Alternative exon 3 splicing of the human major protein zero gene in white blood cells and peripheral nerve tissue

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Received 26 July 1999

Abstract The major protein zero (MPZ) is involved in peripheral myelin folding. Using nested reverse transcription-PCR, we amplified several fragments of *MPZ* mRNAs in white blood cells and in peripheral nerve tissue. Cloning of PCR products revealed the existence of three alternative splicing patterns: one resulted in the complete loss of exon 3 and two others induced partial skipping of the exon 3 sequence. All three alternative splicing mechanisms produced a frame-shift and created an identical premature stop codon in exon 4. We conclude that the existence of these *MPZ* RNA transcript variants may be the result of deliberate splicing decisions and may have functional implications in the cell.

Key words: Major protein zero; Myelin; Splicing; Premature termination codon

1. Introduction

Peripheral myelin is a highly ordered and compact extension of the Schwann cell membrane [1]. The major protein zero (MPZ) is an abundant transmembrane adhesion molecule [2]. Homophilic interaction between its extracellular part leads to a tight wrapping of myelin around the axon [3,4]. The *MPZ* gene is located on chromosome 1q21.3-23 and has a size of 6 kb [5]. This gene contains six exons [6] and encodes a 1948 nucleotides long mRNA [5]. At least 56 mutations in the *MPZ* gene have been described as implicated in a peripheral neuropathy called Charcot-Marie-Tooth disease type 1B [7]. The majority of mutations are more particularly located in exons 2 and 3 of the *MPZ* gene and affect the extracellular domain of the protein. One silent polymorphism has been reported in exon 3 in a French family affected by CMT1 [8]. Such an observation may suggest that this silent polymorphism may promote *MPZ* missplicing and could be involved in the etiology of the disease. To the best of our knowledge, no information is available concerning the composition of human *MPZ* mRNA transcripts and no secondary transcription products have previously been reported in rat [3]. Since the silent polymorphism occurs in exon 3, we decided to investigate the splicing of this exon.

2. Materials and methods

2.1. Patient selection

Four subjects from the Hôpital Neurologique of Lyon (52 ± 12 year old men) were studied. They suffered from non-hereditary neuropathies and were not classified as CMT patients. Single strand conformation polymorphism analysis of *MPZ* exons revealed no alteration in the *MPZ* gene (data not shown). This result permitted us to select them as control subjects for studying splicing of the *MPZ* mRNA in exon 3.

2.2. Sample preparation

Peripheral blood and fragments of peripheral nerve tissue (sural nerve biopsies) were collected from each subject after informed consent. Genomic DNA was purified from 10 ml of frozen blood using a QIAamp blood kit (Qiagen GmbH, Hilden, Germany). PolyA+ mRNAs from white blood cells (WBCs) were extracted in duplicate from 200 μl of fresh blood with the PolyAtract system 1000 kit (Promega, Madison, WI, USA) according to manufacturer's protocol. In the final step, mRNAs were eluted at 72°C in 30 μl of RNAse-free water. Total cellular RNAs were isolated from peripheral nerve tissue using RNAxel solution (Eurobio, Les Ulis, France). Due to poor quantities of nerve tissue, extractions were not carried out in duplicate. Absence of genomic DNA in the different RNA preparations was checked by two successive amplifications of β-actin DNA with primers chosen in different exons of this gene.

2.3. 5′ End labelling

Labelling was for 1 h at 37°C on 20 pmol of primer, 2 μCi of [α-^{32}P]ATP (3000 Ci/mmol, Amersham Pharmacia Biotech Europe, Freiburg, Germany) and 2 U of T4 polynucleotide kinase (Promega). Labelled primer was then purified using a Microbiospin 6 chromatography column (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol.

2.4. Amplification and quantification of MPZ mRNAs

cDNA was synthesised by the oligo-dT primer method, with Murine Moloney Leukemia Virus Reverse Transcriptase (Eurobio), using either 10 μl of polyA+ mRNA or 750 ng of the total RNA preparation. cDNA was then diluted with water to a final volume of 100 μl.

MPZ cDNA was amplified using two steps of nested PCR. In the first step, the PCR reaction (PCR1) was in a final volume of 50 μl using primer set 1 (Table 1). The applied reaction conditions were 30 cycles of 45 s at 95°C, 45 s at 56°C and 1 min at 72°C, using 10 μl of denatured cDNA template, 20 pmol of forward and reverse primers, 1 U of inorganic pyrophosphatase (Eurobio) and 0.25 U of Eurobluetaq (Eurobio). PCR was completed by a terminal elongation of 7 min at 72°C. The number of cycles was chosen in order to be in the exponential phase of PCR. First step PCR products were purified in order to remove enzymes and primers using the QIAquick PCR purification kit (Qiagen).

In the second nested PCR (PCR2), the applied conditions were 25 cycles of 1 min at 95°C, 1 min at 56°C and 1 min and 30 s at 72°C in order to be in the exponential part of the PCR amplification. PCR2 was done on 0.3 μl of the purified and denatured PCR1 products, in the presence of 20 pmol of forward primer, 18 pmol of reverse primer and 2 pmol of labelled reverse primer (Table 1) and 0.25 U of Eurobluetaq.

Electrophoresis of 10 μl of PCR2 products was conducted on a

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PII: S0014-5793(99)01069-8

3.5% acrylamide gel for 2 h at 120 V. The gel was then air-dried and exposed for 5 h at −80°C to a BioMax MS film using a BioMax MS intensifying screen (Eastman Kodak Company, Rochester, NY, USA) for autoradiography. Quantification was done on a Phosphoimager SI (Molecular Dynamics, Sunnyvale, CA, USA). Air-dried gels were exposed for 2 h in an exposure cassette (Molecular Dynamics). Quantification of radioactivity was then carried out with Image Quant v2.1 software (Molecular Dynamics).

2.5. Amplification of β-actin mRNAs

β-Actin RNA was amplified from 1 μl of cDNA templates by PCR. Reaction was in a final volume of 25 μl using β-actin-specific primers (Table 1). The applied reaction conditions were 30 cycles of 1 min at 95°C, 1 min at 62°C, 1 min and 30 s at 72°C using 1 μl of denatured cDNA templates, 20 pmol forward primer, 18 pmol reverse primer, 2 pmol labelled reverse primer and 0.25 U Eurobluetaq. These conditions were chosen in order to be in the exponential part of the PCR amplification. Electrophoresis and quantification were proceeded as previously described.

2.6. DNA sequencing

PCR2 products were cloned in the pGEM-T easy vector (Promega) according to the manufacturer's protocol. Plasmids containing different inserts were sequenced with the ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction kit and an ABI Prism 310 Genetic Analyser automatic sequencer (Perkin Elmer, Branchburg, NJ, USA).

2.7. Statistical analysis

Each PCR was realised in duplicate on each RNA preparation. The quantity of radioactivity signals for each *MPZ* mRNA variant was adjusted with the β-actin signal as reference. Results were then analysed using the non-parametric Mann-Whitney test.

3. Results and discussion

Primer set 2 (PCR2, Table 1) used to amplify first step PCR products revealed a complex PCR pattern (Fig. 1). In WBCs, a signal with a length of 365 bp corresponding to a normally spliced transcription product was visible (E3N RNA variant: Fig. 1). In addition, shorter fragments were also found. In nerve tissue, as expected, the predicted fragment for *MPZ* RNA was amplified abundantly. As seen in blood, the same shorter transcription fragments were observed (Fig. 1).

Illegitimate transcription of tissue-specific genes has been demonstrated in a large number of cell types, especially WBCs [9–12]. In our work, we demonstrate that the peripheral myelin gene *MPZ* is expressed in WBCs. Comparison with the β-actin signals revealed that the concentration of the normally spliced *MPZ* mRNA was 25 times more abundant in peripheral nerve tissue than in WBCs ($P < 0.001$, Table 2).

In order to characterise the shorter transcription fragments, PCR products from the second amplification step were cloned in the pGEM-T easy vector. More than 100 clones were size-selected and four of them with inserts corresponding to 365, 151, 190 and 220 bp were sequenced. In addition to the normal 365 bp splicing product of *MPZ* RNA (E3N), we obtained three other sequences corresponding to (i) the complete removal of exon 3 (E3°), (ii) partial exon 3 skipping between nucleotides 274 (nucleotide +1 is A of initiator AUG) and 448 (E3Δ274–448) and (iii) partial exon 3 skipping between nucleotides 304 and 448 (E3Δ304–448) (Fig. 2). These different splicing patterns lead to the amplification of four PCR2 fragments with respective lengths of 365, 151, 190 and 220 bp.

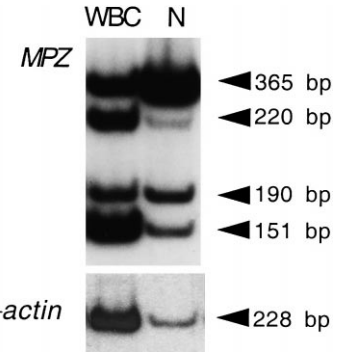

Fig. 1. Exon 3 splicing pattern of *MPZ* RNAs. Reverse-transcribed RNAs of WBCs and peripheral nerve tissue (N) were amplified (a) by nested PCR with *MPZ*-specific primers chosen to amplify exon 3, (b) by a single PCR with β-actin-specific primers. ^{32}P-labelled reverse primer was used either in the second round of PCR for *MPZ* or in the single PCR for β-actin. PCR products were electrophoresed in 3.5% acrylamide and exposed for autoradiography.

All three small RNA variants were expressed in WBCs and peripheral nerve tissue (Fig. 1). In WBCs, only the E3° variant was slightly more expressed than the other RNAs ($P < 0.01$, Table 2). In peripheral nerve tissue, the normally spliced RNA was much more abundant than the three small RNA variants ($P < 0.001$, Table 2). Comparison between the levels of expression for the three small RNA variants revealed no significant difference between WBCs and peripheral nerve tissue. This finding allows us to suggest that (i) illegitimate transcripts of *MPZ* detected in blood are also present in peripheral nerve tissue of control subjects. This result is consistent with aberrant splicing also found for genes encoding argininosuccinate lyase and adenosine deaminase, among others [11]. For the dystrophin gene, only one case of an alternative splicing product has been found in WBCs but not in muscle [13]. Thus, we may conclude that, with differences in quantity, the illegitimate transcription of the *MPZ* gene in WBCs reflects tissue-specific transcription of *MPZ*. (ii) The *MPZ* gene is submitted to different alternative splicing patterns. It has previously been described that only one isoform of *MPZ* RNA is observed in rat peripheral nerve [3], but this result is probably due to the limited resolution of the applied Northern blotting technique. In humans, *MPZ* transcription may differ by expression of three additional small RNA variants, which are not differently and significantly expressed between WBCs and peripheral nerve tissue.

Table 1
Primer sequences

	Forward primers	Reverse primers
PCR1	TGAGTGGGTCTCAGATGACA (bases 165–184 in exon 2)	TCTTATCCTTGCGAGACTCC (bases 742–723 in exon 6)
PCR2	GTCTCAGATGACATCTC (bases 172–188 in exon 2)	ACCACGTAGAAAAGCAGCAG (bases 536 to 517 in exon 4)
PCR β-actin	GCACTCTTCCAGCCTTCC (bases 779–795 in exon 4)	GCGCTCAGGAGGAGCAAT (bases 1005–988 in exon 6)

Bases correspond to RNA sequences, +1 is the A of the initiation codon. *MPZ* GenBank accession number: D14583, β-actin GenBank accession number: X00351.

Each of the two new alternative splicing sites described here occurred at a consensus acceptor intron site: GU. Sequence analysis of the three small RNA variants for *MPZ* revealed a reading frame-shift, which leads in each case to the same premature termination codon (PTC) at position 482–484 of exon 4. Transcripts containing a PTC in their sequence have been described in the *hMSH2* gene by Marshall et al. [14]. They showed the existence of two additional transcripts for the *hMSH2* gene where alternative splicing mechanisms produced the appearance of two different PTCs. They pointed out that the presence of sequences which show similarity to previously described mRNA destabilising motifs downstream of each of the two PTCs might bring transcription variants to a rapid degradation [14]. Moreover, many reports have shown that mutations in several genes like the dystrophin gene [10], the Fanconi anaemia group C gene [15], the *CFTR* gene [16], the fibrillin gene [17] and the neurofibromatosis type 1 gene [18] may produce PTCs, but also that alternative splicing of PTC containing exons may occur. These splicing alterations result in mRNAs that either lack the PTC or disrupt the frame-shift generated by the PTC so as to encode an almost normal but shortened protein. Concerning the *MPZ* gene, normal alternative splicings described in control subjects lead to PTCs containing small RNA variants. However, none of the described destabilising motifs [19–22] is found downstream of the PTC. Thus, the existence of these PTC containing RNAs for the *MPZ* gene may reflect deliberate splicing mechanisms, suggesting a functional role of these transcripts in the cell.

Moreover, a silent polymorphism (GTG→GTA) at position +276 in CMT1 patients has been described in a French family (family CMT-R, in [8]). This silent polymorphism was present in three patients as well as in two asymptomatic subjects. Using the HSPL version 2 (prediction of splice sites in human DNA sequences) program available at http://gc.bcm.tmc.edu:8088/search-launcher.html [23,24], it was possible to calculate splicing scores (0.76 is the threshold of the program), which give a value for the likelihood to use a nucleotide as a site for splicing [23,24] for the *MPZ* gene. The HSPL program does not predict the splicing site at position +274, although we found this site to be used in one of the alternative transcripts. But, the program indicates the creation of this splicing site when the GTG→GTA polymorphism occurs (splicing score 0.80). Thus, we hypothesise that this polymorphism may enhance the efficiency of the splicing site at position +274. Unfortunately, no nerve tissue from these CMT1 patients was available and we were unable to confirm this hypothesis.

Table 2
Quantification of the expression of exon 3 splicing *MPZ* variants in nerve and WBCs in four subjects

Splicing variant	WBCs	Peripheral nerve tissue
E3N	1 ± 0.35	25 ± 7.44***
E3Δ274–448	0.94 ± 0.46	0.43 ± 0.14
E3Δ304–448	0.80 ± 0.37	0.90 ± 0.44
E3°	1.98 ± 0.50**	0.84 ± 0.31

PCR signals for each splicing variants were quantified on a Phosphoimager SI. Values for each sample were standardised with the measurements obtained for β-actin and arbitrarily expressed as a function of the signal for E3N splicing variant in WBCs.
***Mann-Whitney test: $P < 0.001$, E3N versus other variants in peripheral nerve tissue and versus E3N in WBCs.
**Mann-Whitney test: $P < 0.01$, E3° versus other variants in WBCs.

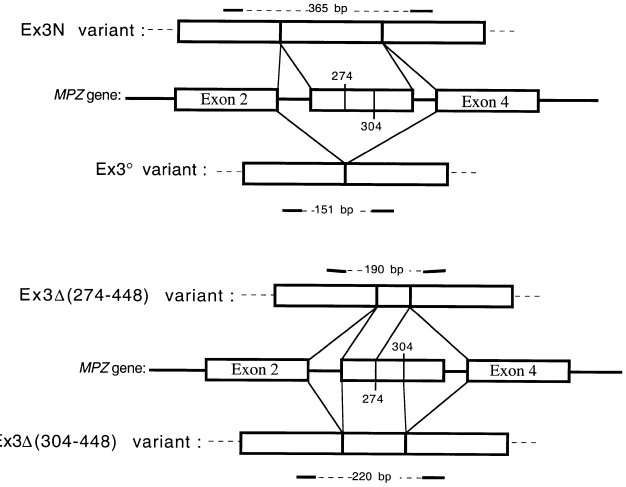

Fig. 2. Schematic representations of the alternative splicing mechanisms that involve exon 3 of the *MPZ* gene. E3N: normal splicing of exon 3, E3°: total removal of exon 3, E3Δ274–448: partial exon 3 skipping between nucleotides 274 and 448, E3Δ304–448: partial exon 3 splicing between nucleotides 304 and 448. Primer positions and amplification product lengths are respectively indicated on each small RNA variant.

Also, a recent report has shown the existence of high titres IgG auto-antibodies in sera of patients with inflammatory neuropathies which are able to recognise, in the peripheral but not the central nervous system, a protein of 36 kDa related to MPZ [25]. As MPZ proteins associate to form tetramers, the encoded proteins by the *MPZ* small RNA variants might assemble and form this 36 kDa MPZ-related protein.

To our knowledge, this is the first description of a polymorphism that may alter the transcription of a small RNA variant of a gene. It remains necessary to further investigate the translation of the small RNA variants of the *MPZ* gene and their possible function.

Acknowledgements: We thank Mrs. Susan Gamon for linguistic assistance. The Hospices Civils de Lyon and the Association Française contre les Myopathies supported this work. The laboratory of 'Génétique Moléculaire Humaine' is a member of the European CMT Consortium sponsored by the EU Biomed 2 Grants CT 961614 and CT 960055.

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