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Regulation of differential processing of mouse immunoglobulin μ heavy-chain mRNA

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ABSTRACT

The switch between the synthesis of membrane-bound and secreted IgM during B cell differentiation is accomplished by producing, from a single gene, two alternative forms of μ heavy-chain mRNA that differ only in their 3' termini. The precursor μ RNA is either polyadenylated at the first poly(A) site, for secreted μ mRNA, or spliced between the C4 and M1 exons, for membrane-bound μ mRNA, in a mutually exclusive manner. To elucidate the molecular mechanism of the differential processing of mouse μ mRNA, we analyzed the expression of various mouse μ gene constructs stably transfected into mouse cell lines. In B cell lines, processing of the exogenously transfected μ gene transcripts accurately reflected the developmental stage of the recipient cells: both secreted and membrane-bound μ mRNAs are produced in early-stage B cells while secreted μ mRNA is primarily produced in late-stage B cells. In fibroblast cell lines, μ mRNAs transcribed from the Moloney murine sarcoma virus LTR promoter were processed primarily to the secreted form. Thus, production of the secreted form seems to be the non-regulated processing pattern. When the splicing signal of the C4-M1 intron was mutagenized, polyadenylation at the first poly(A) site occurred efficiently regardless of the recipient cell lines. On the other hand, when the polyadenylation signal was mutagenized, the splicing occurred efficiently in early-stage B cells, but only weakly in late-stage B cells and fibroblast cells. These results suggest that the splicing of the C4-M1 intron is stimulated in early-stage B cells.

INTRODUCTION

During differentiation, B lymphocytes undergo a shift from producing a membrane-bound IgM to making primarily a secreted form. These two forms of IgM differ at the carboxyl terminal of the μ heavy chain; the membrane-bound form ends in a hydrophobic anchor peptide, and the secreted form ends in a hydrophilic sequence (1,2). Both forms of μ heavy-chains are encoded by a single gene which has two alternative 3' coding regions ending in polyadenylation (3-5, see Fig. 1A). In early-stage B cells both secreted and membrane-bound μ mRNAs are produced while secreted μ mRNA is primarily produced in late-stage B cells.

Yuan and Tucker (6) and Ruether et al. (7) reported that no decrease in

RNA synthesis was observed between the first and second poly(A) sites in the μ gene in B cell lines regardless of their developmental stage. This indicates that transcription termination does not play a significant role in the switching mechanism. Further, Danner and Leder (8) and Peterson and Perry (9) showed that neither secreted nor membrane-bound μ mRNA was preferentially degraded in B cells. This suggests that differential RNA degradation is not involved in the switching. Therefore, it seems likely that the switching is controlled at the level of post-transcriptional processing of the precursor μ RNA.

When the precursor RNA is processed at the first poly(A) site, the resulting mature mRNA produces the secreted form of μ heavy chain. On the other hand, when the first poly(A) site in the C4-M1 intron is spliced out and the second poly(A) site is used, the mature μ mRNA produces the membrane-bound form. Thus, polyadenylation at the first poly(A) site and splicing between the C4 and M1 exons are mutually exclusive. Peterson and Perry (9) suggested that the differential processing is regulated through the control of alternative use of these two processing pathways. To elucidate the molecular mechanism of the differential processing of μ mRNA, we analyzed expression of various mouse μ gene constructs stably transfected into mouse cell lines and determined the processing pattern of μ mRNA in the non-regulated state and the roles of the alternatively used processing signals.

In B cell lines, processing of μ RNAs, whether transcribed from the authentic heavy-chain promoter or the Moloney mouse sarcoma virus LTR (Mo-LTR) promoter (10), reflected the developmental stage of the recipient B cells; both secreted and membrane-bound form of μ mRNAs were produced in the M12.4.1 lymphoma (11), whereas primarily the secreted form was produced in the SP2/0 myeloma (12). In fibroblast L and NIH-3T3 cell lines, most of the μ mRNAs transcribed from the Mo-LTR promoter were polyadenylated at the first poly(A) site, suggesting that processing of precursor μ RNA to the secreted form is the non-regulated processing pattern.

To examine the roles of the alternatively used processing signals, we introduced point mutations into the first poly(A) site and the 5' splice site of the C4-M1 intron. When the splicing signal was mutagenized, the polyadenylation site was efficiently used in M12.4.1, SP2/0 and L cells. When the polyadenylation signal was mutagenized, the splicing site was efficiently used in M12.4.1 but only weakly in SP2/0 and L cells. These

results suggest that splicing between the C4 and M1 exons for membrane-bound μ mRNA is positively regulated in early-stage B cells.

MATERIALS AND METHODS

Cells and media

Mouse lymphoma M12.4.1 (11) and myeloma SP2/0 (12) were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum and 50 μM 2-mercaptoethanol. Mouse fibroblast cell lines L(tk$^-$) and NIH-3T3 were maintained in DME media supplemented with 10% fetal calf serum.

Plasmid constructions

DNA manipulation was carried out according to Maniatis et al. (13).

Plasmid pμ1 was constructed as follows: pSV2gpt DNA (14), with the BamHI site converted into an XhoI site, was digested with EcoRI and XhoI. The large fragment from the digestion was ligated with the 3.1 kb EcoRI fragment, containing the functionally rearranged VDJ segment of the mouse hybridoma 17.2.25 (15), and the EcoRI-XhoI mouse genomic DNA fragment which contains the constant region of the μ gene.

Plasmid pMoμ was constructed by digesting pUC13 DNA (16) with HindIII and EcoRI, and ligating the resulting large fragment with the 0.45 kb HindIII-BamHI fragment of pEMSV33 (17), containing the LTR promoter of Moloney mouse sarcoma virus (10), and the 5.2 kb BamHI-EcoRI mouse genomic DNA fragment containing part of the μ gene constant region (18-20). Then, the 2.6 kb PvuII-EcoRI fragment containing the gpt gene of pSV2gpt was inserted into the EcoRI site using EcoRI linkers.

To make pμΔA, where a part of the C4-M1 intron is deleted, pμ1 DNA was partially digested with HaeII and KpnI, treated with DNA polymerase I large fragment, and religated with ClaI linkers.

Site-directed mutagenesis

Synthetic oligodeoxynucleotides 23-mer (5'-GTACAGTGTGGGTTTGCCAGTGG) and 32-mer (5'-CCAGTGACGTTTGAATGGATTTTTTTTCTTTC) containing a one base pair mismatch with the mouse μ gene at the underlined positions were prepared using the Beckman DNA synthesizer. M13 single-stranded DNA containing the 1.7 kb BamHI-FspI fragment of the mouse μ gene constant region (see Fig. 1A) was hybridized to either the 23-mer or 32-mer, and converted to double-stranded DNA by *E. coli* DNA polymerase III. After ligation, the double-stranded DNA was transfected into *E. coli* JM101 (16). Several phage plaques were picked and examined for base substitution by sequencing using the chain

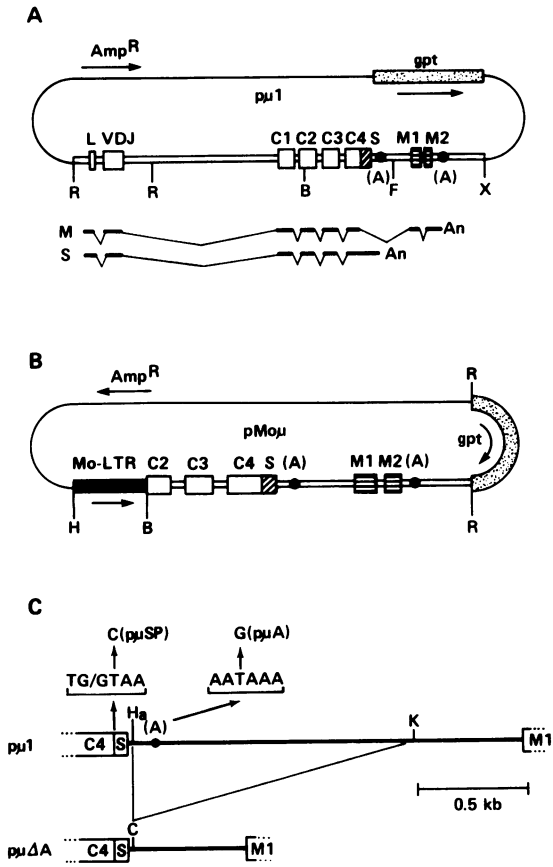

Figure 1. (A) Structure of mouse μ gene recombinant plasmid pμ1. Open and hatched boxes represent mouse immumoglobulin μ gene sequences. Dotted box represents the E. coli gpt gene derived from pSV2gpt (14). Arrows show direction of transcription. M and S show processing pattern of membrane-bound and secreted μ mRNAs, respectively. Symbols: L, leader segment; VDJ, variable, diversity and joining segments; C1-C4, constant regions of mouse μ gene; S, 3' exon for secreted μ mRNA; M1 and M2, 3' exons for membrane-bound μ mRNA; (A) with a filled circle, polyadenylation site; Amp^R, β-lactamase gene encoding ampicillin resistance; R, EcoRI site; B, BamHI; F, FspI; X, XhoI. (B) Structure of hybrid μ gene construct pMoμ. Filled box represents the Moloney murine sarcoma virus genome fragment containing the LTR promoter. H, HindIII site. Other symbols are described above. The diagrams in Figs. 1A and 1B are not drawn to scale. (C) Mutations in the μ gene. Construction of mutant μ genes is described in Materials and Methods. HaeII (Ha) and KpnI (K) sites used to delete the first poly(A) site lie 118 bp upstream and 1,272 bp downstream of the poly(A) addition site, respectively (20), and a ClaI site (C) was generated at the junction point in pμΔA. S, M1 and C4 are described above.

termination method of Sanger et al. (21). In both cases, about 50% of randomly picked clones had the desired mutation. The BamHI-AccI fragment carrying the mutation was used to replace the wild-type sequence. The details of the in vitro mutagenesis will be published elsewhere (N. Tsurushita et al., manuscript in preparation).

DNA transfection

Stable transformants of B cell lines were obtained by the protoplast fusion method of Oi et al. (22). Transformed cells were selected with 1 to 4 μg/ml of mycophenolic acid. For fibroblast cell lines, the $CaPO_4$ method was used according to Kavathas and Herzenberg (23). Transformants were selected by expression of the cotransfected chicken thymidine kinase gene (24) or hygromycin B phosphotransferase gene (25).

RNA extraction and S1-nuclease mapping

Total cellular RNA was extracted by the hot phenol method described by Queen and Stafford (26). S1-nuclease mapping was performed essentially according to Tso et al. (27). A uniformly-labeled single-stranded DNA probe was prepared using M13 single-stranded DNA containing the 0.59 kb PstI-HindIII fragment of the μ gene constant region. Total cellular RNA was hybridized with the probe at 51°C overnight in 80% formamide. Digestion was performed by incubating with 90 units of S1-nuclease (BRL) at 20°C for 60 min. The protected fragments were analyzed by 5% polyacrylamide/7.5 M urea gel electrophoresis followed by autoradiography.

RESULTS

Expression of a cloned mouse μ gene in B cell lines

A cloned mouse μ heavy-chain gene (pμ1, Fig. 1A) was stably transfected into the mouse myeloma SP2/0 and the lymphoma M12.4.1. Endogenous μ mRNA is not produced in either cell line (Fig. 2B, lanes 1 and 6), which facilitates the analysis of μ mRNA transcribed from transfected μ genes. Expression and post-transcriptional processing of μ mRNA in the transformants were analyzed by S1-nuclease mapping. The probe, which is uniformly labeled, covers the 5' splice site of the C4-M1 intron and the first poly(A) site (Fig. 2A). As shown in Fig. 2B, lane 2, the M12.4.1 transformants produced both secreted and membrane-bound μ mRNAs with a molar ratio of about three to two. On the other hand, the SP2/0 transformants produced primarily secreted μ mRNA (Fig. 2B, lane 7). The processing patterns of the μ mRNA observed accurately reflected the developmental stage of the recipient B cell lines; the M12.4.1

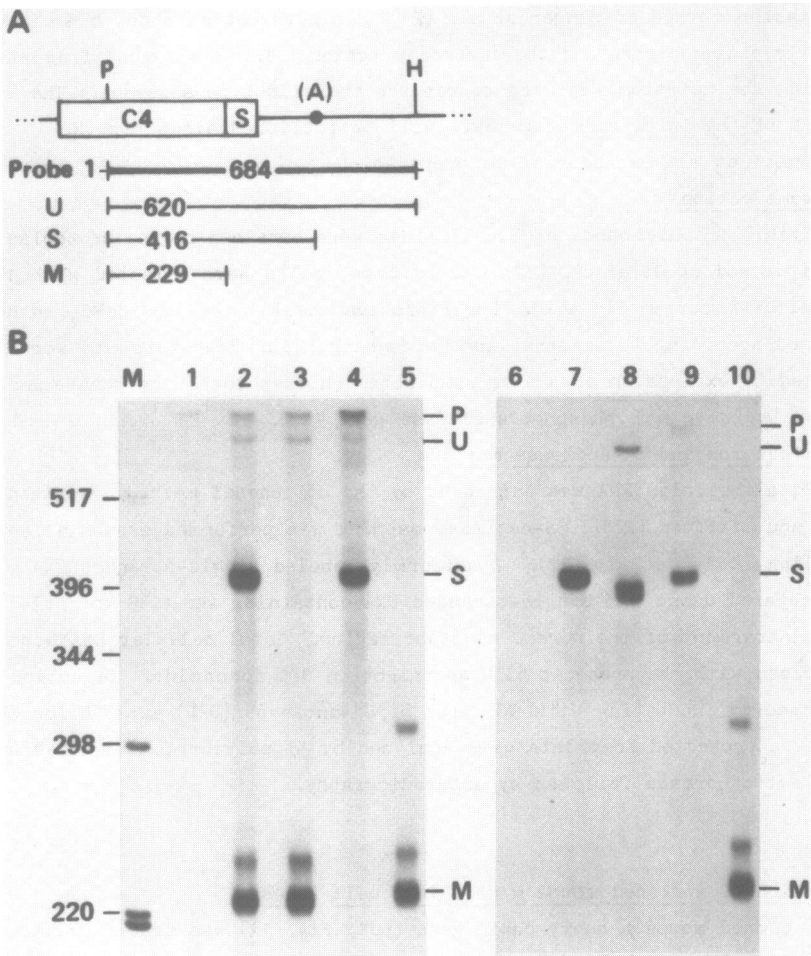

Figure 2. (A) Structure of S1 probe and protected fragments. Numbers show nucleotide lengths of fragments. The probe has short M13 phage DNA sequences at both ends. U, protected by unprocessed μ mRNA; S, protected by secreted μ mRNA; M, protected by membrane-bound μ mRNA; P, PstI site. Other symbols are described in Fig. 1. (B) Expression of various μ gene constructs in B cell lines. S1-nuclease analysis of μ gene transcripts in the stable transformants is shown. Recipient cells: lanes 1 to 5, the M12.4.1 lymphoma; 6 to 10, the SP2/0 myeloma. Plasmids used for transformation: lanes 1 and 6, none; 2 and 7, pμ1; 3 and 8, pμA; 4 and 9, pμSP; 5 and 10, pμΔA. For the analysis, 10 μg (M12.4.1) or 5 μg (SP2/0) of total cellular RNA was hybridized with the probe. S1-nuclease analysis was performed as described in Materials and Methods. Lane M shows positions and lengths (in nucleotides) of end-labeled HinfI-digested pBR322 DNA fragments. P shows the position of undigested probe. U, S and M show positions of fragments protected by unprocessed, secreted and membrane-bound μ mRNA, respectively.

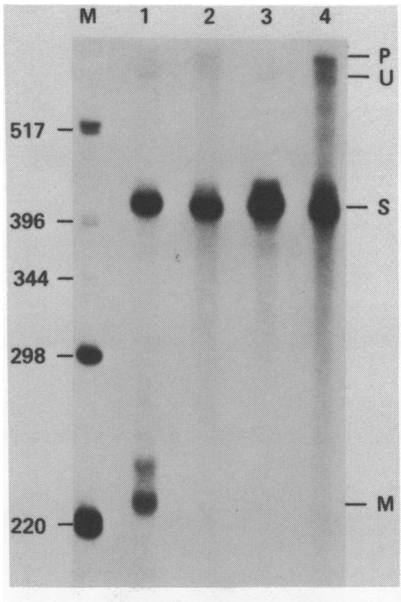

Figure 3. Expression of μ gene in fibroblast cell lines. S1-nuclease analysis of μ gene expression in cells stably transformed with pMoμ is shown. The cell lines used are SP2/0 in lane 1, M12.4.1 in lane 2, L in lane 3 and NIH-3T3 in lane 4. For the analysis, 20 μg (SP2/0, M12.4.1 and NIH-3T3) or 10 μg (L) of total cellular RNA was used. Other symbols are described in Fig. 2.

lymphoma represents an early-stage B cell (11) and the SP2/0 myeloma represents a terminally differentiated plasma cell (12).

When the hybrid μ gene in pMoμ (Fig. 1B), where the μ gene constant region is connected to the Mo-LTR promoter, was stably transfected into M12.4.1 and SP2/0, the processing pattern of the hybrid μ mRNA in either cell line (Fig. 3, lanes 1 and 2) was essentially the same as seen with the whole μ gene (Fig. 2B, lanes 2 and 7). Thus, as was observed previously (7,9), the promoter and enhancer regions of the heavy-chain gene are not necessary for the proper processing of μ mRNA.

Processing of μ mRNA in fibroblast cell lines

In order to examine the non-regulated processing pattern of μ mRNA, we analyzed μ gene expression in mouse fibroblast L cells which were stably transformed with pMoμ (Fig. 1B). As shown in Fig. 3, lane 3, μ mRNAs were processed primarily to the secreted form in the L cells. A similar result was obtained with another mouse fibroblast cell line NIH-3T3 (Fig. 3, lane

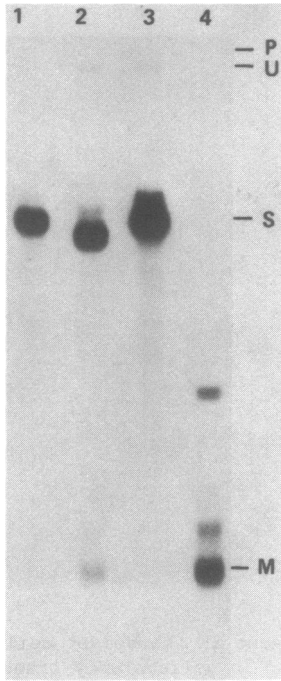

Figure 4. Processing of mutant μ gene transcripts in L cells. 10 μg of total mRNA was used for S1-nuclease analysis. Plasmids used for transformation: lane 1, pMoμ; 2, pMoμA; 3, pMoμSP; 4, pMoμΔA. Symbols are described in Fig. 2. Plasmids pMoμA, pMoμSP and pMoμΔA were constructed by replacing of the BamHI-XhoI fragment of pMoμ with the counterpart of pμA, pμSP and pμΔA, respectively.

4). Since these fibroblasts seem to have no B-cell-specific regulation of the differential processing of μ mRNAs, it is likely that the precursor μ RNA is basically processed to the secreted form in the non-regulated state. Consequently, B cells at an early stage may possess a specific mechanism to increase the ratio of membrane-bound to secreted μ mRNA.

Role of alternatively used processing signals

When precursor μ RNA is cleaved and polyadenylated at the first poly(A) site for the secreted form, they lose the acceptor site for splicing between the C4 and M1 exons for the membrane-bound form. Conversely, once splicing between the C4 and M1 exons occurs, the first poly(A) site is spliced out. Therefore, it seems likely that the switching between the two μ mRNAs is regulated by an alternative use of polyadenylation at the first poly(A) site and splicing of the C4-M1 intron.

The increase in the ratio of membrane-bound to secreted μ mRNA in early-stage B cells could result either from inhibition of polyadenylation at the first poly(A) site or stimulation of splicing between the C4 and M1 exons. To distinguish between these alternatives, we analyzed μ genes containing mutations that affect the processing signals. Three mutations were used (Fig. 1C): [1] a point mutation in the consensus sequence of the 5' splice site (28) of the C4-M1 intron (TG/GTAA to TG/GCAA) for membrane-bound μ mRNA (pμSP), [2] a point mutation in the consensus sequence of the polyadenylation signal (28) at the first poly(A) site (AATAAA to AAGAAA) for secreted μ mRNA (pμA), and [3] a deletion of the first poly(A) site (pμΔA). The mutations were separately introduced into the μ gene on pμ1 and the plasmids were stably transfected into M12.4.1 and SP2/0.

When the 5' splice site of the C4-M1 intron was destroyed by the point mutation (pμSP), polyadenylation at the first poly(A) site occurred efficiently in M12.4.1 (Fig. 2B, lane 4) as well as in SP2/0 (Fig. 2B, lane 9). If the polyadenylation is inhibited in early-stage B cells, one would expect the appearance of unprocessed μ mRNA, which is neither polyadenylated at the first poly(A) site nor spliced between the C4 and M1 exons, together with the secreted form in the M12.4.1 lymphoma. However, the level of the unprocessed RNA was extremely low in M12.4.1 (Fig. 2B, lane 4) Thus, it seems unlikely that polyadenylation at the first poly(A) site is negatively regulated in early-stage B cells.

When the first poly(A) site was destroyed by the point mutation (pμA), splicing between the C4 and M1 exons still occurred efficiently in M12.4.1 (Fig. 2B, lane 3). In SP2/0, however, splicing was hardly detectable and a new major band, which was not seen with the wild-type μ gene, appeared (Fig. 2B, lane 8). This new μ mRNA in the pμA-transformed SP2/0 cells is polyadenylated and does not contain the M1 and M2 exons (data not shown). We presume that precursor μ RNA was polyadenylated at a cryptic poly(A) site lying slightly upstream of the first poly(A) site. The 'ATAAA' sequence which lies 12 bp upstream of the 'AATAAA' sequence for the first poly(A) site (20) may be a part of the putative cryptic poly(A) site. It seems that in the SP2/0 myeloma splicing of the C4-M1 intron occurs even less readily than polyadenylation at the cryptic poly(A) site. Since the splicing of the C4-M1 intron occurs efficiently in the pμA-transformed M12.4.1 lymphoma cells (Fig. 2B, lane 3), it is likely that the splicing between C4 and M1 exons is already stimulated in early-stage B cells.

When the first poly(A) site was deleted together with the cryptic

poly(A) site (pμΔA), membrane-bound μ mRNA was primarily produced in both M12.4.1 and SP2/0 (Fig. 2B, lanes 5 and 10). This result excludes the possibility that membrane-bound μ mRNA is unstable in the SP2/0 myeloma. In addition, the level of unprocessed μ mRNA, whose protected fragment was 298-nucleotides long, remained low (Fig. 2B, lanes 5 and 10).

Expression of mutant μ genes in L cells

To analyze the expression of mutant μ genes in fibroblast cells, the three mutations in the μ gene were transfered to pMoμ. These mutant μ genes were stably transfected into L cells. The processing pattern of each mutant μ mRNA in L cells was essentially the same as observed in the SP2/0 myeloma (Fig. 4). This result supports the idea that the processing of μ mRNA in the M12.4.1 lymphoma is specifically regulated.

DISCUSSION

We have shown that mouse immunoglobulin μ heavy-chain RNA is processed primarily to the secreted form in mouse fibroblast cell lines. Nishikura and Vuocolo (29) obtained a similar result when they analyzed the expression of the mouse μ gene connected to the SV40 early promoter in Cos cells. In contrast, Ruether et al. (7) reported that nearly equal amounts of secreted and membrane-bound μ mRNAs were produced in the human fibroblast KB18 which was transiently transfected with an adenovirus 5 construct containing the mouse μ gene under the E1B promoter. There are a number of possible explanations for the discrepancy between Ruether et al. (7), and Nishikura and Vuocolo (29) and our observation. For example, the differential processing might be controlled in a delicate manner so that the regulation is disturbed in a heterologous species. Alternatively, the expression of viral genes in the experiment by Ruether et al. (7) might have affected cellular polyadenylation and/or splicing. In our experiments, we have used a homologous system; mouse μ genes were transfected into mouse cell lines. Further, stable transformants of two mouse fibroblast cell lines, L and NIH-3T3, gave the same result. Thus, we conclude that μ RNA is almost exclusively polyadenylated at the first poly(A) site in the non-regulated state.

In early-stage B cells, both secreted and membrane-bound μ mRNA are produced. To explain the observation that μ mRNA is processed to the secreted form in the non-regulated state, we have suggested that there is a mechanism to increase the ratio of membrane-bound to secreted μ mRNA in early-stage B cells. Since polyadenylation at the first poly(A) site, for

secreted μ mRNA, and splicing between the C4 and M1 exons, for membrane-bound μ mRNA, occurs in a mutually exclusive manner, the increase in the ratio could be accomplished either by inhibition of polyadenylation or stimulation of splicing. To distinguish between these two possibilities, we have analyzed the processing pathways by introducing mutations into each processing signal. The results obtained suggest that polyadenylation at the first poly(A) site is not regulated and that splicing of the C4-M1 intron is stimulated in the M12.4.1 lymphoma. Thus, it is likely that the switching of the synthesis between membrane-bound and secreted μ mRNAs is regulated through the control of splicing of the C4-M1 intron.

It has been proposed that the differential processing of μ mRNA is controlled at the level of poly(A) site selection between the first site, for the secreted form, and the second site, for the membrane-bound form (5,30). This model implies that the choice of splice site follows automatically from the choice of polyadenylation site. If this is the case, a mutation in the 5' splice site of the C4-M1 intron (pμSP) should not affect the choice between the two μ mRNAs and one would expect that the pμSP-transformed M12.4.1 lymphoma should produce secreted μ mRNA and the membrane-bound form in which the C4-M1 intron is not spliced. However, as shown in Fig. 2B, lane 4, only the secreted form was produced in the pμSP-transformed M12.4.1 cells. Although we can not exclude the possibility that such an unspliced μ mRNA is unstable, it seems unlikely that polyadenylation at the second poly(A) site is involved in the regulation of differential processing.

When a resting B cell differentiates to a plasma cell, it produces primarily secreted μ mRNA. The fusion of a B lymphoma, producing both secreted and membrane-bound μ mRNAs, with a myeloma gave rise to a hybridoma which produced primarily secreted μ mRNA (31). These observations suggest the presence of a dominant control mechanism for the regulation of μ mRNA processing in a terminally differentiated plasma cell. We assume that the expression of the factor(s) which stimulates splicing of the C4-M1 intron is turned off in plasma cells. Alternativley, the activity of the factor(s) might be inhibited by additional factors.

Recently, Peterson and Perry (9) and our group (32) reported that there is a correlation between the length of the C4-M1 intron and the molar ratio of membrane-bound to secreted μ mRNAs: the shorter the C4-M1 intron, the higher the ratio. It seems that when the 5' and 3' splice sites become closer, splicing of the C4-M1 intron occurs more readily. These results

support an idea that the ratio between the two μ mRNAs can be regulated through the control of the splicing efficiency of the C4-M1 intron. However, the length of the C4-M1 intron is constant throughout B-cell development. We speculate that the formation of the "spliceosome" complex (33,34) in the C4-M1 intron is enhanced in early-stage B cells, resulting in more efficient splicing of the C4-M1 intron and therefore an increase in the ratio of membrane-bound to secreted μ mRNA.

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