Int J Legal Med (1996) 108:313–315

ORIGINAL ARTICLE

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Failed PCR amplifications of MBP-STR alleles due to polymorphism in the primer annealing region

Received: 16 October 1995 / Received in revised form: 5 February 1996

Abstract The PCR-based STR system MBP-B (myelin basic protein locus B) has been reported to exhibit a high rate of mutations. Using a newly designed pair of primers we present evidence that this is due to failed amplifications caused by a polymorphism in the annealing region of the reverse primer originally designed. With the new reverse primer described here no exclusions were found (out of 59 mother/child pairs analysed) while one was detected with the old set of primers. The results obtained with both pairs of primers in a random population sample (n = 112) from North Portugal are compared. In this sample 13 individuals typed as homozygotes with the pair of primers originally described, were found to be heterozygous when the amplifications were performed with the new reverse primer. By sequence analysis, a substitution in the reverse primer binding sequence originally described was determined. This substitution is located upstream from the repetition site and consists of G→A transition. This variation reaches polymorphic frequency and is responsible for the relatively frequent null alleles due to failed amplifications when the previously designed primers are used.

Key words MBP · STR · Sequence data · Population genetics · Paternity investigations · Exclusions

Introduction

A repetitive tetrameric sequence in the myelin basic protein gene (MBP, 18q23-qter) was first described by Boylan et al. (1990). In 1992 Polymeropoulos et al. described one pair of primers which simultaneously amplifies two adjacent STRs (locus A and B, Fig. 1) inside this region, due to a double annealing site for the reverse primer. Using these primers Möller et al. (1994) reported two germline mutations for locus B and none for locus A. We have also detected a mother/child exclusion (out of 59 pairs) as well as an isolated paternity exclusion in a case with a very high paternity probability in the other systems tested. For both exclusions opposite homozygosities in locus B, but compatible heterozygosities for locus A, were observed. In order to verify if these apparent exclusions were caused by failed locus B amplifications (due to genetic variation in the reverse primer annealing sequence), instead of *de novo* mutations, an alternative reverse primer specific for locus B was designed.

In this work we describe this (reverse) primer for locus B, and new genetic data on this system, comparing the amplification results of the two sets of primers. The variation responsible for the failed amplifications was further characterised by sequence analysis performed on MBP amplified products.

Material and methods

Samples (blood and buccal swabs) were collected from unrelated individuals from North Portugal and from mother/child pairs involved in paternity investigations. Blood samples were obtained by venipuncture; samples were processed as previously described (Gusmão et al. 1995). DNA was extracted using the method of Lareu et al. (1994).

Two sets of primers were used for PCR amplification as described by Polymeropoulos et al. (1992):

Primer pair 1: 5′GGA CCT CGT GAA TTA CAA TC 3′(forward)
5′ATT TAC CTA CCT GTT CAT CC 3′ (reverse)

and a new set, with a different reverse primer:

Primer pair 2: 5′GGA CCT CGT GAA TTA CAA TC 3′(forward)
5′ CTC ATG TAT CCA TCT ATT TAC C 3′
(reverse).

The PCR mixture was prepared as previously described (Gusmão et al. 1995).

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...CGAGGACAA[1]**GGACCTCGTGAATTACAATC**ACTGTAACTCAGATATTTAGAATGGTACA

TTACACCTATTTGGTACTCATAAATAA(TGGA)nTGGATGAACAG[A/G]TA**GGTAAA**[2]**TAGATG**

GATACATGAG(TGGA)nTGAATGGATAGATGGATGGATGAACAGGTAGGTAAA[3]TGGATG

AATACATGAGT...

Fig. 1 Forward sequence of the 5′ flanking region of MBP locus (GenBank Accession No. J04746). Annealing regions for Polymeropoulos et al. (1992) primers are underlined (Pair 1: 1 annealing region for the forward primer, 2 for the reverse primer defining locus A between 1 and 3, and locus B between 1 and 2). The sequences in bold type correspond to primers presented in this work (Pair 2)

Thermocycling conditions (Perkin-Elmer 480, 30 cycles):

Pair 1: 5 min at 93°C, followed by 94°C-1 min, 63°C-0.5 min, 72°C-1 min;

Pair 2: 5 min at 93°C, followed by 94°C-1 min, 58°C-0.5 min, 72°C-1 min.

PCR amplifications were always performed with negative and positive controls. At least two independent amplifications were performed for all individuals. Amplified DNA fragments were separated by horizontal electrophoresis in polyacrylamide gels (T:10%; C:5%) according to Luis and Caeiro (1995) and visualised by silver staining (Budowle et al. 1991). Alleles were named according to the number of TGGA repeats, following the recommendations of the DNA Commission of the International Society of Forensic Haemogenetics (1994). Genotyping was performed by side-by-side comparison with an allelic ladder made from previously typed samples containing alleles 7 and 9 to 12.

Allele frequencies were estimated by gene counting and population conformity with Hardy-Weinberg expectations was tested according to Guo and Thompson (1992) using the statistical software package GENEPOP (Raymond and Rousset 1995). Unbiased heterozygosity under Hardy-Weinberg assumptions was estimated according to Nei (1978) and the difference between observed and expected numbers of homo- and heterozygotes was used to calculate χ^2 with one degree of freedom. Allele size determinations were made on an Automatic laser fluorescent (ALF) DNA sequencer (Pharmacia, Uppsala).

After electrophoresis PCR products were eluted using a chelating resin protocol adapted from Singer-Sam et al. (1989). Eluted DNA fragments were reamplified and purified with Microspin S-200 HR columns (Pharmacia) before cycle sequencing. The purified products were used for the dideoxy cycle sequencing reactions using Taq DNA polymerase. The sequence determination was carried out on the 5′-3′ chain using the 5′ labelled forward primer of the PCR reaction. Promega sequencing Kit (fmol DNA Sequencing System -Q4100) was used and the fragments were run on an Automatic laser fluorescent (ALF) DNA sequencer (Pharmacia). Sequence analysis was determined automatically using the ALF Manager software.

Results

PCR amplifications under the described conditions were regularly successful although the two loci MBP-A and MBP-B were amplified with both primer pairs.

A random sample of 112 individuals autochthonous to North Portugal was typed using the two primer sets. The genotype distribution obtained with the originally described primers does not deviate significantly from Hardy-Weinberg equilibrium expectations, but the agreement is poor ($P = 0.103$; s.e. = ± 0.007). A much better agreement ($P = 0.336$; s.e. = ± 0.004) is obtained with the new primers.

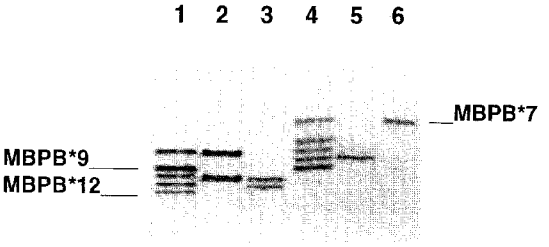

Fig. 2 MBPB phenotypes using primer pairs differing in the 3′ annealing region (see Methods). Lanes 1–3 using primer set 2 and lanes 4–6 using primer set 1. Lane 1: ladder; lane 2: 7–10; lane 3: 10–11; lane 4: ladder; lane 5: 11–11; lane 6: 7–7. Lanes 2/3 and 6/5 are from the same mother/child pair, showing that allele 10 was not amplified with primer pair 1. Allelic ladders were made up from previously typed samples containing alleles 7 and 9–12

Genotypings were concordant with both methods, except for 13 homozygotes (out of 41) with primer pair 1, that were found to be heterozygous when using primer pair 2. A striking example is shown in Fig. 2: an apparent mother/child exclusion by opposite "homozygosity" (lanes 5 and 6) was shown to be compatible by typing the PCR products using the second primer pair (lanes 2 and 3).

In Table 1 we compare the allele frequencies, observed and expected heterozygosities, and forensic efficiency values of MBP-B in our sample resulting from the 2 genotyping methods.

The results on the sequence analysis of MBP-B locus products found in our sample have shown a simple repeat motif, $(TGGA)_n$. The substitution responsible for the null allele observed at the MBP-B locus occurred in the 13 bp

Table 1 MBP-B: allele frequencies (%) in North Portugal, observed and expected heterozygotes and forensic efficiency values obtained with the two genotyping methods (see Methods section for details). H = heterozygosity; PIC = polymorphic information content; Pex = a priori exclusion chance

Allele	Pair 1	Pair 2
7	35.3	33.0
9	4.5	4.5
10	10.2	15.6
11	35.3	32.6
12	13.8	13.4
13	0.9	0.9
Heterozygotes		
observed	71	84
expected	80.91	83.30
χ^2	4.373	0.023
P	0.037	0.879
H	0.722	0.744
PIC	0.672	0.697
Pex	0.461	0.487

sequence upstream to the TGGA motif corresponding to the non-overlapping region between the two alternative reverse primers used. In the null allele, an adenine was found in the 8th position upstream to the repeat, while all other alleles exhibited a guanine (Fig. 1).

Discussion

The new set of primers presented in this work allowed to understand the basis for the apparent mother/child and paternity exclusions previously detected when employing the original pair of primers (Fig. 2). On the other hand, by retyping all the "homozygotes" in the North Portugal population sample a substantial fraction (31.7%) was in fact revealed to correspond to true heterozygotes. This is due to a failure in the amplification owing to genetic polymorphism in the internal annealing sequence for the reverse primer of Polymeropoulos et al. (1992). The mismatch was restricted to the 13 bp non-overlapping region between the two alternative reverse primers upstream to the TGGA motif. Sequencing results confirmed this interpretation and revealed a polymorphic G→A substitution.

The high frequency of this polymorphism (0.058, estimated from the 13 detected failed amplifications in 224 chromosomes analysed) explains the false exclusions detected by our group and referred to by Möller et al. (1994) as well as the highly significant deficiency of observed heterozygotes (Table 1). It is worth mentioning that out of 13 adenine substitutions, 12 were associated with 10 repeats, while only 1 had 12 repeats. Surprisingly, consistent amplifications of both A and B locus regions were obtained with the new primer, which was designed according to the sequence published by Boylan et al (1990) in order to amplify specifically only locus B. It seems, therefore, that this sequence needs revision.

The use of the primers now reported therefore seems to substantially improve the forensic applicability of this system, which has previously been of doubtful use for practical purposes, due to an unexpectedly high (apparent) mutation rate.

Acknowledgements This work was partially supported by JNICT (Junta Nacional de Investigação Científica e Tecnológica, BD/2849/93-ID and PBIC/C/CEN/1174/92).

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