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Exon skipping of exonuclease 1 in MRL/MpJ mice is caused by a nucleotide substitution of the branchpoint sequence in intron eight

Yuka Namiki1), Yasuhiro Kon2)*, Nobuya Sasaki3), Takashi Agui3), Daiji Endoh3)

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Abstract

In MRL/MpJ mice, there is a genetic mutation of exonuclease 1 (Exo1), in which the exon 9 is sometimes deleted. In the present study, to check the generation of the spliced exons, exon 8-intron 8-exon 9 (pCX/Ex/EIE/B and pCX/Ex/EIE/M) plasmids were temporally transfected in vitro into BALB 3T3 cells, and RT-PCR using appropriate primer pair was carried out 1 day after transfection. In these constructions, pCX/Ex/EIE/B was derived from genomic sequence of C57BL/6 mice, and pCX/Ex/EIE/M was from MRL/MpJ. A spliced band was detected in pCX/Ex/EIE/B, but was present little or very weakly in pCX/Ex/EIE/M. Next, the same spliced band was demonstrated in the pCX/Ex/EIE/M(T) plasmid, in which the branchpoint sequence (BPS) of pCX/Ex/EIE/M including the exon 9 was changed into that of pCX/Ex/EIE/B. The splicing did not occur in the del1/B mutant, in which 1960 nucleotides of the intron 8 were deleted, whereas it was detected in the del2/B plasmid deleted 1036 nucleotides in its middle region. These results suggest that the nucleotide T to A mutation of the BPS in the intron 8 is at least a sufficient for generation of splice variants (tr-1 and tr-2 Exo1).

Key words: branchpoint sequence; exonuclease 1; exon skipping; MRL mice; splicing

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Exon skipping of Exo1 in MRL mice

Introduction

It has been reported that the MRL/MpJ strain is strikingly unique in its capacity for regenerative wound-healing, as shown by the closure of ear punches and cardiomyocyte regeneration with normal tissue architecture reminiscent of amphibian regeneration as opposed to scarring. It has furthermore been indicated that the development of several autoimmune diseases, such as systemic lupus erythematosus, polyarteritis nodosa, rheumatoid arthritis, Sjögren’s syndrome and systemic sclerosis are dependent on the MRL genetic background, but not on the lpr allele.

Recently, we found three unique characteristics in the testis of the MRL/MpJ mouse: metaphase-specific apoptosis of meiotic spermatocytes (msa), heat-shock resistance in spermatocytes found in experimental cryptorchidism (hsr) and the appearance of oocyte-like cells during postnatal development (unpublished data).

In a previous study, we found that msa was mapped to the telomeric region of chromosome 1 and was significantly linked with Exo1. The mouse Exo1 gene consists of 14 exons and 13 introns spanning about 40 kb of DNA, possessing a translational start site in the exon 2 and a stop codon in the exon 14. On the Exo1 cDNA in strain MRL/MpJ, but not in other strains, it was noted that truncated forms (tr-1 Exo1 and tr-2 Exo1) were expressed in all tissues examined. These were shown to be due to the skipping of exon 9 by comparing their nucleotide sequences with normal one. Additionally, in tr-1 Exo1, the stop codon (TGA) appeared in exon 10, leading to prediction of truncated protein. To identify the cause of abnormal splicing of Exo1, nucleotide sequencing of the 8th intron was performed paying attention to the splicing signals. As a result, nucleotide substitution in the branchpoint sequence (BPS) was detected, namely, GGTTAA in C57BL/6 was substituted to GGGAAT in MRL/MpJ.

These results revealed that the cause of abnormal splicing of Exo1 was nucleotide substitution of BPS in the intron 8. To check and confirm the generation of the spliced variants, an expression plasmid containing the nucleotide sequence from the exon 8 to the exon 9 of the Exo1 gene was constructed and introduced transiently into the mouse 3T3 cell line. In the present study, the results of RT-PCR clearly showed that the mutation of BPS in the intron 8 caused abnormal splicing of Exo1 gene in the MRL/MpJ mouse.

Materials and Methods

Animals

Male inbred mice, C57BL/6 and MRL/MpJ-+/+ were purchased from an animal breeding company (Japan SLC, Inc., Hamamatsu, Japan) and maintained in our facility with feeding and drinking ad libitum. In the experimental animal care and handling, the investigators adhered to the “Guide for the Care and Use of Laboratory Animals, Hokkaido University, Graduate School of Veterinary Medicine.”

RT-PCR and nucleotide sequence of Exo1

In order to confirm the previous study, total RNA was prepared from the testis, spleen and thymus of C57 BL/6 and MRL mice by acid guanidium thiocyanate-phenol-chloroform extraction (TRIZOL, Life Tech., Gaithersburg, USA) and subjected to reverse transcription (ReverTra Ace, Toyobo, Osaka, Japan), according to the manufacturers’ instructions. PCR for Exo1 was performed using the primer pair, consisting of the forward: 5'-CAGGATGAAATATAACGGGTGCC-3' and the reverse: 5'-ATTGCCTGGGAATCTGTGAGGC-3', in which the amplified length was 919 bp in the posi-
PCR was carried out on a Biorad PCR thermal cycler (iCycler, Tokyo, Japan) with the cycling sequence of 94°C for 5 min (one cycle), followed by 35 cycles consisting of denaturation at 94°C for 40 sec, primer annealing at 60°C for 30 sec, and extension at 72°C for 150 sec. The PCR mixture and enzymes, in which there was sufficient proofreading ability, were purchased from Takara (EX Taq Polymerase, Tokyo, Japan) or Toyobo (KOD Dash Polymerase, Osaka, Japan). The amplified samples were electrophoresed with 1% agarose gel, stained with ethidium bromide, and finally photographed under an ultraviolet lamp. For nucleotide sequencing, the amplified products cloned into the TA-vector (pGEM-T Easy vector, Promega, Madison, USA) were used with a cycle sequencing kit containing fluorescent terminators employing standard methods (Applied Biosystems, Foster City, USA) and then analyzed on a model 377 automatic sequencer (Applied Biosystems, Foster City, USA).

Construction of plasmids

Genomic DNA was prepared from the spleen of each animal. These tissues were incubated in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 20 mM EDTA, 1% sodium dodecyl sulfate and 100 μg/ml protease K, overnight at 56°C, and then treated with two phenol extractions and one phenol:chloroform extraction. Finally, genomic DNA was purified by ethanol precipitation and dissolved in TE (10mM Tris-HCl, 1mM EDTA, pH8.0).

To amplify the exon8-intron8-exon 9 fragment of Exo1 spanning about 2.6kb in each strain (Ex/EIE/B for C57BL/6 and Ex/EIE/M for MRL/MpJ), the primer pair (forward: 5'-ATGTACGTCGGTGACTCTGT-3' annealed on the 5' site of exon 8, reverse: 5'-CATTTCTTGGTCTTTTCAACACAC-3' annealed on the 3' site of exon 9) was constructed according to a previous report (Accession No. AC073663)\(^{\text{10}}\). The products amplified by PCR was ligated with TA-vector, and the nucleotide sequence was confirmed on the automatic sequencer. To make original expression plasmids, the expression vector, pCXN 2 (a kind gift from Dr. J. Miyazaki)\(^{\text{11}}\) was ligated with either the Ex/EIE/B or Ex/EIE/M fragment digested by EcoRI from pGEM-T plasmid.

Construction of pCX/Ex/EIE plasmids and their mutants is shown in Figure1. Three typed mutants were constructed from pCX/Ex/EIE/B or pCX/Ex/EIE/M plasmids. First, an exchanged mutant was obtained by cutting original plasmids with XhoI and HindIII, and the fragments were exchanged with each other. In these, the BPS (GGGTTAA) originating from B6 mice was changed into GGGAAA (pCX/Ex/EIE/B(A)), whereas that (GGGAAA) from MRL mice was changed into GGGTTAA (pCX/Ex/EIE/M(T)). Second, in deletion mutant 1 (del 1/B, del 1/M) on the pCX/Ex/EIE/B or pCX/Ex/EIE/M plasmid, the proximal and middle regions of 1960 nucleotides between BglII and XhoI in the intron 8 were deleted. Third, in deletion mutant 2 (del 2/B) the middle region of 1036 nucleotides between EcoT221 and XhoI in the intron 8 was deleted from pCX/Ex/EIE/B.

Transfection into BALB3T3 cells and RT-PCR

The pCX/Ex/EIE plasmids and their mutants from both strains were temporally transfected into BALB3T3 cells by the lipofection method (FuGene 6, Roche Diagnostics, Tokyo, Japan). After 1 day of transfection, RT-PCR was performed using the appropriate primer pair, consisting of the forward: 5'-GGC TTCTTTCGTACATGTGTGT-3' (located just before the exon 8) and the reverse: 5'-CATTTCTTGGTCTTTTCAACACAC-3' (located on the
Figure 1. Construction of pCX/Ex/EIE and mutant plasmids. The 2268th nucleotide starting from the 5' site of the exon 8 is inside of the BPS, producing T in pCX/Ex/EIE/B derived from C57BL/6 or A in pCX/Ex/EIE/M derived from MRL. The numbers presented below each restriction enzyme are the distances from the 5' site of the exon 8. The large arrows showing Forward and Reverse indicate the primer site used for RT-PCR.

end of the exon 9) primers, in which the length of amplified product was deduced to be 0.3kb in normal splicing of the intron 8. On the other hand, if not abnormally spliced, its length would be 2.6kb in pCX/Ex/EIE plasmid, 0.7kb in the del1 mutant, and 1.6kb in the del2 mutant, respectively. PCR was carried out with the same protocol described above and the samples were electrophoresed with 2% agarose gel.

Results

Three types of products were at least detected by RT-PCR for Exo1 in all tissues of MRL mice, whereas there was just one product in those of C57BL/6 mice (Fig. 2a). Nucleotide sequencing showed that the first type in MRL mice was normal Exo1, in which two nu-
cleotides were substituted between C57BL/6 and MRL mice (Fig. 2b). On the other hand, the second and third types had shorter sequences with deletion of 226bp (tr-1 Exo1), and with insertion of 205 bp and deletion of 21bp (tr-2 Exo1), respectively. The deduced amino acids suggested that there was a stop codon in tr-1 Exo1 by flame shift, whereas tr-2 Exo1 was in-flame even though different sequence was inserted (Fig. 2c). The comparison of genomic sequence from exon 8 to exon 9 in Exo1 between C57BL/6 and MRL mice showed 8-nucleotide substitutions and 9-nucleotide deletions of MRL mice, in which BPS was changed from GGTTAA in C57BL/6 into GGGAAA in MRL mice (Fig. 2d). These results was corresponded with the previous report10.

In the experiments using the original expression plasmids, a spliced band spanning about 0.3kb could be detected in pCX/Ex/EIE derived from C57BL/6, but it could not be found or was very weak in pCX/Ex/EIE/M isolated from MRL (Fig. 3). Next, the same spliced band was demonstrated in the pCX/Ex/EIE/M(T) plasmid, in which the BPS including the exon 9 was changed from pCX/Ex/EIE/M type to pCX/Ex/EIE/B type. On the other hand, pCX/Ex/EIE/B(A) did not show any spliced band. These results suggested that the nucleotide T to A mutation of the BPS in the intron 8 was at least sufficient for generation of spliced variants tr-1 and tr-2 Exo1.

Splicing expecting 0.7 kb band did not occur in either the del1/B or del 1/M mutated plasmid, in which the 1960 nucleotides of the intron 8 were deleted (Fig. 4). In the del 2/B mutant, in which the 1036 nucleotides of the intron 8 were deleted, a spliced band was detected as a product with the same 0.3kb-length as in the original pCX/Ex/EIE/B plasmid. These results revealed the possibility that other sequence elements located in the proximal region of the intron 8 than the BPS and polypyrimidine tract may be required for normal splicing.

![Figure 2a](image-url)
Exon skipping of \( \text{Exo} \) 1 in MRL mice

**Figure 2 b**

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**Figure 2 c**

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Exon skipping of Exo 1 in MRL mice

**Figure 3.** In vitro splicing assay with original and exchanged plasmids. The deduced 0.3kb-length bands are detected in the cells into which pCX1ExEIEIIE and pCX1ExEIEIME(T) plasmids were introduced by RT-PCR. For internal control, β-actin was used.

**Figure 4.** In vitro splicing assay with del1 and del2 mutant plasmids. The spliced 0.3kb-length bands are detected in pCX1ExEIEIIE and del2B plasmids; however, in del1B and del1M mutants non-spliced 0.7 kb-length bands are detected. For internal control, β-actin was used.

**Discussion**

Previous work has shown that the high fidelity of splice-site recognition in pre-mRNA splicing involves specific networks of RNA-protein, protein-protein, and RNA-RNA interactions\(^{30,31,32}\). These interactions are established on the three intronic sequence elements required for splicing: the 5' splice site, the BPS, and the polypyrimidine tract/AG dinucleotide at the 3' splice site\(^{30,31}\). We organized this study to clarify why the exon 9 is abnormally deleted during pre-mRNA splicing of Exo1 in MRL mice.

The present study and previously sequenced data showed that the above three signals were undoubtedly functional in the pCX1ExEIEIIE plasmid\(^{30}\). Additionally, the results obtained from the exchanged mutants composing pCX1ExEIEIIE(A) and pCX1ExEIEIME(T) plasmids suggested the importance of the BPS for intron splicing. We did not check mutants other than del1 and del2; however, the first determination of whether the intron 8 was spliced was clearly dominated by the sequence in BPS. The splice reaction takes place in two catalytic steps\(^{30}\). In the first reaction, the 2' hydroxyl of the conserved
adenosine residue within the BPS attacks the phosphate at the 5' splice site, producing a free 5' exon and a lariat intermediate. In the second reaction, the 3' hydroxyl of the 5' exon attacks the phosphate at the 3' splice site, generating spliced exons and a lariat intron. In the present study, it was possible that the triple adenosine repeat observed in the BPS of MRL mice could not recognize the 5' splice site of intron 8, with the result that the BPS within the intron 9, not the intron 8, attacked the 5' splice site of intron 8. These results suggest that the intron8-exon9-intron 9 complex is spliced all together from the genomic DNA of MRL mice, resulting to produce tr-1 Exo1 mRNA, in which exon 9 is deleted.

In this study, no intronic splicing occurred on either del1lB or del1/M. These results revealed the possibility that another still unknown nucleotide sequence influenced the normal pre-mRNA splicing. This important signal might be restricted to the proximal region of the intron 8 with 925 bp between BglII and EcoT221. However, it was also assumed that instability of a small sized lariat intermediate was caused not to be able to attack the 5' or 3' spliced site. It was outside of the scope of the present study, to determine why tr-2 Exo1 was produced in MRL mice. However, since in tr-2 Exo1, an optional 205 bp sequence, part of the intron 8 directly introduced from the exon 8, was inserted into the deleted region of tr-1 Exo1, this could be clarified by the experiment constructing a mutant plasmid throughout the 8-10th exons for investigation of tr-2 Exo1 production.

Since the abnormality of Exo1 was associated with mismatch repair for DNA duplication, our results indicated that MRL/MpJ mice are a useful model for investigation of cell proliferation. According to the results from database searches, incomplete abnormal splicing of Exo1 in MRL mice produces tr-1 protein in which a nuclear localization signal, composing KRPR at 417-420 amino acids is deleted. It is unknown whether or not this truncated protein acts as a dominant negative product. In any case, further investigation is necessary to identify the relationship between the production of mismatched DNA and the activity of Exo1 in MRL/MpJ mice.

Acknowledgements

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