A secondary structure at the 3' splice site affects the *in vitro* splicing reaction of mouse immunoglobulin μ chain pre-mRNAs

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ABSTRACT

The expression of the IgM (immunoglobulin μ) heavy chain gene is known to be regulated at the post-transcriptional level. The two isoforms, the membrane-bound and secreted forms, are generated from the same gene by alternative processing at the 3' end of the primary transcript. The processing reactions involved are polyadenylation at the upstream poly(A) site (for the secreted form) and polyadenylation at the downstream poly(A) site coupled with splicing between exon C4 and exon M1 (for the membrane-bound form). The regulatory mechanism underlying these differential processing reactions is still not well understood. We investigated the splicing reaction between exon C4 and exon M1 in a HeLa nuclear extract using model transcripts containing the 5' and 3' splice sites of the C4-M1 intron. We found that the 3' splice site of the C4-M1 intron is sequestered in a stem-loop structure, which inhibits the splicing reaction *in vitro*. The inhibition by the stem-loop structure was also observed with a mouse lymphoma extract.

INTRODUCTION

The diversity of gene expression in eukaryotes is partly achieved by alternative splicing (1,2). The expression of the IgM heavy chain (μ) gene is one of the best-studied cases where primary transcripts undergo alternative processing. During B-lymphocyte differentiation, the two isoforms of IgM, the secreted form and the membrane-bound form are generated from a single gene by alternative processing at the 3' end of the mRNA precursor (pre-mRNA) (3-5). If the pre-mRNA is polyadenylated at the upstream poly(A) site (the secreted site), secreted type mRNA is produced. If, on the other hand, splicing occurs between C4 and M1 exons, the upstream polyadenylation site is eliminated and the downstream poly(A) site (the membrane-bound site) is used to produce membrane-bound type mRNA. The relative amount of these two types of mRNAs is known to be regulated during B-cell differentiation: in the early stage, both the membrane-bound and secreted types of mRNA are produced, whereas the secreted type mRNA is predominantly produced in the differentiated plasma cells.

Thus, the differential production of the two types of mRNA is thought to be achieved at the post-transcriptional level, not by transcription termination or by cell-type specific degradation of respective mRNAs (6,7). Two models have been proposed based on previous transfection experiments. One is the poly(A) site model, in which competition for polyadenylation at the secreted site and at the membrane-bound site is thought to determine the type of mRNA (8,9). The second model is the secreted site-splice model (10-12). According to this model, polyadenylation at the secreted site competes with C4-to-M1 splicing rather than with polyadenylation at the membrane-bound site. If the former model is correct, polyadenylation at the membrane-bound site is expected to precede C4-to-M1

splicing. Peterson and Perry, however, have recently reported that splicing can occur with non-polyadenylated precursors (10). They also showed that mutations at the 5' splice site of the C4-M1 intron dramatically shifted μ mRNA production in plasmacytoma cells from predominantly secreted type to exclusively membrane-bound type. These observations clearly show that C4-to-M1 splicing is important in regulating this differential processing event.

The development of *in vitro* RNA splicing systems has made it possible to analyze this processing reaction biochemically (for reviews see 13,14). In an attempt to elucidate the mechanism of the regulation of alternative processing of the μ gene, we analyzed the *in vitro* splicing reaction with model substrates. We were able to show that a possible secondary structure exists, which sequesters the 3' splice site of the intron between exon C4 and exon M1, and which has an inhibitory effect on the *in vitro* splicing reaction.

MATERIALS AND METHODS

Chemicals and Enzymes

³²P-labeled nucleotides were obtained from Amersham. Other nonradioactive nucleotides including cap analogues and RNase A were purchased from Pharmacia. RNases T1, T2 and U2 were from Sankyo. All other enzymes were purchased from Takara Shuzo Co.

Plasmid Construction and Preparation of Pre-mRNAs

Mouse μ gene fragments were obtained from plasmid pMo μ Δ A (kindly provided by Dr. N. Tsurushita; 11, see Figure 1A). The 350 bp *BanI*-*PstI* fragment spanning a 3' portion of exon C3, a 5' portion of exon C4, and the intron between them was gel-purified. This fragment was ligated into the *SmaI*-*PstI* site of pSP65 vector, which gave p μ C3-C4, the template plasmid for μ C3-C4 pre-mRNA. Two fragments, the 252 bp *HinfI* fragment containing exon M1 (115 bp) with some additional intron sequences at both ends and the 160 bp *HpaII*-*Clal* fragment spanning a 3' portion of exon C4, S region, and a portion of the C4-M1 intron were ligated into the *AccI*-*PstI* site of pSP65 vector, which gave p μ Δ C4-M1. The *Clal* site was regenerated between these two fragments. The *EcoRI*-*Clal* fragment of p μ Δ C4-M1 was excised and the 310 bp *EcoRI*-*NlaIV* fragment of p μ C3-C4 was inserted instead, which gave p μ Δ C3-M1. The *NlaIV*-*PstI* fragment containing the 3' splice site was excised from p μ C3-C4 and the 252 bp *HinfI* fragment was inserted, which gave p μ C4-C4. The template plasmids were linearized with *HindIII* and transcribed *in vitro* with SP6 RNA polymerase as described previously (15). The transcripts were purified by electrophoresis on a 5% polyacrylamide gel containing 8 M urea.

Site-directed Mutagenesis

To create mutations in the stem-loop structure at the 3' splice site, the *EcoRI*-*HindIII* fragment (0.5 Kb) of p μ Δ C3-M1 or p μ Δ C4-M1 was subcloned into the M13mp18 vector and mutagenesis was carried out as described previously (16). The following oligonucleotides carrying substitutions (indicated in *italics*) were used as primers: 39 mer (5'-CCTCCTCAGCATTCA***TTGTTTGTG***CTATGAAGGGACACA) for μ Δ C3-M1R9 and μ Δ C4-M1R9, 36 mer (5'-CCTCCTCAGCATTCA***TTGTTTCT***CTATGAAGGGAC) for μ Δ C3-M1R6, 33 mer (5'-CCTCCTCAGCATTCA***TGCCCCCT***CTATGAAGG) for μ Δ C3-M1R3, 36 mer (5'-CCTCCTCAGCATTCA***TGATATCCT***CTATGAAGGGAC) for μ Δ C3-M1R6*. These oligonucleotides were synthesized with an Applied Biosystems DNA synthesizer A380.

Cell and Media

The M12 lymphoma cell line (17) was maintained in RPMI1640 medium supplemented with 10% fetal calf serum and 50 μ M 2-mercaptoethanol.

Preparation of Nuclear Extracts and In Vitro Splicing

HeLa nuclear extracts were prepared as described previously (18). M12 nuclear extract was prepared according to Gorski *et al.* (19) with slight modifications: after centrifugation on a sucrose cushion, the isolated nuclei were extracted with buffer C (0.3 M KCl) according to Dignam *et al.* (18). The splicing reaction with a HeLa nuclear extract was carried out in 25 μ l of the reaction mixture as described previously (20, 21). The splicing reaction with an M12 nuclear extract was carried out in the same way as that with HeLa nuclear extracts except that creatine kinase was present at a concentration of 100 μ g/ml in the reaction mixture, which proved to be critical to prevent non-specific degradation of RNAs.

Secondary Structure Analysis using Partial RNase Digestion

RNA transcripts were 3' end-labeled with [32 P]pCp and T4 RNA ligase (22) and were gel-purified. Nuclease digestions were carried out as follows. Each reaction mixture contained 10 μ g of carrier yeast tRNA in 25 μ l of the digestion buffers given below. For RNases T1, T2 and A, 20 mM Tris-HCl (pH 7.5), 60 mM KCl and 1.6 mM MgCl₂. For RNase U2, 30 mM sodium acetate (pH 4.6), 60 mM KCl and 1.6 mM MgCl₂. For S1 nuclease, 50 mM sodium acetate (pH 4.6), 280 mM NaCl, 4.5 mM ZnSO₄. After the addition of nuclease, the reaction mixture was incubated at 30°C for 10 min, then the reaction was stopped by the addition of 100 μ l of 2 $\times$ PK buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 2% SDS), 5 μ l of 20 mg/ml proteinase K and water to give a final volume of 200 μ l. After 30 min incubation at 37°C, the RNAs were extracted with phenol, precipitated with ethanol, and then electrophoresed on a 4% polyacrylamide gel containing 8 M urea.

RESULTS AND DISCUSSION

In Vitro Splicing of Mouse μ Gene Transcripts in a HeLa Nuclear Extract

In an attempt to analyze processing of μ RNA precursors *in vitro*, we carried out the splicing reaction with a HeLa nuclear extract using the model pre-mRNAs illustrated in Figure 1B. They all contain one intron with different combinations of 5' and 3' splice sites. Although this is a heterologous system, we can expect to get information that might provide some insight into the basic property of the substrates, because splicing machinery of eukaryotes is widely conserved especially among mammals.

To see if mouse μ gene transcripts can be spliced efficiently with a HeLa nuclear extract, the μ C3-C4 pre-mRNA, spanning parts of exon C3 and exon C4 with the intron between them, was synthesized. The $\mu\Delta$ C4-M1 pre-mRNA is a model substrate for the splicing reaction between exon C4 and exon M1, and contains parts of exon C4 and exon M1 with a shortened version of the intron between them. The secreted type polyadenylation site which is located in this intron is deleted for simplicity. The splicing reactions of these two transcripts in a HeLa nuclear extract were analyzed and the splicing efficiency of each transcript was quantified by measuring the radioactivity of the spliced products (Figure 1C). As expected, the μ C3-C4 pre-mRNA was efficiently spliced in this system (Figure 1C lanes 1–3), with a yield of the spliced products of about a quarter of the input precursor on a molar basis after 60-min incubation. In contrast, the splicing reaction of the $\mu\Delta$ C4-M1

pre-mRNA occurred very poorly; the splicing efficiency was reduced more than 10-folds compared to that of the μ C3-C4 pre-mRNA (Figure 1C lanes 4–6).

In order to examine which splice site is responsible for the low splicing efficiency of the $\mu\Delta$ C4-M1 pre-mRNA, we constructed templates for two model pre-mRNAs, μ C4-C4 and $\mu\Delta$ C3-M1. As shown in Figure 1B, either the 5' (for μ C4-C4) or the 3' (for $\mu\Delta$ C3-M1) splice site of the μ C3-C4 pre-mRNA is replaced by the corresponding segment of the C4-M1 intron. The time course analyses of the splicing reactions of these transcripts in a HeLa nuclear extract showed that both the μ C4-C4 and $\mu\Delta$ C3-M1 pre-mRNAs were spliced less efficiently than the μ C3-C4 pre-mRNA (about 5-fold reduction in each case; Figure 1C lanes 7–9, 10–12). The most straightforward explanation for these results is that both the 5' and 3' splice sites of the $\mu\Delta$ C4-M1 pre-mRNA are responsible for the low level of the splicing reaction. The recent report by Peterson and Perry showed that the 5' splice site of C4-M1 intron is suboptimal with regard to splicing efficiency (10). This is consistent with our *in vitro* results.

We took a close look at the sequence of the 3' splice site to see if there was any structure which might interfere with the splicing reaction. Previous studies (24, 25) indicated that when splice sites are sequestered in stem-loop structures, these structures can inhibit splicing *in vitro*. We found a potential stem-loop structure at the 3' splice site (Figure 2A). This structure consists of a stem of 14 bp (including 8 G-C pairs) and a loop of 6 nucleotides. The free energy was calculated to be -22.7 kcal (23), which represented the most stable stem-loop structure located by the computer search in the sequence spanning exon C4 to exon M2.

The Secondary Structure Probed by Nuclease Sensitivity

To determine how the potential stem-loop structure affects splicing at the 3' splice site, we constructed templates for mutant pre-mRNAs, $\mu\Delta$ C4-M1R9 and $\mu\Delta$ C3-M1R9. Base substitutions were introduced at the bottom part of the stem in the exon M1 sequence. As shown in Figure 2A and B, 9 bases of exon M1 (AGGGGGAGG) are replaced by CACAAACAA, so that the potential stem structure is largely distorted. To demonstrate that the stem structure actually exists in $\mu\Delta$ C3-M1 but not in $\mu\Delta$ C3-M1R9, these transcripts were partially digested with various RNases after labeling at their 3' ends. We used five kinds of nucleases with different base specificities: RNase T1, RNase U2, RNase A, RNase T2 and nuclease S1 (note that under the conditions we employed, RNases U2 and T2

Figure 1. (A) Partial map of the mouse μ gene recombinant plasmid pMo μ Δ A (10). The location of the deleted *HaeII-KpnI* fragment containing the secreted-type polyadenylation site is shown. Open, hatched and filled boxes represent μ chain exon sequences. Coding regions are enlarged. C3, C4: constant regions (open box); S: 3' exon for the secreted μ mRNA (hatched); M1 and M2: 3' exons for the membrane-bound μ mRNA (filled); As, Am with arrowheads: polyadenylation sites for the secreted and the membrane-bound type μ mRNAs, respectively. Restriction sites used are as follows: B, *BanI*; N, *NlaIV*; P, *PstI*; Hp, *HpaII*; C, *Clat*; Ha, *HaeII*; K, *KpnI*; Hf, *HinfI*. (B) Schematic representation of the model pre-mRNAs. The boxes represent exon sequences and the lines between them show intron sequences. These sequences contain short leader and trailer sequences derived from pSP65. The lengths (in nucleotides) of the exons and introns are indicated above them. The letters in the boxes represent individual μ mRNA exons. The plasmids used for the *in vitro* synthesis of these pre-mRNAs were constructed as described in MATERIALS AND METHODS. (C) *In vitro* splicing of the IgM model pre-mRNAs in a HeLa nuclear extract. The pre-mRNAs (10 fmol each) were incubated in a HeLa nuclear extract at 30°C for the duration indicated on the top of each lane. The bands for the pre-mRNAs (P), spliced products (S), and the 5' exons (E) identified by their lengths are indicated on the right of each panel. Lanes 1–3, μ C3-C4; lanes 4–6, $\mu\Delta$ C4-M1; lanes 7–9, μ C4-C4; lanes 10–12, $\mu\Delta$ C3-M1. The gel pieces corresponding to these RNAs were cut out and their radioactivities were measured by Cerenkov counting.

preferentially attacked ApN and RNase A preferentially attacked CpN). By comparing the digestion patterns of the different nucleases, we were able to identify the regions of the RNA protected by secondary structures. Digestions with RNases T1, T2 and A were carried out under the same conditions as in the splicing reaction, so that the configuration of the pre-mRNA in the digestion reactions was expected to be comparable to that in the splicing reaction. Though S1 and U2 digestions were performed in low pH, the results obtained were consistent with those from T1, T2 and A digestions.

Figure 2C shows the partial digestion patterns of $\mu\Delta C3$ -M1 and $\mu\Delta C3$ -M1R9. The digestion patterns of $\mu\Delta C3$ -M1 support the existence of the predicted secondary structure. Nucleotides contained in both sides of the stem structure (indicated by the arrows) were protected from all five of the nucleases, while the nucleotides in the loop sequence between the two sides of the stem (indicated by the thick bar) were attacked. The change in the digestion patterns of $\mu\Delta C3$ -M1R9 indicates that the secondary structure was substantially altered by the substitutions in the stem. The 3' side of the stem protected in $\mu\Delta C3$ -M1 was attacked by all five of the nucleases in $\mu\Delta C3$ -M1R9. The substituted sequence CACAAACAA was not attacked by RNase T1, due to the base specificity of this nuclease. However, G -1 of $\mu\Delta C3$ -M1R9 was attacked, indicating that this region was no longer protected by the stem structure. Curiously, its counterpart, the pyrimidine cluster region, which is contained in the 5' side of the stem structure in $\mu\Delta C3$ -M1, was still protected in $\mu\Delta C3$ -M1R9. It remains to be elucidated why this region of $\mu\Delta C3$ -M1R9 is resistant to the nucleases. A plausible explanation would be that the pyrimidine cluster region may form a different secondary structure with other part of the pre-mRNA sequence, if the region cannot pair with the 3' side sequence of the stem structure in Figure 2A.

Effect of Substitution on In Vitro Splicing

The mutant pre-mRNAs, $\mu\Delta C4$ -M1R9 and $\mu\Delta C3$ -M1R9, were examined for their splicing efficiency *in vitro* (Figure 3). A remarkable increase in splicing efficiency was observed with both of these two substrates (Figure 3, lanes 4–6, 10–12). The amount of spliced product from each mutant pre-mRNA was about 5 times greater than that from corresponding wild-type pre-mRNA at 30 minutes.

In order to analyze the effect of the secondary structure of the stem region on splicing in more detail, we constructed templates for several other $\mu\Delta C3$ -M1 mutant pre-mRNAs.

Figure 2. (A) Schematic representation of the stem-loop structure predicted to be present at the 3' splice site of the C4-M1 intron. The G positions attacked by RNase T1 are indicated by numbers (the first nucleotide in exon M1 is +1). The sequence of exon M1 is enclosed in a box. The arrows indicate the positions which were attacked when $\mu\Delta C3$ -M1 was partially digested with single-strand specific nucleases. BP shows the branchpoint determined in primer extension experiments (data not shown). The bars on the right side of the stem show the segments replaced by the mutant pre-mRNAs indicated on the top of each bar. (B) The sequences in the mutant pre-mRNAs which replace part of the stem-loop structure. R9: for $\mu\Delta C3$ -M1R9 and $\mu\Delta C4$ -M1R9, R6: for $\mu\Delta C3$ -M1R6, R3: for $\mu\Delta C3$ -M1R3, R6*: for $\mu\Delta C3$ -M1R6*. (C) Partial digestion patterns of $\mu\Delta C3$ -M1 and $\mu\Delta C3$ -M1R9. The nucleases used are indicated on the top of the lanes. The amount of nuclease added to each reaction mixture (containing 10 μ g RNA in 25 μ l) is as follows: lanes 1 and 12, control (no enzyme); lanes 2 and 13, RNase T1, 0.0025 units; lanes 3 and 14, RNase T1, 0.005 units; lanes 4 and 15, RNase T2, 0.01 units; lanes 5 and 16, RNase T2, 0.02 units; lanes 6 and 17, nuclease S1, 5 units; lanes 7 and 18, nuclease S1, 10 units; lanes 8 and 19, RNase U2, 0.005 units; lanes 9 and 20, RNase U2, 0.01 units; lanes 10 and 21, RNase A, 20 pg; lanes 11 and 22, RNase A, 40 pg. Lanes 1–11 $\mu\Delta C3$ -M1, lanes 12–22, $\mu\Delta C3$ -M1R9. All reactions were carried out at 30°C. The arrows show the location of the predicted stems and the thick bar on the right of the arrows indicates the regions attacked by the nucleases. The numbers on the left side of each panel show the G positions attacked by RNase T1 which were shown schematically in panel A. Their positions were calibrated on the basis of the sequence ladder electrophoresed on the same gel (data not shown).

Figure 3. *In vitro* splicing of wild type and mutant pre-mRNAs. $\mu\Delta C4$ -M1, $\mu\Delta C3$ -M1 (W) and their R9 mutant (M) pre-mRNAs were incubated with a HeLa nuclear extract for the duration indicated on the top of each lane and electrophoresed on a 5% denaturing polyacrylamide gel. Each RNA species which was identified by the size analysis and by the debranching assay is represented schematically on the right side of each panel. The junction point of the spliced product for $\mu\Delta C3$ -M1R9 was analyzed by S1 mapping, and this showed that the splicing of the mutant pre-mRNAs occurred correctly (data not shown). Lanes 1–3, $\mu\Delta C4$ -M1; lanes 4–6, $\mu\Delta C4$ -M1R9; lanes 7–9, $\mu\Delta C3$ -M1; lanes 10–12, $\mu\Delta C3$ -M1R9

They are all mutated on the 3' side of the stem but the consensus sequence at the 3' splice site remains the same (see Figure 2A). Whereas $\mu\Delta C3$ -M1R9 contained a 9-base substitution (Figure 2B, R9), $\mu\Delta C3$ -M1R6 and $\mu\Delta C3$ -M1R3 contain 6- and 3-base substitutions, respectively (Figure 2B, R6 and R3). $\mu\Delta C3$ -M1R6* also contains a 6-base substitution, but the substituted sequence is different from that of $\mu\Delta C3$ -M1R6 (Figure 2B, R6*). Figure 4A shows the time course analyses of the *in vitro* splicing reaction of these $\mu\Delta C3$ -M1 mutant pre-mRNAs. By comparing $\mu\Delta C3$ -M1 (lanes 2–4), $\mu\Delta C3$ -M1R3 (lanes 5–7), and $\mu\Delta C3$ -M1R6 (lanes 8–10), it became clear that as the number of bases involved in base pairing in the stem region decreased, the efficiency of the splicing reaction increased. For instance, at 30 min the amount of spliced product from the $\mu\Delta C3$ -M1R3 and $\mu\Delta C3$ -M1R6 pre-mRNAs was about three times and five times greater, respectively, than that from $\mu\Delta C3$ -M1. This probably reflects the increasing destabilization of the stem-loop structure caused by the mutations. However, the efficiency of the splicing of $\mu\Delta C3$ -M1R9

Figure 4. (A) *In vitro* splicing of $\mu\Delta C3-M1$ mutant pre-mRNAs. $\mu\Delta C3-M1$ wild type and the four mutant pre-mRNAs indicated were incubated with a HeLa nuclear extract for the duration indicated on the top of each lane and electrophoresed on a 6% denaturing gel. Lane 1, the Hpa II digests of pBR322 as size marker; lanes 2–4, $\mu\Delta C3-M1$; lanes 5–7, $\mu\Delta C3-M1R3$; lanes 8–10, $\mu\Delta C3-M1R6$; lanes 11–13, $\mu\Delta C3-M1R9$; lanes 14–16, $\mu\Delta C3-M1R6^*$. (B) *In vitro* splicing of $\mu\Delta C3-M1$ (lanes 1–4) and $\mu\Delta C3-M1R9$ (lanes 5–8) in a mouse lymphoma (M12) nuclear extract. $\mu\Delta C3-M1$ and $\mu\Delta C3-M1R9$ were incubated with an M12 nuclear extract at 30°C for the duration indicated on the top of each lane and electrophoresed on a 6% denaturing gel.

(lanes 11–13) is almost the same as that of $\mu\Delta C3-M1R6$, suggesting that the 6-base substitution causes the maximum alteration of the stem-loop structure. This is quite reasonable, since the 6-base substitution eliminates the 4 G-C pairings which seem to represent the core of the secondary structure.

$\mu\Delta C3-M1R6^*$ was used to examine whether the substituted sequence in $\mu\Delta C3-M1R6$, AACAAA, may play a direct role in increasing splicing efficiency. In $\mu\Delta C3-M1R6^*$, AACAAA of $\mu\Delta C3-M1R6$ was replaced by AUAUCA, which could also destabilize the stem-loop structure. If the secondary structure, but not the RNA sequence, is of primary importance, $\mu\Delta C3-M1R6^*$ will be spliced as efficiently as $\mu\Delta C3-M1R6$. This was shown to be the case (Figure 4A, lanes 14–16), although the splicing efficiency of $\mu\Delta C3-M1R6^*$ was slightly lower than that of $\mu\Delta C3-M1R6$.

In order to examine the effect of the secondary structure in a homologous system, we prepared a nuclear extract from M12 mouse lymphoma cells; this represented the mature B-cell type. Figure 4B shows the time course of the *in vitro* splicing reactions of $\mu\Delta C3-M1$ (lanes 1–3) and of $\mu\Delta C3-M1R9$ (lanes 4–6) with an M12 nuclear extract. As was the case with the HeLa nuclear extract, the mutations in the stem region increased the splicing efficiency of $\mu\Delta C3-M1$ by several folds. Thus, the inhibitory effect of the secondary structure at the 3' splice site of the C4-M1 intron on the *in vitro* splicing reaction was also observed with the homologous system. As far as we know, this is the first example of a naturally-occurring secondary structure affecting the splicing reaction at least *in vitro*, although a similar phenomenon was reported previously by several groups, using transcripts with artificially introduced stem-loop structures (24,25,27–29). We demonstrated that the secondary structure was actually formed in the reaction mixture in which *in vitro* splicing

occured, and that the *in vitro* splicing reaction was enhanced as the secondary structure was destroyed by the mutation. It appears, therefore, that both the 3' and 5' splice sites of the C4-M1 intron are suboptimal for the splicing reaction *in vitro*.

The salient feature of the C4-M1 intron is that it contains key sequences which are involved in differential processing to produce the two isoforms of IgM. We do not know whether the stem-loop structure at the 3' splice site of the C4-M1 intron is indeed formed in cells and inhibits the splicing reaction of the intron *in vivo*. In this connection, it has been shown that a stem-loop structure sequestering the splice site can affect splice site selection *in vivo* (24,25,27–29). Undoubtedly, *in vivo* experiments are required to clarify whether the potential stem-loop structure at the 3' splice site of the C4-M1 intron may have any relevance to the alternative processing of the μ gene transcript. We are in the process of analyzing transient expression of the μ gene carrying the mutations in the stem region in mouse mature B and plasma cells.

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