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Citation	Japanese Journal of Veterinary Research, 52(3), 125-134
Issue Date	2004-11
DOI	10.14943/jjvr.52.3.125
Doc URL	http://hdl.handle.net/2115/10518
Type	bulletin (article)
File Information	52(3)_125-134.pdf

Instructions for use

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

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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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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[7),9)]. 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[12),17)].

Recently, we found three unique characteristics in the testis of the MRL/MpJ mouse : metaphase-specific apoptosis of meiotic spermatocytes (*msa*)[2),4)], heat-shock resistance in spermatocytes found in experimental cryptorchidism (*hsr*)[1),3)] 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*[10)]. 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, GGG<u>T</u>AA in C57BL/6 was substituted to GGG<u>A</u>AA 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'-CAGGATG AATATAACGGTGCC-3' and the reverse : 5'-ATTGCCTGGAATCTGAGAGC-3', in which the amplified length was 919 bp in the posi-

tion of 799-1717 (Accession No. AJ 23823).

PCR was carried out on a Biorad PCR thermal cycler (iCycler, Tokyo, Japan) with the cycling sequence of 94℃ for 5 min (one cycle), followed by 35 cycles consisting of denaturation at 94℃ for 40 sec, primer annealing at 60 ℃ for 30 sec, and extension at 72℃ 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 proteinase K, overnight at 56℃, 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, 1 mM 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'-CATTTCT

TGGTCTTTTCAACACAC-3' annealed on the 3' site of exon 9) was constructed according to a previous report (Accession No. AC073663)[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)[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 (GGG<u>T</u>AA) originating from B6 mice was changed into GGG<u>A</u>AA (*pCX/Ex/EIE/B(A)*), whereas that (GGG<u>A</u>A A) from MRL mice was changed into GGG<u>T</u>AA (*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 *EcoT 221* 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 TTCTGGCGTGTCACC-3' (located just before the exon 8) and the reverse : 5'-CATTTCTT GGTCTTTTCAACACAC-3' (located on the
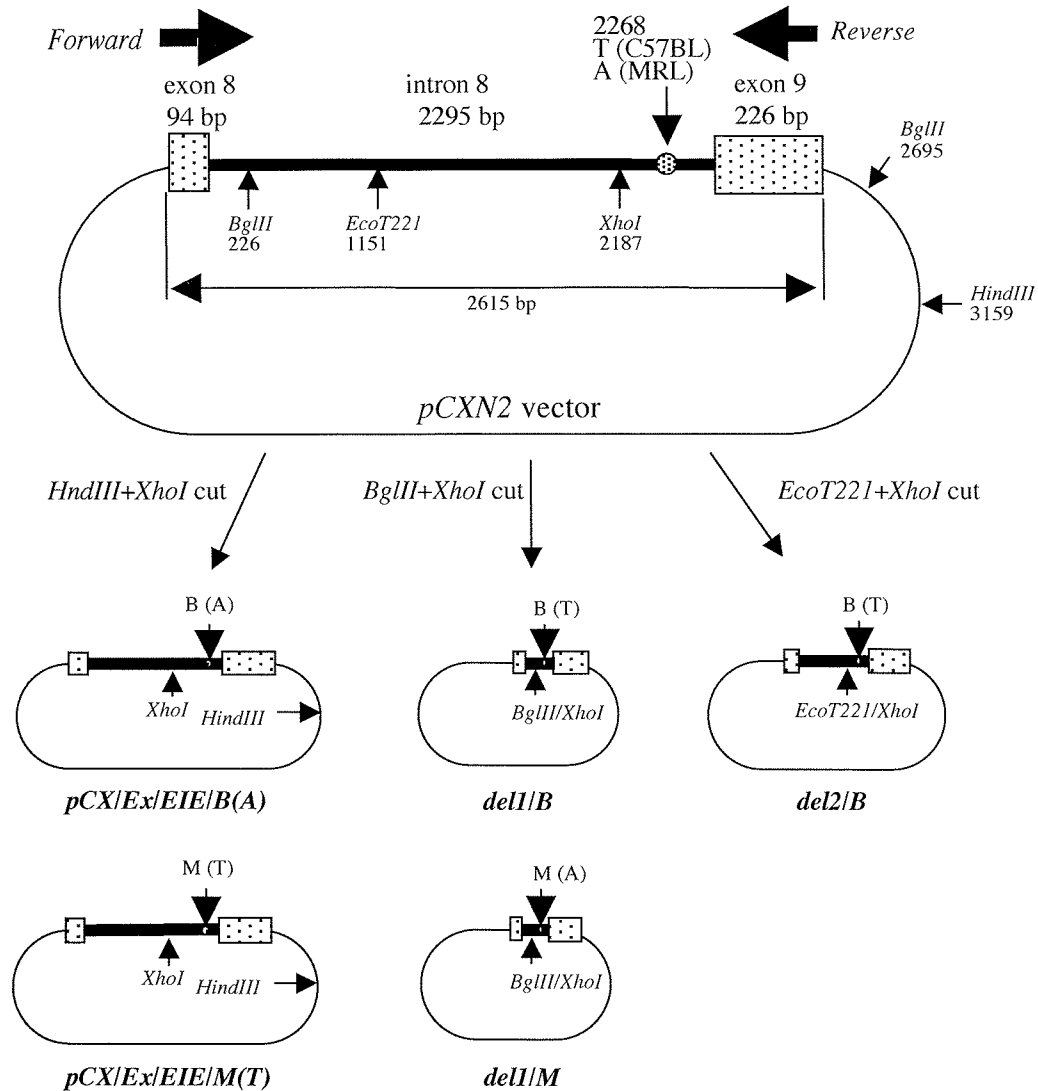
Figure 1 . Construction of *pCX/Ex/EIE* and mutant plasmids. The 2268 th nucleotide starting from the 5' site
of the exon 8 is inside of the BPS, producing T in *pCX/Ex/EIE/B* derived from C57BL/6 or A in *pCX/
Ex/EIE/M* derived from MRL. The numbers presented below each restriction enzyme are the dis-
tances 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* plas-
mid, 0.7kb in the *del1* mutant, and 1.6kb in
the *del2* mutant, respectively. PCR was car-
ried out with the same protocol described
above and the samples were electrophoresed

with 2% agarose gel.

Results

Three types of products were at least de-
tected by RT-PCR for *Exo1* in all tissues of
MRL mice, whereas there was just one prod-
uct in those of C57BL/6 mice(Fig. 2a). Nucleo-
tide 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 C 57 BL/6 and MRL mice showed 8-nucleotide substitutions and 9-nucleotide deletions of MRL mice, in which BPS was changed from GGG<u>T</u>AA in C57BL/6 into GGG<u>A</u>AA in MRL mice (Fig. 2d). These results was corresponded with the previous report[10].

In the experiments using the original expression plasmids, a spliced band spanning about 0.3kb could be detected in *pCX/Ex/EIE/B* 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 *del1/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.
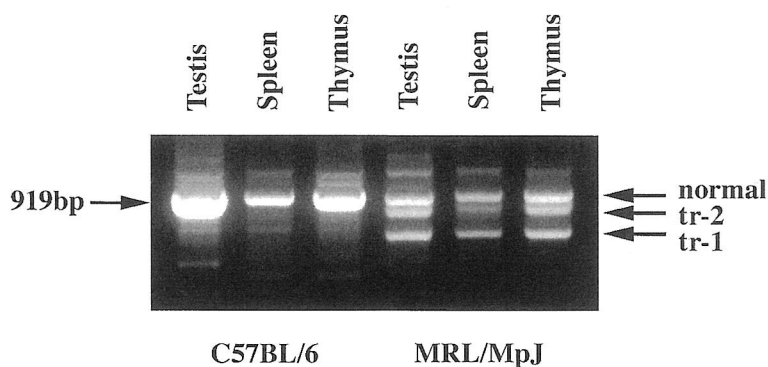
Figure 2 a

Figure 2 . Comparison of partial nucleotide/amino acid sequence of *Exo1* between C57BL/6 and MRL mice. a : Expression of *Exo1* by RT-PCR in various tissues. Primer pair used is mid region of *Exo1* cDNA as described in materials and methods. Three type of products are detected in MRL mice, but not in C 57BL/6 mice. b : Nucleotide sequence of the products amplified in Fig. 2a. Forward and reverse primer sequences are underlined. Identical nucleotides are indicated by dots (.), and the numbers with each line indicate the nucleotide position. Nucleotide deletion shown by dashed lines is detected in *tr-1* and *tr-2 Exo1*. Additionally, different sequence is inserted in *tr-2 Exo1*. c : Putative amino acid sequence in C57BL/6 and MRL mice. Stop codon indicated by asterisks is present in *tr-1 Exo1*. d : Genomic sequence from exon 8 into exon 9 in C57BL/6 and MRL mice. Underlines indicate exon 8 and 9. BPSs are shown by outlined box.

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C57BL/6   CAGGATGAAT ATAACGGTGC CCGAGGATTA CATCACAGGA TTTATTCGTG CCAACAATAC TTTCCTCTAC CAGCTCGTGT TCGACCCCAT CCAAAGGAAG   100
MRL-nor   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
MRL-tr1   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
MRL-tr2   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

          CTGGTCCCTC TGAATGCCTA CGGAGATGAC GTTAATCCCG AAACACTGAC TTACGCTGGG CAGTACGTCG GTGACTCTGT AGCTCTTCAG ATCGCCCTTG   200
          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

          GAAATAGAGA TGTAAATACT TTTGAACAGA TTGATGACTA CAGTCCGAC ACCATGCCAG CCCACTCAAG AAGCCACAGC TGGAATGAGA AAGCAGGTCA   300
          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
          .......... .......... .......... .......... ......----  ---------- ---------- ---------- ----------
          .......... .......... .......... .......... .......... ......GTAA CATTTAACAA CTGTTACATA AGCGTGTTTG AGTTTTGTCT

          GAAACCACCT GGTACCAACA GCATTTGGCA CAAGAATTAT TGTCCTAGAC TTGAGGTGAA CAGTGTCTCC CACGCTCCTC AACTGAAGGA AAAGCCAAGC   400
          .......... ...G...... .......... .......... .......... .......... .......... .......... .......... ..........
          ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
          CAAGAGCTTT GAGGCGGGTC TTTCCTTGCT CAGCAGCCGT GAAGTACTTC CGTACTCAGC GCTATGCAGA GCGTGCATGA AAATGACTTC ACCTGGTGTG

          ACTTTGGGCC TTAAACAAGT GATTAGTACT AAAGGGTTAA ATCTTCCCAG GAAGTCTTGT GTGTTGAAAA GACCAAGAAA TGAAGCGCTG GCTGAAGATG   500
          .......... .......... .......... .......... .......... .......... .......... .......... --......... ..........
          ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --......... ..........
          GCAGGCGTCT GTAATCCCAG GACTGGGGAC GCTGAGACAG GAGAATCGCC ACAAGTTCAA ---------- ---------- -......... ..........

          ACCTGTTGAG CCAGTATTCG TCAGTTTCAA AGAAGATCAA GGAAAATGGC TGTGGGGATG GCACATCACC TAACTCTTCT AAAATGTCCA AGTCCTGCCC   600
          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

          CGATTCTGGG ACTGCTCACA AGACAGATGC ACACACCCCG TCTAAGATGA GGAATAAATT TGCAACGTTC TTACAGAGGA GGAATGAAGA AAGCGGTGCA   700
          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

          GTCGTGGTTC CAGGGACCAG AAGCAGGTTT TTTTGCAGTT CTCAGGATTT TGACAATTTC ATACCAAAAA AAGAAAGCGG CCAGCCTCTA AATGAAACTG   800
          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

          TGGCCACTGG CAAAGCCACC ACCAGCCTCC TCGGGGCACT GGACTGTCCA GACACGGAAG GCCACAAGCC GGTTGATGCA AATGGGACGC ACAATCTGAG   900
          .......... .......... .......... .......... .......... .......... .......... .......... .......T.. ..........
          .......... .......... .......... .......... .......... .......... .......... .......... .......T.. ..........
          .......... .......... .......... .......... .......... .......... .......... .......... .......T.. ..........

          CTCTCAGATT CCAGGCAAT       919
          .......... .........
          .......... .........
          .......... .........
```

Figure 2 b

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C57BL/6   RMNITVPEDY ITGFIRANNT FLYQLVFDPI QRKLVPLNAY GDDVNPETLT YAGQYVGDSV ALQIALGNRD VNTFEQIDDY   80
MRL-nor   .......... .......... .......... .......... .......... .......... .......... ..........
MRL-tr1   .......... .......... .......... .......... .......... .......... .......... ..........
MRL-tr2   .......... .......... .......... .......... .......... .......... .......... ..........

          SPDTMPAHSR SHSWNEKAGQ KPPGTNSIWH KNYCPRLEVN SVSHAPQLKE KPSTLGLKQV ISTKGLNLPR KSCVLKRPRN   160
          .......... .......... ....A..... .......... .......... .......... .......... ..........
          .....----- ---------- ---------- ---------- ---------- ---------- ---------- ----------
          .....VTFNN CYISVFEFCL KSFEAGLSLL SSREVLPYSA LCRACMKMTS PGVAGVCNPR TGDAETGESP QVQ-------

          EALAEDDLLS QYSSVSKKIK ENGCGDGTSP NSSKMSKSCP DSGTAHKTDA HTPSKMRNKF ATFLQRRNEE SGAVVVPGTR   240
          .......... .......... .......... .......... .......... .......... .......... ..........
          KRWLKMTC*A SIRQFQRRSR KMAVGMAHHL TLLKCPSPAP ILGLLTRQMH TPRLR*GINL QRSYRGGMKK AVQSWFQGPE
          .......... .......... .......... .......... .......... .......... .......... ..........

          SRFFCSSQDF DNFIPKKESG QPLNETVATG KATTSLLGAL DCPDTEGHKP VDANGTHNLS SQIPGN   306
          .......... .......... .......... .......... .......... ......M... ......
          AGFFAVLRIL TISYQKKKAA SL*MKLWPLA KPPPASSGHW TVQTRKATSR LMQMGCTI*A LRFQA
          .......... .......... .......... .......... .......... ......M.... ......
```

Figure 2 c

```
C57BL/6  ATGTACGTCGGTGACTCTGTAGCTCTTCAGATCGCCCTTGGAAATAGAGATGTAAATACTTTTGAACAGATTGATGACTACAGTCCAGACACCATGGTAA   100
MRL      ....................................................................................................

         CATTTAACAACTGTTACATAAGCGTGTTTGAGTTTTGTCTCAAGAGCTTTGAGGCGGGTCTTTCCTTGCTCAGCAGCCGTGAAGTACTTCCGTACTCAGC   200
         ....................................................................................................

         GCTATGCAGAGCGTGCATGAAAATGACTTCACCTGGTGTGGCAGGCGTCTGTAATCCCAGGACTGGGGACGCTGAGACAGGAGAATCGCCACAAGTTCAA   300
         ....................................................................................................
                                                        BglII
         GGTTAGCCTGGGCTATACAGTGAGATCTTGTCTTTCTGGTTTTGTTTTAAGACAGGTTTTCACTGTAAAGTCCTAGCTGTCAAGAAACTCATTGTGTAGA   400
         ....................................................................................................

         CCAAGATGGCCTCGAACTCGTGGAGATTTGTCTGACTCTGTCTCCTGGGTACTGGGCATGTGCCACCACATCTAGCCAATTACTTTTAAAAAAATGATTAA   500
         ...........................................TG.......................................................

         CTTGATCCGAGAGAAATTATCTATTAAATTATAGGTGTATTATAATACTAAAAACTGATTACATGATATTTTTAAGACCAGCATATGATACATTCAGCAC   600
         ...........................................G......................................................

         CTTTTGTCTCATCTTACGTCATTCAGACTTTGGCACTTGGCATTGTTGAAAACCCAAAACATGGAGTACCAGCTGTCTTAGGCTAATCCGTCAAATACTC   700
         ....................................................................................................

         AGTGAGCTGCCCTCCCTTGTGTGTCTGAACTCTTCACTGCTCTCTTTGTTGTATACCCTCCTGGCAGCGTCTGCCACCTCTTCAGGTTATGTTTATGTCT   800
         ....................................................................................................

         TCAATAAGTTCTTTTAGCTACTGTAATTTCTTTTGTTCTTTGAGAACGCCATATATGTATAGTGTTAGGCCATTTATGCACAGAACCTGAGTTAGTTTAT   900
         ....................................................................................................

         CTTATTGTTGGGAATCATTACCGAGATGAAAGTCTGGAAACACTGGCTGTGTTGTCATTTACACATCTGAATTAGCAATTCAGTAATTTTACCAGGAGTG   1000
         ....................................................................................................

         ATGCTGAAGGTGTTTGTCTTAAACCCACAAATTGTCGCACCGACCCTGTAGGTTCTGAGCTGCTAAGGTGATCCTGATTCAGAAACCCAAATCTATGCCT   1100
         ....................................................................................................

         CATCCTTTTTATAGGGAAACTATACAGCAAACGATATGATCTAAAGTGTGGGCTTCCTGGGTTTGTTTTGGTTTTTTGGGTTTGTTTTCGGTTTTTGTTT   1200
         ....................................................................................................
                                                        EcoT22I
         TTTTGGTCTTTTTTTTCTTTGCCTATTTCTTTTTCTCAGGAAATGCATCTGTGGGATTTAATTCTGAGAGCCATGCAGCGGCTGAGGCTGTTTGTTGTTC   1300
         ....................................................................................................

         TCAAGCCTTAAGTTAACCTTCCTTGGCAATTCTCAGAGCCTGTTTTGAATGCTGTCTGTTTTTTTTTCCCTTTTACTTTCAGCAAAAAGACTTTTTTTTT   1400
         ....................................................................................................

         TTTTTTTTTTGGTTTTAGTACATTTGAGAATTGGTTATGTGACAGTCATACTTCGAGATTTTCTGTTTTGTTCTTGTTTTTTTGAGACAGGATCTCTCCCCA   1500
         ....................................................................................................

         GCTCTGGCCGTTCTGGAACTCACTGTGTAGAACCGCCTGCCTCTGCCTCTCAAGTGCTGGGGTTAAATGCGTGTGCCACCACACAGCCCAATTTTCTGTG   1600
         .........A..................................T......................................................

         TCTTCACTTTTTGGTCTTGGCTGCCTTTGTCTATTGTAATAGCGTTACCGATGTTTCTAGCGGTCTTCTGTAACAGTTGTAATCATAAATCTATCAGTGA   1700
         ....................................................................................................

         GACTGTTACCTTGTGGGTGTTAGCTGGCGTCAACCCTTTCTCCAAAGTTTTATGCACGATTCAGTGTCAGCCAGAACAGCTTTCTCTTTTCCAGCATGGT   1800
         ....................................................................................................

         ACCAAGTGAGTCCGATACAACAAACAAGAACTTTCTAACCTAGGTTGTTCTGGTTTGAACTGAACAAATTTTTATCCAGGCTTGCGAGCTTATGGTTATA   1900
         ....................................................................................................

         ATCCCAGCATGTGGGAGACTGAGGCAGGAGAATAAATTCAGCTAGCCTAGACTACAGGAGACCCTGTCTCAAACAAATAATTAAAATTAAACACATTTCA   2000
         ...T................................................................................................

         GATTGGAGAGATGGCTCAGCGGTTAAGAGCACTGACTGCTCTTCCAGAAGAAAGCAAAACGTGTTATTAGAGCAGAGACCATGAGATCCCTTCCCTCTCT   2100
         ....................................................................................................

         CTCTCTTCCTTCTTCTCCCACCAGGCCCCACATGTGCTAGGCAAGTACTGCGCCCCTGAGCCATTTTTAATTCAAAATAAAATAATTAGTGCTCAGAAGT   2200
         ..........................................--.---------......................................
                                                                                     XhoI
         CACCTAGACTAGTGACTAGTCTGAGCTCACTGTAACCTAAGCAGCCTTTGAATTTTCAATCCTCCTGCCTCACCAGGCCAGCCTCGAGAGTTTTTCTCTT   2300
         ....................................................................................................

         GCACACTAATTGAACATCACTATAAGCAGACACAGGAGGAAATGGTCACGTCTTCTGTTCCGGTAACCTTTTTCTTTGTTCCTTTCTAGCCAGCCCACTC   2400
         ........................................................[GGTAAC]..A..................CCAGCCCACTC
                                                                .A..

         AAGAAGCCACAGCTGGAATGAGAAAGCAGGTCAGAAACCACCTGGTACCAACAGCATTTGGCACAAGAATTATTGTCCTAGACTTGAGGTGAACAGTGTC   2500
         ............................................G.......................................................

         TCCCACGCTCCTCAACTGAAGGAAAAAGCCAAGCACTTTGGGCCTTAAACAAGTGATTAGTACTAAAGGGTTAAATCTTCCCAGGAAGTCTTGTGTGTTGA   2600
         ....................................................................................................

         AAAGACCAAGAAATG
         ...............
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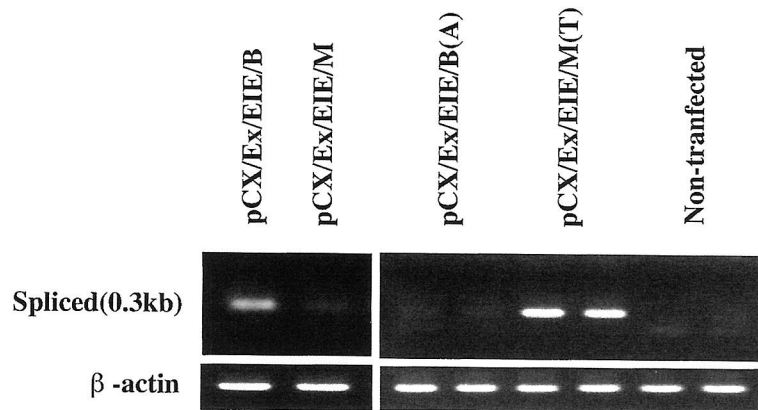
Figure 2 d

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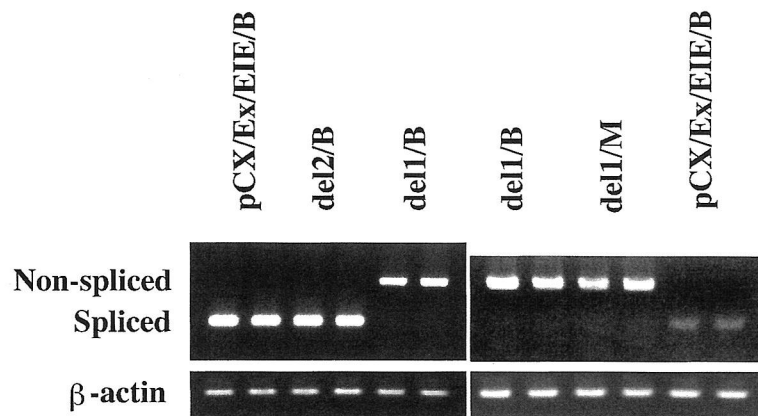

Figure 4 . *In vitro* splicing assay with *del1* and *del2* mutant plasmids. The spliced 0.3kb-length bands are detected in *pCX/Ex/EIE/B* and *del2/B* plasmids; however, in *del1/B* and *del1/M* mutants non-spliced 0.7 kb-length bands are detected. For internal control, β-actin was used.

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[13), 15, 16) 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[5), 14). 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 *pCX/Ex/EIE/B* plasmid[10). Additionally, the results obtained from the exchanged mutants composing *pCX/Ex/EIE/B(A)* and *pCX/Ex/EIE/M(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[15). 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 *del1/B* 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 *EcoT22I*. 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[6),8)]. 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.

References

1) Kazusa, K., Namiki, Y., Asano, A., Kon, Y., Endoh, D. and Agui, T. 2004. Strain difference of spermatogenesis in cryptorchid testis in mice. *Comp. Med.,* 54:179-184.

2) Kon, Y. and Endoh, D. 2000. Morphological study of metaphase-specific apoptosis in MRL mouse testis. *Anat. Histol. Embryol.,* 29:313-319.

3) Kon, Y. and Endoh, D. 2001. Heat-shock resistance in experimental cryptorchid testis of mice. *Mol. Reprod. Develop.,* 58: 216-222.

4) Kon, Y., Horikoshi, H. and Endoh D. 1999. Metaphase-specific cell death in meiotic spermatocytes in mice. *Cell. Tissue. Res.,* 296:359-369.

5) Krämer, A. 1996. The structure and function of proteins involved mammalian pre-mRNA splicing. *Ann. Rev. Biochem.,* 65: 367-409.

6) Lee, BI., Nguyen, L.H., Barsky, D. and Fernandes, M. and Wilson, D.M. III. 2002. Molecular interactions of human Exo 1 with DNA. *Nuc. Acids. Res.,* 30:942-949.

7) Leferovich, J.M., Bedelbaeva, K., Samule-

wicz, S., Zhang, X.M., Zwas, D., Lankford, E.B. and Heber-Katz E. 2001. Heart regeneration in adult MRL mice. *Proc. Natl. Acad. Sci. USA.*, 98:9830-9835.

8) Marti, T.M., Kunz, C. and Fleck, O. 2002. DNA mismatch repair and mutation avoidance pathways.*J.Cell.Physiol.*, 191: 28-41.

9) McBreaty, B.A., Clark, L.D., Zhang, X.M., Blankenhorn, E.P. and Heber-Katz, E. 1998. Genetic analysis of a mammalian wound-healing trait. *Proc. Natl. Acad. Sci. USA.*, 95:11792-11797.

10) Namiki, Y., Endoh, D. and Kon, Y. 2003. Genetic mutation associated with meiotic metaphase-specific apoptosis in MRL / MpJ mice. *Mol. Reprod. Dev.*, 64:179-188.

11) Niwa, H., Yamamura, K. and Miyazaki, J. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene*, 108:193-200.

12) Nose, M., Terada, M., Nishihara, M., M, Kamogawa, J., Miyazaki, T., Qu, W., Mori, S. and Nakatsuru, S. 2000. Genome analysis of collagen disease in MRL/lpr mice : polygenic inheritance resulting in the complex pathological manifestations. *Int. J. Cardiol.*, 75:S53-S61.

13) Reed R. 1996. Initial splice-site recognition and pairing during pre-mRNA splicing. *Curr. Opin. Genet. Dev.*, 6 : 215-220.

14) Reed R. 2000. Mechanisms of fidelity in pre-mRNA splicing. *Curr. Opin. Cell. Biol.*, 12:340-345.

15) Staley, J.P. and Guthrie, C. 1998. Mechanical devices of the spliceosome : motors, clocks, springs, and things. *Cell,* 92: 315-326.

16) Tacke, R. and Manley, J.L. 1999. Determinants of SR protein specificity. *Curr. Opin. Cell. Biol.*, 11:358-362.

17) Yamada, A., Miyazaki, T., Lu, L.M., Ono, M.R., Ito, M., Terada, S., Mori, K., Hata, Y., Nozaki, S., Nakatsuru, Y., Nakamura, M., Onji, M. and Nose, 2003. Genetic basis of tissue specificity of vasculitis in MRL/lpr mice. *Arthritis. Rheumat.*, 48: 1445-1451.