
| CDI6F       | 6    | 5'-CCTCCTGTACTAGAATTCTTCAC-3'          |
| CDI6R       |      | 5'-CACACAAACAGAAAAAATCCCG-3'           |
| CDI7F2      | 7    | 5'-CTGTAAGTGGTGTATGTATGTGC-3'          |
| E7aF (DM4)  |      | 5'-GTTAAAGCCCGGAGGAAGATTGATTTG-3'      |
| E7aF'       |      | 5'-AAATGAAGCCTCTGATGGG-3'              |
| CDE7cR      |      | 5'-GCTCTGTAGTCTAATGTCTTAAAATCACACCC-3' |
| E7aR' (DM3) |      | 5'-GGACCACTGCTTTTCTTATCTG-3'           |
| VNTRF       | 8    | 5'-CTTTAAGGGAGCATGAATAAATGGG-3'        |
| VNTRR       |      | 5'-GTTGCCCTCATATTATGTAAAGG-3'          |

0.05% bromophenol blue, and 0.05% xylene cyanol. The samples were then incubated at 95°C for 5 min and immediately placed on ice. Conformation polymorphisms of single-stranded DNA fragments were analyzed using nondenaturing polyacrylamide gels (40 × 30 × 0.04 cm) made in one of the following ways. Gels containing 0.5 × mutation detection enhancement (MDE) (FMC BioProducts, Rockland, Maine, USA) with 10% glycerol were run in 0.6 × Tris-borate electrophoresis buffer (TBE) buffer at 5 W overnight at room temperature using a BioRad Power Pac 3000 (Madison, WI, USA). Gels containing 0.5 × MDE without glycerol were run in 0.6 × TBE buffer at 8°C for 6 h at 15 W. The gel was dried under vacuum for 1 h at 70°C and the DNA fragments were visualized by autoradiography (Kodak X-OMAT AR film, Rochester, NY, USA) at room temperature for 1–2 days. When signal intensity was weak, a 1- to 2-day exposure was done at –80°C with an intensifying screen. To scan the entire coding region for additional polymorphisms of the *PCMT1* gene, primers were designed for PCR amplification to span each exon but also to include the splice site consensus sequences (Table 1). To maintain PCR products of less than 200 bp for the subsequent SSCP analysis, multiple overlapping primer sets were often used to span a single exon.

In the analysis of the dinucleotide repeat polymorphism in the 3'-untranslated region, the procedure of Litt et al. (1993) was followed to avoid the problem of "shadow banding", which can make it difficult to score alleles reliably. The VNTRF primer was end-labeled with [ $\gamma$ -<sup>32</sup>P] ATP (>6000 Ci/mmol; ICN) and T<sub>4</sub> polynucleotide kinase (New England Biolabs, Beverly, MA, USA) for 1 h at 37°C. The 50- $\mu$ l PCR mixture contained 1 × buffer (Promega), a 3.5-mM MgCl<sub>2</sub>, HotWax bead (Invitrogen), 200  $\mu$ M of each dNTP (GIBCO BRL), 320 ng each of the unlabeled primers VNTRF and VNTRR (Table 1), 40 ng of the <sup>32</sup>P-labeled primer, 5  $\mu$ l of buccal genomic DNA lysate, and 2 U of Taq DNA polymerase (Promega). The PCR cycling parameters were carried out as described above for the PCR-SSCP. A 5- $\mu$ l aliquot of the PCR reaction was mixed with 10  $\mu$ l of a loading buffer containing 95% formamide, 10 mM ethylene diamine tetraacetic acid (EDTA), 0.05% bromophenol blue, and 0.05% xylene cyanol and incubated at 95°C for 5 min prior to loading. From this diluted mixture, 5  $\mu$ l of each sample was run on 7% acrylamide, 30% formamide, 6 M Urea, 0.6 × TBE gel (40 × 30 × 0.04 cm) for 2 h at 50 W. The gel was subsequently dried down under vacuum for 1 h at 70°C and exposed to autoradiographic film (Kodak X-OMAT AR).

For the purposes of restriction digests and DNA sequencing, DNA was amplified in a 50- $\mu$ l PCR reaction using 40 pmol of each exon-specific primer, 1 × reaction buffer (Promega), 200  $\mu$ M of a dNTP mix containing all four deoxyribonucleotide triphosphates, a 3.5-mM Hotwax MgCl<sub>2</sub> bead, 2 U Taq DNA polymerase, and 10  $\mu$ l buccal lysate containing genomic DNA. The PCR cycling conditions consisted of the parameters described above. DNA was isolated from the reaction mixture using the Wizard PCR Prep DNA Purification System (Promega). Restriction digest analyses for the codon 119 polymorphism were carried out with 10 U of the enzyme *DdeI* (GIBCO BRL) at 37°C for

2 h and visualized on a 1.2% agarose gel stained with ethidium bromide. PCR reactions to be sequenced were submitted to UCLA's DNA Sequencing Facility for analysis with the ABI Fluorescent Sequencing System (Perkin-Elmer, Norwalk, CT, USA).

#### Methyltransferase activity assays

Methyltransferase activity was examined in red blood cell cytosolic fractions prepared from individuals of known genotype for the polymorphism at amino acid position 119. Blood (approximately 200  $\mu$ l) was collected after informed consent by pricking the finger with a sterile microlancet, and was transferred to a 1-ml solution of phosphate-buffered saline containing 2 mg/ml disodium EDTA at pH 7.4. The red blood cells were isolated by centrifugation at 14,000 g at 4°C for 2 min. After approximation of the volume of packed red blood cells and discarding of the supernatant, the cells were resuspended in 5 volumes of lysis buffer containing 5 mM sodium phosphate (monobasic), 1 mM ethyleneglycoltetraacetic acid (EGTA) at pH 7.4, and 25  $\mu$ M phenylmethylsulfonyl fluoride (PMSF). The resuspended cells were frozen in dry ice for 30 min to aid lysis and then centrifuged at 14,000 g at 4°C for 5 min to pellet the membrane fraction. The cytosol was transferred to a new tube and the protein concentration was determined by a modification of the Lowry assay, using bovine serum albumin as a standard (Bailey 1967).

The activity of the protein L-isoaspartyl (D-aspartyl) methyltransferase was determined using the vapor-phase diffusion method (Macfarlane 1984). The L-isoaspartyl containing protein ovalbumin (5  $\mu$ l of an 80 mg/ml stock; Grade V; Sigma, St. Louis, MO, USA) was added to 5  $\mu$ l red blood cell cytosol and 10  $\mu$ l of 0.2 M Bis-Tris buffer pH 6, in a microcentrifuge tube. Following the addition of 10  $\mu$ l of 30  $\mu$ M S-adenosyl-[methyl-<sup>14</sup>C]-methionine (in 0.2 M bis-Tris, pH 6; 55 mCi/mmol; Amersham, Arlington Heights, IL, USA) the reaction mixture was incubated at 37°C for 20 min and the reaction was terminated by placing the tube on dry ice. The reaction mixture was then thawed and 60  $\mu$ l of 0.2 M NaOH was added to convert the radiolabeled methyl esters to [<sup>14</sup>C]methanol. Immediately after the addition of base, the reaction was spotted onto a 1.9 cm × 10.2 cm piece of thick filter paper (BioRad gel dryer paper no. 1650962) that had been folded in an accordion pattern. This filter paper was then placed in the neck of a scintillation vial suspended above 5 ml of Safety-Solve Counting Fluid (Research Products International, Mount Prospect, IL, USA) and capped tightly. After a 2-h incubation time at room temperature, the filter paper was discarded and the scintillation fluid was counted three times for 5-min intervals in a Beckman LS 6500 scintillation counter. Specific activities were measured as pmol of methyl groups transferred per min per mg of red blood cell cytosolic protein.

The thermal stability of methyltransferase variants was determined by preincubating the red blood cell cytosols at a protein concentration of 23–33 mg/ml with 0.2 M Bis-Tris buffer (pH 6.5) at 0°C, 37°C, 48°C, 50°C, and 52°C for a period

of 15 min prior to measuring enzyme activity as described above. Similarly, oxidative stability was determined by preincubating the red blood cell cytosols at a protein concentration of 46–78 mg/ml in various concentrations of H<sub>2</sub>O<sub>2</sub> and 1 mg/ml sodium azide (to inhibit catalase activity) for 15 min.

### Statistical analysis

$\chi^2$  analysis was utilized in comparisons of allele and genotype frequencies between ethnic groups in the population study, as well as in comparisons between young and old populations in the successful aging study. Analysis of variance was performed to compare the activities of the repair methyltransferase enzyme between individuals of different genotype, and a Student's *t*-test was used to compare both  $V_{\max}$  and  $K_m$  means between homozygous genotypes in kinetic studies. Values represent the means  $\pm$  SEM. These calculations were carried out with the statistical programs Excel (Microsoft, Redmond, WA, USA) and Prism (GraphPad, San Diego, CA, USA).

## Results

### Population study of the amino acid 119 polymorphism in the human protein L-isoaspartate (D-aspartate) methyltransferase

The frequency of variation at the amino acid 119 polymorphic site within this methyltransferase was determined in a population study of 440 individuals. Using genomic DNA, individuals were genotyped using PCR-SSCP analysis for the AAT codon leading to isoleucine<sub>119</sub> or the GAT codon leading to valine<sub>119</sub> (Fig. 2). The sequence-specific single strand conformations of the Ile<sub>119</sub> homozygous genotype are represented by a doublet banding pattern and the Val<sub>119</sub>

homozygous genotype is represented by a single band. The presence of both banding patterns represents a heterozygous genotype. These banding patterns were initially correlated with specific genotypes by sequence analysis (Fig. 2) and restriction digestion (data not shown). The presence of a G nucleotide in the third position of codon 119 creates a recognition site for the *DdeI* restriction enzyme. The population distributions found for each genotype are given in Table 2. Although no gender-specific differences were observed, significant differences in the distribution of genotypes were observed between certain ethnic groups for which we had an adequate number of samples for comparison. Specifically, the Caucasian allele and genotype frequencies were significantly different from those in both the Asian and African ethnic groups. The allele frequency in the Caucasian population was 0.45 for Ile<sub>119</sub> and 0.55 for Val<sub>119</sub>, while in the Asian population the allele frequency was 0.88 for Ile<sub>119</sub> and 0.12 for Val<sub>119</sub> ( $P < 0.0001$ ). Similar to the Asian population, the African population had allele frequencies of 0.81 and 0.19 for Ile<sub>119</sub> and Val<sub>119</sub>, respectively, that were significantly different from Caucasians ( $P < 0.0001$ ). The differences between Asian and African were not significant ( $P = 0.14$ ). Based on the observed allele frequencies, the calculated best fit population distribution was very similar to the observed distribution in all ethnic groups, confirming a Hardy-Weinberg equilibrium. Three homogeneous populations within the Caucasoid group (Indian, Ashkenazi Jewish, and Norwegian) showed similar genotypic distributions and were not significantly different from the total Caucasian group ( $P = 0.80$ ;  $P = 0.77$ ; and  $P = 0.32$ , respectively). Within the Asian group, however, there appear to be some differences between the individual populations. The Chinese and Korean populations, the only two with adequate numbers for comparison in this group, were significantly different from each other ( $P = 0.016$ ). The number of individuals in other ethnic groups was insufficient to make any other comparisons.

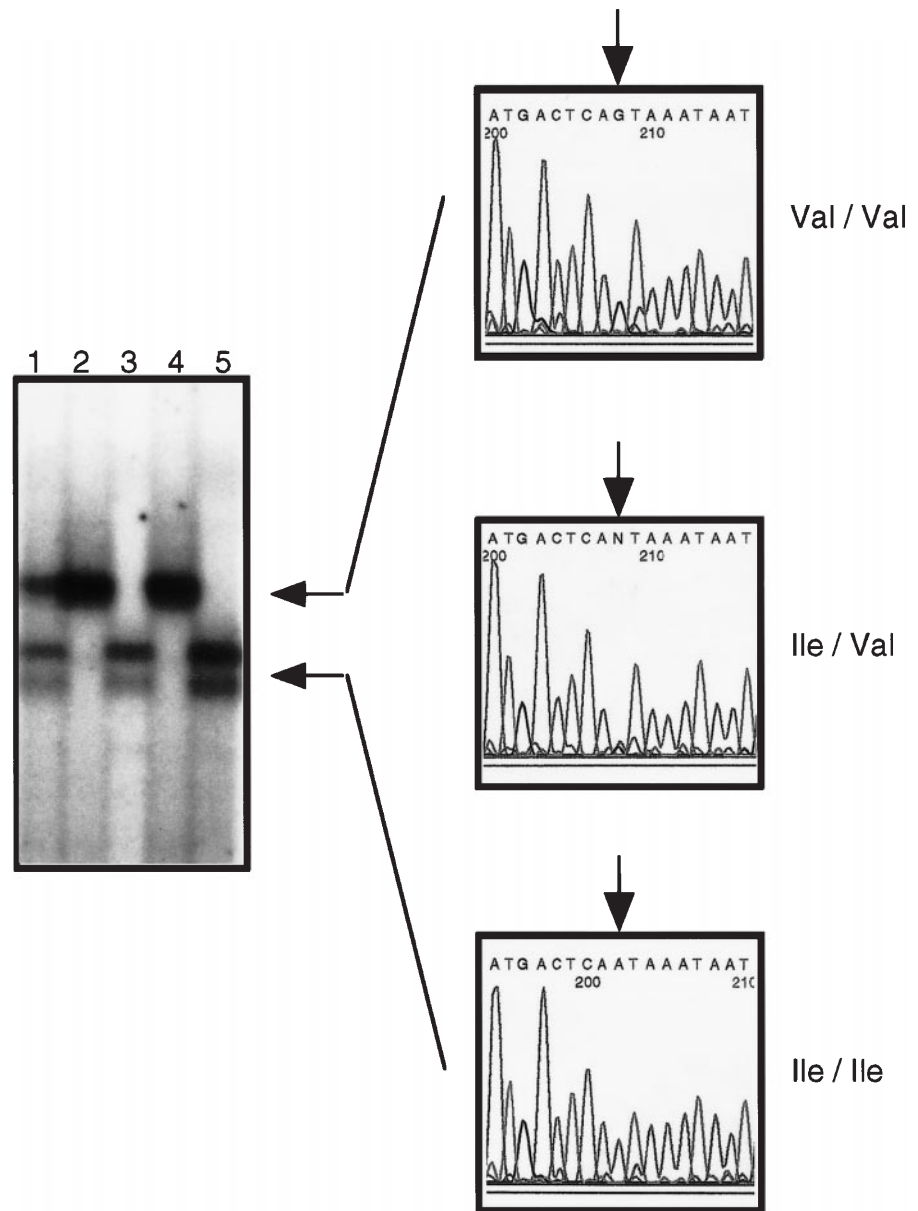
**Table 2.** Population study of the polymorphism at amino acid position 119

| The study population ( <i>n</i> = 440)  | No. of individuals/genotype (% of total) |            |           | Allele frequency <sup>a</sup> |       |
|---|--|------------|-----------|-------------------------------|-------|
|   | Ile/Ile                                  | Ile/Val    | Val/Val   | Ile                           | Val   |
| Caucasian ( <i>n</i> = 284)             | 55 (19.4)                                | 144 (50.7) | 85 (29.9) | 0.447                         | 0.553 |
| Ashkenazi Jewish ( <i>n</i> = 80)       | 15 (18.8)                                | 44 (55.0)  | 21 (26.2) | 0.462                         | 0.538 |
| Indian ( <i>n</i> = 31)                 | 6 (19.3)                                 | 14 (45.2)  | 11 (35.5) | 0.419                         | 0.581 |
| Norwegian <sup>b</sup> ( <i>n</i> = 25) | 8 (32.0)                                 | 11 (44.0)  | 6 (24.0)  | 0.540                         | 0.460 |
| Other Caucasian ( <i>n</i> = 148)       | 26 (17.5)                                | 75 (50.7)  | 47 (31.8) | 0.429                         | 0.571 |
| Asian ( <i>n</i> = 96)                  | 72 (75.0)                                | 24 (25.0)  | 0         | 0.875                         | 0.125 |
| Chinese ( <i>n</i> = 39)                | 35 (89.7)                                | 4 (10.3)   | 0         | 0.949                         | 0.051 |
| Korean ( <i>n</i> = 33)                 | 22 (66.7)                                | 11 (33.3)  | 0         | 0.833                         | 0.167 |
| Japanese ( <i>n</i> = 6)                | 2 (33.3)                                 | 4 (66.7)   | 0         |                               |       |
| Vietnamese ( <i>n</i> = 5)              | 4 (80.0)                                 | 1 (20.0)   | 0         |                               |       |
| Other Asian ( <i>n</i> = 13)            | 9 (69.2)                                 | 4 (30.8)   | 0         |                               |       |
| African ( <i>n</i> = 40)                | 25 (62.5)                                | 15 (37.5)  | 0         | 0.812                         | 0.188 |
| Hispanic ( <i>n</i> = 14)               | 3 (21.4)                                 | 7 (50.0)   | 4 (28.6)  |                               |       |
| Mixed ( <i>n</i> = 6)                   | 2 (33.3)                                 | 3 (50.0)   | 1 (16.7)  |                               |       |

<sup>a</sup> Allele frequency only reported in population  $n \geq 25$

<sup>b</sup> Norwegian samples collected by Dr. Richard Weinshilboum at the Mayo Clinic in Rochester, Minnesota

**Fig. 2.** Detection of amino acid polymorphism at codon position 119 of *PCMT1* by polymerase chain reaction-single chain reaction-single-stranded conformational polymorphism (PCR-SSCP) and DNA sequencing. A 117-bp region of exon 5 was amplified by PCR with the primers DM5 and DM6 (Table 1). The labeled PCR products were separated by polyacrylamide gel electrophoresis (PAGE) following the SSCP protocol as described in "Materials and Methods". Individuals homozygous for the Val<sub>119</sub> allele presented a single band (lanes 2 and 4), while individuals homozygous for Ile<sub>119</sub> presented a doublet banding pattern (lanes 3 and 5). Heterozygous individuals demonstrated a combination of both banding patterns (lane 1)



#### Enzymatic differences of the human PCMT1 polymorphic variants

To determine the functional consequence of the Ile<sub>119</sub>/Val<sub>119</sub> polymorphism, a series of enzymatic analyses were conducted with cytosolic extracts from red blood cells of individuals of known genotype. Specific activity differences were determined by measuring the initial velocity of a reaction with saturating levels of the methyl-accepting substrate ovalbumin and the methyl-donating substrate S-adenosylmethionine, using a vapor phase diffusion assay. In four independent experiments with representatives of each genotype ( $n = 4$ ), a small but significant difference was observed between the means of each homozygous genotype group ( $P = 0.013$ ), as determined by a one-way analysis of variance (ANOVA) (Fig. 3A). The average activity for the

four Ile<sub>119</sub> representatives of  $4.4 (\pm 0.19 \text{ SEM})$  pmol/min per mg was approximately 20% higher than that of the Val<sub>119</sub> individuals, which averaged  $3.4 (\pm 0.19 \text{ SEM})$  pmol/min per mg. Intermediate values of  $4.0 (\pm 0.15 \text{ SEM})$  were observed for the heterozygotes. A Tukey's multiple comparison test of the data found a significant difference of means specifically between the Ile<sub>119</sub>/Ile<sub>119</sub> and Val<sub>119</sub>/Val<sub>119</sub> homozygous genotypes ( $P < 0.01$ ), with a mean difference of  $0.96$  pmol/min per mg (95% confidence interval [CI]: 0.26–1.7).

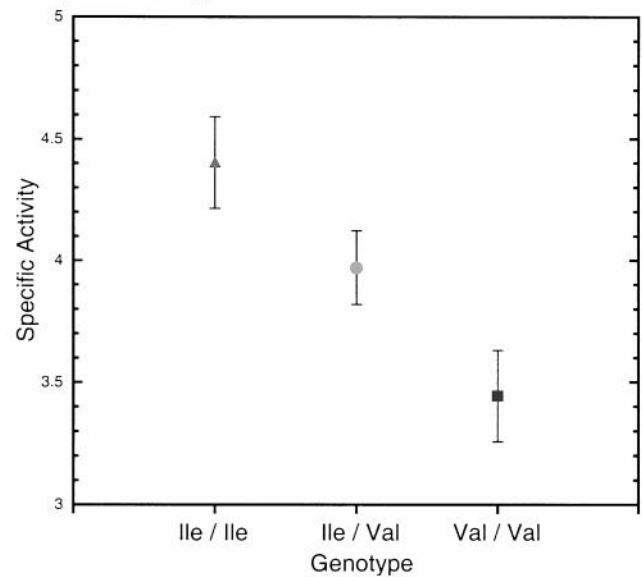
Such differences in the specific activity of the enzyme in red blood cells can result from differences in the stability of the enzyme, as well as from its intrinsic ability to methylate ovalbumin (cf. refs. Frosst et al. 1995; Lotta et al. 1995). We thus measured the effect this amino acid substitution has on the temperature stability of the enzyme by preincubating representative red blood cell cytosolic fractions from four

individuals of each genotype for 15 min at a range of temperatures (Fig. 3B). This preincubation was immediately followed by initial velocity assays, using the substrate ovalbumin as described above. A two-way ANOVA established that genotype had a significant effect on thermostability ( $P < 0.0001$ ). The results showed that the Ile<sub>119</sub> enzyme was significantly more thermotolerant than the Val<sub>119</sub> enzyme at all preincubation temperatures (48°C, 50°C, and 52°C) ( $P = 0.0005$ ). After preincubation at 50°C, the Ile<sub>119</sub> homozygous cytosol maintained an approximately four fold higher activity than the Val<sub>119</sub> homozygous cytosol. The Ile<sub>119</sub>/Ile<sub>119</sub> cytosol retains 41% of its initial activity at 50°C while the Val<sub>119</sub>/Val<sub>119</sub> cytosol retains only 11% of its initial activity. The greater stability of the Ile<sub>119</sub> enzyme may account for the higher activity of this enzyme as compared with the Val<sub>119</sub> enzyme in the red blood cell, which has an average age of about 60 days in the circulation (Bartosz 1990). Similar stability assays were performed with oxidative stress in which red blood cell cytosols from two representatives of each genotype were preincubated with various concentrations of H<sub>2</sub>O<sub>2</sub> in three independent experiments (Fig. 3C). The genotype did effect the result (two-way ANOVA;  $P = 0.0024$ ), with the activity of the Val<sub>119</sub> enzyme being consistently lower than that of the Ile<sub>119</sub> enzyme after a 15-min preincubation at final concentrations of 7.7, 15.4, 30.8, 76.9, and 153.8 mM. However, the genotype did not have the same level of effect at all values of H<sub>2</sub>O<sub>2</sub> concentration. The H<sub>2</sub>O<sub>2</sub> concentration with greatest effect was 30.8 mM, at which an approximately 10% difference in activity was observed.

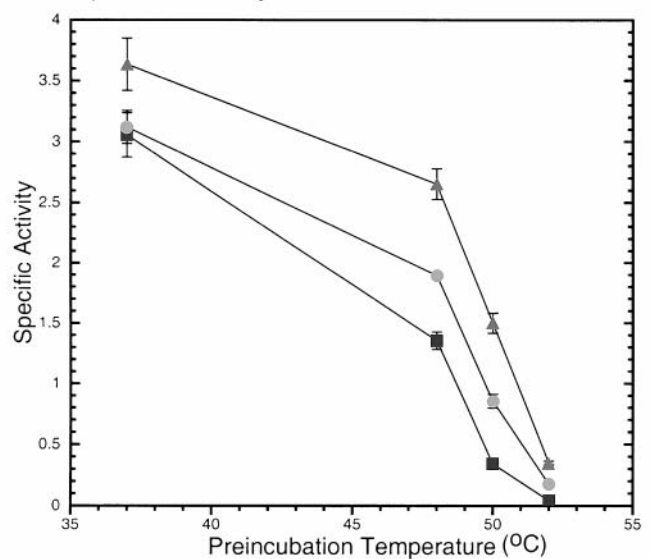
To investigate the possibility of differences in substrate recognition specificities between the codon 119 variants, activity assays were performed with a number of different isoaspartyl-containing peptide substrates for which the repair methyltransferase is known to have varying affinity (Lowenson and Clarke 1991). The reactions were carried out at saturating concentrations for each peptide. After normalizing for the differences observed with ovalbumin that might be attributable to enzyme levels associated with the differences in stability, there was no appreciable differ-

**Fig. 3A–C.** Differences in L-isoaspartyl methyltransferase activity and stability between PCMT1 polymorphic variants of amino acid position 119. **A** The specific activity was measured as pmol/min per mg protein with red blood cell cytosolic extracts prepared from individuals ( $n = 4$ ) of each genotype, using ovalbumin as substrate. *Error bars* indicate the standard error of the mean in four independent experiments. **B** Red blood cell cytosols from four unrelated individuals of each genotype were preincubated at either 37°C, 48°C, 50°C, or 52°C for 15 min prior to the methyltransferase assay, using ovalbumin as a methyl acceptor as described above. The average specific activity of the group in each genotype is shown with *error bars* representing the standard error of the mean. The specific activity is measured in pmol/min per mg protein in the red blood cell cytosolic extract. Ile<sub>119</sub>/Ile<sub>119</sub> homozygotes are represented by *triangles*, Ile<sub>119</sub>/Val<sub>119</sub> by *circles*, and Val<sub>119</sub>/Val<sub>119</sub> by *squares*. **C** Methyltransferase stability under oxidative stress was measured in red blood cell cytosolic extracts of two representatives for each homozygous genotype. In three independent experiments, extracts were preincubated at either 0, 7.7, 30.8, 76.9, or 153.8 mM H<sub>2</sub>O<sub>2</sub> with 1 mg/ml sodium azide for 15 min prior to the methyltransferase assay with ovalbumin. The specific activity given is measured relative to the activity maintained in the absence of H<sub>2</sub>O<sub>2</sub>. *Error bars* represent the standard error of the mean. Ile<sub>119</sub> homozygous cytosols are represented by *triangles*; Val<sub>119</sub> homozygous cytosols are represented by *squares*.

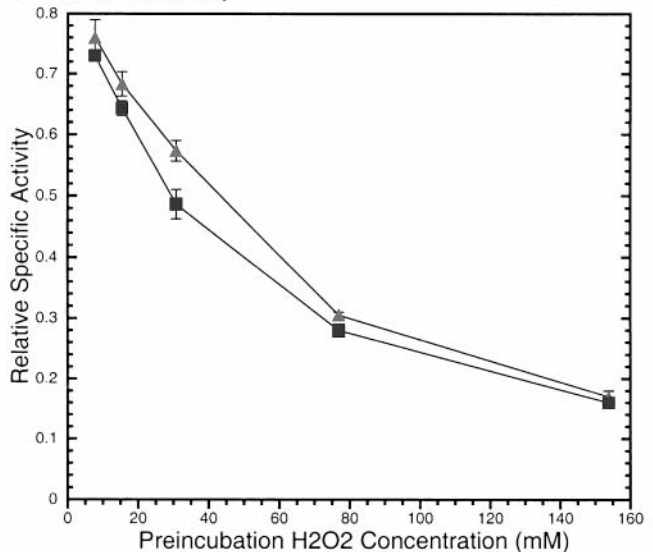
### A. Specific Activity



### B. Temperature Stability



### C. Oxidative Stability

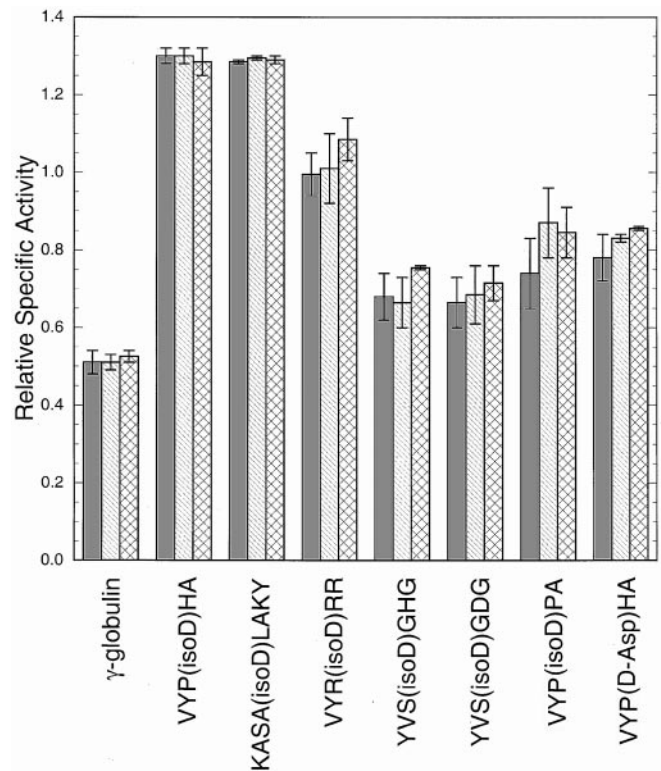


ence in the ability of either enzyme variant to recognize any of the representative peptide substrates (Fig. 4).

We next attempted to more accurately examine kinetic differences between the codon 119 variants with a range of substrate concentrations, using ovalbumin, two different isoaspartyl-containing peptides, and the brain cytosolic extract from a *Pcmt-1*-deficient mouse (Kim et al. 1997). The absence of the repair enzyme in these mice leads to the accumulation of proteins with damaged sites, particularly in the brain (Kim et al. 1997). Therefore, the *Pcmt-1* deficient mouse strain was useful as a more representative source of endogenous methyl-accepting protein substrates in the damaged state. In three independent experiments, assays of red blood cell cytosolic extracts from representatives for each homozygous genotype ( $n = 3$ ) using brain cytosol from *Pcmt-1* deficient mice revealed a significant difference in both the  $V_{\max}$  and  $K_m$  values between the Ile<sub>119</sub> and Val<sub>119</sub> polymorphic variants (Fig. 5A). The mean  $V_{\max}$  established for the Ile<sub>119</sub> enzyme was  $3.8 (\pm 0.16\text{SEM})$  pmol/min per mg protein, while the Val<sub>119</sub> enzyme gave a mean value of  $2.9 (\pm 0.21\text{SEM})$  pmol/min per mg protein ( $P = 0.004$ ). This was consistent with the difference in specific activity observed with ovalbumin between these variants (Fig. 3A). Interestingly, the  $K_m$  determined for the methyltransferase with the *Pcmt-1* deficient mouse brain cytosol as a methyl-accepting substrate was  $1.9 \pm 0.19\text{mg/ml}$  protein for the Val<sub>119</sub> enzyme and  $2.6 \pm 0.18\text{mg/ml}$  protein for the Ile<sub>119</sub> enzyme ( $P = 0.011$ ). This difference represents a 30% increase in affinity of the Val<sub>119</sub> enzyme for this collection of endogenous substrates. A difference between the amino acid 119 variants was also observed in kinetic experiments with ovalbumin (Fig. 5B). The  $V_{\max}$  values of ovalbumin for the Ile<sub>119</sub> and Val<sub>119</sub> homozygotes were significantly different, as expected, with values of  $3.9 \pm 0.25\text{pmol/min}$  per mg protein and  $2.8 \pm 0.13\text{pmol/min}$  per mg protein, respectively ( $P = 0.007$ ). Affinity differences were also observed for ovalbumin, with the  $K_m$  values of  $3.4 \pm 0.38\text{mg/ml}$  for the Ile<sub>119</sub> enzyme and  $2.3 \pm 0.35\text{mg/ml}$  for the Val<sub>119</sub> enzyme ( $P = 0.078$ ). Again the Val<sub>119</sub> enzyme showed an increase in affinity, although not quite meeting the 95% significance level. With respect to substrate affinity, no differences were observed for methyl-accepting peptides (Fig. 5C). The  $V_{\max}$  values established for the VYR(isoD)RR peptide were  $3.7 \pm 0.36\text{pmol/min}$  per mg protein with the Ile<sub>119</sub> cytosols and  $3.2 \pm 0.38\text{pmol/min}$  per mg protein with the Val<sub>119</sub> cytosols (not significantly different;  $P = 0.37$ ). The  $K_m$  values for the VYR(isoD)RR peptide were  $17.5 \pm 1.5\mu\text{M}$  and  $17.1 \pm 2.4\mu\text{M}$  for the Ile<sub>119</sub> and Val<sub>119</sub> cytosols, respectively (not significantly different;  $P = 0.90$ ). The peptide YVS(isoD)GHG also had similar  $K_m$  values with respect to the two isoforms (data not shown).

#### Protein L-isoaspartate (D-aspartate) methyltransferase alleles in successful longevity

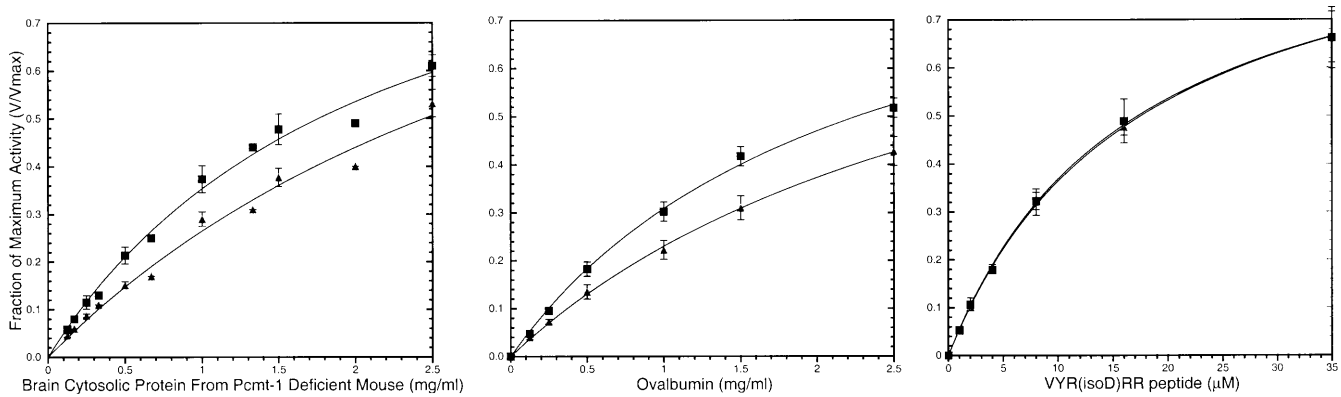
Because the potential accumulation of damaged proteins may have detrimental effects over the human lifespan, an analysis of a group of “successfully aged” individuals was



**Fig. 4.** Methyl-acceptor substrate specificity comparisons between the PCMT1 amino acid 119 polymorphic variants, using various protein and peptide substrates. Red blood cell cytosols from a homozygous Ile<sub>119</sub> individual (solid bars), a heterozygous Ile<sub>119</sub>/Val<sub>119</sub> individual (striped bars), and a homozygous Val<sub>119</sub> individual (cross-hatched bars), were assayed in two independent experiments for methyltransferase activity. The following concentrations were used for each peptide: 30  $\mu\text{M}$  VYP(isoD)HA, 50  $\mu\text{M}$  KASA(isoD)LAKY, 35  $\mu\text{M}$  VYR(isoD)RR, 155  $\mu\text{M}$  YVS(isoD)GHG, 4.89 mM YVS(isoD)GDG, 40 mM VYP(isoD)PA, and 30 mM VYP(D-Asp)HA. These concentrations are roughly ten fold greater than the  $K_m$  for these peptides, except for VYP(isoD)HA and KASA(isoD)LAKY, for which 100-fold concentration was used. The specific activity given is measured relative to ovalbumin at  $V_{\max}$  concentrations of substrate to normalize for isoform stability differences. Error bars represent the standard error of the mean

initiated, with the assumption that this population might be enriched in the most beneficial alleles (including those of the PCMT1 methyltransferase) for a long and healthy life. A preliminary study was carried out with 40 individuals of Ashkenazi Jewish descent that met our “successfully aged” phenotype by being age 75 or older and in good mental and physical health. The 40 healthy older individuals in this study ranged in age from 75–104, with an average age of 87.2 years, and were compared with a younger control population of the same ethnic background consisting of 40 individuals ranging in age from 21–74, and averaging 36.2 years. We found that the overall allele frequencies were nearly identical between the two groups and very similar to the expected values for the Caucasian ethnic background (Table 3). Surprisingly, however, the distribution of genotypes revealed that, within the older population, the heterozygous genotype was represented at an unusually high frequency of 65%, given the 45% frequency in ethnically





**Fig. 5A–C.** Kinetic analysis of methyltransferase enzyme activity in the amino acid 119 polymorphic variants. In three independent experiments, red blood cell cytosols from three unrelated representatives of each homozygous genotype (Ile<sub>119</sub>/Ile<sub>119</sub>; Val<sub>119</sub>/Val<sub>119</sub>) were used in enzymatic assays with three different methyl-accepting substrates. **A** brain cytosolic extract from a *Pcmt-1* deficient mouse; **B** ovalbumin; **C** L-isoaspartyl-containing synthetic peptide. The specific methyltransferase activity is shown as a fraction of the maximum rate to normalize the  $V_{max}$  differences associated with Ile<sub>119</sub> and Val<sub>119</sub> genotypes.

*Squares* represent data from Val<sub>119</sub>/Val<sub>119</sub> homozygous individuals; *triangles* represent data from individuals homozygous for the Ile<sub>119</sub>-containing enzyme. The *solid lines* are drawn based on the best fit of the Michaelis-Menton equation to the data yielding  $K_m$  values of  $1.9 \pm 0.19$  mg/ml (Val<sub>119</sub>) and  $2.6 \pm 0.18$  mg/ml (Ile<sub>119</sub>) for brain cytosolic protein,  $2.3 \pm 0.35$  mg/ml (Val<sub>119</sub>) and  $3.4 \pm 0.38$  mg/ml (Ile<sub>119</sub>) for ovalbumin, and  $17.1 \pm 2.4$   $\mu$ M (Val<sub>119</sub>) and  $17.5 \pm 1.5$   $\mu$ M (Ile<sub>119</sub>) for the synthetic peptide VYR(isoD)RR. *Error bars* represent the standard error of the mean

**Table 3.** Polymorphism in younger and older Ashkenazi Jewish populations

| Younger Ashkenazi Jewish (Average age, 36.2 years; range, 21–74 years) |         |         | Healthy older Ashkenazi Jewish (Average age, 87.2 years; range, 75–104 years) |         |         |         |
|--|---------|---------|---|---------|---------|---------|
| Ile/Ile  | Ile/Val | Val/Val |   | Ile/Ile | Ile/Val | Val/Val |
| 9  | 18      | 13      | No. of individuals  | 6       | 26      | 8       |
| 36   |         | 44      | No. of alleles  | 38      |         | 42      |
| 0.450  |         | 0.550   | Observed allele frequency   | 0.475   |         | 0.525   |
| 0.203  | 0.495   | 0.303   | Expected Hardy-Weinberg distribution  | 0.226   | 0.499   | 0.276   |
| 0.225  | 0.450   | 0.325   | Observed population distribution  | 0.150   | 0.650   | 0.200   |

matched younger controls. While comparison of the two populations directly did not reveal statistical significance at the  $P < 0.05$  level ( $P = 0.20$ ), a significant difference was observed when the genotype frequencies in the old population were compared with the population frequencies expected by Hardy-Weinberg equilibrium based on the allele frequencies observed in the younger population ( $P = 0.049$ ). This result suggests the possibility of a heterozygote advantage and that a selection for having both forms of the gene may occur for individuals that have “successfully aged”, but further studies will be needed to validate this hypothesis. This putative hybrid vigor may offer a protective advantage for the Ile<sub>119</sub>/Val<sub>119</sub> genotype in the repair of protein damage associated with aging.

#### Additional polymorphisms identified in the human *PCMT1* gene

To determine whether additional polymorphisms in the *PCMT1* gene exist, a comprehensive screen of the entire coding region was undertaken. Fifty randomly chosen individuals of various ethnic backgrounds were genotyped by PCR-SSCP with primer sets specific for each of the seven

major coding exons and flanking intron sequence of the gene (DeVry et al. 1996) (Table 1). Using the two different gel electrophoresis conditions described in the “Materials and Methods” section, three additional polymorphisms were identified, but all were located in noncoding regions of the gene (Fig. 6). A single nucleotide substitution was identified in intron 4, 79bp upstream of exon 5, with a T-to-C transition base substitution. An insertion/deletion of a T nucleotide was identified in intron 6 in a region of 12 sequential T nucleotides, 8–19bp upstream of exon 7. In the 3′-untranslated region (3′-UTR), a GT dinucleotide repeat was observed approximately 2kb downstream of exon 8, with repeat length variation limited to either 8 or 14 repeats. Interestingly, all three of these noncoding polymorphisms demonstrate nonrandom association with the Ile/Val polymorphism at amino acid position 119, resulting in just two different haplotypes being present (Fig. 7). Although the genotyping at the non-coding polymorphic sites initially comprised 50 individuals, further attempts to find a recombination event involved genotyping additional individuals at the site most distant to the Ile<sub>119</sub>/Val<sub>119</sub> polymorphism, the 3′-UTR dinucleotide repeat. A total of 122 individuals (39 Ile<sub>119</sub> homozygotes, 27 Val<sub>119</sub> homozygotes, and 56 Ile<sub>119</sub>/Val<sub>119</sub> heterozygotes) have subsequently been genotyped at

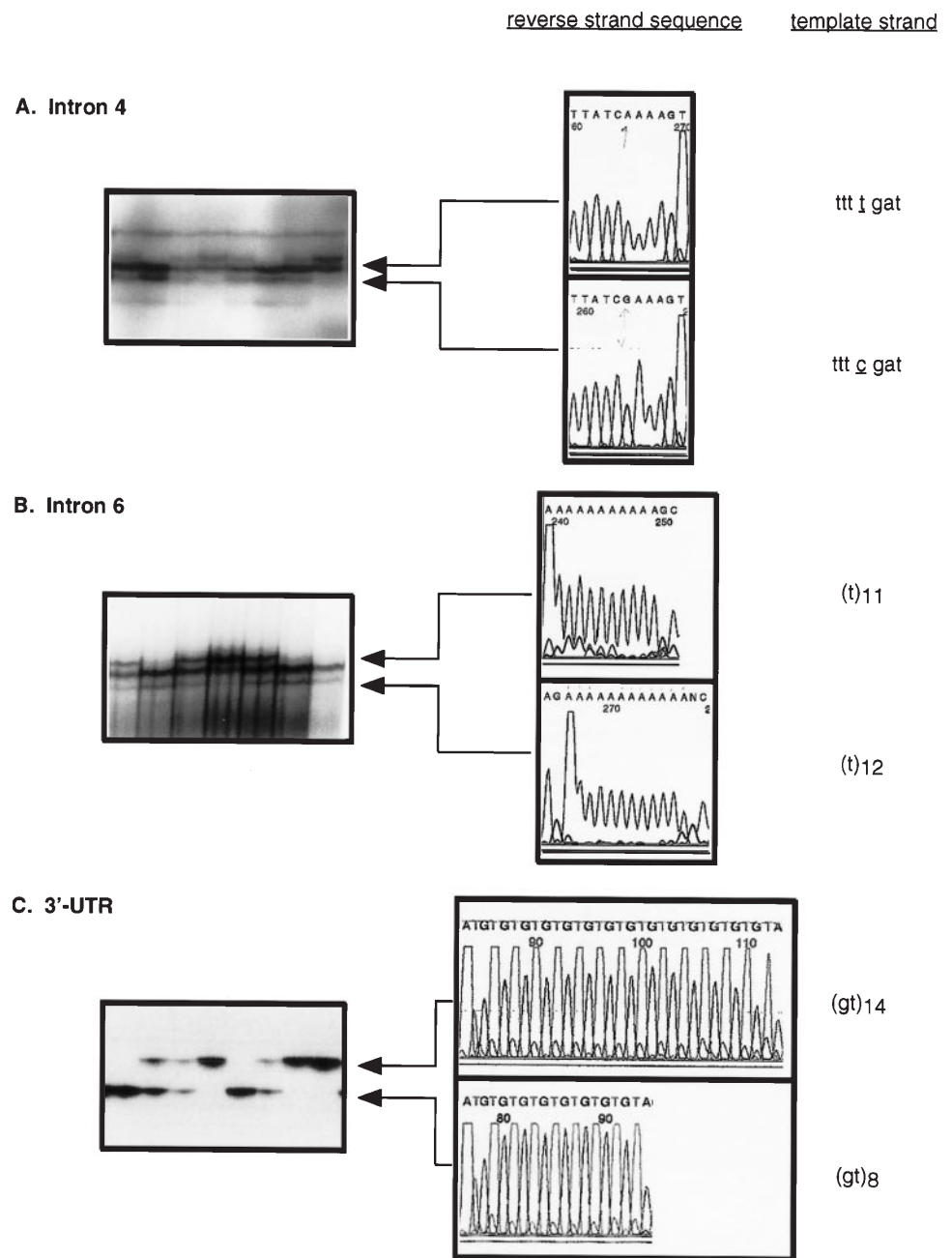
both of these two loci, separated by almost 20kb, and no recombinants have been identified, demonstrating complete linkage disequilibrium.

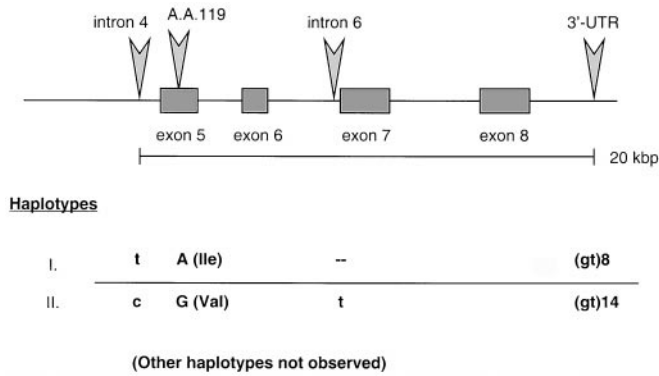
An additional base substitution was observed in intron 2 for a single individual out of the 50 genotyped in the polymorphism screen described above (Fig. 8A). However, analysis of an additional 50 individuals revealed no further cases of this mutation. Sequencing the PCR product in this individual revealed a mutation upstream of exon 3 in the branch site consensus splicing sequence (Fig. 8B). The genotype at this position for the individual was heterozygous with a C-to-A transversion in one copy of the gene. We then

genotyped 17 additional members of this individual's family (Fig. 8C). We found five more cases of the mutation in a heterozygous genotype that followed a Mendelian pattern of inheritance through three generations (Fig. 8C). However, enzymatic analysis of the red blood cells from a heterozygous individual revealed no differences in the level of repair methyltransferase activity (data not shown), suggesting little, if any, effect of this mutation on enzyme expression.

Finally, the variation in the coding region of exon 7 identified by Tsai and Clarke (1994), that apparently resulted in an Arg residue at amino acid position 205, was not observed in 50 individuals genotyped, nor could it be con-

**Fig. 6A-C.** Additional polymorphisms identified in the noncoding regions of the *PCMT1* gene. PCR-SSCP analysis of the entire coding region and flanking intronic sequence revealed three newly identified polymorphisms. The PAGE banding pattern of the SSCP for each polymorphism is shown on the *left* and the descriptive sequence analysis for the homozygous individuals singled out is shown to the *right*. **A** Intron 4 contains a single nucleotide transition polymorphism that is *underlined*. **B** Intron 6 contains a single nucleotide insertion/deletion polymorphism resulting in a repeated stretch of either 11 or 12T residues. **C** The 3'-untranslated region (*UTR*) contains a biallelic dinucleotide repeat of either 14 or 8 GT residues. The sequence data given are representative of the reverse strand for the intron 4 and intron 6 polymorphisms, but the template strand sequence is shown for the 3'-UTR polymorphism





**Fig. 7.** Four identified polymorphisms of the *PCMT1* gene demonstrate complete linkage disequilibrium. Each allele associates with a specific set of alleles at the other polymorphic loci. The nonrandom association pattern is illustrated by the presence of only two types of chromosomes. The position of these polymorphisms relative to one another is depicted with arrowheads, over a distance spanning approximately 20 kb. A total of 122 individuals have been genotyped at both the Ile<sub>119</sub>/Val<sub>119</sub> and 3'-UTR dinucleotide repeat polymorphisms without a single recombination event being identified

firmed for the individual in which it was originally identified (data not shown). In addition, the Ile/Leu variation at amino acid position 22, originally seen by protein sequence analysis in Ingrosso et al. (1989), was not identified in the 50 individuals genotyped in our experiments.

## Discussion

The Ile/Val polymorphism at codon 119 in the protein L-isoleucyl (D-aspartate) methyltransferase is the only amino acid substitution in this enzyme that occurs with any appreciable frequency, as determined in this comprehensive screen of the entire coding sequence by PCR-SSCP analysis. We show here that the allele frequencies for these codon 119 variants differ between ethnic groups and that phenotypic differences are observed in both enzyme thermostability and substrate affinity. While the Ile<sub>119</sub> isoform is a more stable enzyme, as determined by thermostability, the Val<sub>119</sub> isoform has a higher affinity for protein substrates. These compensatory attributes observed in vitro are consistent with our findings of a possible association between "successful aging" and selection of the heterozygous genotype at amino acid position 119.

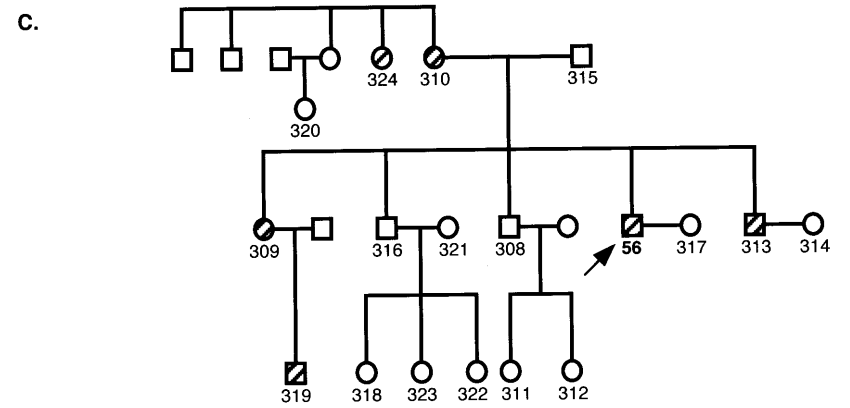
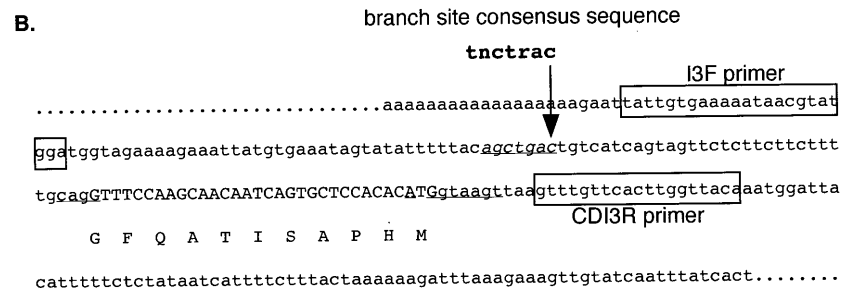
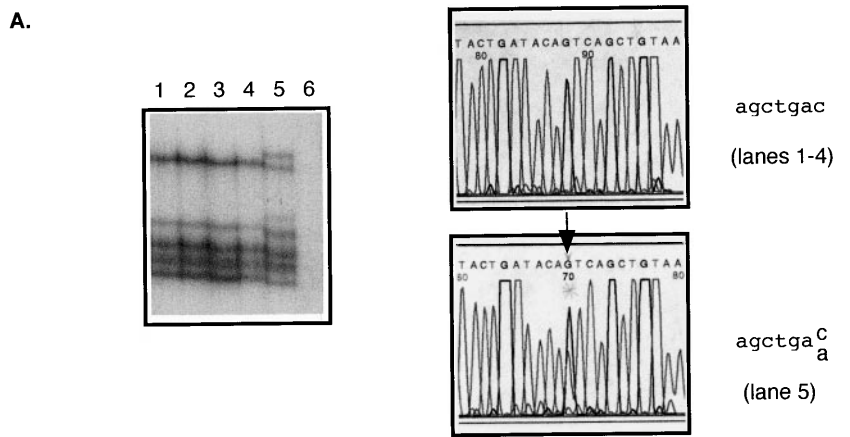
The activity differences observed between the polymorphic isoforms of this methyltransferase are subtle but significant, as might be expected from such a conserved amino acid substitution. The higher thermostability of the Ile<sub>119</sub> enzyme may result in a greater amount of functional enzyme in red blood cell cytosols of individuals homozygous for that genotype as compared with those from Val<sub>119</sub> homozygotes. The consistently higher enzyme activity and thermostability observed in multiple individuals of the same genotype would indicate that it is not merely a difference in enzyme expression levels. Moreover, our data are in agreement with results observed by David et al. (1997) in both parameters of activity and stability. Stability at high tem-

peratures and oxidative stress may reflect greater stability under physiologically stressful conditions. The novel difference in substrate affinity between the amino acid 119 variants may balance out these stability differences. Interestingly, the affinity differences are specific for protein substrates, and are not seen in the small methyl-accepting peptide substrates. Although the structure for the L-isoleucyl methyltransferase has yet to be determined, comparisons with other AdoMet-binding methyltransferases suggest that this polymorphic region may be in the same planar face as the active site. The presence of an additional methyl group in the isoleucine side chain could possibly contribute a small amount of steric hindrance at the substrate-interacting surface. This may explain the affinity differences observed between the Ile<sub>119</sub>/Val<sub>119</sub> variants of the repair methyltransferase for protein but not peptide substrates. Previous studies have demonstrated that the accumulation rate of damaged proteins is inversely proportional to the substrate affinity of the methyltransferase (Lowenson and Clarke 1991). In mathematical models of a typical erythrocyte protein, it was shown that, after 120 days, the number of damaged sites decreased almost linearly as the  $K_m$  for methylation decreased. Therefore, a 30% difference in substrate affinity between the Ile<sub>119</sub> and Val<sub>119</sub> isoforms of the methyltransferase (Fig. 5) could account for a substantial difference in the level of isoaspartyl damage accumulation.

The idea that protein repair may play a role in the multifaceted process of aging led us to search for a possible selection with the Ile<sub>119</sub>/Val<sub>119</sub> polymorphism in a healthy old population, similar to other studies on the genetics of longevity (Schachter et al. 1994; Mannucci et al. 1997). The initial findings for a small Ashkenazi Jewish population indicate a possible selection for the heterozygous genotype in the "successfully-aged" individuals, suggesting a putative hybrid vigor. This would correlate with our findings on the compensatory benefits of higher activity/stability in the Ile<sub>119</sub> enzyme and higher substrate affinity in the Val<sub>119</sub> enzyme. Thus, the combination of a higher stability and a higher affinity enzyme in the heterozygous individual may be most advantageous for preventing the accumulation of damaged proteins and mitigating the aging process. We are currently acquiring DNA samples from a group of Japanese individuals also meeting our "successfully-aged" requirements to see if they corroborate our previous findings in the Jewish population. The skewed allele frequency in the Asian population should make a heterozygote selection even more pronounced if the hybrid vigor hypothesis is correct.

The ethnic differences in allele frequencies for the Ile<sub>119</sub>/Val<sub>119</sub> polymorphism were significant between some of the populations for which there were adequate numbers to compare. The high allele frequency for the Ile<sub>119</sub> enzyme in the African and Asian populations (0.81 and 0.88, respectively) is suggestive of these populations being nearly homogeneous for this isoform of *PCMT1*. The rare presence of the Val<sub>119</sub> allele in these populations, in which no Val<sub>119</sub>/Val<sub>119</sub> homozygotes were identified, may simply reflect admixture in the lineages of our representatives. This is a strong possibility, considering that most of our participants

**Fig. 8A–C.** Mutation identified within the branch site consensus sequence upstream of exon 3 in the *PCMT1* gene. **A** Normal banding pattern (lanes 1–4) and alternative banding pattern (lane 5) observed for exon 3 by SSCP analysis in 5 individuals out of 100 genotyped. Sequencing of the reverse strand of exon 3 in this individual (in lane 5) revealed a heterozygous genotype with a C-to-A transversion in one copy of the *PCMT1* gene. The template strand sequence for branch site is given at the right for the DNA of the individual in lane 5 (below) and for the normal DNA samples (above). Lane 6 of the SSCP gel represents a negative control. **B** Sequence of exon 3 and flanking introns. Lowercase lettering represents intronic sequence and uppercase lettering represents exon sequence. Primers used in PCR-SSCP and sequencing are boxed. Amino acids encoded by exon 3 are depicted below the exon sequence. The underlined sequence identifies the branch site splicing sequence motif compared with the consensus sequence identified by Green (1991). **C** Analysis of the branch site mutation in family members of the individual identified with the exon 3 mutation above. PCR-SSCP analysis of numbered individuals revealed a Mendelian inheritance pattern. The *propositus* is individual number 56 (arrow and bold). Only heterozygotes of the C→A branch site mutation were observed in this family (individuals with striped symbols). Circles represent females and squares represent males



are North American, and dilution of ancestral gene pools is common in this group (Chakraborty et al. 1992). In sharp contrast, the Val<sub>119</sub> allele appears to be quite well established in the Caucasian population. This geographic distribution might suggest that the Val<sub>119</sub> allele was a mutation derived from the original Ile<sub>119</sub> allele, based on the evolutionary expansion of modern humans out of Africa 100,000 years ago (Cavalli-Sforza et al. 1994). If the mutational event has not spread to Asia this would suggest that it was not yet present in the population when Paleolithic humans moved westward into Europe and eastward into Asia 35,000–40,000 years ago (Cavalli-Sforza et al. 1994). Alternatively, a founder effect may have contributed to this large discrepancy in allele frequencies, but any selective effects

for maintaining the even distribution in the Caucasian population remain to be determined. The genotyping of more individuals from other races, particularly genetically homogeneous peoples from different parts of the world, may enable us to more accurately hypothesize about the origin, evolution, and dispersion of this polymorphism.

Our findings here can be compared to the situation seen with the human catechol O-methyltransferase (COMT) (Weinshilboum 1992; Lotta et al. 1995). A valine-to-methionine substitution at codon 108 in COMT results in a high and low activity enzyme, respectively. Similar to the L-isoaspartyl methyltransferase, this activity difference may be attributable to differences in thermostability. Interestingly, the ethnic differences in allele frequency seem to

parallel our findings as well, with the low-activity allele being more prevalent in the Caucasian population as compared with Asian and African populations (McLeod et al. 1998). The availability of a three-dimensional structure of rat COMT (Vidgren et al. 1994), coupled with the conservation of sequence motifs between S-adenosylmethionine-utilizing methyltransferases, led us to ask whether the PCMT1 polymorphic site at codon 119 was located in a similar position to the COMT polymorphic site. Alignment of the PCMT1 and COMT sequences showed that both the PCMT1 and COMT polymorphic sites appeared to be located in a loop connecting the putative S-adenosylmethionine-binding sequences of motif post I and II (Kagan et al. 1997).

The physiological importance of such a thermostability change in an enzyme has been demonstrated most dramatically in the methylene tetrahydrofolate reductase (van der Put et al. 1996), in which an alanine for valine substitution results in decreased folate metabolism, elevated homocysteine levels, and a significant risk for having spina bifida offspring. In the case of PCMT1 however, we find a compensating increase in affinity of the more thermolabile enzyme.

The identification of three additional polymorphisms is not unexpected considering that approximately 2400bp of sequence was screened in our analysis, with approximately one polymorphic site expected per 500 nucleotides of DNA sequence (Cavalli-Sforza et al. 1994). However, it was surprising to find complete allelic association at all four sites, demonstrating that these four different polymorphisms were in total linkage disequilibrium. Although some degree of linkage disequilibrium is common (Antonarakis et al. 1988; Heizmann et al. 1991) we have found no evidence at all for recombination within this 20-kb region of the *PCMT1* gene. Based on the assumption that 1Mb is equal to 1cM, we would have expected a 0.02% recombination rate per generation over this segment of DNA. In the 66 homozygous individuals (132 chromosomes) that were genotyped at both the codon 119 and 3'-UTR polymorphic sites we would thus expect one recombination event each 38 generations. An estimate of 25 years per generation would put this expected recombination frequency at 1 every 950 years. The absence of recombination would suggest that either the emergence of this haplotype occurred recently (Stephens et al. 1998) or that the *PCMT1* gene is located in a recombination "cold-spot" of the chromosome.

We are also interested in addressing questions about the nature of selective forces that underlie this variation and allow it to be maintained in such an evolutionarily conserved enzyme (Kagan et al. 1997). New mutations can establish polymorphism in a population by the processes of natural selection and random genetic drift. A new mutation that is maintained by natural selection must eventually take over the entire population, establishing homozygosity for the mutation (Falconer 1989). The more recent origin of the Val allele, as predicted by the geographic distribution of allele frequencies, could be in the process of establishing itself in such a way within the European population. That is, the allele frequencies we are currently observing might possibly be a transitional stage. Alternatively, a balanced polymorphism can become established in a population if the

heterozygote has a greater fitness than either homozygote (Falconer 1989). Although it is often difficult to measure fitness differences for most single-locus polymorphisms, we have attempted to draw some conclusions about fitness based on phenotypic differences in methyltransferase repair activity. The compensating benefits of the Ile<sub>119</sub> stability and the Val<sub>119</sub> affinity could possibly fulfill requirements for maintaining a selection for heterozygotes, but the effect this would have on fitness is still unknown.

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