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Repositioning of an alternative exon sequence of mouse IgM pre-mRNA activates splicing of the preceding intron

Akiya Watakabe, Hiroshi Sakamoto, and Yoshiro Shimura

Department of Biophysics, Faculty of Science, Kyoto University, Kyoto, Japan

Using a transient expression system of mouse IgM mini-gene constructs in mouse B-cell lines and in fibroblast L cell, we investigated splicing of the IgM transcript. We observed that the efficiency of splicing between exons C4 and M1 (C4-to-M1 splicing), the splicing reaction leading to the production of membrane-bound form (μm) mRNA, was drastically affected by mutations in a specific portion of the downstream exon (M2). The results show that the specific exon M2 sequence activates the C4-to-M1 splicing. This activation was not observed when splicing between exons M1 and M2 was abolished by base substitutions at the splice sites. These results indicate that positioning of the downstream exon is crucial for efficient splicing of the preceding intron.

Splicing of mRNA precursors (pre-mRNAs) in eukaryotes is a process that requires remarkable fidelity in two aspects (for reviews see Green, 1986; Krainer and Maniatis, 1988; Padgett et al., 1986). First, the 5′ and 3′ splice sites must be accurately recognized and ligated without an error of a single nucleotide. Second, for pre-mRNAs containing multiple introns, the cognate 5′ and 3′ splice sites to be ligated should be correctly selected from multiple splice sites. In general, the closest pairs of the 5′ and 3′ splice sites are selected, so that all exons are incorporated in order in mature mRNAs. In the case of alternative splicing, however, non-adjacent splice sites are optionally utilized, and mRNAs containing different sets of exons are produced from a single primary transcript (for reviews see Breitbart et al., 1987; Leff et al., 1986; Smith et al., 1989). Such alternative use of exons generates protein isoform diversity. Moreover, developmental regulation of some genes is achieved by tissue- or developmental stage-specific splicing. Thus, the analysis of the molecular basis of alternative splicing not only elucidates the basic mechanism of splice site selection but also contributes to our understanding of the control mechanism of gene expression.

Previous studies have revealed that the consensus sequences located at both ends of an intron play an important role in splicing (Green, 1986; Krainer and Maniatis, 1988; Padgett et al., 1986). Multiple factors including U snRNPs recognize these signal sequences and assemble to form a large complex designated the spliceosome (Brody and Abelson, 1985; Frendewey and Keller, 1985; Grabowski et al., 1985). Mutational analyses suggest that the affinity of such general splicing factors to the consensus sequences is important for the splice site selection (Eperon et al., 1986; Fu et al., 1988; Lowery and Van Ness, 1988; Peterson and Perry, 1989). Some alternative splice sites contain

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Correspondence: Yoshiro Shimura, Department of Biophysics, Faculty of Science, Kyoto University, Kyoto 606, Japan Tel 81-075-753-4230 Fax 81-75-753-4235

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"weak" consensus sequences, and mutations in such sequence elements change the mode of the pre-mRNA splicing reactions (Lowery and Van Ness, 1988; Peterson and Perry, 1989). Besides the consensus sequences, various cis elements and trans-acting factors are known to affect the splice site selection. As the cis-acting elements, portions of exon (Furdon and Kole, 1988; Hapson et al., 1989; Helfman et al., 1988; Mardon et al., 1987; Nagoshi and Baker, 1990; Reed and Maniatis, 1986; Streuli and Saito, 1989) and intron sequences (Emeson et al., 1989; Helfman et al., 1990) as well as secondary structure of pre-mRNAs (Clouet d'Orval et al., 1991; Eperon et al., 1988; Libri et al., 1991; Solnick, 1985; Watakabe et al., 1989) are known to affect the splice site selection. In the case of tissue- or developmental stage-specific alternative splicing, trans-acting factors have been postulated to control the splicing reaction (Breitbart et al., 1987; Leff et al., 1986; Smith et al., 1989). In several cases, the presence of specific regulatory factors has been suggested (Bell et al., 1988; Burtis and Baker, 1989; Inoue et al., 1990; Zachar et al., 1987). In addition to such regulatory factors, it has been suggested that cell-specific differences in the activities of general splicing factors may control alternative splicing (Maniatis, 1991).

With regard to alternative RNA processing, the IgM gene system is one of the best studied systems in which regulated use of alternative exons plays an important role in gene expression (Alt et al., 1980; Early et al., 1980). The mRNAs for two isoforms of IgM, the secreted form (μs) and the membrane-bound form (μm), are produced by differential processing of the IgM transcript. If splicing occurs between C4 and M1 exons, the upstream μs poly(A) site located within the C4-M1 intron is eliminated, and the downstream μm poly(A) site is used to produce μm mRNA. If, on the other hand, the μ pre-mRNA is polyadenylated at the μs poly(A) site, μs mRNA is produced. It is known that the relative amount of these two forms of mRNAs is regulated during B-cell differentiation: in the early stage, both the μm and μs mRNAs are produced, whereas μs mRNA is predominantly produced in the differentiated plasma cells. Although the mechanism for such developmental regulation is not well understood, it has been suggested that a balance between the polyadenylation reaction at the μs site and the C4-to-M1 splicing reaction is important for the proper regulation (Peterson and Perry, 1989; Tsurushita et al., 1987). Unexpectedly, such subtle balance was found to be disturbed by deletions around the 3′ splice site of downstream exon M2: μm mRNA production was drastically reduced by such deletions (K. Tomizuka and N. Tsurushita, personal communication). This finding prompted us to examine the role of the two μm-specific exons, M1 and M2, in the C4-to-M1 splicing. In transient expression experiments using two mouse B-cell lines and fibroblast L cell, we found that efficient splicing between exons C4 and M1 depended on the presence of a portion of exon M2, irrespective of the cell type used for transfection. However, such efficient C4-to-M1 splicing was not observed when splicing between exons M1 and M2 (M1-to-M2 splicing) was abolished by base substitutions. These results suggest that the exon M2 sequence may activate the C4-to-M1 splicing if positioned downstream of exon M1, and that this activation is not directly related to the developmentally regulated differential processing of IgM pre-mRNA.

Materials and methods

Plasmid construction

Mouse μ gene fragments were obtained from plasmids pMoμ and pMoμΔA, kindly provided by Dr. Naoya Tsurushita (Tsurushita et al., 1987); see Figure 1A. To construct pμW, several cloning steps were performed. First, pMoμ was digested with Spe I, treated with DNA polymerase I large fragment, and ligated with Sal I linker (GGTCGACC). This pMoμ derivative was designated pMoμ2. Next, the 0.9-kb Acc I-Kpn I fragment was deleted from C4-M1 intron of this plasmid and ligated with Cla I linker (CATCGATG). This construct was designated pMoμΔAK. The 3.5-kb Xho I fragment from pμ2, a gift from Dr. Naoya Tsurushita (Tsurushita and Korn, 1987), which contains the replication origin and the T antigen gene of polyomavirus, was inserted into pMoμΔAK to construct pμW. We constructed (μs⁻)/W in a similar manner, except that pMoμΔA (Tsurushita et al., 1987) was used as the starting plasmid.

The deletion mutants of exon M2 were constructed by subcloning appropriate portions of the mutant μ gene plasmids constructed by K. Tomizuka and N. Tsurushita (construction of

these original plasmids will be described elsewhere) either into pμW or (μs⁻)/W. The structures of the resulting plasmids—ΔA, ΔB, and ΔC, or (μs⁻)/ΔA, (μs⁻)/ΔB, and (μs⁻)/ΔC—are as described in the legend to Figure 2.

To construct M1-M2 splicing mutants MD and ML, the 0.9-kb Kpn I-Sal I fragment of pMoμ2 was subcloned into M13mp19, and Cla I linker was inserted into the Kpn I site of this recombinant phage DNA, which was designated M13CS. Oligonucleotide-directed mutagenesis was carried out using M13CS (Kunkell, 1985). The 19-bp oligonucleotides 5′-AACCATACTTCCTTGAACA-3′ (for the 5′ splice site) and 5′-TCATTTCACGTG-CAGGTGA-3′ (for the 3′ splice site) were used for MD, and the 40-bp oligonucleotide 5′-CAT-GCTGAGAGTCATTTCACCTTGAACAGGGTG-ACGGTGG was used for ML. The mutations were confirmed by sequencing and then cloned into the Cla I-Sal I cut of pμW.

Cells and DNA transfection

Mouse lymphoma M12.4.1 (M12) and myeloma P3X63Ag8U1 (P3U1) were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum and 50 μM 2-mercaptoethanol. Mouse fibroblast cell lines L(tk⁻) were maintained in HAM medium supplemented with 10% fetal calf serum. For mouse B cell lines, the DEAE-dextran transfection method (Tsurushita et al., 1987) was used for transient expression experiments. For L cells, the $CaPO_4$ method was used (Inoue et al., 1990).

RNA extraction and S1 nuclease analysis

Total cellular RNA was extracted by the hot phenol method described previously (Tsurushita et al., 1987). For S1 nuclease analysis, the 791-bp Pst I-Pvu II fragment containing 610-bp of the μ gene sequence plus 181-bp of the vector sequence (pUC119) was used for the probe (Fig. 1B). The probe was 3′ end labeled with T4 DNA polymerase and [α-^{32}P]dCTP. Total cellular RNA was hybridized with the probe at 51°C overnight in 80% formamide. S1 nuclease digestion was performed at 25°C for 60 minutes. The digests were analyzed on a 6% polyacrylamide gel containing 8M urea. For electrophoresis, one-third of the S1 nuclease digests was used on the first run. After quantification, the remainder of the reaction products was electrophoresed so that all lanes contained approximately equal amounts of μs mRNA. For the experiments shown in Figures 3 and 5, 5μg (M12) or 20μg (P3U1 and L cell) of total cellular RNAs were used for the S1-nuclease analysis, and all the reaction mixtures were electrophoresed.

Results

Deletions in the acceptor site region of exon M2 lead to reduction of μm mRNA level

For transient expression experiments, we used a hybrid μ gene, pμW, as a basic construct, in which the μ gene constant region (C2-M2) is under the control of Moloney murine sarcoma virus LTR promoter (Fig. 1A). A portion of the intron sequence between C4 and M1 exons is deleted in this construct. Although deletion in the C4-M1 intron changes the ratio of μm mRNA relative to μs mRNA (μm/μs ratio), it does not abolish the regulation (Peterson and Perry, 1986; Tsurushita and Korn, 1987). The pμW plasmid also contains the polyoma virus sequence, which allows the plasmid to replicate in mouse cells (Tsurushita and Korn, 1987). The RNA expressed from this plasmid was analyzed by S1 nuclease analysis using a probe, shown in Figure 1B. This probe distinguishes mRNA that has undergone the C4-to-M1 splicing (μm mRNA) from mRNA that has been cleaved at the upstream μs poly(A) site (μs mRNA). Two mouse B-cell lines, the M12.4.1 lymphoma (M12) and the P3X63Ag8U1 myeloma (P3U1), were used as recipients in transfection experiments; the former represents an early-stage B-cell, and the latter represents a terminally differentiated plasma cell. Since neither cell line produces endogenous μ mRNAs, expression of μ mRNAs transcribed from transfected mini-gene constructs was readily detectable. The developmental difference between the two cell lines was clearly observed in this assay system, the μm/μs ratio being higher in M12 (Fig. 2B, M12 and P3U1, lanes 1 and 5). These results are consistent with the previous reports that the region upstream of exon C2 is not required for the regulated processing of μ pre-mRNA (Peterson and Perry, 1989; Tsurushita et al., 1987).

To examine sequences that might be involved in the differential processing, portions of exon M2 and the adjacent intron sequence were deleted from pμW plasmid (Fig. 2A, ΔA). As shown in Figure 2B, the production of μm mRNA was drastically reduced when ΔA plasmid was trans-

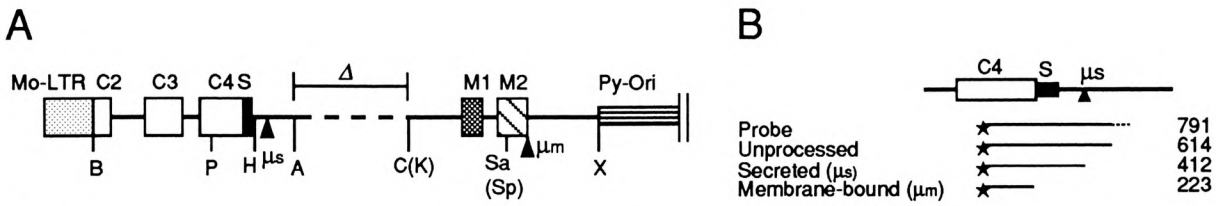

Figure 1. Schematic representation of the hybrid μ gene construct pμW and the probe used for S1 nuclease analysis. **A.** The hybrid Mo-LTR-μ gene construct, pμW. Solid lines represent introns. Open, filled, and hatched boxes represent exons. The dotted box represents the Moloney murine sarcoma virus genome fragment containing the LTR promoter (Mo-LTR). Filled triangles indicate polyadenylation sites: μs, secreted form polyadenylation site; μm, membrane-bound form polyadenylation site. Δ denotes the portion of the C4-M1 intron deleted in pμW; A, Acc I site; B, BamH I; C(K), Cla I linker inserted into the original Kpn I site; P, Pst I; Sa(Sp), Sal I linker inserted into the original Spe I site; X, Xho I. **B.** Diagram of the probe used for S1 nuclease analysis. The sizes (in nucleotides) of the full-length probe and protected fragments are indicated.

fected into M12. The μm/μs ratio decreased approximately 6-fold compared with the ratio obtained with pμW (lanes 1 and 2). As ΔA plasmid retains the 5′ portion of the M1-M2 intron, two other deletion mutants, ΔB and ΔC, were examined for their expression (Fig. 2A). Deletions extend to 3-bp and 36-bp upstream from the 3′ end of exon M1 in ΔB and in ΔC, respectively, resulting in removal of the entire M1-M2 intron sequence. When these mutant μ gene constructs were transfected into M12, the μm/μs ratios were lower than that with ΔA (Fig. 2B, lanes 3 and 4). Similar results were obtained with P3U1 (Fig. 2B, lanes 5–8). The results obtained with ΔB and ΔC plasmids exclude the possibility that the presence of the intron sequence causes the decrease in the relative μm mRNA level. Since the probe used for S1 mapping covers only the

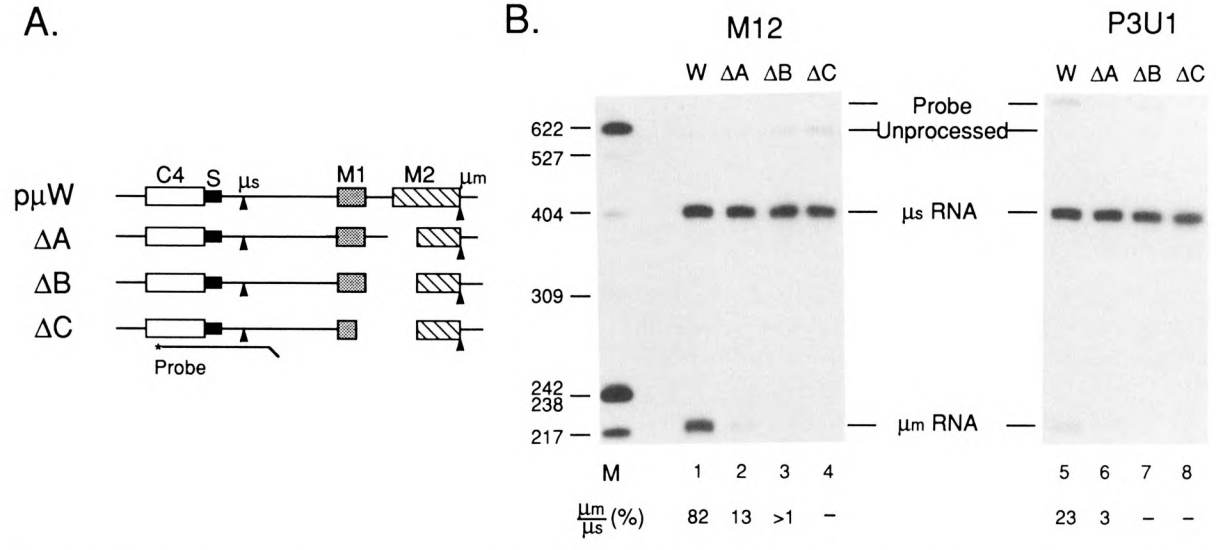

Figure 2. Structures and expression of μ gene deletion mutant plasmids. **A.** The 3′ terminal structures of the deletion mutant plasmids, ΔA, ΔB, and ΔC. The symbols are the same as in Figure 1. The stepwise deletions start from the Sal I site in exon M2 in the 3′-to-5′ direction. In ΔA, the deletion removes 166-bp of exon M2 and 29-bp of the 3′ end of M1-M2 intron. In ΔB, the deletion removes 166-bp of exon M2 and the entire M1-M2 intron sequences, as well as 3-bp of exon M1. In ΔC, 36-bp of the 3′ end of exon M1 is deleted, along with the M1-M2 intron and exon M2 sequences. **B.** S1 nuclease analysis of RNA from M12 and P3U1 transfected with the plasmids indicated on the top of each lane. Lane M shows ^{32}P-labeled pBR322 DNA digested with Hpa II used as size markers. The probe used is the same as in Figure 1B. The positions of the bands corresponding to the probe, unprocessed, μs, and μm mRNAs are indicated. The gel pieces corresponding to these RNAs were cut out, and their radioactivity was measured by Cerenkov counting. The radioactivity of the bands corresponding to μm mRNA relative to that of μs mRNA is indicated under each lane (μm/μs).

5′ splice site region of the C4-M1 intron, we analyzed splicing in the acceptor site region of this intron in more detail. Both S1 mapping using different probes and PCR amplification of μ cDNAs showed that splicing occurs accurately between exons C4 and M1, and between exons M1 and M2 (data not shown). We also found that the efficiency of the C4-to-M1 splicing was reduced by the deletions in the acceptor site region of exon M2, even though there was no deletion in the C4-M1 intron (data not shown).

We could think of four possible mechanisms to explain the effect of the exon M2 deletions. (1) The deletions activate polyadenylation at the μs site and increase the level of μs mRNA with concomitant decrease of μm mRNA. (2) The deletions reduce the efficiency of the C4-to-M1 splicing, leading to decrease of μm mRNA production. (3) The deletions inhibit polyadenylation at the μm site, and the level of μm mRNA decreases due to either instability of μm mRNA precursor or defective transport into cytoplasm. (4) μm mRNAs from mutant plasmids are unstable due to some conformational change caused by the deletions.

Deletions in the exon M2 acceptor site region reduce the efficiency of C4-to-M1 splicing

To distinguish the possibilities described above, we constructed double deletion mutants in which both the upstream μs poly(A) site and the sequences around the exon M2 acceptor site were deleted (see Fig. 3A: (μs$^-$)/W, (μs$^-$)/ΔA, and (μs$^-$)/ΔC are derived from pμW, ΔA, and ΔC, respectively). Because these mutant μ gene constructs lack the μs poly(A) site, all of the transcripts expressed from these constructs are polyadenylated at the μm poly(A) site. Thus, two RNA species are expected to be produced: the RNA molecule produced by splicing between exons C4 and M1 (spliced RNA), and the RNA molecule which is not spliced between these exons (unspliced RNA). Both of these RNA species contain the μm-specific exons M1 and M2. We examined the ratios and the total amounts of these two RNA species (spliced vs unspliced). If deletions in the exon M2 acceptor site region affect the polyadenylation at the μs site, neither the ratio of the two RNA species nor the total amount of μ mRNAs would be affected significantly. If the deletions affect the C4-to-M1 splicing, the total amount of μ mRNAs would not be affected, but the amount of the spliced RNA relative to the unspliced RNA is expected to be reduced. On the other hand, if the deletions inhibit μm polyadenylation, or affect the stability of μm RNA, the total amount of μ mRNAs expressed from the double deletion mutants would be reduced, but there would be no change in the relative amount of the spliced RNA. We transfected these mutant constructs into either M12 or P3U1 cells, and analyzed by S1 nuclease mapping with the same probe as in Figure 2. By using this probe, we could distinguish the RNA spliced between exons C4 and M1 from the unspliced RNA (Fig. 3B).

As consistent with the previous reports (Galli et al., 1987; Peterson and Perry, 1986; Tsurushita et al., 1987), the pre-mRNAs from the (μs$^-$)/W which lacks the μs poly(A) site were efficiently spliced between exons C4 and M1 in both M12 and P3U1 cells (Fig. 3C, lanes 1 and 4). By contrast, the RNA spliced between exons C4 and M1 decreased drastically, with concomitant increase of the unspliced RNA when either (μs$^-$)/ΔA or (μs$^-$)/ΔC was transfected (Fig. 3C, lanes 2, 3, 5, and 6): in the case of (μs$^-$)/ΔA, the ratio of the spliced vs unspliced RNAs was approximately 7.5% in M12 and 7.1% in P3U1 (Fig. 3C, lanes 2 and 5), and in the case of (μs$^-$)/ΔC, spliced RNA was not detectable in either cell (Fig. 3C, lanes 3 and 6). Thus, the decrease of the relative amounts of the spliced RNA with (μs$^-$)/ΔA, and (μs$^-$)/ΔC correlated closely with the decrease of the μm/μs ratios with ΔA, and ΔC (Fig. 2B), respectively. These results indicate that decrease of the μm/μs ratio caused by the exon M2 deletions (ΔA and ΔC) is ascribed to the decrease of the efficiency of C4-to-M1 splicing.

Moreover, we observed that the total amount of μ mRNAs expressed from either (μs$^-$)/ΔA or (μs$^-$)/ΔC was approximately equivalent to that from (μs$^-$)/W (Fig. 3C). Further analyses showed that most of the μ transcripts including the unspliced RNA exist in the cytoplasm (data not shown), indicating that the RNA which was not spliced between exons C4 and M1 was efficiently transported into and stably present in the cytoplasm. We also found that cleavage/polyadenylation occurred efficiently at the μm poly(A) site of double deletion mutants, (μs$^-$)/ΔA and (μs$^-$)/ΔC, as judged by RNase protection assay using a probe that covers this site (data not shown). These results exclude the pos-

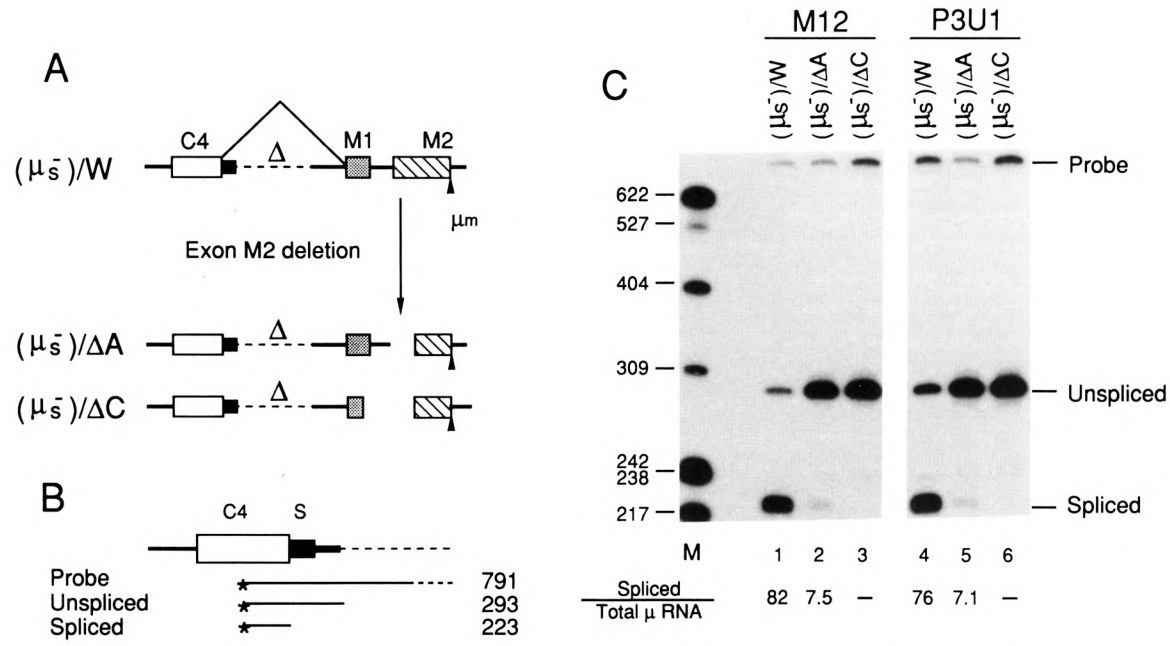

Figure 3. Structure and expression of double deletion mutants of the μ gene plasmid. **A.** The 3′ terminal structures of the deletion mutants, (μs⁻)/W, (μs⁻)/ΔA, and (μs⁻)/ΔC. A portion of C4-M1 intron containing the μs polyadenylation site is deleted in these constructs (denoted by Δ and break lines). The deletions in the M1-M2 region of (μs⁻)/ΔA and (μs⁻)/ΔC are the same as those of ΔA and ΔC in Figure 2. **B.** The diagram of the S1 probe and the protected fragments. **C.** S1 nuclease analysis of RNA from M12 and P3U1 transfected with the plasmids indicated on the top of each lane. Lane M is the same as that in Figure 2. The positions of the bands corresponding to the probe, the μ RNA which was not spliced between exons C4 and M1 (Unspliced), and the μ RNA which was spliced (Spliced) are indicated. The gel pieces corresponding to these RNAs were cut out, and their radioactivity was measured by Cerenkov counting. The radioactivity of the bands corresponding to the spliced RNA relative to the total μ RNA is indicated under each lane.

sibility that the deletions around exon M2 affect μm polyadenylation and/or the stability of μm mRNA significantly.

Exon M2 sequence enhances the C4-to-M1 splicing when joined to exon M1

The results described above suggest that the deleted region in ΔA (29-bp of the 3′ half of the M1-M2 intron and 166-bp of the 5′ half of exon M2) may contain essential sequences that activate the C4-to-M1 splicing. To examine whether splicing between exons M1 and M2 (M1-to-M2 splicing) plays a role in activating the C4-to-M1 splicing, we constructed a mutant μ gene construct having base substitutions at both the splice sites of M1-M2 intron (Fig. 4A, MD). These base substitutions abolished the M1-to-M2 splicing (data not shown). When MD was transfected into M12, the μm/μs ratio decreased approximately 5-fold, as compared to that with pμW (Fig. 4B, W and MD). These results suggest that the M1-to-M2 splicing is important for the C4-to-M1 splicing to occur efficiently.

We further examined the role of the M1-to-M2 splicing by constructing another mutant μ gene plasmid, ML, in which the M1-M2 intron sequence is totally deleted (Fig. 4A). When ML was transfected into M12 cells, μm mRNA was efficiently produced, although the μm/μs ratio was slightly lower compared to that with pμW (Fig. 4B, W and ML). This shows that the M1-M2 intron sequence itself is not necessary for activation of the C4-to-M1 splicing, and that the presence of the 5′ portion of exon M2 is important. Because such effect of exon M2 was not observed if the M1-to-M2 splicing was abolished by the base substitutions in the splice site regions, we conclude that exon M2 activates the C4-to-M1 splicing only when it is ligated to exon M1. Similar results were obtained when MD and ML were transfected into P3U1 (data not shown).

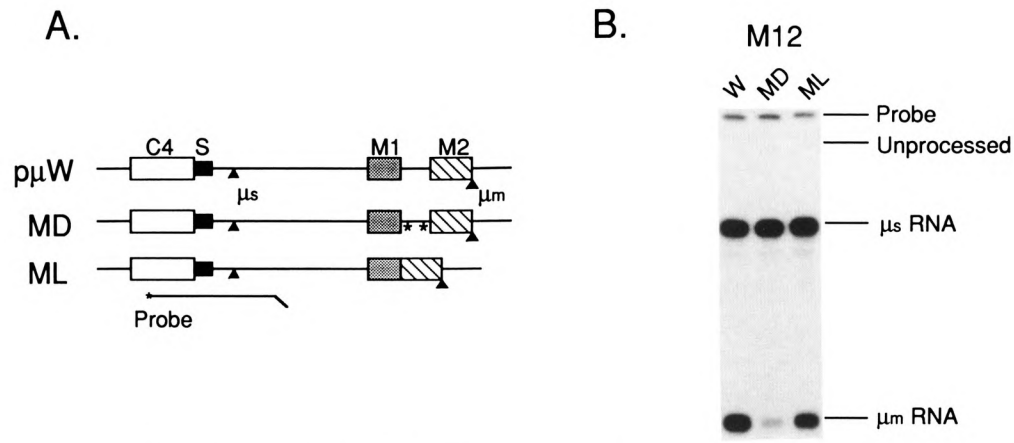

Figure 4. Structure and expression of μ gene mutant plasmids. **A.** The 3′ terminal structures of the mutant plasmids, MD and ML. MD contains base substitutions at both 5′ and 3′ splice sites (*): /GT to/GA, at the 5′ splice site; AG to AC/, at the 3′ splice site. In ML, M1-M2 intron is deleted. **B.** S1 nuclease analysis of RNA from M12 transfected with the plasmids indicated on the top of each lane. The positions of the bands corresponding to the probe, unprocessed, μs, and μm mRNAs are indicated.

Effect of mutations in exon M2 in non-lymphoid cells

Finally, mutant μ gene constructs were transfected into mouse fibroblast L cells to examine whether mutations in exon M2 have similar effects on the C4-to-M1 splicing in non-lymphoid cells. When the wild-type construct pμW was transfected, both μs and μm mRNAs were produced, although μs mRNA was predominant (Fig. 5B, lane 1). However, if a 5′ portion of exon M2 is deleted (ΔB and ΔC; lanes 2 and 3), or if base substitutions occurred at the splice sites of the M1-M2 intron (MD; lane 4), the μm/μs ratio dropped sharply. These results suggest that activation of the C4-to-M1 splicing by the exon M2 sequence may occur irrespective of cell types.

Discussion

We showed that deletions of a portion of exon M2 immediately downstream of the 3′ splice

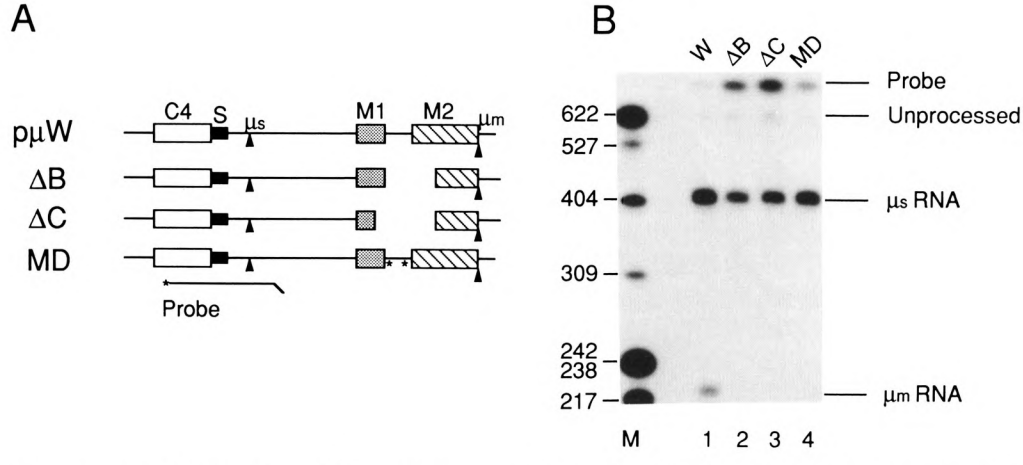

Figure 5. Structures and expression of the mutant μ genes in L cells. **A.** The 3′ terminal structures of the mutant μ genes, ΔB, ΔC, and MD. **B.** S1 nuclease analysis of RNA from mouse fibroblast L cells transfected with the plasmids indicated on the top of each lane. The positions of the bands corresponding to the probe, unprocessed, μs, and μm mRNAs are indicated.

site drastically reduce the μm/μs ratio. When the μs poly(A) site was deleted from the M2 deletion constructs, most of the pre-mRNAs remained unspliced between exons C4 and M1, but the total level of μ mRNAs did not change significantly. This indicates that the reduction of the μm/μs ratio is due to the decrease of efficiency of the C4-to-M1 splicing. We also examined the effect of the M1-to-M2 splicing on activation of the C4-to-M1 splicing. The results show that activation of the C4-to-M1 splicing can be observed if exon M2 sequence is ligated to exon M1. Such activation was not observed when the M1-to-M2 splicing was abolished by base substitutions, suggesting that repositioning of exon M2 is important in activating the C4-to-M1 splicing. These results were obtained with fibroblast L cell as well as with two B-cell lines (lymphoma and myeloma), suggesting that the phenomenon we observed is not directly related to the differential processing of μ pre-mRNAs.

As to the regulatory mechanism involved in differential processing of μ pre-mRNAs, two models have been proposed. One is the "poly(A) site choice model," which proposes that competition for polyadenylation at the μm and μs poly(A) sites determines the form of μ mRNAs (Galli et al., 1988; Galli et al., 1987). The other is the "μs site-splice model" (Peterson and Perry, 1989; Tsurushita et al., 1987), which claims that polyadenylation at the μs site competes with the C4-to-M1 splicing rather than with polyadenylation at the μm site. It remains to be clarified which of these models is more likely. It has been argued that the latter model could not explain the observation that insertion of heterologous sequences into the M1-M2 intron decreased the μm/μs ratio (Galli et al., 1988). In this connection, our results suggest that the C4-to-M1 splicing may well be affected by such mutations because the efficiency of the C4-to-M1 splicing seems to be dependent on the downstream M1-to-M2 splicing. Thus, the effect of heterologous sequences inserted into the M1-M2 intron could be explained in the context of the μs site-splice model. Moreover, correlation between the decrease of the μm/μs ratio and the decrease of the efficiency of the C4-to-M1 splicing would support the latter model. Therefore, our results strongly favor the μs site-splice model.

The most remarkable finding in the present study is that the downstream exon sequence (exon M2) activates the splicing of the intron which is not immediately upstream but one further upstream (C4-to-M1 splicing). It is curious how a sequence in a downstream exon affects the splicing of an intron not immediately upstream. Our data suggest that the 5′ portion of exon M2 affects the C4-to-M1 splicing if positioned downstream of exon M1. In several other cases, a specific portion of an exon is known to affect the splicing of an intron immediately upstream (Furdon and Kole, 1988; Hapmson et al., 1989; Mardon et al., 1987; Nagoshi and Baker, 1990; Reed and Maniatis, 1986; Streuli and Saito, 1989). In the case of the human LCA gene (Streuli and Saito, 1989) and the Drosophila doublesex gene (Hoshijima et al., 1991; Nagoshi and Baker, 1990), tissue- and sex-specific factors that act on specific exon sequences may regulate splice site selection, respectively. In our case, such cell-specific regulatory factors do not seem to be involved, because deletions of exon M2 affected the μm/μs ratio even in mouse fibroblast L cells. It is possible, however, that interaction of one of the general splicing factors with exon M2 may enhance the C4-to-M1 splicing.

Recent in vitro experiments suggest that binding of splicing factors to the downstream 5′ splice site enhances the spliceosome assembly at the 3′ splice site of the preceding intron (Robberson et al., 1990; Talerico and Berget, 1990). Based on these observations, Berget and co-workers proposed the exon definition model (Robberson et al., 1990; Talerico and Berget, 1990), in which exons are recognized as units by the interaction of factors bound at the 3′ splice site (5′ end of exon) and also at the downstream 5′ splice site (3′ end of exon). The role of downstream 5′ splice site was also demonstrated in the in vitro splicing system using preprotachykinin pre-mRNA (Kuo et al., 1991; Nasim et al., 1990). Splicing between the common exon E3 and the optional exon E4 of preprotachykinin pre-mRNA, it has been suggested, is activated by the splicing between exon E4 and exon E5 and by the 5′ splice site sequence immediately downstream of exon E5. In this mechanism, the key event seems to be replacement of the "relatively poor" downstream 5′ splice site of exon E4 by the efficient 5′ splice site of exon E5. It has been suggested that increased binding of U1 snRNP

to the 5′ splice site immediately downstream of the E4 exon might be responsible for promoting the E3-to-E4 splicing. In the case of μ pre-mRNA splicing, our data suggest that the C4-to-M1 splicing is activated by the downstream splicing event. Although the sequence important for the activation is not the 5′ splice site sequence but a portion of a downstream exon in this case, the activation of the C4-to-M1 splicing by the exon M2 sequence could occur by a similar mechanism.

Based on the model described above, the C4-to-M1 splicing would be inefficient in the absence of exon M2 due to the weak definition at the downstream 5′ splice site of exon M1. When exon M2 is joined to exon M1, the downstream side of exon M1 would be defined more efficiently, thereby resulting in enhancement of the spliceosome assembly at the 3′ splice site of exon M1. We have shown that the efficiency of the C4-to-M1 splicing was reduced further when the deletions from within exon M2 extended to the 3′ end of exon M1 (Fig. 2B, lanes 4, 5, 8, and 9). This implies that the downstream 5′ splice site of exon M1 might be required—at least in part—for the upstream splicing event. Moreover, when the 5′ splice site sequence of exon M1 (G/GTAGTA) was mutated to an exact consensus match (G/GTAAGTA) by one base insertion, the C4-to-M1 splicing occurred efficiently in the absence of exon M2. Conversely, when the 5′ splice site of exon M1 was downmutated, free of other changes, splicing occurred exclusively between exons C4 and M2, skipping exon M1 (data not shown). These results suggest that the downstream 5′ splice site of exon M1 plays an important role in the C4-to-M1 splicing, consistent with the exon-definition model.

Alternatively, exon M2 sequence might influence the splicing efficiency by changing the secondary structure of pre-mRNAs. Previous studies demonstrated that secondary structures of pre-mRNAs could potentially affect the efficiency of splicing (Eperon et al., 1988; Solnick, 1985; Solnick and Lee, 1987). We have shown that a stem-loop structure exists at the 3′ splice site region of the C4-M1 intron, which inhibits in vitro splicing of the intron by sequestering the 3′ splice site (Watakabe et al., 1989). This structure might inhibit splicing even in vivo, and the repositioning of the exon M2 sequence could alter the secondary structure of μ pre-mRNA, unfolding the stem-loop structure so that the 3′ splice site of the C4-M1 intron can be utilized efficiently.

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