B-cell and plasma-cell splicing differences: A potential role in regulated immunoglobulin RNA processing

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ABSTRACT

The immunoglobulin μ pre-mRNA is alternatively processed at its 3′ end by competing splice and cleavage-polyadenylation reactions to generate mRNAs encoding the membrane-associated or secreted forms of the IgM protein, respectively. The relative use of the competing processing pathways varies during B-lymphocyte development, and it has been established previously that cleavage-polyadenylation activity is higher in plasma cells, which secrete IgM, than in B cells, which produce membrane-associated IgM. To determine whether RNA-splicing activity varies during B-lymphocyte development to contribute to μ RNA-processing regulation, we first demonstrate that μ pre-mRNA processing is sensitive to artificial changes in the splice environment by coexpressing SR proteins with the μ gene. To explore differences between the splice environments of B cells and plasma cells, we analyzed the splicing patterns from two different chimeric non-Ig genes that can be alternatively spliced but have no competing cleavage-polyadenylation reaction. The ratio of intact exon splicing to cryptic splice site use from one chimeric gene differs between several B-cell and several plasma-cell lines. Also, the amount of spliced RNA is higher in B-cell than plasma-cell lines from a set of genes whose splicing is dependent on a functional exonic splice enhancer. Thus, there is clear difference between the B-cell and plasma-cell splicing environments. We propose that both general cleavage-polyadenylation and general splice activities are modulated during B-lymphocyte development to ensure proper regulation of the alternative μ RNA processing pathways.

Keywords: Cleavage-polyadenylation; RNA splicing; SR proteins

INTRODUCTION

Alternative RNA processing is a common strategy for regulating gene expression, yet the mechanisms governing alternative RNA production in the appropriate cell types are not well-understood. RNA from the immunoglobulin (Ig) μ gene is alternatively processed at its 3′ end to encode the secreted and membrane-associated forms of the IgM protein, and this gene has been studied extensively as a model system for regulated alternative RNA processing (for review, see Peterson 1994b; Edwalds-Gilbert et al. 1997). An RNA encoding the secreted form of IgM (μs) is produced when a promoter-proximal cleavage-polyadenylation site is used, whereas an mRNA encoding the membrane-associated IgM (μm) is spliced to remove the μs poly(A) site and is cleaved and polyadenylated at the downstream μm poly(A) site. The relative amount of these two mRNAs changes during B-lymphocyte maturation; B cells produce similar amounts of the two mRNAs, whereas plasma cells produce 10- to 20-fold more μs than μm mRNA. The efficiencies of the competing cleavage-polyadenylation and splice reactions are balanced, which is critical for regulated μ pre-mRNA processing (Peterson and Perry 1989; Peterson 1992). RNA from a non-Ig gene that was modified to contain a poly(A) site within an intron was regulated in B-cell and plasma-cell lines (Peterson 1994a) and when it was expressed as a transgene in resting and activated mouse splenic B cells (Seipelt et al. 1998). These experiments (1) demonstrated that μ-gene sequences are not specifically required for processing regulation, (2) suggested that the regulatory mechanism involves changes in general RNA-processing factors, and (3) validated the use of tissue culture cell lines for studying this regulatory mechanism.

There is good evidence that cleavage-polyadenylation ac-

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tivity differs between B cells and plasma cells, and that these changes contribute to μ mRNA-processing regulation. Tandem poly(A) sites are differentially used in the two cell types (Peterson et al. 1991; Matis et al. 1996), the amount of Cleavage-stimulatory Factor (CstF) 64K subunit cross-linked to poly(A) sites differed between extracts from B-cell and plasma-cell lines (Edwalds-Gilbert and Milcarek 1995), the levels of CstF 64K increased when resting mouse B cells were stimulated with LPS (Takagaki et al. 1996), and overexpression of CstF 64K in a chicken B-cell line altered the endogenous μs/μm mRNA ratio (Takagaki et al. 1996). Thus, CstF 64K is likely to be a component of μ-processing regulation. However, the exact role of the CstF 64K protein in μs/μm regulation is not fully resolved; although μs/μm processing is regulated in established B-cell and plasma-cell lines, CstF 64K protein levels do not consistently differ between them (Edwalds-Gilbert and Milcarek 1995). This suggests that additional factors must impact μs/μm RNA-processing regulation when B cells mature to plasma cells. It is possible that cleavage-polyadenylation activity is modulated between B-cell and plasma-cell lines (Edwalds-Gilbert and Milcarek 1995; Yan et al. 1995; Martincic et al. 1998; Phillips et al. 2001; Veraldi et al. 2001). But, it is equally possible that the splicing environment is altered as B cells mature; this is a reasonable hypothesis, as the μs/μm mRNA ratio is determined by competing splice and cleavage-polyadenylation reactions that have balanced efficiencies.

Whether alterations in splicing are involved in μ RNA-processing regulation has been addressed previously, but individual studies reached different conclusions. When the μ gene was coinjected into *Xenopus* oocytes with nuclei from either B-cell, plasma-cell, or nonlymphoid-cell lines, the Cμ4-M1 splice reaction that removes the μs poly(A) site was seen only when B-cell nuclei were injected (Tsurushita et al. 1988). This suggested that B-cell nuclei contain a diffusable *trans*-acting factor(s) that stimulates μm mRNA splicing. Additional evidence of RNA metabolism differences between B cells and plasma cells has been reported. A lymphoma (B-cell line) was found to have a six- to sevenfold higher nuclear to cytoplasmic ratio of mature mRNA for both the Ig γ and κ genes and to accumulate more nuclear precursor RNA than a matched hybridoma cell line (Milcarek et al. 1998); similar results also were seen for the Ig μ RNAs (Nelson et al. 1983). Also, partially spliced transcripts from chimeric sarco/endoplasmic reticulum Ca^{2+}-ATPase genes were detected in transfected B-cell, but not plasma-cell lines (van den Bosch et al. 1996). These results suggest that B cells and plasma cells differ both in nuclear-cytoplasmic transport and general pre-mRNA-processing efficiencies. On the other hand, when two different RNAs, each containing a suboptimal 5′ splice site in competition with a more efficient 5′ splice site, were expressed in B-cell and plasma-cell lines, the B cells did not specifically activate the suboptimal splice site (Peterson et al. 1991). Also, a μ gene that lacked the μs poly(A) site produced some unprocessed RNA in addition to spliced μm mRNA, but the ratio of spliced RNA to this other RNA did not vary substantially between B-cell and plasma-cell lines (Watakabe et al. 1991). One interpretation of these latter results is that splicing is the same between the two cell types. However, another explanation for these results is that the competing reactions in these substrates were not balanced within the range necessary to detect differences in the cellular splice environment. Therefore, they do not rule out the possibility that differences in splicing activity contribute to μs/μm regulation, and this question remains unresolved.

To determine whether μ pre-mRNA-processing regulation is sensitive to splicing changes, we artificially modified the splicing environment by transiently coexpressing the μ gene with several serine/arginine-rich (SR) proteins that can function in alternative splicing regulation (Fu 1995; Manley and Tacke 1996; Graveley 2000). We found that the μs/μm mRNA ratio was altered by some of the SR proteins, indicating that the balance between the splice and cleavage-polyadenylation reactions in the μ pre-mRNA can be modified by splicing factors. To explore whether the splicing environments of B cells and plasma cells do differ, we analyzed the splicing patterns from two different chimeric non-Ig genes that can be alternatively spliced, but do not contain a competing cleavage-polyadenylation reaction. If these genes are differentially spliced in B-cell and plasma-cell lines, this would establish that the splicing environments differ functionally between these two cell types. Here, we show that several B-cell lines splice a chimeric gene differently than several plasma-cell lines. As an initial attempt to identify potential *cis*-acting splice regulatory elements in the μ gene, we inserted multiple overlapping fragments from the Cμ4 exon into a gene whose splicing is dependent on a functional exonic splice enhancer (ESE). We found that multiple fragments activated splicing and, in support of our model, the amount of spliced RNA was always higher in B-cell than plasma-cell lines. Thus, we demonstrate that there is clear difference between the B-cell and plasma cell-splicing environments, and that the μ gene is sensitive to artificial changes in splicing-factor expression. Taken together, these results suggest a model wherein changes in both general cleavage-polyadenylation and splice activities during B-lymphocyte development ensure proper regulation of alternative μ mRNA-processing pathways.

RESULTS

Splicing environment changes affect μ RNA processing

If our model that splice environment differences between B cells and plasma cells contributes to the developmental changes in μs/μm mRNA processing is correct, then μ mRNA processing may be sensitive to artificial changes in SR protein levels. Whereas individual SR proteins have been

shown to alter splice patterns when overexpressed in vivo with splice substrates that contain competing 5′ splice sites (Caceres et al. 1994; Screaton et al. 1995; Wang and Manley 1995), competing 3′ splice sites (Bai et al. 1999) and exons that could be either included or excluded (Caceres et al. 1994; Jiang et al. 1998), it is not known whether the μ pre-mRNA, which contains a splice reaction in competition with a cleavage-polyadenylation reaction (Fig. 1A), responds to changes in SR protein expression. To assess this, we transiently cotransfected the M12 B-cell and the S194 plasma-cell lines with the μ gene and several SR protein-expression vectors (Screaton et al. 1995) and quantitated the μs/μm mRNA ratio by S1 nuclease protection analysis (Fig. 1B,C). SF2/ASF (SF2), SRp30c, and SRp55, when coexpressed with the μ gene, decreased the μs/μm ratio about twofold compared with the pCDM8 vector control in both cell lines (Fig. 1B,C). This suggests that these SR proteins enhanced the Cμ4-M1 splice reaction relative to cleavage-polyadenylation at the μs poly(A) site. Although some coexpressed SR proteins have been shown to dramatically shift the RNA-splicing patterns of specific substrates in vivo (Caceres et al. 1994; Du et al. 1997; Bai et al. 1999; ten Dam et al. 2000), a twofold change in splice ratios has also been reported with other splice substrates (Caceres et al. 1994; Screaton et al. 1995; Jiang et al. 1998; Bai et al. 1999). There was no significant change in μ processing with coexpressed SRp40 and SC35 in either cell line (Fig. 1B,C). Because the Cμ4 exon contains an SC35-responsive ESE required to splice the constitutive Cμ3–Cμ4 intron in vitro (Chandler et al. 1997), the fact that overexpression of this SR protein did not affect the μs/μm mRNA ratio suggests that this ESE participates in Cμ3–Cμ4 splicing without substantially affecting Cμ4–M1 splicing. Interestingly, SRp20 coexpression increased the μs/μm ratio twofold in the plasma-cell line; a slight increase was also observed in the B-cell line (Fig. 1B,C). SRp20 has been identified to be a part of a cleavage-polyadenylation enhancer complex (Lou et al. 1998), but it also often affects alternatively spliced substrates differently than other SR proteins (e.g., Screaton et al. 1995; ten Dam et al. 2000). Thus, in the μ pre-mRNA, it is not clear whether SRp20 is enhancing cleavage-polyadenylation or repressing splicing. It is likely that the SR proteins were expressed in excess in these transient transfection assays, as altering the ratio of SR expression construct to μ gene con-

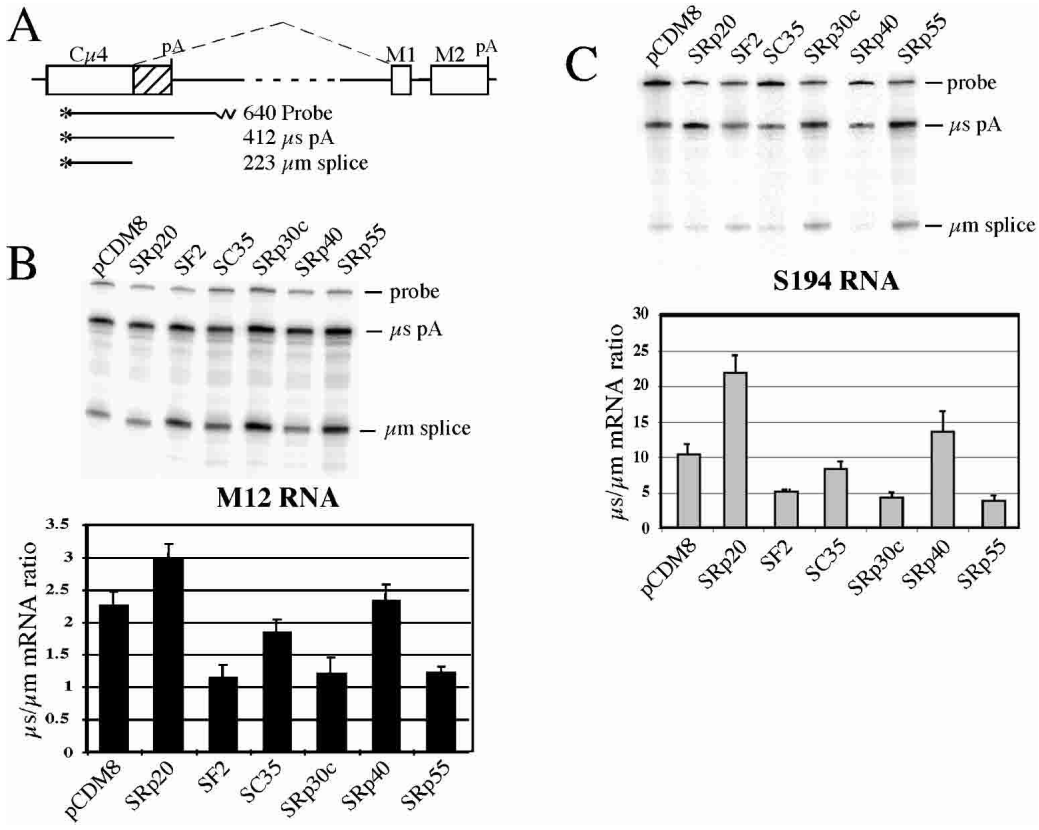

FIGURE 1. μ gene–SR protein cotransfections. (*A*) Diagram of the alternative RNA-processing reactions at the 3′ end of the Cμ gene, and the S1 probe used to distinguish cleaved-polyadenylated μs mRNA from spliced μm mRNA; the sizes of the protected fragments are shown. (*B*) Equal amounts of pSV5Cμ and the SR protein constructs shown above each lane (pCDM8 is the empty vector) were cotransfected into the M12 B-cell line, and RNA was analyzed by S1 nuclease protection assay. The probe and protected fragments are identified on the *right*. The S1 protection assays were quantitated on a PhosphorImager and expressed as a ratio of μs to μm mRNA. Each bar represents at least two independent transfections analyzed two or more times. (*C*) Cotransfection analysis in the S194 plasma-cell line as described in *B*.

struct from 1:7 to 7:1 did not affect μ expression substantially (data not shown). However, because only 5%–10% of the cells are transfected, we are not able to measure the level of overexpression achieved in the transfected cells. When the μ gene and SF2 were coexpressed with either SRp30c or SRp55 in M12 B cells, the μs/μm ratio was reduced below that seen with either individual SR protein (data not shown). Taken together, these results show that μs/μm pre-mRNA processing can respond to artificial changes in the splicing environment.

The splicing environment of B cells and plasma cells is different

Having established that the μ gene is able to respond to splice environment changes, we tested whether the splice environments of B cells and plasma cells differ by expressing chimeric non-Ig genes that could be alternatively spliced, but did not have a competing cleavage-polyadenylation reaction in the M12 B-cell and S194 plasma-cell lines. We have shown previously that the unusually large fourth exon from the mouse polymeric Ig receptor (pIgR) gene, when placed in the third intron of the mouse major histocompatibility complex (MHC) class I D^d gene (D^d-pIgR or pIgR, Fig. 2A), is constitutively spliced into the mRNA in the human HepG2 liver-cell line (Bruce et al. 1999; Bruce and Peterson 2001). However, when the 5′ splice site of pIgR exon 4 was weakened by a point mutation (D^d-pIgR 5′SS or 5′SS, Fig. 2A), a cryptic 5′ splice site 158 nucleotides into the pIgR exon was partially activated (Fig. 2B, Bruce and Peterson 2001). We transiently transfected D^d-pIgR and D^d-pIgR 5′SS into M12 B cells and S194 plasma cells and quantitated the alternatively spliced RNAs by S1 nuclease protection assays using a probe that will distinguish RNA that contains the intact exon from RNA that has been spliced at the cryptic 5′ splice site (Fig. 3C). We also monitored expression by RT–PCR (data not shown), which provides information on the size of the pIgR exon spliced into the D^d RNA, as described previously (Bruce et al. 1999; Bruce and Peterson 2001). The ratio of full-length to cryptic RNA expressed in the S194 plasma-cell line was similar to that seen previously in HepG2 cells; in pIgR, exon 4 was spliced intact, whereas the 5′SS mutation partially activated the previously identified upstream cryptic 5′ splice site (Fig. 3A). When these constructs were expressed in the M12 B-cell line, however, very different results were obtained. Surprisingly, RNA spliced at the cryptic 5′ splice site was detected even when the pIgR exon was not mutated (Fig. 3A). The cryptic 5′ splice site was further activated by the 5′SS mutation; the cryptic splice site was used more frequently than the authentic 5′ splice site in M12 cells (Fig. 3A). For each construct, the ratio of full-length to cryptically spliced RNA was 7- to 11-fold higher in the plasma cells than in the B cells. Several other pIgR mutations (Bruce and Peterson 2001) were also expressed in M12 and S194 cells with similar results; in all cases, the upstream cryptic splice site was recognized more frequently in the M12 B cells than in the S194 plasma cells (data not shown). These results were also confirmed by stably transfecting pIgR and 5′SS into the M12 and S194 cell lines (data not shown). Thus, these two cell lines clearly differentially splice the chimeric D^d-pIgR gene.

To assure these processing differences were due to a true cell-type difference in splicing environment and were not specific to these particular cell lines, we transiently expressed both pIgR and 5′SS in two other B-cell lines and two other plasma-cell lines. Like the M12 B cells, both the A20 B-cell and the 70Z/3 early-B-cell lines recognized the cryptic 5′ splice site in pIgR, whereas the J558L and the MPC11 plasma-cell lines spliced this exon intact, similar to the S194 plasma cells (Fig. 3B). The cryptic splice site was further activated in the A20 and 70Z/3 B-cell lines by the 5′SS mutation; the expression ratios are similar to those in the M12 cell line. In the J558L and MPC11 plasma-cell lines, 5′SS also activated some cryptic splice site use as it had in the S194 cell line (Fig. 3B). The similarity in pIgR and 5′SS expression patterns among the three B-cell and three plasma-cell lines confirms that there are functional differences in the splicing environments between these two cell types.

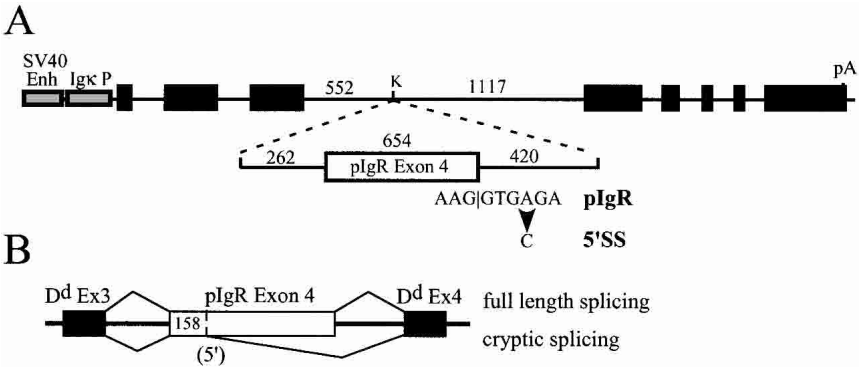

FIGURE 2. Chimeric D^d-pIgR construct. (*A*) The 654-bp pIgR exon 4, with 262 bp and 420 bp of surrounding intron sequence, was placed into the *Kpn*I (K) site in intron 3 of the D^d gene. The open box represents the pIgR exon, the black boxes are D^d exons, and thin lines are introns. The 5′ splice-site sequence at the end of pIgR exon 4 is shown; the A at the +4 position was mutated to C in the construct 5′SS (Bruce and Peterson 2001). (*B*) Diagram of the splicing patterns of the D^d-pIgR RNA and the 5′SS mutant in a nonlymphoid cell line. The full exon is spliced in D^d-pIgR (splice pattern shown above) and a cryptic 5′ splice site 158 nucleotides into exon 4 is activated in 5′SS (splice pattern shown below) (Bruce et al. 1999; Bruce and Peterson 2001).

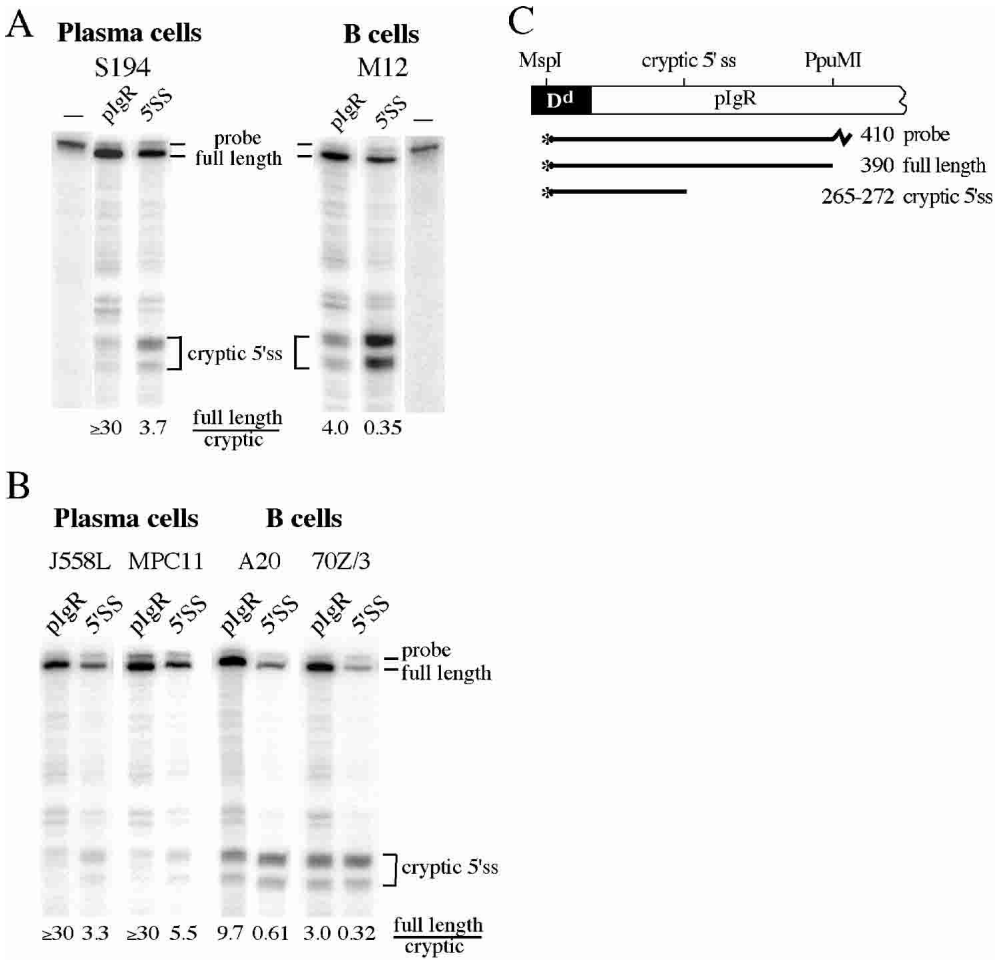

FIGURE 3. D^d-pIgR constructs are differentially spliced in B-cell and plasma-cell lines. (*A*) RNA from S194 plasma cells and M12 B cells mock transfected (−) or transiently transfected with the constructs shown above each lane was analyzed by S1 nuclease protection. The probe and protected fragments are labeled. The S1 reactions were quantitated by PhosphorImager analysis and expressed as a ratio of full-length to cryptically spliced RNA. Multiple protected bands are observed with the cryptic 5′ splice-site RNA due to fortuitous homology between the probe and the sequences in D^d exon 4; the fragments were combined for quantitation. The values shown below each lane are the mean of least two independent transfections, analyzed two or more times; the standard deviation of each mean was <10%. Because of variable lane background, we cannot reliably measure ratios above 30 (Bruce and Peterson 2001); other bands in the lanes are considered background because they do not correspond to bands seen by RT–PCR analysis of this RNA. (*B*) D^d-pIgR and D^d-5′SS were transiently expressed in the J558L and MPC11 plasma-cell lines and the A20 and 70Z/3 B-cell lines, and the RNA was analyzed by S1 nuclease protection. The probe and protected fragments are labeled and the reactions were quantitated as described above. (*C*) Diagram of the D^d-pIgR S1 nuclease protection analysis probe that distinguishes full-length from cryptically spliced RNA. RNA that has spliced the full-length pIgR exon into D^d protects the probe to the *Ppu*MI site, and cryptically spliced RNA protects the probe to the cryptic 5′ splice site; the expected sizes of each product are indicated.

Multiple μ gene sequences respond to the splicing environment differences of the B cells and plasma cells

Because the splice environments of B cells and plasma cells differ and the μs/μm mRNA ratio could be altered by co-expressing the μ gene with some SR proteins, we proposed that there may be exonic sequences in the μ gene that contributed to RNA-processing regulation. The possibility that specific exonic sequences contribute to μs/μm mRNA regulation does not conflict with our model that global changes in RNA-processing factors modulate μ alternative processing, as ESE sequences are degenerate and are likely to be found throughout most pre-mRNAs (Liu et al. 1998; Schaal and Maniatis 1999). Exonic splice silencer (ESS) sequences are less well-defined, but are also likely to appear frequently throughout genes (Cartegni et al. 2002). We reasoned that if we could find a fragment from the μ gene that differentially activated splicing in B cells and plasma cells, we could identify the interacting protein and thus, a component of μs/μm regulation. Although an ESE that affects M1–M2 splicing and, indirectly, Cμ4–M1 splicing, has been identified in M2, there is no evidence that this element contributes to μs/μm regulation (Watakabe et al. 1991, 1993). Thus, we first examined sequences within the Cμ4 exon. We used the minigene μAVWT.BSC (μAV; Fig. 4A; Xu et al. 1993), which contains a truncated avian sarcoma virus (ASV) *env* intron that requires an ESE downstream from

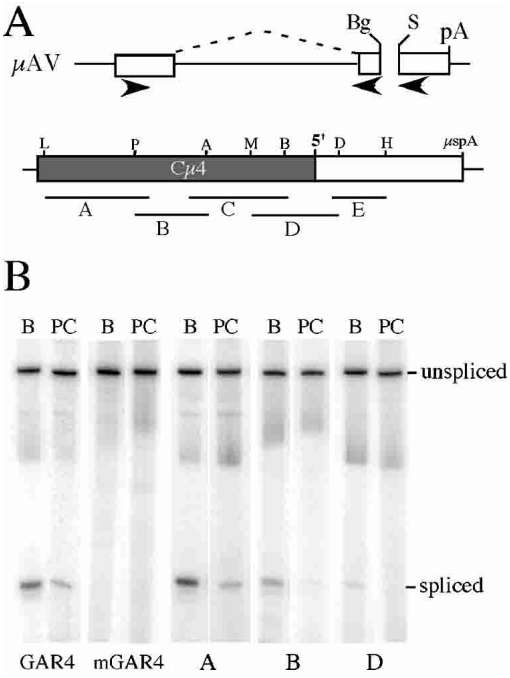

FIGURE 4. μAV-derived RNAs are differentially spliced between B cells and plasma cells. (*A*) The μAVWT.Bsc vector (μAV) that requires an exonic splice enhancer to be spliced is diagrammed. The arrows below are the PCR primers used to analyze the spliced vs. unspliced RNA; the downstream-most primer was used to monitor the size of the two RNAs, whereas the upstream two primers were used in quantitation. The diagram below identifies the overlapping fragments from the Cμ4 exon that were inserted between the *Bgl*II (Bg) and *Spe*I (S) sites of μAV to test for ESE activity; (L) *ApaLI*; (P) *Pst*I; (A) *Apa*I; (M) *Msp*I; (B) *BstE*II; (D) *Dpn*I; (H) *Hae*II. (*B*) Representative RT–PCR reactions of RNA from M12 B cells (B) and S194 plasma cells (PC) that were stably transfected with the construct shown below each pair of lanes. The bands representing the unspliced (308 nucleotides) and spliced (82 nucleotides) RNAs are shown.

the 3′ splice site to be spliced, to monitor Cμ4 fragments for splice-enhancing activity. We cloned overlapping 60–127-bp fragments from the Cμ4 exon into μAVWT.Bsc (Fig. 4A); the A fragment contains SC35-dependent ESE activity that is required for the Cμ3–Cμ4 intron to be spliced in vitro (Chandler et al. 1997). These μAV derivatives were stably transfected into the M12 B-cell and S194 plasma-cell lines, total RNA was isolated, and the spliced and unspliced RNAs were quantitated by RT–PCR using an end-labeled primer. Splicing was activated when four copies of a synthetic purine-rich sequence was inserted in the downstream exon, but not when a mutant version of this sequence was present (GAR4 vs. mGAR4; Fig. 4B; Table 1; Xu et al. 1993). We found that, in addition to the A fragment, several others had ESE activity that was above background in M12 B cells (Fig. 4B; Table 1). Interestingly, rather than finding one fragment that directed μAV to be differentially spliced between B cells and plasma cells, we found that most of the μAV constructs with measurable splicing activity were more efficiently spliced in B cells than plasma cells. This was also true for the positive control GAR4 (Fig. 4B; Table 1). Several fragments that had weak ESE activity in M12, D and E, did not activate detectable spliced RNA in S194 cells (Fig. 4B; Table 1). The C fragment was only weakly spliced in both cell types (Table 1). Total RNA was assayed in these experiments, but we have also analyzed both nuclear and cytoplasmic RNA fractions from several transfected lines; relatively more of the spliced RNA was found in the cytoplasm compared with the nucleus, but the unspliced RNA was also clearly present in the cytoplasm, and the differences in "% spliced RNA" observed between B-cell and plasma-cell lines were seen in both cellular compartments (data not shown). Thus, this cell-type difference in expression is due to splicing activity, and nuclear-cytoplasmic RNA transport variations do not contribute substantially.

Whereas some of the differences in activity among the Cμ4 subfragments may be due to the strength of an ESE element, there could also be multiple enhancer and/or silencer elements within the fragments. The ESEfinder program (http://exon.cshl.org/ESE/) identified numerous potential sites for ASF/SF2, SRp40, SC35, and SRp55 within the Cμ4 fragments. However, the number of predicted sites did not correlate with the splicing activity stimulated by each fragment. For example, fragment C was predicted to have nearly twice as many motifs for each SR protein as fragment A, including more with high-homology scores, yet it was substantially less active than fragment A in stimulating μAV splicing. We do not yet know enough about ESS sequences to be able to identify them by sequence, but the variable presence of these elements may also affect the overall activities within the Cμ fragments. Nevertheless, it is clear from our experimental analysis that the Cμ4 exon contains multiple sequences that can enhance μAV splicing. But more importantly, most of the μAV derivatives were differentially spliced between B-cell and plasma-cell lines. Because SR proteins are known to interact with ESE se-

TABLE 1. μAV derivatives are differentially spliced in B cells and plasma cells

	% Spliced	
Fragment (bp)	B cell	Plasma cell
Bsc (–)	–	–
GAR4 (37)	++++	++
mGAR4 (37)	–	–
A (121)	++++	++
B (85)	+++	+/–
C (127)	+	+
D (110)	+	–
E (60)	+/–	–

% spliced ranges: –, <1%; +/–, 1%–2%; +, 2%–8%; ++, 8%–16%; +++, 16%–32%; ++++, >32%

The % spliced RNA for each construct expressed in the M12 B cell and S194 plasma cell was quantitated by RT–PCR as detailed in Materials and Methods. The range of values are shown below.

quences to modulate splice activity (Fu 1995; Manley and Tacke 1996; Graveley 2000), one interpretation of these results is that the levels of multiple SR proteins may differ between the B-cell and plasma-cell lines. However, it is also possible that the functional splice environment differences that we detect with both the D^d-pIgR and μAV plasmids may be due, not to changes in the SR proteins themselves, but rather to changes in factors that modify the activity of, cooperatively interact with, or compete with the SR proteins or other components of the splice machinery. In either case, the difference between the B-cell and plasma-cell splice environment is likely to be more complex than can be approached using a standard biochemical strategy to identify individual RNA-binding proteins involved in μs/μm regulation.

DISCUSSION

Previous studies have shown that the regulated changes in μs/μm mRNA processing during B-lymphocyte development do not require specific μ gene sequences (Peterson 1994a; Seipelt et al. 1998). Thus, μ processing is likely controlled by general RNA-processing factors; theoretically, this could be due to changes in general cleavage-polyadenylation activity, general splice activity, or a combination of both. Evidence has accumulated to suggest that cleavage-polyadenylation activity is higher in plasma cells, which preferentially use the μs poly(A) site, than in B cells (Peterson et al. 1991; Edwalds-Gilbert and Milcarek 1995; Matis et al. 1996; Takagaki et al. 1996; Veraldi et al. 2001). By using two different RNA substrates that could be alternatively spliced, with no competing cleavage-polyadenylation reaction, we have demonstrated here that the splice environment is also clearly different between B cells and plasma cells. B cells spliced the large pIgR exon 4 using an internal cryptic 5′ splice site more frequently than plasma cells, and they spliced the μAV-derived RNAs more frequently than did plasma cells. These results appear to contradict the conclusion of a previous experiment that found no evidence for a change in splicing activity between B-cell and plasma-cell lines (Peterson et al. 1991). However, this previous experiment tested the limited hypothesis that a splice factor, such as a novel U1 small nuclear RNA, was present in B cells that specifically recognized the suboptimal Cμ4 5′ splice site or the mutant adenovirus E1A 12S 5′ splice site. In fact, an adenovirus E1A splice substrate that contained the competing wild-type 12S and 13S 5′ splice sites was included in these experiments as a control for the RNase protection analysis, but the expression wasn't quantitated (Fig. 2 in Peterson et al. 1991). We recently quantitated this data and found that, in two independent experiments, the E1A 12S:13S ratio was 1.7- to 2.1-fold higher in the 3-1 pre-B cells than in the S194 plasma cells. This is similar to the change in use of tandem poly(A) sites that was measured in these same cells (Peterson et al. 1991) and in a similar independent experiment (Matis et al. 1996). This, then, is a third example of an alternative splice reaction that is differentially modulated between B cells and plasma cells.

Because the splicing environment of B cells and plasma cells differ, we propose that this contributes to the mechanism that regulates alternative μ mRNA processing during B-lymphocyte development. If this is true, then the μ pre-mRNA, which has a splice reaction in competition with a cleavage-polyadenylation reaction, should respond to changes in the splice environment, independently of changes in cleavage-polyadenylation efficiency. We artificially altered the cellular-splicing environment by transiently overexpressing SR proteins with the μ gene and found four different SR proteins that altered the μs/μm ratio. However, although these cotransfection studies demonstrate that μs/μm mRNA processing can respond to changes in SR protein levels, this does not mean that changes in these specific SR proteins are responsible for μs/μm regulation during B-lymphocyte maturation or for the difference in splicing environment that we observed between B cells and plasma cells. This only indicates the potential for splicing regulation to occur through an SR protein-dependent mechanism. In fact, regulation is unlikely to be mediated by gross changes in the SR protein levels. Obvious changes in SR protein levels were not detected in populations of normal resting B cells and cells induced to differentiate with LPS, although minor differences in the amount of SRp55 could be observed (Takagaki et al. 1996). Therefore, the artificially overexpressed SR proteins may substitute for factors that normally change during lymphocyte development or alter the balance among different RNA-processing factors. For example, the overexpressed SR proteins could be acting in an exon-independent manner by stabilizing and/or bridging interactions between factors bound at the 3′ and 5′ splice sites or the 3′ splice site and the μs poly(A) site (for review, see Graveley 2000). Alternatively, they could affect the balance between an SR protein and an antagonizing activity (e.g., Mayeda and Krainer 1992; Hanamura et al. 1998; Barnard and Patton 2000; Eperon et al. 2000; Cowper et al. 2001) or affect a modification or subcellular localization step that modulates SR protein activity (e.g., Colwill et al. 1996; Prasad et al. 1999).

On the basis of the data presented here and reported previously (Peterson et al. 1991; Peterson 1994a; Edwalds-Gilbert and Milcarek 1995; Takagaki et al. 1996), we propose that changes in both general cleavage-polyadenylation and splicing activities during B-cell maturation ensure proper regulation of the alternative μ RNA-processing pathways. Our data are consistent with B cells splicing weaker splice sites more efficiently than plasma cells; the Cμ4 5′ splice site is known to be suboptimal (Peterson and Perry 1989), the cryptic 5′ splice site in pIgR exon 4 is weaker than the authentic pIgR 5′ splice site (Bruce and Peterson

2001), and the μAV 3′ splice site is suboptimal and requires a downstream ESE. This enhanced splice activity in B cells, in combination with lower cleavage-polyadenylation activity, would result in Cμ4–M1 splicing competing successfully with cleavage-polyadenylation at the suboptimal μs poly(A) site in B cells. In plasma cells, we propose that cleavage-polyadenylation activity is increased, whereas weaker splice site use is no longer augmented. Thus, the suboptimal Cμ4 5′ splice site does not effectively compete with the increased use of the μs poly(A) site and, in the D^d-pIgR pre-mRNA, only the authentic pIgR 5′ splice site is used, and the large exon is spliced intact. This model, wherein both splice and cleavage-polyadenylation activities are modulated relative to each other, does not require that there be large changes in either activity, as the Ig genes, which have balanced competing processing signals, would be able to respond effectively to modest changes in each reaction.

Firm proof of this model will require that the processing factors whose activity or expression differs between B cells and plasma cells be isolated and shown to affect μs/μm processing. Candidate factors include hnRNP F and hnRNP H; the ratio of these two proteins was found to differ between B-cell and plasma-cell lines and to modulate in vitro cleavage-polyadenylation activity (Veraldi et al. 2001). Also, the amount of U1A associated with U1 snRNP decreased ~25% in a human B-cell line after it was stimulated to differentiate, whereas the amount of U1A in a snRNP-free complex did not change (Milcarek et al. 2003). It is not yet clear whether changes in hnRNP F and hnRNP H (Min et al. 1995; Gamberi et al. 1997) or U1snRNP contribute to the developmental changes in RNA-splicing activity that we have detected. To obtain a more complete view of the μ regulatory mechanism, a more global approach to identifying differentially expressed genes is required. Because a large body of evidence indicates that changes in a cascade of transcription factors drives B cells to terminally differentiate to plasma cells (for review, see Calame 2001), it is likely that some of the transcriptionally regulated genes will encode proteins that influence μs/μm RNA-processing regulation. Microarray analyses of RNA from stimulated and unstimulated B cells can be used to identify candidate regulatory factors, which then must be overexpressed and underexpressed to verify their roles in μ-processing regulation. Characterizing the RNA-processing factor differences between B cells and plasma cells should uncover new information not only on μ RNA-processing regulation, but also on the competitive and/or cooperative interactions that may take place between the splicing and cleavage-polyadenylation machinery.

MATERIALS AND METHODS

Plasmid constructions

The cDNAs for the human splicing factors SRp20, SF2/ASF, SC35, SRp30c, SRp40, and SRp55 are cloned into the pCDM8 expression vector (Screaton et al. 1995) and were a generous gift from Gavin Screaton (Institute of Molecular Medicine, John Radcliff Hospital).

The Ig μ plasmid pSV5Cμs-m (Peterson and Perry 1986) and the D^d-pIgR chimeric gene and its derivatives (Bruce et al. 1999; Bruce and Peterson 2001) have been described previously. As with the Cμ construct, a 3.8-kb polyomavirus fragment that enables the plasmids to replicate was added to the D^d-pIgR constructs (Peterson and Perry 1989).

The μAV series of constructs were derived from the plasmid μAVWT.BSC (Xu et al. 1993) that was provided to us, along with positive (GAR4) and negative (mGAR4) control plasmids, by Tom Cooper (Baylor College of Medicine). This plasmid contains a truncated ASV *env* intron and flanking exons that requires an ESE to be spliced; fragments containing putative ESE sequences are cloned between *Bgl*II–*Spe*I sites in the downstream exon. We cloned overlapping fragments from the Cμ4 exon into this vector in two ways. First, restriction fragments *Pst*I–*Apa*I (PA) and *Msp*I–*Dpn*I (MD) were made blunt with Klenow and cloned into the vector. Second, we generated PCR fragments using primers that contained *Bgl*II or *Spe*I restriction sites at their ends, digested the PCR products with these enzymes, and directionally cloned them into the vector: 5′-CGAGATCTGCACAAACATCCACCT-3′ and 5′-CTACTAGTGCACACTGATGTCTGC-3′ for LP, 5′-CGAGAT CTGCCCCGATGCCAGA-3′ and 5′-GTACTAGTGGCAGGGCC TCGT-3′ for AB and 5′-CGAGATCTCCCTGATCATGTCTGAC-3′ and 5′-GTACTAGTTGAGCGCTAGCATGGT-3′ for DH. All constructs were confirmed by DNA sequencing.

Cell culture

The mouse B-cell lines M12 and A20 and the early-B-cell line 70Z/3 were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 50 U/mL penicillin/streptomycin (all from Life Technologies) and 50 μM 2-mercaptoethanol. The mouse plasmacytoma cell lines S194 and MPC11 were grown in Dulbecco's modified Eagles medium supplemented with 10% heat-inactivated horse serum and 50 U/mL penicillin/streptomycin (all from Life Technologies). The J558L cell line was grown in Iscove's modified Dulbecco's medium supplemented with 5% heat-inactivated horse serum and 50 U/mL penicillin/streptomycin.

Transfections and RNA preparation

The B-cell and plasma-cell lines were transiently transfected using the DEAE Dextran procedure and 8 μg plasmid DNA for $3–4 \times 10^7$ cells (Grosschedl and Baltimore 1985). Transfected M12, S194, J558L, and MPC11 cells were harvested after 40 h. Transfected A20 and 70Z/3 cells were harvested after 24 h. For the SR protein cotransfection studies, equal amounts of pSV5Cμ and SR protein expression plasmids were used, although plasmid ratios ranging from 1:7 to 7:1 gave similar expression results (data not shown). For the triple transfections, 2 μg pSV5Cμ was cotransfected with 3 μg of each SR protein. Cytoplasmic RNA was prepared as described previously (Schibler et al. 1978).

The M12 and S194 cell lines were stably transfected with the μAV series of constructs by electroporation. The μAV plasmids were linearized and mixed with linear pSV2neo in a ratio of 2:1,

electroporated into cells, and the cells were allowed to recover overnight before being put into selective medium. Stable pools of cells were selected with G418 at 300 μg/mL for M12 and 500 μg/mL for S194. Total RNA was isolated using Trizol Reagent (Invitrogen).

RNA analysis

The S1 probe to distinguish μs from μm mRNA was 3′ end labeled at a *Pst*I site using Klenow and [α-^{32}P]dCTP and the analysis performed as described previously (Fig. 1A; Peterson and Perry 1989). The S1 probe used to differentiate the D^{d}-pIgR transcripts was derived from a D^{d}-pIgR cDNA subclone in pGEM4. The probe was 3′ end-labeled at an *Msp*I site using Klenow and [α-^{32}P]dCTP and extends to the *Eco*RI site in the vector (Fig. 3C). A total of 100 μg of RNA, a combination of 50–100 μg of specific RNA and carrier RNA, was hybridized overnight at 50°C with the labeled probe. The reactions were treated at 37°C for 30 min with 60 units of S1 nuclease (Pharmacia). The protected fragments were separated on a 6% acrylamide, 7 M urea gel and quantitated by PhosphorImager analysis. Multiple protected fragments are observed with the cryptically spliced RNAs due to fortuitous homology between the probe and the D^{d} exon 4 sequences to which the cryptic site is spliced; by changing S1 digestion temperatures, all bands could be combined into one.

A RT–PCR assay was developed to quantitate spliced and unspliced μAV mRNA. A total of 2 μg of RNA from stably transfected cells was reverse-transcribed using oligo dT and SuperScript (Life Technologies) according to the manufacturer's instructions. A total of 1 μL of the RT reaction was used in a 50-μL PCR reaction with 2.5 U Taq polymerase (Life Technologies), 2 mM $MgCl_2$, 200 μM dNTPs, 200 nM each primer, plus 5×10^5 cpm (4–10 nM) of the top primer end-labeled with [γ^{32}P]ATP. The PCR cycles were 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Tubes were removed sequentially from the cycler after 21–33 cycles, 40 μL of each reaction was ethanol precipitated and run on a 6% acrylamide, 7 M urea gel. The gels were dried and the products were quantitated by PhosphorImager analysis. The cycle titration of each reaction identified the cycles during which both reaction products were within the linear phase of amplification. The products were quantitated from the maximum cycle number within this window, and these values were used to calculate the "% spliced RNA". The primers used for quantitation were as follows: top strand, 5′-CATTCACCACATTGGTGTGC-3′, and bottom strand, 5′-CCAGGGTATCCAGTCAGAACTGC-3′. To ensure that the size of the RNAs made from each construct reflected the size of the insert and that no cryptic splice reactions occurred within the inserted sequence, we also used a primer downstream of the insertion site in the vector (5′-GATTGTCATCCTGAGTGT GG-3′) with the top strand primer (data not shown).

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REFERENCES

Bai, Y., Lee, D., Yu, T., and Chasin, L.A. 1999. Control of 3′ splice site choice in vivo by ASF/SF2 and hnRNP A1. *Nucleic Acids Res.* **27:** 1126–1134.

Barnard, D.C. and Patton, J.G. 2000. Identification and characterization of a novel serine-arginine-rich splicing regulatory protein. *Mol. Cell. Biol.* **20:** 3049–3057.

Bruce, S.R. and Peterson, M.L. 2001. Multiple features contribute to efficient constitutive splicing of an unusually large exon. *Nucleic Acids Res.* **29:** 2292–2302.

Bruce, S.R., Kaetzel, C.S., and Peterson, M.L. 1999. Cryptic intron activation within the large exon of the mouse polymeric immunoglobulin gene: Cryptic splice sites correspond to protein domain boundaries. *Nucleic Acids Res.* **27:** 3446–3454.

Caceres, J.F., Stamm, S., Helfman, D.M., and Krainer, A.R. 1994. Regulation of alternative splicing in vivo by overexpression of antagonistic splicing factors. *Science* **265:** 1706–1709.

Calame, K.L. 2001. Plasma cells: Finding new light at the end of B cell development. *Nat. Immunol.* **2:** 1103–1108.

Cartegni, L., Chew, S.L., and Krainer, A.R. 2002. Listening to silence and understanding nonsense: Exonic mutations that affect splicing. *Nat. Rev. Genet.* **3:** 285–298.

Chandler, S.D., Mayeda, A., Yeakley, J.M., Krainer, A.R., and Fu, X.-D. 1997. RNA splicing specificity determined by the coordinated action of RNA recognition motifs in SR proteins. *Proc. Natl. Acad. Sci.* **94:** 3596–3601.

Colwill, K., Pawson, T., Andrews, B., Prasad, J., Manley, J.L., Bell, J.C., and Duncan, P.I. 1996. The Clk/Sty protein kinase phosphorylates SR splicing factors and regulates their intracellular distribution. *EMBO J.* **15:** 265–275.

Cowper, A.E., Caceres, J.F., Mayeda, A., and Screaton, G.R. 2001. Serine-arginine (SR) protein-like factors that antagonize authentic SR proteins and regulate alternative splicing. *J. Biol. Chem.* **276:** 48908–48914.

Du, K., Peng, Y., Greenbaum, L.E., Haber, B.A., and Taub, R. 1997. HRS/SRp40-mediated inclusion of the fibronectin EIIIB exon, a possible cause of increased EIIIB expression in proliferating liver. *Mol. Cell. Biol.* **17:** 4096–4104.

Edwalds-Gilbert, G. and Milcarek, C. 1995. Regulation of poly(A) site use during mouse B-cell development involves a change in the binding of a general polyadenylation factor in a B-cell stage-specific manner. *Mol. Cell. Biol.* **15:** 6420–6429.

Edwalds-Gilbert, G., Veraldi, K.L., and Milcarek, C. 1997. Alternative poly(A) site selection in complex transcription units: means to an end? *Nucleic Acids Res.* **25:** 2547–2561.

Eperon, I.C., Makarova, O.V., Mayeda, A., Munroe, S.H., Caceres, J.F., Hayward, D.G., and Krainer, A.R. 2000. Selection of alternative 5′ splice sites: Role of U1 snRNP and models for the antagonistic effects of SF2/ASF and hnRNP A1. *Mol. Cell. Biol.* **20:** 8303–8318.

Fu, X.-D. 1995. The superfamily of arginine/serine-rich splicing factors. *RNA* **1:** 663–680.

Gamberi, C., Izaurralde, E., Beisel, C., and Mattaj, I.W. 1997. Interaction between the human nuclear cap-binding protein complex and hnRNP F. *Mol. Cell. Biol.* **17:** 2587–2597.

Graveley, B.R. 2000. Sorting out the complexity of SR protein functions. *RNA* **6:** 1197–1211.

Grosschedl, R. and Baltimore, D. 1985. Cell-type specificity of immunoglobulin gene expression is regulated by at least three DNA sequence elements. *Cell* **41:** 885–897.

Hanamura, A., Caceres, J.F., Mayeda, A., Franza Jr., B.R., and Krainer, A.R. 1998. Regulated tissue-specific expression of antagonistic pre-mRNA splicing factors. *RNA* **4:** 430–444.

Jiang, Z.H., Zhang, W.J., Rao, Y., and Wu, J.Y. 1998. Regulation of Ich-1 pre-mRNA alternative splicing and apoptosis by mammalian splicing factors. *Proc. Natl. Acad. Sci.* **16:** 9155–9160.

Liu, H.-X., Zhang, M., and Krainer, A.R. 1998. Identification of functional exonic splicing enhancer motifs recognized by individual SR proteins. *Genes & Dev.* **12:** 1998–2012.

Lou, H., Neugebauer, K.M., Gagel, R.F., and Berget, S.M. 1998. Regulation of alternative polyadenylation by U1 snRNPs and SRp20. *Mol. Cell. Biol.* **9:** 4977–4985.

Manley, J. and Tacke, R. 1996. SR proteins and splicing control. *Genes & Dev.* **10:** 1569–1579.

Martincic, K., Campbell, R., Edwalds-Gilbert, G., Souan, L., Lotze, M.T., and Milcarek, C. 1998. Increase in the 64-kDa subunit of the polyadenylation/cleavage stimulatory factor during the Go to S phase transition. *Proc. Natl. Acad. Sci.* **95:** 11095–11100.

Matis, S.A., Martincic, K., and Milcarek, C. 1996. B-lineage regulated polyadenylation occurs on weak poly(A) sites regardless of sequence composition at the cleavage and downstream regions. *Nucleic Acids Res.* **24:** 4684–4692.

Mayeda, A. and Krainer, A.R. 1992. Regulation of alternative splicing by hnRNP A1 and splicing factor SF2. *Cell* **68:** 365–375.

Milcarek, C., Suda-Hartman, M., and Croll, S.C. 1998. Changes in abundance of IgG 2a mRNA in the nucleus and cytoplasm of a murine B-lymphoma before and after fusion to a myeloma cell. *Mol. Immunol.* **33:** 691–701.

Milcarek, C., Martincic, K., Chung-Ganster, L.-H., and Lutz, C.S. 2003. The snRNP-associated U1A levels change following IL-6 stimulation of human B cells. *Mol. Immunol.* **39:** 809–814.

Min, H., Chan, R.C., and Black, D.L. 1995. The generally expressed hnRNP F is involved in a neural-specific pre-mRNA splicing event. *Genes & Dev.* **9:** 2659–2671.

Nelson, K.J., Haimovich, J., and Perry, R.P. 1983. Characterization of productive and sterile transcripts from the immunoglobulin heavy-chain locus: Processing of μm and μs mRNA. *Mol. Cell. Biol.* **3:** 1317–1332.

Peterson, M.L. 1992. Balanced efficiencies of splicing and cleavage-polyadenylation are required for μs and μm mRNA regulation. *Gene Expr.* **2:** 319–327.

———. 1994a. Regulated immunoglobulin (Ig) RNA processing does not require specific cis-acting sequences: Non-Ig genes can be alternatively processed in B cells and plasma cells. *Mol. Cell. Biol.* **14:** 7891–7898.

———. 1994b. RNA processing and the expression of immunoglobulin genes. In *Handbook of B and T lymphocytes* (ed. E.C. Snow), pp. 321–342. Academic Press, San Diego, CA.

Peterson, M.L. and Perry, R.P. 1986. Regulated production of μm and μs mRNA requires linkage of the poly(A) addition sites and is dependent on the length of the μs-μm intron. *Proc. Natl. Acad. Sci.* **83:** 8883–8887.

———. 1989. The regulated production of μm and μs mRNA is dependent on the relative efficiencies of μs poly(A) site usage and the Cμ4-to-M1 splice. *Mol. Cell. Biol.* **9:** 726–738.

Peterson, M.L., Gimmi, E.R., and Perry, R.P. 1991. The developmentally regulated shift from membrane to secreted μ mRNA production is accompanied by an increase in cleavage-polyadenylation efficiency but no measurable change in splicing efficiency. *Mol. Cell. Biol.* **11:** 2324–2327.

Phillips, C., Jung, S., and Gunderson, S.I. 2001. Regulation of nuclear poly(A) addition controls the expression of immunoglobulin M secretory mRNA. *EMBO J.* **20:** 6443–6452.

Prasad, J., Colwill, K., Pawson, T., and Manley, J.L. 1999. The protein kinase Clk/Sty directly modulates SR protein activity: Both hyper- and hypophosphorylation inhibit splicing. *Mol. Cell. Biol.* **19:** 6991–7000.

Schaal, T.D. and Maniatis, T. 1999. Selection and characterization of pre-mRNA splicing enhancers: Identification of novel SR protein-specific enhancer sequences. *Mol. Cell. Biol.* **19:** 1705–1719.

Schibler, U., Marcu, K.B., and Perry, R.P. 1978. The synthesis and processing of the messenger RNAs specifying heavy and light chain immunoglobulins in MPC-11 cells. *Cell* **15:** 1495–1509.

Screaton, G.R., Caceres, J.F., Mayeda, A., Bell, M.V., Plebanski, M., Jackson, D.G., Bell, J.I., and Krainer, A.R. 1995. Identification and characterization of three members of the human SR family of pre-mRNA splicing factors. *EMBO J.* **14:** 4336–4349.

Seipelt, R.L., Spear, B.T., Snow, E.C., and Peterson, M.L. 1998. A nonimmunoglobulin transgene and the endogenous immunoglobulin μ gene are coordinately regulated by alternative RNA processing during B-cell maturation. *Mol. Cell. Biol.* **18:** 1042–1048.

Takagaki, Y., Seipelt, R.L., Peterson, M.L., and Manley, J.L. 1996. The polyadenylation factor CstF-64 regulates alternative processing of IgM heavy chain pre-mRNA during B cell differentiation. *Cell* **87:** 941–952.

ten Dam, G.B., Zilch, C.F., Wallace, D., Wieringa, B., Beverley, P.C., Poels, L.G., and Screaton, G.R. 2000. Regulation of alternative splicing of CD45 by antagonistic effects of SR protein splicing factors. *J. Immunol.* **164:** 5287–5295.

Tsurushita, N., Ho, L., and Korn, L.J. 1988. Nuclear factors in B lymphoma enhance splicing of mouse membrane-bound μ mRNA in *Xenopus* oocytes. *Science* **239:** 494–497.

van den Bosch, L., Mertens, L., Cavaloc, Y., Peterson, M., Wuytack, F., and Eggermont, J. 1996. Alternative processing of the sarco/endoplasmic reticulum Ca2+-ATPase transcripts during muscle differentiation is a specifically regulated process. *Biochem. J.* **317:** 647–651.

Veraldi, K.L., Arhin, G., Martincic, K., Chung-Ganster, L.-H., Wilusz, J., and Milcarek, C. 2001. hnRNP F influences binding of a 64-kilodalton subunit of cleavage stimulation factor to mRNA precursors in mouse B cells. *Mol. Cell. Biol.* **21:** 1228–1238.

Wang, J. and Manley, J.L. 1995. Overexpression of the SR proteins ASF/SF2 and SC35 influences alternative splicing in vivo in diverse ways. *RNA* **1:** 335–346.

Watakabe, A., Sakamoto, H., and Shimura, Y. 1991. Repositioning of an alternative exon sequence of mouse IgM pre-mRNA activates splicing of the preceding intron. *Gene Expr.* **1:** 175–184.

Watakabe, A., Tanaka, K., and Shimura, Y. 1993. The role of exon sequences in splice site selection. *Genes & Dev.* **7:** 407–418.

Xu, R., Teng, J., and Cooper, T.A. 1993. The cardiac troponin T alternative exon contains a novel purine-rich positive splicing element. *Mol. Cell. Biol.* **13:** 3660–3674.

Yan, D.-H., Weiss, E.A., and Nevins, J.R. 1995. Identification of an activity in B-cell extracts that selectively impairs the formation of an immunoglobulin μs poly(A) site processing complex. *Mol. Cell. Biol.* **15:** 1901–1906.