A secondary structure at the 3' splice site affects the \textit{in vitro} splicing reaction of mouse immunoglobulin $\mu$ chain pre-mRNAs

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Received June 23, 1989; Revised and Accepted September 15, 1989

ABSTRACT
The expression of the IgM (immunoglobulin $\mu$) 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 \textit{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 ($\mu$) 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
Nucleic Acids Research

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 pMOμΔA (kindly provided by Dr. N. Tsurushita; 11, see Figure 1A). The 350 bp Ban1-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 Smal-PstI site of pSP65 vector, which gave pμC3-C4, the template plasmid for μC3-C4 pre-mRNA. Two fragments, the 252 bp Hinfl fragment containing exon M1 (115 bp) with some additional intron sequences at both ends and the 160 bp HpaII-ClaI 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 pμΔC4-M1. The Clal site was regenerated between these two fragments. The EcoRI-ClaI fragment of pμΔC4-M1 was excised and the 310 bp EcoRI-NlaIV fragment of pμC3-C4 was inserted instead, which gave pμΔC3-M1. The NlaIV-PstI fragment containing the 3’ splice site was excised from pμC3-C4 and the 252 bp Hinfl fragment was inserted, which gave pμC4-C4. The template plasmids were linearized with HinflIII 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 pμΔC3-M1 or pμΔC4-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’-CCTCCTCAGCATTCTTTTGTCTATGAAGGGACACA) for μΔC3-M1R9 and μΔC4-M1R9, 36 mer (5’-CCTCCTCAGCATTCTTTTCTCTCTATGAAGGGAC) for μΔC3-M1R6, 33 mer (5’-CCTCCTCAGCATTCTTTTCTCTCTATGAAGGG) for μΔC3-M1R3, 36 mer (5’-CCTCCTCAGCATTCTTTTCTCTCTATGAAGGG) for μΔC3-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 μM 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 μl 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 μg/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 [32P]pCp and T4 RNA ligase (22) and were gel-purified. Nuclease digestions were carried out as follows. Each reaction mixture contained 10 μg of carrier yeast tRNA in 25 μl 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 μl of 2 × PK buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 2% SDS), 5 μl of 20 mg/ml protease K and water to give a final volume of 200 μl. 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 μC3-C4 pre-mRNA, spanning parts of exon C3 and exon C4 with the intron between them, was synthesized. The μΔC4-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 μC3-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 μΔC4-M1
pre-mRNA occurred very poorly; the splicing efficiency was reduced more than 10-folds compared to that of the \( \mu \)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 \( \mu \)AC4-M1 pre-mRNA, we constructed templates for two model pre-mRNAs, \( \mu \)C4-C4 and \( \mu \)AC3-M1. As shown in Figure 1B, either the 5′ (for \( \mu \)C4-C4) or the 3′ (for \( \mu \)AC3-M1) splice site of the \( \mu \)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 \( \mu \)C4-C4 and \( \mu \)AC3-M1 pre-mRNAs were spliced less efficiently than the \( \mu \)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 \( \mu \)AC4-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 is suboptimal with regard to splicing efficiency (10). This is consistent with our \textit{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 \textit{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.

\textbf{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, \( \mu \)AC4-M1R9 and \( \mu \)AC3-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 CACAAACAAA, so that the potential stem structure is largely distorted. To demonstrate that the stem structure actually exists in \( \mu \)AC3-M1 but not in \( \mu \)AC3-M1R9, these transcripts were partially digested with various RNases after labeling at their 3′ ends. We used five kinds of nuclease 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

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure1}
\caption{(A) Partial map of the mouse \( \mu \) gene recombinant plasmid pM0\( \mu \)A (10). The location of the deleted \textit{HaeII-KpnI} fragment containing the secreted-type polyadenylation site is shown. Open, hatched and filled boxes represent \( \mu \) chain exon sequences. Coding regions are enlarged. C3, C4: constant regions (open box); S: 3′ exon for the secreted \( \mu \) mRNA (hatched); M1 and M2: 3′ exons for the membrane-bound \( \mu \) mRNA (filled); As, Am with arrowheads: polyadenylation sites for the secreted and the membrane-bound type \( \mu \) mRNAs, respectively. Restriction sites used are as follows: B, \textit{BanI}; N, \textit{NlaIV}; P, \textit{PstI}; H, \textit{HpaII}; C, \textit{ClaI}; Ha, \textit{HaeII}; K, \textit{KpnI}; Hf, \textit{HindIII}. (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 \( \mu \) mRNA exons. The plasmids used for the \textit{in vitro} synthesis of these pre-mRNAs were constructed as described in MATERIALS AND METHODS. (C) \textit{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, \( \mu \)C3-C4; lanes 4–6, \( \mu \)AC4-M1; lanes 7–9, \( \mu \)C4-C4; lanes 10–12, \( \mu \)AC3-M1. The gel pieces corresponding to these RNAs were cut out and their radioactivities were measured by Cerenkov counting.}
\end{figure}
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 $\mu$AC3-M1 and $\mu$AC3-M1R9. The digestion patterns of $\mu$AC3-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 $\mu$AC3-M1R9 indicates that the secondary structure was substantially altered by the substitutions in the stem. The 3' side of the stem protected in $\mu$AC3-M1 was attacked by all five of the nucleases in $\mu$AC3-M1R9. The substituted sequence CACAAACAAA was not attacked by RNase T1, due to the base specificity of this nuclease. However, G-1 of $\mu$AC3-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 $\mu$AC3-M1, was still protected in $\mu$AC3-M1R9. It remains to be elucidated why this region of $\mu$AC3-M1R9 is resistant to the nucleases. A plausible explanation would be that the pyrimidine cluster region may form a different secondary structure with other parts 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, $\mu$AC4-M1R9 and $\mu$AC3-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 $\mu$AC3-M1 mutant pre-mRNAs.
**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 SI 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 μΔC3-M1 mutant pre-mRNAs. μΔC3-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, μΔC3-M1; lanes 5–7, μΔC3-M1R3; lanes 8–10, μΔC3-M1R6; lanes 11–13, μΔC3-M1R9; lanes 14–16, μΔC3-M1R6*. (B) In vitro splicing of μΔC3-M1 (lanes 1–4) and μΔC3-M1R9 (lanes 5–8) in a mouse lymphoma (M12) nuclear extract. μΔC3-M1 and μΔC3-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 μΔC3-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.

μΔC3-M1R6* was used to examine whether the substituted sequence in μΔC3-M1R6, AACAAA, may play a direct role in increasing splicing efficiency. In μΔC3-M1R6*, AACAAA of μΔC3-M1R6 was replaced by AUUAUCA, which could also destabilize the stem-loop structure. If the secondary structure, but not the RNA sequence, is of primary importance, μΔC3-M1R6* will be spliced as efficiently as μΔC3-M1R6. This was shown to be the case (Figure 4A, lanes 14–16), although the splicing efficiency of μΔC3-M1R6* was slightly lower than that of μΔC3-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 μΔC3-M1 (lanes 1–3) and of μΔC3-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 μΔC3-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
occurred, 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 \( \mu \) gene transcript. We are in the process of analyzing transient expression of the \( \mu \) gene carrying the mutations in the stem region in mouse mature B and plasma cells.

ACKNOWLEDGEMENTS

We are grateful to Dr. Naoya Tsurushita for the gift of pM0\( \mu \Delta \)A plasmid and the cell line M12 as well as for valuable advice. We thank Dr. Taka-aki Tamura for providing us the procedures for the preparation of nuclear extracts developed by Gorski et al (19). We also thank Karin Knisly for proofreading the manuscript. This work was supported by a Grant-in-Aid for Scientific Research (Grant No. 62065009) from the Ministry of Education, Science and Culture of Japan.

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