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Effect of upstream RNA processing on selection of μS versus μM poly(A) sites

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ABSTRACT

All of the regulatory factors responsible for augmenting μS mRNA levels preceding the dramatic increase in secretory IgM production upon B cell activation has not been totally elucidated. Whereas previous experiments have centered on the region of the gene specifying the choice between splicing to μM exons versus selection of the μS poly(A) site, we have found that upstream sequences within the Cμ gene, specifically the Cμ4 acceptor splice site together with intronic sequences between the Cμ3 and Cμ4 exons, play an important role in dictating the precision or the extent of splicing to the μM exons even under conditions in which functional polyadenylation factors should be in excess. Therefore, splicing of upstream exons can affect remotely located downstream exons. These findings suggest that regulation of differential μS/μM mRNA expression may involve general processing enzymes that recognize specific *cis*-regulatory sequences residing within the body of the μ gene and account for the unique ability of activated B cells to secrete copious amounts of IgM.

INTRODUCTION

The differentiation of resting B lymphocytes expressing predominantly cell surface IgM to a secretory stage is accompanied by a dramatic increase in the initiation of heavy chain as well as L chain gene transcription (1,2). A number of mechanisms can be evoked to account for the preferential usage of the μS poly(A) site in the newly induced transcripts. Because selection of the μS poly(A) site excludes the possibility of splicing to the downstream μM exons, early work suggested that the strength of the μM 5′-splice site, which is embedded within the μS exon, is of critical importance for maintaining the balance of μS versus μM expression (3). Alternatively, increased production of termination factors that prevent full extension of the primary transcript to the μM poly(A) site would favor usage of the upstream μS poly(A) site (4,5). Finally, based on the finding that the μM poly(A) site is intrinsically stronger than the μS poly(A) site, increased μS processing could be induced by increasing the level of polyadenylation factors (6,7).

Strong support for the latter mechanism was shown in experiments demonstrating that overexpression of cleavage factor CstF-64 in chicken B lymphoma cells results in an amplification of μS mRNA expression suggesting that it is the increase in functional polyadenylation enzymes that allows preferential usage of an intrinsically weaker poly(A) site (8). These findings, which are consistent with earlier reports of increased binding activity of CstF-64 in plasmacytomas (9), led to the hypothesis that the regulation is independent of any *cis*-regulatory sequences other than those that specify the affinity of the poly(A) sites for polyadenylation factors. Indeed, there is evidence that replacement of the specific μS or γS poly(A) sites with similarly weak heterologous poly(A) sites, regardless of sequence composition, does not perturb appropriate regulation in lymphoma versus plasmacytomas (10,11). On the other hand, there is increasing evidence that many steps involved in processing of primary transcripts are linked to polymerase initiation as well as progression across the template (12–14) and it is likely that upstream processing signals modify polymerases as they transit across these sites. In this regard, the relevance of specific μ gene sequences was recently tested in normal B cells by examination of a non-Ig transgene that contains competing splice versus polyadenylation sites (15). Interestingly, it was found that upon B cell activation increased processing to the proximal poly(A) site was only 50% of that for the μS poly(A) site. Therefore, the possibility remains that increased concentrations of cellular CstF-64 can operate more effectively on the μS poly(A) site in the endogenous context.

To test this hypothesis we prepared a number of constructs. These constructs all contain the endogenous IgH promoter and enhancer as well as at least the Cμ1 acceptor splice site to ensure that only polymerases destined to be spliced to the μ exons are assessed. This point is important because the μ exons reside within a complex transcriptional unit with many potential alternative, downstream, exons to which the VDJ segment can be spliced in a developmentally regulated manner. In these constructs the segment of the Cμ4 exon containing sequences surrounding the μMI donor splice site as well as the distance between the two poly(A) sites are maintained in the germline configuration. Superimposed on this backbone a number of alterations were made in the exons in between. Examination of the ratio of μS versus μM mRNA processed from these transcripts assayed in transfectants allowed us to conclude that the terminal Cμ4 exon, as well as its upstream intron, must be maintained in its native configuration to permit appropriately regulated splicing to the

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μM exons. Thus, generation of intact μM mRNA can be elevated or depressed when these elements are disturbed, even under conditions that presumably favor μS poly(A) site usage. These observations suggest that appropriate splicing of upstream sequences is also important for regulating the alternative usage of the μS versus μM polyadenylation sites and that increased μS mRNA production may not be solely dependent on differential expression of a polyadenylation factor.

MATERIALS AND METHODS

Cell lines and transfections

J558L (16) and M12.4 (17) cells were maintained in RPMI supplemented with 10% FCS (Gibco BRL, Grand Island, NY). Transient transfections were performed essentially as previously described (18).

Constructs

All of the constructs are recombinants of the Pμ (19) vector which contains an Ig μ gene rearranged to the 17.2.25 V region, extending up to the *Xho*I site within the μ-δ intron, the polyoma virus early region and a modified histone gene. Plasmid 155 contains in addition a 5760 bp fragment containing CδH, Cδ3, δMI and δMII, derived from pLLn5R (20) ligated into the *Xho*I site. RNA derived from transfectants of this construct in J558L and M12.4 cells exhibits a μS/μM phenotype that is essentially identical to Pμ. All remaining constructs are derivatives of plasmid 155. Plasmid 171 was constructed by deleting the 1165 bp fragment between two unique *Bst*EII sites, resulting in the obliteration of part of Cμ2 and Cμ4 and all of Cμ3. Plasmid 202 was constructed by replacing 1242 bp of the μS–μM interval starting from the *Bcl*I site located 130 bp 5′ of the poly(A) addition signal with a 1115 bp fragment containing a 165 bp *Xmn*I–*Bam*HI segment surrounding the poly(A) signal for δS and 950 bp of pGem4 vector sequence. This mutation results in the altered poly(A) addition signal being located at a site 100 bp closer to the Cμ4–μM splice site but does not alter the distance between the μS and μM poly(A) sites. To construct plasmid 218 a 180 bp PCR fragment derived from amplification of genomic sequences encompassing 55 bp upstream of the Cμ4 acceptor splice site and extending 125 bp into the Cμ4 exon was inserted into the *Pvu*II site located at the Cμ2 acceptor splice site of plasmid 171. This resulted in the restoration of the Cμ4 acceptor site and branch point and increased the exon size of the Cμ2–Cμ4 chimeric exon by 125 bp. Plasmid 231 was constructed by first subcloning a fragment, containing the entire Cμ4 exon and 80 bp upstream of the acceptor splice site, amplified from the genomic sequence, into the *Pvu*II site upstream of Cμ2 in 171 to create an intermediate vector. From this construct the *Bst*EII–*Sma*I fragment was excised and cloned into the same sites in plasmid 171, thus restoring an intact Cμ4 exon. Plasmid 233 was made by the same strategy as that used for plasmid 231 except that a 2.1 kb fragment containing the entire Cμ3 and Cμ4 exon and extending 100 bp upstream of the Cμ3 acceptor splice site was first cloned into the intermediate vector prior to excision of the *Bst*EII–*Sma*I fragment, which was then cloned into plasmid 171. Plasmid 255 was constructed by amplifying a 670 fragment containing 198 bp of Cμ3 and all of Cμ4 from Pμ into an intermediate vector from which an *Sma*I–*Bst*EII fragment was obtained and cloned into plasmid 171 that had been digested with the same enzymes. For plasmid 275 additional Cμ1 sequence immediately 3′ of the *Sma*I site was restored by inserting a 114 bp fragment, obtained by PCR amplification, into the *Sma*I site. The resulting construct contained a chimeric Cμ1–Cμ3 exon that is 78 bp larger than the genomic Cμ1 exon.

RNA isolation and S1 analysis

Total RNA was prepared from transfected cells by extraction in the presence of guanidinium thiocyanate and S1 analysis was performed as described (21). Optimal hybridization temperatures were established empirically for each probe. Samples were analyzed on denaturing polyacrylamide gels, dried and exposed to the phosphorimager screen and quantified using the Imagequant software package (Molecular Dynamics, Sunnyvale, CA). The S1 probe to evaluate μS versus μM expression in 155 transfectants was a 467 bp *Hpa*II–*Hin*dIII fragment isolated from Pμ that had been 3′-end-labeled by filling in with [α-^{32}P]dCTP using the Klenow fragment of DNA polymerase. μS versus μM expression was evaluated in 171 transfectants using a similarly labeled 381 bp fragment isolated from a pBlueScript KS (Stratagene, La Jolla, CA) subclone that contains the 82 bp *Hin*fI–*Bcl*I fragment isolated from plasmid 171. The S1 probe used for measuring relative stability of μS mRNA in 155 versus 171 transfectants was generated by annealing a ^{32}P-end-labeled μS (5′-GACATGATC-AGGGAGACATTGTAC) primer to a plasmid containing the *Pst*I–*Hin*dIII fragment from Cμ4 cloned into pGem 4 followed by primer extension and digestion of the product at a unique enzyme site within the plasmid to yield a fragment of 300 bp which was isolated on a 6% denaturing gel. The glyceraldehye dehydrogenase probe (GAPDH) was a 600 bp *Nco*I–*Pvu*II fragment isolated from a rat GAPDH cDNA clone (22). Hybridization up to the region of greatest homology with mouse GAPDH yields a protected fragment of 240 nt (21).

Semi-quantitative RT–PCR analyses

Total RNA was primed with oligo(dT)$_{15}$ and hexadeoxyribonucleotide random primers (Promega, Madison, WI) and reverse transcribed with MMLV reverse transcriptase (Gibco BRL, Long Island NY). In some experiments mRNA was primed with only oligo(dT)$_{15}$, but no detectable difference in the quality or quantity of amplification products was found for the two priming conditions. For RT–PCR to quantify the ratio of μS to μM mRNA the same forward primer corresponding to sequence within J_H4 (5′-TATGGACTACTGGGTCAG) was used for all samples. The reverse primer was either specific for the μS exon (5′-GACAT-GATCAGGGAGACATTGTAC) or the μMII exon (5′-GAAAG-AAGACCATCTCAGAG). By varying the cycle time 25 cycles was determined to fall within the logarithmic increase phase for both primer sets for cDNA derived from all transfectants. The amplification conditions were 94°C for 3 min, followed by 25 cycles of 94°C for 45 s, 58°C for 1 min and 72°C for 2 min, with a final extension cycle of 72°C for 7 min. For quantification of products one of the primers was end-labeled to high specific activity with [^{32}P]γATP and added to the reaction mixture containing optimal concentrations of unlabeled primers. PCR products were run on 1% agarose gels and either transferred to nitrocellulose or dried directly and exposed to the phosphoimager screen and quantified.

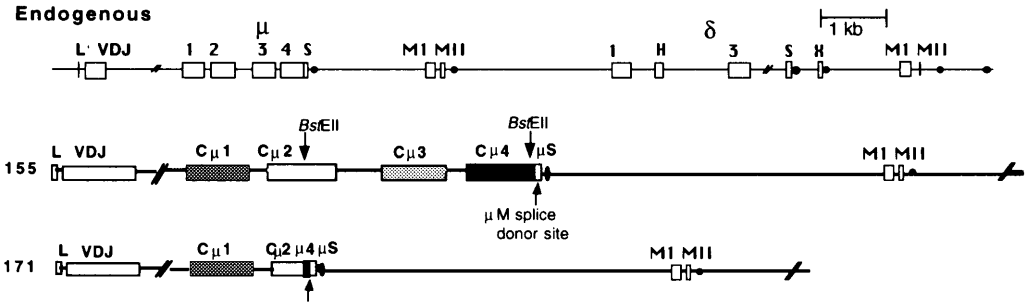

Figure 1. Map of the μ-δ transcription complex and constructs 155 and 171. The genomic context of the entire Cμ gene up to 1000 bp beyond the μM poly(A) site is maintained in construct 155. Construct 171 only differs from 155 by the deletion of the gene segment between the two *Bst*EII sites indicated.

Sequencing

Sequencing of DNA or cDNA was performed using an AmpliCycle sequencing kit (Perkin-Elmer, Branchburg, NJ) according to the manufacturer's instructions. Amplified products were either sequenced directly or first cloned into the KS vector.

Inhibition of RNA synthesis

Two days after transfection 6-dichloro-1β-D-iribofuranosyl-benzimidazole (DRB; Sigma Chemical Co., St Louis, MO) was added at a final concentration of 100 μM. Aliquots were harvested at the indicated times and RNA extracted. Run-on analysis performed in previous experiments (5) have shown that Pol II polymerase progression was completely eliminated at this concentration.

RESULTS

Deletion of upstream sequences alters the relative usage of the μS poly(A) site

The impetus for the first construct made to investigate the role of upstream sequences was derived from a finding of Peterson *et al.* (23) showing that a *Bst*EII–*Bst*EII deletion within the μ gene, encompassing a segment between Cμ2 and Cμ4 (Fig. 1), resulted in highly deregulated expression of μS versus μM in all cell types in that even in plasmacytomas the ratio of μM made is much higher than that found when a plasmid expressing the wild-type gene is transfected. Despite extensive analysis they could not decipher the basis for the effect. However, one aspect not tested was the effect of this mutation in the context of the endogenous gene, since all of the constructs used contained an SV40 *neo* promoter and were devoid of the VDJ or Cμ1 exons. In view of the evidence indicating an effect of distance between the cap site and the poly(A) site on 3′-end formation (24) we wished to determine if more regulated expression can be achieved by making the same alteration in a construct that contains an intact μ gene promoter as well as the VDJ and Cμ1 exons (171, Fig. 1). This construct was transfected into J558L plasmacytoma cells that do not express endogenous μ genes and the relative expression of μS versus μM was compared with the same cells transfected with 155 (Fig. 1), a construct containing all of the Cμ exons in the endogenous configuration. S1 analysis of the RNA showed that the ratio of μS versus μM expression was dramatically altered (Fig. 2) such that instead of μS being expressed at much higher levels than μM, as would be expected in a plasmacytoma, the expression of μM now far exceeded that of μS. A virtually identical phenotype was found in transfectants of M12.4 (data not shown), a lymphoma cell line that allows expression of higher μM/μS ratios from wild-type constructs. Thus, despite differences in the upstream as well as downstream regions, the phenotype of mRNA expressed from this particular construct appears to be similar to that previously found by Peterson *et al.* (23). Therefore, restoring the upstream exons did not alter the deregulated expression.

Alterations in the μM/μS ratio in 171 are not due to effects on RNA stability

Previous experiments have shown that the stabilities of μS and μM RNA are not differentially regulated in either normal B cells (21) or in tumor cells induced to differentiate to a secretory state (5). Thus even though the stability of mRNA encoding the secretory and membrane forms of IgM and IgG increases significantly in fully differentiated B cells or plasmacytomas (25–27), their decay rates relative to each other do not change. A possibility remains, however, that the alterations in the structure of RNA derived from the mutated construct may have affected the stability in comparison with the wild-type. Subtle changes in stability may, however, not be easily detectable in J558L cells, since the half-life of μ mRNA has been shown to be >12 h in plasmacytoma cells (25; data not shown). The extensive measurement time required after inhibition of RNA synthesis can adversely affect cell viability and, correspondingly, mRNA stability. Therefore, we transfected M12.4 cells, a lymphoma cell line in which the decay rate of μ mRNA is much shorter and therefore should provide a better window for detection of possible differences. Figure 3A shows results of a representative experiment in which equal amounts of DNA of plasmids 155 and 171 were transfected. Two days after transfection, the cells were treated with DRB to inhibit RNA elongation. Samples were removed at the time points indicated and S1 analysis was performed using a probe that can hybridize to the μS species made from both constructs but yields different protected sizes. Quantification of the relative amounts of RNA remaining (Fig. 3B) shows no difference between the decay rates of the two species, both having a half-life of ~3 h. Therefore, μS mRNA derived from the 171 construct is not less stable and cannot account for the decreased μS/μM ratio. The cells remained >90% viable for the entire period of drug treatment and measurement of mRNA levels for a housekeeping gene, GAPDH, showed no significant decrease up to the last time point (data not shown). However, the mRNA level of GAPDH includes that from

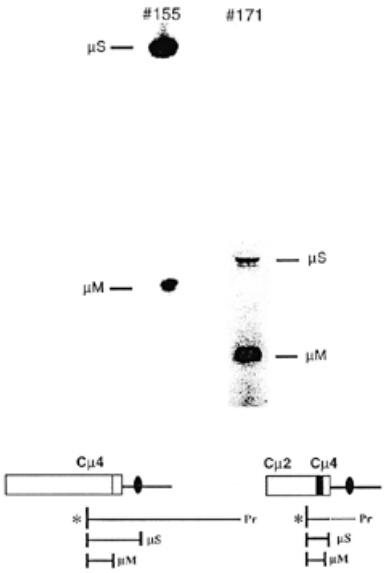

Figure 2. Relative abundance of μS versus μM mRNA in J558L transfectants of plasmid 155 versus 171 as determined by S1 analysis. Derivations of probes are indicated below the gel image for each transfectant. The lightly shaded section of the line designated by Pr indicates plasmid sequences.

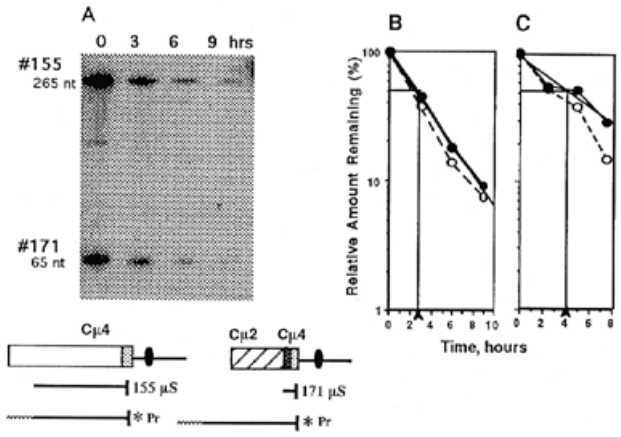

Figure 3. Comparison of stability of μS mRNA from J558L cells co-transfected with plasmid 155 or 171. After transient co-transfection of the two plasmids the amount of end-labeled probe protected from S1 digestion at each time point (**A**) was quantified and plotted (**B**) as a percentage of the amount at $t = 0$. Half-life of each mRNA was determined from the best fit line for each transcript (shown only for RNA from plasmid 171, closed circles). (**C**) A stable 155 transfectant of J558 (open circles) was transiently transfected with plasmid 171 and relative amounts of protection for each probe was plotted as in (B).

transfected and non-transfected cells. The possibility exists that the half-life measurements may be affected by differential viability of transfected cells. Therefore, a M12.4 stable transfectant of plasmid 155 was transiently transfected with the 171 construct and the relative decay rates of the mRNA derived from the two genes were compared as before. Figure 3C shows that the stability of μS mRNA from 155 is indeed somewhat greater in the stable transfectants, suggesting that the decay rate measured in transfected cells may be influenced by greater susceptibility to inhibition of RNA synthesis. Nevertheless, even under these conditions mRNA from 171 is clearly not more labile than that from 155. Therefore, a difference in stability due to the altered RNA structure cannot account for the difference in μS/μM ratios observed.

Alteration of the μS poly(A) site results in aberrant splicing to μM

In attempts to restore the increased usage of the μS poly(A) site in a construct similar to 171, Peterson *et al*. mutated either the μM poly(A) site or the Cμ4–M1 splice site (23). Neither change affected the effect of the *Bst*EII deletion. Based on the reasoning that an alteration of the μS poly(A) site might be needed to restore the μM/μS ratio, we substituted this site with the δS poly(A) site and created the construct labeled as 202 (Fig. 4A). Whether this site is intrinsically weaker than that of the μS poly(A) site has not been previously tested. In order to avoid using different probes to compare RNA derived from different constructs as was done in Figure 2, we elected to analyze the processed RNA by RT–PCR so that the same primer sets spanning the entire μ gene could be used for cDNA from all constructs. Figure 4B shows representative RT–PCR products from each construct analyzed (Fig. 4A), along with the expected sizes for the amplified products from μS or μM mRNA. Lanes 1–4 show that the use of these primers resulted in the amplification of appropriately sized products from cells transfected with plasmids 155 and 171. Whereas the relative ratio of μM to μS RNA from 155 falls within the range of the values previously determined by S1 analysis, the increase in the ratio of μM to μS mRNA from 171 is not as dramatic as that quantified by S1 analysis, although it is still significantly higher than that from 155 (Table 1). In order to justify estimation of μS versus μM ratios by RT–PCR amplification we ascertained that amplification cycles were maintained within a titratable range for all sets of primers and for cDNA from all constructs. Thus, even though different transfections yielded variable amounts of cDNA that can be amplified by the primers, the relative μS versus μM amplification did not change for any given cDNA as long as the amplification cycle was maintained at or below 25. Therefore, we concluded that this is an appropriate method to measure relative μM/μS usage.

Table 1. Effect of μ gene exonic organization on the ratio of μM to μS mRNA expression in J558-L cells

Plasmid	*n*	μM/μS[a] (mean ± SD)
155	9	0.11 ± 0.02
171	17	0.52 ± 0.04
231	11	0.50 ± 0.05
233	11	0.18 ± 0.09

[a]The ratio of μM to μS mRNA expression was determined by quantification of PCR-amplified products as described in Materials and Methods.
n indicates the total number of determinations based on at least four independent transfections per construct.

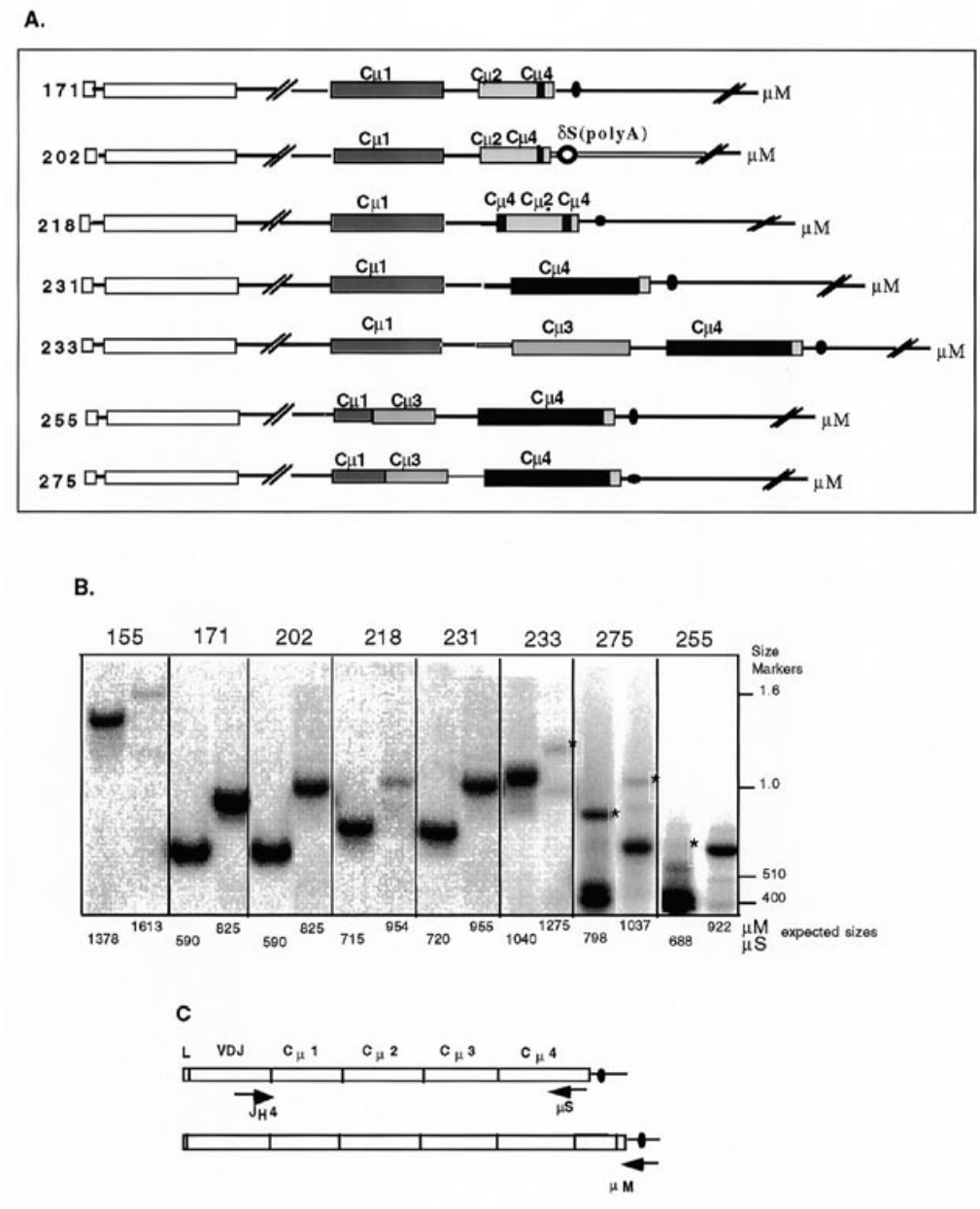

Figure 4. Mutant constructs used for the analysis and the expression of μS and μM mRNA derived from J558L transfectants thereof. (**A**) Map of the constructs. Cμ exons, indicated by patterned blocks, were altered as described in Materials and Methods. μM exons as well as the intronic distance between μS and μM were maintained in the genomic context. Lines represent introns maintained in genomic context. Lines with narrow shaded blocks indicate chimeric introns with the shaded section drawn to correspond to the part originally associated with the downstream exon. (**B**) RT–PCR-amplified products from each transfectant using oligos as indicated in (**C**) were size fractionated on agarose gels which were subsequently dried, visualized and quantified by phosphorimager analysis. In lanes containing more than one species, the ones migrating at the expected sizes are indicated by stars. The figure presented is a composite of two different gels that ran at the same mobility to allow easier comparison. Expected sizes for amplified products from μS or μM RNA from each construct are indicated below each lane.

Using this approach we found that transfection of plasmid 202 containing the altered μS site yielded an appropriately sized amplified μS product. However, the μM product was ~90 bp larger than expected (Fig. 4B). Thus, whereas μS mRNA appears to be appropriately polyadenylated, the majority of μM mRNA was aberrantly processed. μM mRNA was found also to have an aberrant size in M12.4 cells (data not shown). Thus it appears that a change in the proximal poly(A) site affected downstream processing, although the Cμ4–μM donor site itself was not altered.

Placement of the Cμ4 acceptor splice site in an abnormal context results in aberrant splicing to the μMII exon

The result of disturbing processing of the μM mRNA by an alteration in the μS poly(A) site suggested that sequences that

regulate μS polyadenylation may indirectly affect downstream events in a manner that is independent of poly(A) site usage *per se*. Thus the increase in μM expression in RNA from 171 transfectants may be a more subtle reflection of disturbance in μS polyadenylation. According to the Exon Definition Model (28,29), which points to the importance of the terminal splice for proper utilization of the proximal poly(A) site, the presence of a chimeric proximal exon in plasmid 171 may have affected the regulation of μS polyadenylation even in the face of excess poly(A) factors. Therefore, we investigated whether replacing the Cμ2 acceptor splice site with the Cμ4 acceptor splice site including upstream branch point sequences would restore the wild-type phenotype (218, Fig. 4A).

Analysis of mRNA from transfectants of plasmid 218 (Fig. 4B) showed that processing of RNA to μS mRNA is normal since the expected 715 bp μS product is amplified. Processing to μM RNA was, however, aberrant, resulting in the amplification of two very closely migrating species (not resolved in the gel shown in Fig. 4B), only one of which corresponded to the expected size. Sequencing of the major species showed that the amplified product was derived from transcripts that spliced directly from Cμ4 to μMII, resulting in the deletion of μMI. In addition, whereas the donor splice site was correctly utilized, the 3′-splice site used in μMII was not a concensus acceptor site (Fig. 5A). A similarly high fraction of mispliced transcripts was also found when the construct was transfected into M12.4 cells; therefore, the occurrence of aberrant splicing was not restricted to plasmacytoma cells. The sequence of the minor amplified species revealed, on the other hand, normal splicing to μMI and μMII, indicating that this process can occur, albeit at a much lower frequency. S1 gel analysis of RNA using a μMII-specific probe indicated that the ratio of correctly spliced RNA was <10% of that of the aberrant species (data not shown). Plasmid 218 itself was partially sequenced and confirmed to have retained all of the normal donor and acceptor splice sites. Sequences surrounding the Cμ4 fragment insertion site were also verified. Likewise, cDNA derived from the μS mRNA was found to have the appropriate sequence. Restoration of the Cμ4 acceptor splice site therefore appears to remove the enhancement of splicing to μM in RNA from 171 transfectants. However, the aberrant downstream splicing suggests that not all of the regulatory sequences have been replaced.

Presence of the Cμ4 exon and acceptor splice sites is insufficient for preferred selection of the μS poly(A) site

To restore the terminal exon to the wild-type configuration the entire Cμ2–Cμ4 chimeric terminal exon was replaced with Cμ4 along with all of the presumably necessary branch point sequences immediately upstream (80 bp). Transfection of the construct (231, Fig. 4A) into J558L cells resulted in both μS and μM RNAs that were correctly spliced (Fig. 4B), confirming that the abnormal splicing to μM was a result of alteration of the Cμ4 exon content. However, it is interesting that the μM to μS mRNA ratios remained the same as that found for construct 171.

Table 1 shows the results of multiple transfections using the same constructs. Clearly, the μM/μS phenotype of mRNA transcribed from plasmid 231 always resembled more that of mRNA transcribed from 171 than from 155, which contains the wild-type configuration. Therefore, whereas replacement of the entire terminal exon restored the fidelity of splicing to μM, regulation of the Cμ4–μM splice efficiency is still aberrant.

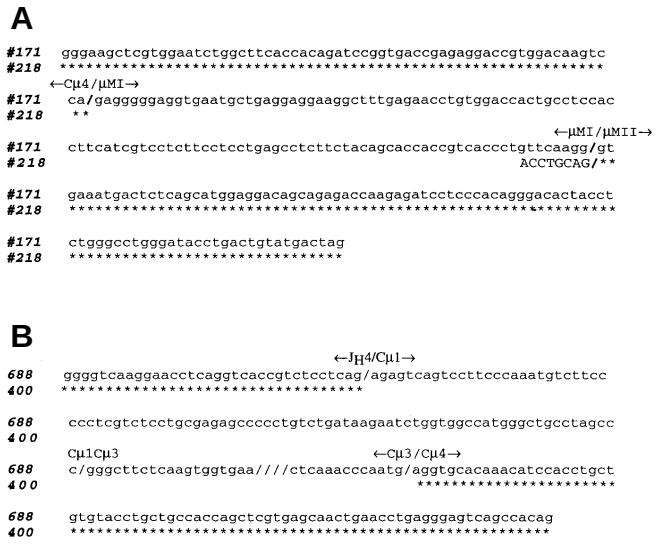

Figure 5. Sequence analysis of cDNA from aberrant transcripts. (**A**) Sequence of amplified product of cDNA derived from J558L cells transfected with plasmids 171 and 218 in the region of Cμ2/Cμ4/μM. The sequence shown for plasmid 171 mRNA corresponds to the predicted sequence of appropriately spliced RNA. Starred line indicates sequence identity found for the major amplified product of cDNA derived from cells transfected with plasmid 218. Bases denoted in upper case letters indicate differences. These bases correpond to the intronic sequence that should have been spliced out. The forward primer used was located just upstream of the *Bst*EII site (5′-GTGGAATCTGG-CTTCACCACAGAT). The reverse oligo was the μM oligo described in Figure 4C. (**B**) Sequence in the region of the chimeric Cμ1–Cμ3 exon of both of the J_H4-μS primer-amplified products (400 and 688 bp) of cDNA derived from J558 cells transfected with plasmid 255. The region indicated by //// was not sequenced. The starred line indicates sequence identity and the breaks between the stars indicate deleted sequences. The break between the dotted line for the sequence derived from the 400 bp fragment indicates deleted bases. The forward primer was located at the V–D junction (5′-GCTAGATACTATAGGTACC). The reverse primer was the μS oligo described in Figure 4C.

Restoration of the proximal exon and intron results in appropriate expression of μS in plasmacytoma cells

Although the entire Cμ4 exon was replaced as the terminal exon in 231, it should be noted that the proximal intron is still chimeric in nature and contains all of the Cμ1–Cμ2 intron. Therefore, in the next construct, plasmid 233, we tested whether restoration of the Cμ3 exon as well as the intronic region between Cμ3 and Cμ4 would alter the phenotype. Figure 4B and Table 1 show that mRNA from this construct yielded a more appropriate ratio of μS/μM expression in that now μS expression far exceeds μM expression. Other species in addition to the appropriately sized one were amplified by the μM primer (940 bp, in the experiment shown); however, the presence as well as size of these species varied between experiments and may represent inappropriately amplified products. Furthermore, the relative abundance of these species decreased upon increased cycle number.

To determine if the acceptor splice site of Cμ3 is needed for the restoration of the phenotype we examined the mRNA encoded by plasmid 275, in which the splice between Cμ1 and Cμ3 in 233 was eliminated. RT–PCR amplification using the μS-specific

primer resulted in two dominant bands, one of them migrating at a position some 390 bp smaller than the predicted size, with only a minor species migrating at the appropriate size. In an earlier construct, in which a 141 bp fragment within the Cμ1 exon was deleted (plasmid 255) the smaller bands were even more pronounced. Partial sequencing of the 400 bp species showed that it is derived from mRNA that has spliced directly from the VDJ exon to Cμ4, deleting the entire chimeric exon (Fig. 5B). Partial sequencing of the poorly amplified 688 bp μS product showed appropriate inclusion of the chimeric exon. Thus, exon skipping does not occur in all of the mRNA made from this construct. The size of the detectable μM product was also decreased by the same increment, showing that a similar skipping of the chimeric exon occurred in μM mRNA. At present the reason for deletion of the chimeric exon in these two constructs is not clear, since the exon size has been altered by <25%. Nevertheless, it can be noted that the ratio of correctly spliced μS to μM mRNA from plasmid 275 indicates that it is much more similar to that from plasmid 155 than from plasmid 171 (μM/μS ratio of 0.3–0.18 in two independent transfections). Therefore, the μS poly(A) site is now utilized more, suggesting that neither the Cμ3 exon nor the donor 5′-splice site is crucial.

DISCUSSION

The major conclusion that can be drawn from our experiments is that processing of μ gene transcripts is not only regulated *in trans* by changes in the level of polyadenylation factors that allow differential utilization of poly(A) sites with different intrinsic affinities for these factors, but that upstream sequences within the gene can have a significant impact on the regulation. Inasmuch as the choice between μS polyadenylation and splicing to μM exons has always been viewed as a competition between two mutually exclusive events, the observation that overexpression of rate limiting polyadenylation factors increases μS polyadenylation (8) provides a relatively simple mechanistic explanation for the increase in μS mRNA expression upon B cell differentiation. However, the lack of specificity of general polyadenylation factors fails to explain, in a completely satisfactory manner, how this effect is targeted towards the μ gene selectively in B cells. In the experiments described here we show that alteration of specific μ gene sequences can affect processing to the μM exons even in plasmacytoma cells that have been documented to express an excess of CstF-64 (9). In two separate types of mutations (202 and 218) that should impinge on appropriate usage of the proximal poly(A) site, it is not processing at this site that is affected but rather processing to μM is perturbed. Whereas these mutations targeted either the μS poly(A) site itself or the terminal exon for processing to μS, Cμ4, other mutations involving the terminal exon and the immediately preceding intron yielded more subtle, yet detectable effects on processing to μM. In plasmids 171 and 231 the precision of splicing to μM is not disturbed but the level is much increased when compared with the unmutated gene. It is only when the entire Cμ3–Cμ4 intron as well as the Cμ4 exon is restored that proper μM/μS regulation is restored. The Exon Definition Model (28) predicts that the terminal exon and immediate upstream splice sequences constitute important elements for utilization of the proximal poly(A) site. This hypothesis is not violated in mRNA encoded by these constructs since the μS poly(A) site in all of the mutants is always properly utilized. In the case of μM mRNA synthesis, however, the presence of an unmutated terminal μM exon does not ensure proper processing of the mRNA. Rather, the evidence presented herein suggests that choice of the μM exons is affected by more remotely located upstream donor and acceptor splice sites. Since neither intronic nor exonic sequences were disturbed in the μM region it is unlikely that proximal enhancers (30,31) affect the acceptor splice sites.

The conclusion that upstream exon–intron structures are important in dictating whether the μS poly(A) site is preferentially utilized even under conditions where polyadenylation factors may be in excess differs somewhat from that arrived at by Peterson *et al*. (23), whose interpretation was based on a series of constructs which contain, for the most part, only one exon upstream of the terminal exon, thus greatly reducing the extent of upstream processing. Although this strategy reduces the complexity of the system, it may fail to detect the effects of these processing events on the final steps leading to μS or μM mRNA synthesis. However, it should be noted that, based on their evidence for the importance of exon size, we have taken care to ensure that all the exon sizes in the constructs utilized in this study did not differ substantially from the wild-type.

It should be borne in mind that the dramatically higher level of IgH chain gene transcription in activated B cells (1,2) would require a concomittent increase in the rate limiting processing factors such as CsF-64. Clearly this increase would favor the usage of weaker poly(A) sites in general and polyadenylation at these sites may be independent of upstream sequences (10–11,15). However, the discrepancy in the level of increased usage of the proximal poly(A) site between the transgene and the endogenous gene in transgenic animals (15) suggests that specific sequences or exon–intron structures within the μ gene play an additional role in targeting of the general processing factors to ensure that μ transcripts are processed preferentially.

The reasons underlying the propensity for deletion of the chimeric Cμ1–Cμ3 exon in transcripts from 255 and 273 are unclear, since the mutations did not alter donor or acceptor splice sites. Reinsertion of the 141 bp fragment that is deleted in 255 in a reverse orientation resulted in totally aberrant downstream splicing to all of the exons (data not shown). Therefore, possibly an enhancer for downstream splicing may be located within this exon (32) and experiments are in progress to further explore this possibility. The detection of exon deletion by RT–PCR analysis suggests that analysis of only 3′-termini performed for many previous studies may not be sufficient for examining all of the possible splice forms that could result from various mutations. A further case in point is our finding of aberrantly sized RNA derived from constructs 202 and 218 that would have escaped detection by S1 analysis. Indeed, our initial experiments, which utilized S1 analysis, did not reveal the aberrant splicing because the probe (Fig. 2) only detected differences in levels of Cμ4–μM spliced versus non-spliced forms. This possibility may also account for the difference in ratio of μM/μS determined by S1 analysis (Fig. 2) versus PCR analysis. Although alternative amplified sizes were not apparent in mRNA from 171 transfectants, however, some of these species may not have extended into sequences covered by the primers.

Previous experiments have also shown that choice of poly(A) site can be altered in J558L cells by reducing the distance between the μS and μM poly(A) sites (6,33). In this case a decrease in the extent of transcriptional termination was correlated with increased usage of the μM poly(A) site. In all of the constructs analyzed herein this distance has been maintained in the genomic context,

therefore it is unlikely that transcription termination should play a role in the effects observed. Differences in the extent of transcription progression have in fact not been detected between J558L cells transfected with 155 or 171 (D.Yuan, unpublished observations).

Likewise, differences in relative stability between the μS versus μM mRNA processed from these constructs cannot account for the changes in ratios observed, since no indications of half-life differences have ever been shown (5,21,26–27). We have also directly shown that the mutation created in 171 does not affect the stability of μS mRNA. The decay rates of mRNA derived from the other constructs have not been examined; however, it is unlikely that the respective μS versus μM stability will be different, since appropriate splice sites and exonic sequences were preserved in all of the other constructs and we have ascertained that the mutations did not result in the creation of termination codons that may affect their stability (34).

In conclusion, the finding that changes in upstream splice/processing signals can affect the relative utilization as well as fidelity of processing of the downstream μM exons even under conditions of optimal polyadenylation factor binding to the μS poly(A) site indicates that cleavage at this site does not necessarily precede splicing and points more towards a competitive process which is influenced by a number of factors (reviewed in 35). The results further emphasize the importance of considering transcription, splicing and polyadenylation as an integral unit that is connected by RNA polymerase (12–14,36). Thus it is possible that one of the contributors to regulation of the switch from μS to μM poly(A) site usage entails differential expression of splicing factors that modify the polymerases before reaching the final splice–polyadenylation choice.

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