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Poly(A) site choice rather than splice site choice governs the regulated production of IgM heavy-chain RNAs

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ABSTRACT **Alternative processing of the immunoglobulin μ primary transcript results in regulated production of mRNAs encoding the secreted (μ_s) and membrane-bound (μ_m) form of IgM heavy chain during B-cell development. To elucidate the basis for this control, we analyzed the expression of altered forms of the μ transcription unit. Deletion of intron sequence between the μ_s and μ_m exons, which reduces the distance between the two poly(A) sites as well as the distance between μ_m splice sites, enhances production of μ_m RNA. Correct expression is restored by insertion of heterologous sequences, demonstrating that spacing is indeed the critical aspect. The altered spacing appears to affect poly(A) site usage rather than splice site usage, since it was the distance between the poly(A) sites rather than the distance between splice sites that was found to be decisive. Finally, removal of either the $C_\mu 4$ splice donor or the m1 splice acceptor, thus eliminating normal μ_m splicing, does not increase usage of the μ_s poly(A) site. We therefore conclude that the major factor in determining the ratio of μ_s to μ_m is a poly(A) site choice rather than a splicing choice.**

It is clear from a number of studies that alternative RNA processing can alter the output of a transcription unit (see refs. 1 and 2 for review). One of the better-studied examples is the IgM heavy-chain (μ) transcription unit. During B-cell development, two forms of μ mRNA, one encoding the membrane-bound protein (μ_m) and one encoding the secreted protein (μ_s), are produced as a result of alternative processing of a primary transcript (3–5). We previously demonstrated that the switch from μ_m to μ_s production during B-cell differentiation was a complex process involving changes in several steps of mRNA biogenesis (6). One key aspect appears to be the relative strength of the two poly(A) sites as well as their position within the transcription unit. We suggested that part of the control was a change in the amount of a common factor used by the poly(A) sites (6, 7). However, assigning a role for a poly(A) site factor is complicated by the fact that the switch also involves a concomitant change in splicing. Indeed, others have argued that it is a splicing change that dictates poly(A) site choice rather than vice versa (8). Thus, although it is clear from past studies that poly(A) addition precedes splicing (9), it is possible that the poly(A) site choice is dictated by a prior selection of splice sites even though the actual splicing occurs later. In at least one other similar case, the calcitonin/calcitonin gene-related peptide transcription unit, it appears that the critical event is indeed a splice site choice (10). In an attempt to clarify the issue for the immunoglobulin μ transcription unit, we assayed expression of a variety of μ-encoding plasmids with altered splice sites and poly(A) sites. We conclude that the critical regulatory event is poly(A) site choice rather than splice site choice.

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METHODS

Cell Culture and Transfection. The procedures for growth and maintenance of the M12 lymphoma cell line and the J558L plasmacytoma line have been described, as have the methods for DNA transfection (6).

Plasmids. The pμ plasmid is the p$\mu\Delta$3 plasmid described by Grosschedl and Baltimore (11). The p$\mu\Delta$950 plasmid was constructed by BAL-31 exonuclease deletion starting at the single *Eco*RV site in the large intron followed by the addition of *Cla* I linkers. To construct the p$\mu\Delta$950λ plasmid, a 1.1-kilobase *Cla* I λ phage DNA fragment was inserted into the *Cla* I site of p$\mu\Delta$950. The construction of the p$\mu\Delta$300 and p$\mu\Delta$1000 plasmids has been described (6). The pμ1500 and pμ1570 plasmids were generated by inserting the m1 exon, flanked on both sides by 40 nucleotides (nt) of intron sequence and followed by a 1-kilobase fragment of plasmid pBR322 on the 3′ side, into the *Cla* I site of the plasmids p$\mu\Delta$1500 and p$\mu\Delta$1570, respectively. The p$\mu\Delta$1500 plasmid contains 370 nt downstream of the μ_s poly(A) site and 30 nt upstream of the m2 exon. The p$\mu\Delta$1570 plasmid contains 220 nt downstream of the μ_s poly(A) site and 100 nt upstream of the m2 exon.

The p$\mu\Delta$1200, p$\mu\Delta$1600, and p$\mu\Delta$1750 plasmids contain 1200-, 1600-, and 1750-nt intron deletions, respectively, created by BAL-31 nuclease digestions starting at the *Eco*RV site.

S1 Nuclease Assays. The procedures for S1 protection assay for mRNA and histone H4 RNA were described previously (6).

RESULTS

Spacing Within the μ Transcription Unit Affects the μ_s/μ_m Ratio. To assay for regulated expression of the μ transcription unit, we made use of the wild-type plasmid pμ as described previously (11). When pμ is transfected into a transformed analogue of a mature B cell (M12 lymphoma), there is approximately equal production of μ_m and μ_s RNAs, whereas when pμ is expressed in a plasmacytoma (J558L), the ratio shifts to 8:1 in favor of the μ_s RNA (6). We (6) and others (8) have previously demonstrated that deletion of intron sequences between the $C_\mu 4$ and m1 exons resulted in a change in the ratio in favor of μ_m. The underlying basis for this effect is complex and is also cell-specific. In the J558L plasmacytoma, the deletion allowed transcription to proceed through the μ_m poly(A) site rather than terminating upstream. In the M12 lymphoma, deletion of intron sequences had no effect on transcription; instead, the deletions altered RNA processing such that μ_m increased at the expense of μ_s. This result is reproduced in Fig. 1, in which expression of pμ is compared to that of p$\mu\Delta$950. We then assayed the plasmid

Abbreviations: μ_m, membrane-bound form of IgM heavy chain; μ_s, secreted form of IgM heavy chain; nt, nucleotide(s).

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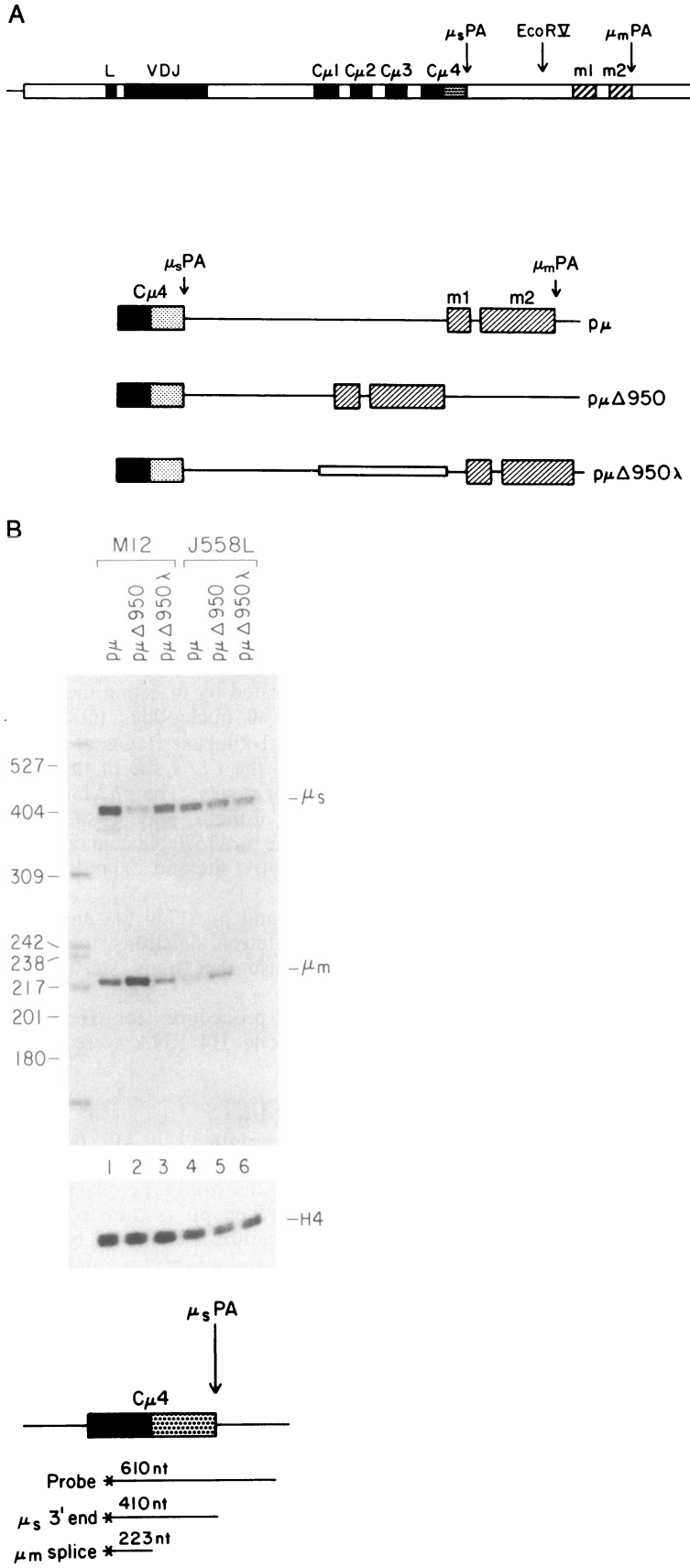

FIG. 1. Expression of μ intron-deletion and -substitution mutants in M12 and J558L cells. (*A*) Structure of the pμ, pμΔ950, and pμΔ950λ plasmids. The pμ plasmid is the pμΔ3 plasmid described by Grosschedl and Baltimore (11). The derivation of the other plasmids is described in *Methods*. Exons encoding the leader (L), variable–diversity–joining (VDJ), and constant ($C_\mu1$–$C_\mu4$) regions of the μ heavy chain are shown. The stippled portion of the $C_\mu4$ exon encodes the carboxyl terminus of the secreted form of the protein. Exons m1 and m2 encode the carboxyl terminus of the membrane-associated form. Poly(A) sites for μ_s and μ_m RNA are indicated μ_sPA and μ_mPA, respectively. (*B*) S1 nuclease protection assays. M12 cells or J558L cells were transfected with each of the indicated plasmids, and RNA was harvested 48 hr later. The μ_s and μ_m RNAs were detected by S1 protection assay using the probe depicted below the autoradiograph. Leftmost lane contained a *Hpa* II digest of pBR322 DNA to provide size markers (lengths given in nt). Transfection and S1 nuclease mapping assays were performed as described (6, 11). Histone H4 RNA was detected by a 5′ S1 assay as described (11).

pμΔ950λ, in which a segment of phage λ DNA has been inserted into pμΔ950 at the site of the original deletion. The inserted DNA restored wild-type expression, thus indicating that the original alteration did not involve a specific sequence element. We also conclude that the effects are directly related to spacing rather than simply a fortuitous alteration of RNA secondary structure, since the change in μ_s/μ_m ratio has been shown to be directly proportional to the size of the deletion (6, 8). In the case of J558L expression, the results of Fig. 1 indicate that transcription termination has been restored, thus preventing use of the μ_m poly(A) site. We conclude that the site of transcription

termination is not sequence-specific and that the signal for termination must reside elsewhere.

Distance Between Poly(A) Sites Is the Critical Factor for Determining Relative μ RNA Levels. In the M12 cell, the size of the $C_\mu 4$/m1 intron has an effect on the RNA processing to yield either μ_s or μ_m RNA. Two models could explain this result. First, if splice site selection were dominant, bringing the $C_\mu 4$ donor and m1 acceptor closer together might favor this reaction over cleavage at the μ_s poly(A) site. Alternatively, the deletion has also brought the μ_s and the μ_m poly(A) sites closer together, potentially positioning the distal μ_m site in a more favorable location in the nascent transcript. To distinguish these possibilities, we made several additional alterations of pμ (Fig. 2*A*). The plasmid pμΔ300, which has a 300-nt deletion of the $C_\mu 4$/m1 intron, is expressed in M12 and J558L cells equivalently to pμ. The distance between the $C_\mu 4$/m1 splice sites is 1560 nt and the distance between the two poly(A) sites is 1900 nt in pμΔ300. In contrast, these distances are reduced to 860 nt and 1200 nt, respectively, in the plasmid pμΔ1000. In the plasmids pμ1500 and pμ1570, the spacing between the poly(A) sites has been restored to that of pμΔ300 but the spacing between the $C_\mu 4$/m1 splice sites has been maintained close to that in pμΔ1000. Thus, if the phenotype of pμΔ1000 were due to bringing the splice sites closer together, we would expect similar expression from pμ1500 or pμ1570. However, if the pμΔ1000 phenotype were due to bringing the poly(A) sites closer together, then we would anticipate that the expression from pμ1500 or pμ1570 would revert to that of pμΔ300. These plasmids were assayed by transfection into either M12 cells or J558L cells (Fig. 2*B*). The ratio of μ_m to μ_s produced from pμΔ300 is 1:1 in M12 cells and 1:7 in J558L cells, similar to that of pμ. In contrast, the same ratios for pμΔ1000 were 5:1 in M12 and 1:1 in J558L. Examination of the expression of pμ1500 and pμ1570 clearly shows a phenotype similar to that of pμΔ300 and not that of pμΔ1000. The μ_m/μ_s ratios for each were 1:1 in M12 and 1:9 in J558L. We presume that the basis for the change in J558L cells is largely due to alleviating transcription termination, as we previously showed to occur as a result of the deletion (6). However, since transcription from pμ does not terminate prior to μ_m in M12 cells and the mutations cannot affect mRNA stability, the expression in M12 cells must be due to a change in processing. We thus conclude that it is the distance between the poly(A) sites rather than the distance between the splice sites that is the important factor.

Impairment of μ_m Splicing Does Not Affect μ_s Poly(A) Site Use. Finally, we probed the role of splicing versus polyadenylylation through the assay of two additional sets of pμ mutants (Fig. 3*A*). The plasmid series pμΔ1200, pμΔ1600, and pμΔ1750 have increasingly larger deletions originating in the $C_\mu 4$/m1 intron. In pμΔ1200 the m1 splice acceptor has been lost and in the other two plasmids, the m1 exon has been completely eliminated. Thus, normal splicing of $C_\mu 4$ to m1 can no longer take place with pμΔ1200, and neither $C_\mu 4$ to m1 nor m1 to m2 splicing can occur with pμΔ1600 and pμΔ1750. If there was a competition between such a splice and selection of the μ_s poly(A) site, one would anticipate an increase in production of μ_s RNA as a function of these deletions in the M12 lymphoma. An assay of these plasmids, compared to pμ, revealed that this was not the case. The production of μ_s RNA, if anything, declined further as a result of these deletions. And apparently, the deletion impaired μ_m processing as evidenced by the appearance of unspliced RNAs (RT bands). As a second approach, we created a small (60-nt) deletion (denoted SP in Fig. 3*B*) encompassing the μ_m splice donor, which would prevent normal μ_m splicing. Indeed, nuclease S1 analysis failed to detect formation of the normal m1 exon (data not shown). Despite this block in normal $C_\mu 4$/m1 splicing, there was little or no increase in the use of the μ_s poly(A) site. The slight increase seen in this analysis is largely nonspecific, as the H4

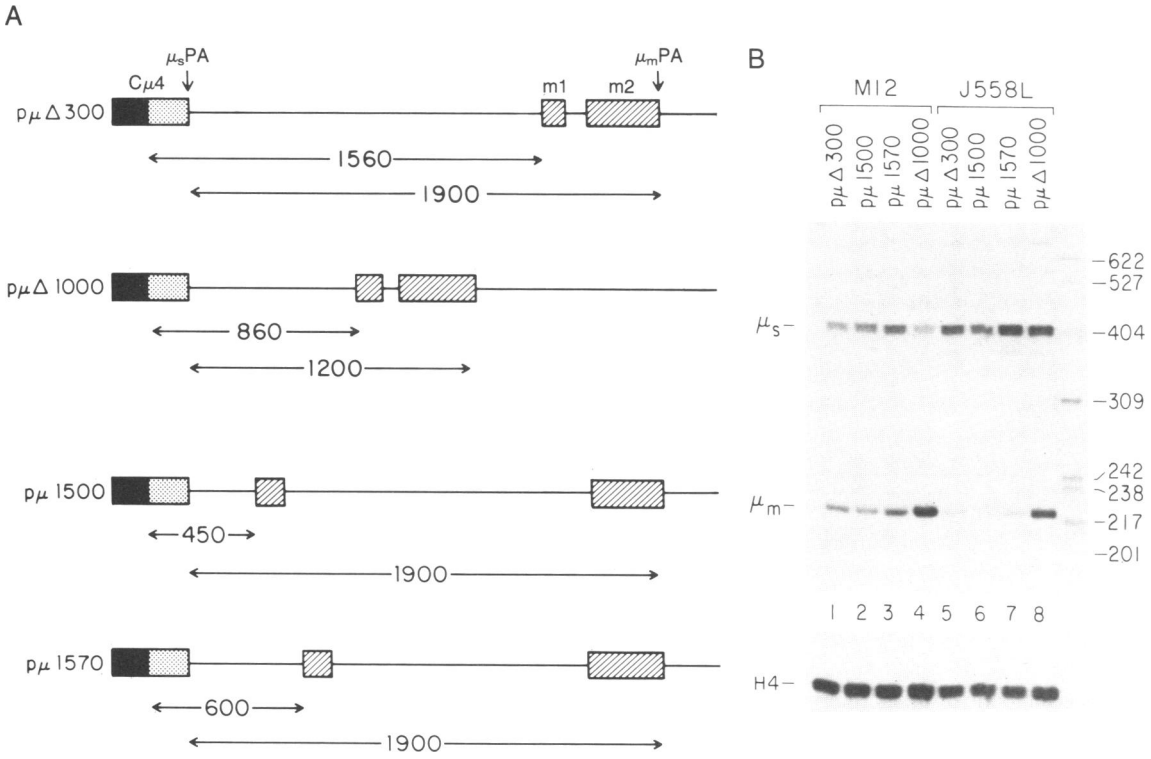

FIG. 2. Structure and expression of pμ spacing mutants. (*A*) Structure of the pμΔ300, pμΔ1000, pμ1500, and pμ1570 plasmids. The spacing (in nucleotides) between the $C_\mu 4$ donor splice site and the m1 splice acceptor and between the μ_s and μ_m poly(A) sites is indicated. (*B*) S1 nuclease protection assays. M12 cells or J558L cells were transfected with each of the indicated plasmids and RNA was prepared 48 hr later. The S1 protection assays were performed as for Fig. 1.

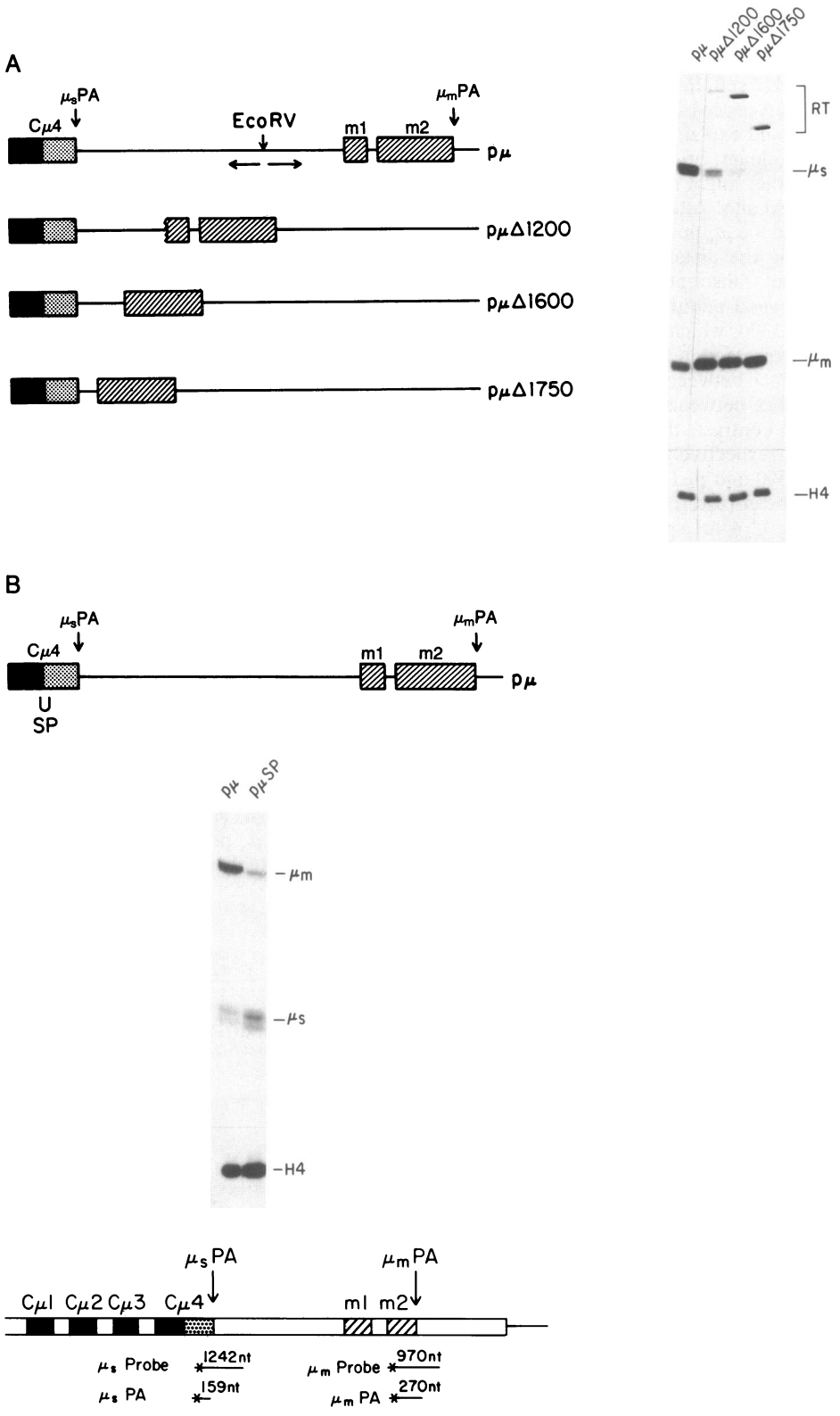

FIG. 3. Structure and expression of pμ splice site mutants. (*A*) Structure of the pμΔ1200, pμΔ1600, and pμΔ1750 plasmids. The S1 protected bands labeled RT are due to RNAs protecting the probe to the site of sequence divergence due to the deletion. Thus, these are RNAs unprocessed at either the $C_\mu 4$ splice donor or the μ_s poly(A) site. S1 nuclease protection assays were performed as for Fig. 1. (*B*) The pμSP plasmid contains a 60-nt deletion, between the *Bst*EII and *Bcl* I restriction sites in the $C_\mu 4$ exon, that removes the splice donor sequence. For assay of μ_s RNA from the pμSP plasmid, a *Bcl* I–*Eco*RV probe was prepared. The μ_m RNA was detected with a *Pst* I–*Xho* I probe. These probes are depicted in the diagram below the autoradiograph.

histone RNA internal control is also increased. We presume that the alteration in normal μ_m splicing is the basis for the reduction in total μ_m RNA. We thus conclude from these results, as well as those presented in Fig. 2, that the dominant factor in μ RNA production is selection of the poly(A) sites and that splicing most likely must follow this selection.

DISCUSSION

The clear result from these and previous experiments (6, 8) is the effect of intron deletions on μ_s/μ_m RNA production and that this is not due to a loss of specific sequence. Furthermore, the analyses of M12 cells—in which the effects must be due to an alteration of RNA processing, since the

mutations do not affect transcription and cannot affect mRNA stability—indicate that the deletions alter proper spacing in the primary transcript that affects poly(A) site use rather than splicing. Thus, in the case of the μ transcription unit, the dominant factor appears to be poly(A) site selection with the splicing pattern following from this initial choice. This is in apparent contrast to the manner in which the expression of another complex transcription unit, that encoding calcitonin/calcitonin gene-related peptide, is regulated (10). It was found that selection of splice sites in a cell-specific manner was the dominant factor in determining the final output of mRNA. Therefore, alternative RNA processing in a complex transcription unit, which in most cases involves both alternative splicing choices as well as alternative poly(A) site choices, can be directed by either splicing or poly(A) site utilization.

How might the spacing of the poly(A) sites within the μ transcription unit affect the relative usage? We previously demonstrated that the μ_m poly(A) site was probably stronger than the μ_s poly(A) site and that if the order of the poly(A) sites in the transcription unit was reversed, then there was exclusive use of the μ_m site (6). This result, together with previous data showing that poly(A) site selection occurred on nascent transcripts (9), argues that there is polarity to the selection process when a transcription unit contains multiple poly(A) sites. The data presented here indicate that the polarity is exhibited not just by the order but also as a function of the distance between the poly(A) sites. We suggest that the spacing of the two poly(A) sites affects the time in which the first poly(A) site is available to be utilized. When the downstream site is brought closer to the upstream site, the window of time in which the first site is available and can be used is made smaller. The probability of using the first site is therefore determined by this available time, the "strength" of the poly(A) site [possibly the affinity for a poly(A) site factor], and the concentration of the "factor." Thus, each of these elements, including the architecture of the transcription unit, would be important for the control of the output of RNAs. Interestingly, a comparison of the sequences of mouse, hamster, and human (ref. 12; P. W. T., unpublished data) reveals that evolution has conserved the spacing between the two poly(A) sites while the sequence of the introns has drifted dramatically. We speculate that this arrangement, along with a changing concentration of a common poly(A) site factor during B-cell differentiation, then determines the final ratio of IgM μ RNAs.

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