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Identification of an Activity in B-Cell Extracts That Selectively Impairs the Formation of an Immunoglobulin μs Poly(A) Site Processing Complex

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The immunoglobulin μ heavy-chain transcription unit is differentially expressed during B-cell development, producing mRNAs that encode secreted (μs) and membrane-bound (μm) forms of the heavy-chain polypeptide. Whereas the μs mRNA and the μm mRNA are produced in approximately equal abundance in B cells, an increase in the utilization of the μs poly(A) site contributes to the production of the μs mRNA as the predominant form in a plasma cell. Previous experiments have demonstrated a correlation between the formation of a stable complex on a poly(A) site and the relative function of the poly(A) site. We have thus investigated the parameters determining the interaction of these factors with the immunoglobulin poly(A) sites. Assays of complex formation involving the two immunoglobulin poly(A) sites by using HeLa cell activities revealed the formation of stable complexes with no apparent difference between the μs site and the μm site. In contrast, the μs-specific complex was markedly less stable when a B-cell extract was used. Fractionation of B-cell extracts has revealed an activity that specifically destabilizes the μs polyadenylation complex, suggesting that the function of this poly(A) site may be regulated by both positive- and negative-acting factors.

The control of gene expression can involve regulated alterations of virtually any step in the process of mRNA biogenesis, including the formation of the 3′ terminus of an mRNA (21). This process, commonly known as poly(A) site formation, involves a recognition of specific sequence elements in the pre-mRNA followed by endonucleolytic cleavage of the pre-mRNA and then the addition of approximately 200 adenylate residues (16, 17, 35, 37). The recognition of essential sequences and accurate processing of the pre-mRNA can be reproduced in cell-free systems, and this has allowed the identification and purification of cellular components that mediate this event (1, 3, 4, 8, 10, 18, 20, 29–34). These studies have demonstrated the involvement of several large, multicomponent activities that recognize sequence elements in the RNA through the formation of RNA-protein complexes (9, 12, 28, 38).

Although in most cases the processing of an RNA at a poly(A) site is an essential but not a regulated event, there are instances where alterations in poly(A) site selection appears to regulate gene expression (15). Perhaps the best-studied example is found in the immunoglobulin (Ig) heavy-chain transcription unit. During B-cell development, expression of the rearranged heavy-chain locus leads to the production of two distinct mRNAs, one encoding a membrane-bound form of the μ heavy-chain polypeptide (μm) and a second encoding the secreted form of the heavy chain (μs) (11). These two RNAs are produced by a combination of alternative RNA splicing (19, 24, 27) as well as alternative use of two poly(A) sites (6, 7, 13, 14, 19). In pre-B and mature B cells, approximately equal amounts of the two RNAs are produced, whereas in an antibody-secreting plasma cell, the μs RNA predominates.

Previous functional analyses of Ig heavy-chain RNA processing have revealed that the distal μm (or γm) poly(A) site is considerably stronger than the proximal μs (or γs) poly(A) site and that the organization of the transcription unit critically determines the regulation (7, 13, 14, 19, 26, 27). Since the selection of a poly(A) site occurs during transcription, thus favoring the promoter proximal site, the spacing of the two sites relative to one another influences their use, and this property is clearly evident with the μ transcription unit (7, 24). Moreover, other assays have demonstrated that the nature of the μs poly(A) site and the μm splice donor appears to allow competition between the two, since each contains sequences that render their use inefficient (27).

Taken together, these previous analyses suggest that the critical aspect of the regulated switch in expression of the Ig heavy-chain transcription unit, either μ or γ, is an alteration in the efficiency of use of the proximal poly(A) site relative to the use of the distal poly(A) site. However, since the regulation of poly(A) site use in this case involves an apparent competition between the two poly(A) sites during the process of transcription, investigations of the basis for the control must focus on the biochemical events that mediate the processing event and that are likely candidates for rate-determining steps. We have sought to understand this process through an analysis of the interaction of processing factors with the Ig μ poly(A) sites. Our results suggest a difference in the interaction of processing factors with the μs and μm sites that reflects their relative efficiency of use.

MATERIALS AND METHODS

Cell culture. Mouse CH12-B-lymphoma cells were maintained, cultured, and stimulated by lipopolysaccharide as described previously (23). HeLa cells were cultured in Joklik-modified minimum essential media (S-MEM; GIBCO BRL) containing 5% bovine serum. The B-lymphoma cell line, C11, originally derived from BCL1-3B3 (2), was cultured in RPMI (GIBCO BRL) containing 5% fetal bovine serum, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and 50 μM β-mercaptoethanol. The B-cell lymphoma cell line, A20, was cultured in S-MEM containing 5% fetal bovine serum, 5% equine serum, 2 mM L-glutamine, and 50 μM β-mercaptoethanol. Nuclear extracts were isolated according to the standard protocol (5).

RNA 3′ processing assays. The in vitro cleavage assays performed in this study were carried out in crude nuclear extracts with [α-^{32}P]GTP-labeled SP6 RNA probes, 20 of μg *Escherichia coli* tRNA per ml, 2% polyvinyl alcohol, and 1 mM

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3′-dATP. After 90 min of incubation at 30°C, the reaction mixture was treated with proteinase K followed by phenol-chloroform extraction and ethanol precipitation to recover the RNAs. The cleavage products were analyzed on an 8% denaturing polyacrylamide gel.

Gel retardation assays. Either crude nuclear extracts or partially purified polyadenylation factors were used in this assay. Partially purified HeLa cleavage polyadenylation specificity factor (CPSF) and cleavage factor 1 (CF1) were Blue-Sepharose and Mono S fractions, respectively; CH12 CPSF and CF1 were both S-Sepharose fractions. These fractions were obtained according to the scheme described previously (9). Off-rate measurements were performed as reported previously (36). Briefly, the binding reaction mixture, including [^{32}P]RNA probes, tRNA (20 μg/ml), 1% polyvinyl alcohol, binding buffer (1 mM ATP, 0.7 mM $MgCl_2$, 0.1 mM EDTA, 0.6 mM dithiothreitol), and nuclear extract, was incubated at 30°C for 10 min, then an aliquot of the binding reaction mixture was removed (designated the 0 time point sample), and 150 ng (approximately 100-fold molar excess of unlabeled AAU plus double-stranded RNAs [9]) was added to the remaining binding reaction mixture. Aliquots of the remaining reaction were removed at various time points thereafter. Upon removal of each aliquot, heparin was added to the aliquot and it was loaded immediately onto a 3% native polyacrylamide gel.

RESULTS

Formation of multicomponent processing complexes on the μs and μm poly(A) sites. Production of the two mRNAs encoding the Ig μ heavy-chain polypeptides involves both alternative splicing and alternative polyadenylation of the primary transcript (Fig. 1A) (11), and it appears that selective polyadenylation plays a significant role in this regulated expression (24). An examination of the sequences found at the μs and μm poly(A) sites reveals canonical AAUAAA elements as well as typical GU-rich sequence downstream of the cleavage sites (Fig. 1B). That these sequences are in fact functional is shown by an in vitro processing assay. In vitro-produced RNAs containing either the μs or the μm poly(A) site were incubated with a nuclear extract prepared from the CH12 B-lymphoma cell line, and processing was then analyzed by gel electrophoresis. As shown in Fig. 1C, each of the RNAs was accurately processed.

It is clear from assays such as those shown in Fig. 1C that accurate processing can be measured by using these poly(A) sites, and it is also evident that the μm poly(A) site is processed more efficiently than the μs poly(A) site. However, attempts to use such assays to probe the basis for the regulation of Ig poly(A) site function based on the differential efficiencies of the two poly(A) sites are hampered by the complexity of the process and the contributions of competition of the two sites during ongoing transcription. An alternative approach is to identify potential rate-determining factors that are involved in the processing and then to study the role of such factors in regulated expression. Previous work has identified a series of factors involved in poly(A) site recognition and processing (3, 4, 8, 10, 18, 29, 31–33). In addition, other studies have established a pathway for the interaction of these factors with the poly(A) site that involves an initial recognition of the AAUAAA element by the CPSF factor, generating a specific but unstable complex (9, 12). Subsequently, a second activity termed CF1 (also known as CstF) recognizes the downstream sequences but only in the context of CPSF previously bound to the RNA. The interaction of CF1 generates a large multicomponent complex with a significantly increased stability over that of the CPSF-RNA complex. Comparison of poly(A) sites that differ in the downstream sequence as well as processing efficiency has shown that the increase in stability imparted by the interaction of CF1 with the CPSF-RNA complex correlates with the processing efficiency of the poly(A) site (36). With these past results in mind, we have used these factors to assess the nature of the interactions on the Ig μ poly(A) sites and, in particular, the possibility that differences in these interactions might reflect the relative function of the poly(A) sites.

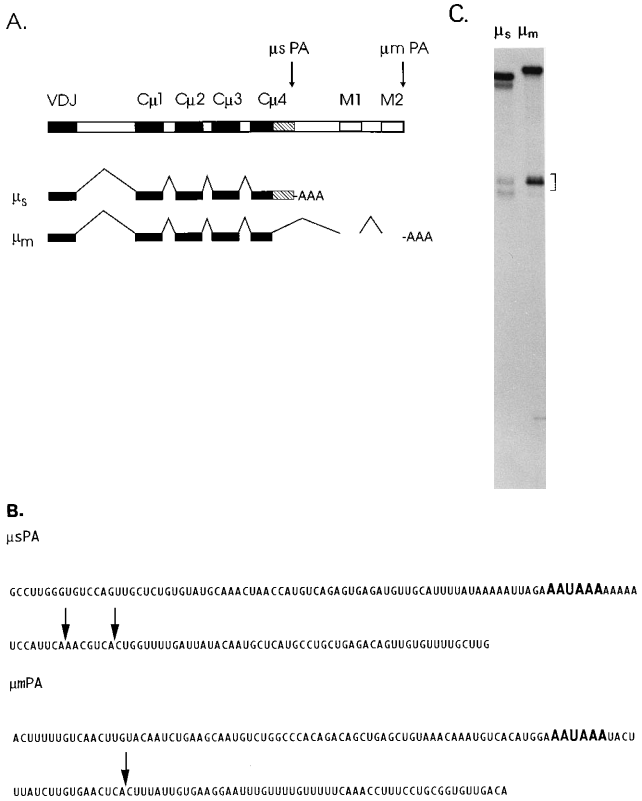

FIG. 1. The Ig μ heavy-chain transcription unit. (A) Organization of the μ transcription unit. A schematic of the rearranged Ig μ heavy-chain locus is shown. The black boxes are exons encoding variable (VDJ) and constant (Cμ1 to Cμ4) regions of the antibody. The open boxes are introns. The shaded boxes represent the exons encoding secreted and membrane-bound specific sequences of the Ig heavy chain. Alternative splicing and alternative polyadenylation of the μ pre-mRNA are responsible for the generation of the μs and μm mRNAs. PA, poly(A) site. (B) The μs and μm poly(A) sites. Sequences of the Ig μs poly(A) site (μsPA) and μm poly(A) site (μmPA) are shown. The canonical polyadenylation signal AAUAAA is shown in boldface, and the cleavage sites are indicated by arrows. (C) In vitro cleavage assay of μs and μm poly(A) sites. ^{32}P-labeled SP6 RNAs were incubated with an equal amount of CH12 crude nuclear extract in the presence of 1 mM 3′-dATP at 30°C for 90 min. The cleavage products were recovered and analyzed in an 8% denaturing polyacrylamide gel. The bracket indicates the 5′ cleavage products from both poly(A) sites.

RNAs containing either the μs or the μm poly(A) site were incubated with the partially purified HeLa cell factors, and then complex formation was assayed by gel electrophoresis in nondenaturing gels. In addition, an excess of unlabeled RNA was then added to each binding assay and the stability of the complex was determined, again by gel electrophoresis assays. As shown in Fig. 2A, addition of the purified CPSF factor generated a specific but unstable RNA-protein complex with both of the RNAs, as indicated by the rapid dissociation of the complexes in the off-rate measurements. Formation of the CPSF-CF1 complex involving both the μs and the μm poly(A) sites was detected, and off-rate measurements indicated that these complexes were stable, each with an off-rate in excess of 60 min (Fig. 2B). Thus, formation of complexes typical of previous assays (9, 36) could be demonstrated, but no apparent difference in the two poly(A) sites could be detected, by these assays.

The μs-specific complex formed with B-cell components is unstable. Although we could detect no differences in the nature of the complexes formed on the two Ig poly(A) sites by using the purified HeLa cell components, it is possible that

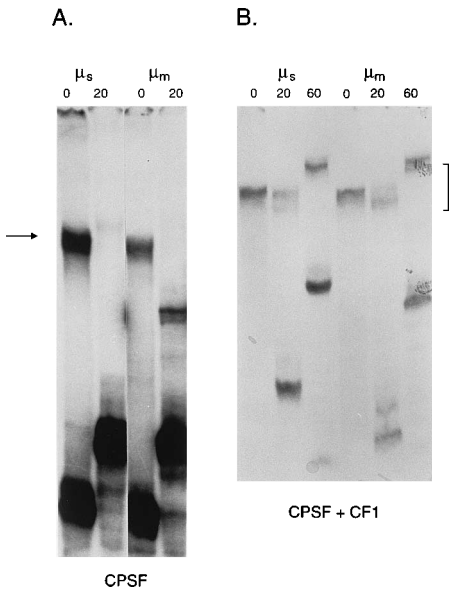

FIG. 2. Formation of poly(A) site processing complexes on the μs and μm sequences. (A) HeLa cell CPSF forms an unstable complex with RNAs containing the μs and μm poly(A) sites. Partially purified HeLa CPSF was incubated with ^{32}P-labeled RNAs containing the μs or μm poly(A) site in a binding assay. Off-rate measurements were carried out as described in Materials and Methods. Following the addition of an excess of the unlabeled competitor RNA, aliquots were removed at the indicated times (minutes) and analyzed in a 3% native polyacrylamide gel. (B) HeLa CPSF and CF1 form stable complexes with μs and μm poly(A) sites. Partially purified HeLa CPSF and CF1 were incubated with ^{32}P-labeled RNAs containing the μs or μm poly(A) site. Off-rate measurements were determined at 0, 20, and 60 min after the addition of the unlabeled competitor RNA. The HeLa CPSF-CF1-RNA complexes are indicated by a bracket.

there are B-cell variants of these factors that recognize the two poly(A) sites in a distinct fashion. We have therefore analyzed complexes formed on the μs and μm poly(A) sites by using a B-cell extract as the source of polyadenylation components.

As shown in Fig. 3, incubation of the RNAs containing the two Ig poly(A) sites with a B-cell nuclear extract generated a complex with mobility equal to that of the previously described

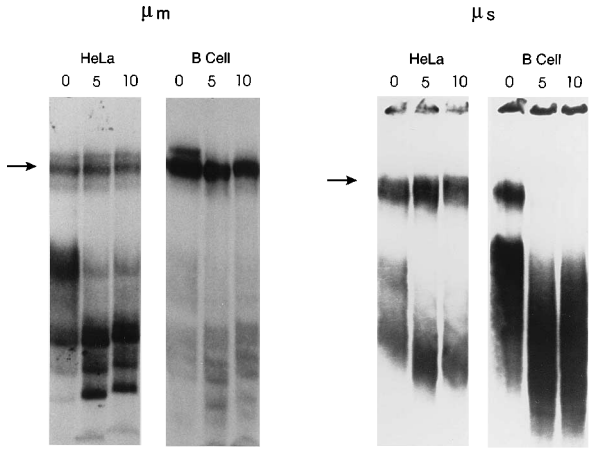

FIG. 3. Formation of processing complexes with B-cell components. HeLa cell nuclear extract or CH12 nuclear extract (B cell) was incubated with μs and μm poly(A) site probes. Off-rate measurements were taken at 0, 5, and 10 min after the addition of unlabeled competitor RNA. The CPSF-CF1-RNA complexes are indicated by arrows.

CPSF-CF1 poly(A) site complex and similar to that formed with the HeLa cell nuclear extract. Although the stability of the complex formed on the μm poly(A) site was similar to that with the HeLa components, exhibiting a half-life in excess of 30 min, the situation was quite different with the μs poly(A) site. The interaction of B-cell factors with the μs poly(A) site generated a complex with a dramatically reduced half-life compared with the HeLa cell complex that formed on the μs poly(A) site. Thus, a distinct difference in the complexes formed on the two poly(A) sites can be observed, correlating stability with relative function and dependent on the source of the extract. In addition, the greater instability of the μs complex than of the μm complex is consistent with the relative inefficiency of μs poly(A) site function that has been measured in previous in vivo assays (7, 13, 14, 19, 26, 27).

A B-cell activity imparts instability to the μs polyadenylation complex. The relative instability of the μs poly(A) site complex formed with the B-cell extract could be due to a difference in the CPSF or CF1 factor in B cells or might possibly be due to an additional activity that somehow destabilizes the complex formed on the μs poly(A) site. An approach to this question was afforded by fractionation of the B-cell extract to achieve a partial purification of the CPSF and CF1 activities. For these subsequent experiments, we have used either the C11 or the A20 B-cell lymphoma cell line for the preparation of extracts because of the ability to grow large numbers of cells. Identical results have been obtained with either of the cell lines. In contrast to the results obtained with the crude B-cell extract, the complex formed on the μs poly(A) site with partially purified fractions was stable (Fig. 4A), equivalent to that seen with the HeLa factors (Fig. 2B) or the HeLa nuclear extract (Fig. 3A) and equivalent to that seen with the μm poly(A) site. Thus, it would appear that the instability of the complex observed with the B-cell extract is not a function of the B-cell CPSF and CF1 activities that create the complex. Rather, it would appear that a component of the B-cell extract that is removed upon fractionation of the CPSF and CF1 activities might be responsible for the instability of the μs poly(A) site complex.

As a further approach to the basis for instability of the μs complex in the B-cell extract, we have attempted to isolate an activity that could impart instability to the complex with the partially purified components. Fractions from the purification of the B-cell extract were recombined with the partially purified CPSF and CF1 fractions, and the stability of the μs complex was measured. As shown in Fig. 4B, the addition of a 0.25 M fraction from the DEAE column resulted in a destabilization of the μs poly(A) site complex. This fraction was further purified on an S-Sepharose column, and as shown in Fig. 4C, the activity again destabilized the μs complex. Importantly, this experiment also shows that the activity had little effect on the stability of the complex formed on the μm poly(A) site. We thus conclude that an activity that specifically destabilizes the complex formed on the μs poly(A) site can be identified in B-cell extracts.

Instability of the μs poly(A) site complex correlates with reduced processing efficiency. Previous experiments have shown a correlation between the stability of the poly(A) site processing complex and the efficiency of poly(A) site use (36). Thus, we might expect that by destabilizing the complex formed on the μs poly(A) site, the B-cell inhibitory activity would reduce the efficiency of processing of the μs poly(A) site. This is, in fact, the case as shown by the assays in Fig. 5. RNAs containing the μs and μm poly(A) sites were incubated in a B-cell nuclear extract with or without the addition of partially purified inhibitory fraction. Poly(A) site processing

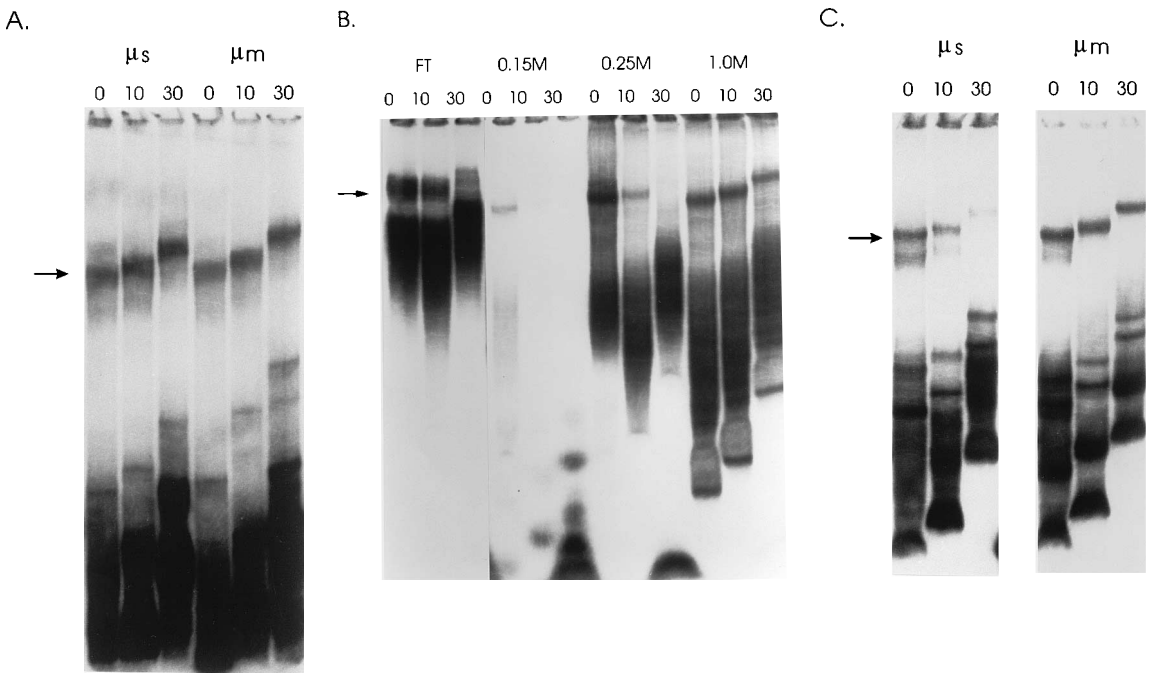

FIG. 4. Fractionation of B-cell extracts identifies an inhibitor of the μs poly(A) site complex. (A) Partially purified CPSF and CF1 from a B-cell extract form a stable complex with RNAs containing the μs and μm poly(A) sites. CPSF and CF1, obtained from DEAE and S-Sepharose fractionation of CH12 cell extracts as described in Materials and Methods, were incubated with μs and μm poly(A) site probes in binding reactions. Off-rate measurements were determined at 0, 10, and 30 min after the addition of unlabeled competitor RNA. The CPSF-CF1-RNA complex is indicated by an arrow. (B) The B-cell DEAE 0.25 M fraction contains an inhibitory activity. DEAE fractions obtained from the fractionation of a C11 B-lymphoma cell extract were assayed for their inhibitory activity by adding an aliquot of each fraction to a binding reaction mixture containing partially purified CH12 cell CPSF and CF1 and μs poly(A) site probe. Off-rate measurements were determined at 0, 10, and 30 min after the addition of unlabeled competitor RNA. The C11 lymphoma cell line was used for the preparation of extracts for fractionation because of the need for large amounts of material. The CPSF-CF1-RNA complex is indicated. FT, flowthrough. (C) The inhibition of stable complex formation is specific for the μs poly(A) site. The C11 cell DEAE 0.25 M fraction was further fractionated through an S-Sepharose column in which the inhibitory activity was eluted by 0.5 M KCl. The concentrated S-Sepharose 0.5 M fraction was added to a binding reaction mixture containing partially purified CH12 CPSF and CF1 and a μs or μm poly(A) site probe. Off-rate measurements were determined at 0, 10, and 30 min after the addition of unlabeled competitor RNA. The CPSF-CF1-RNA complex is indicated.

was then scored by analysis of the RNAs in acrylamide gels. As shown by the assays in Fig. 5, the addition of increasing amounts of the inhibitory fraction caused a marked decrease in the efficiency of μs poly(A) site processing. In contrast, the addition of increasing amounts of the inhibitor had little effect on processing of the μm RNA, resulting in an approximately 2.5-fold difference in the effect of the inhibitory component on the processing of the two RNAs. Thus, the effects of the B-cell inhibitory activity on μs poly(A) site complex stability can be seen reflected in a functional assay as a differential effect on the processing of the μs RNA.

DISCUSSION

Several studies have demonstrated that the recognition and processing of a poly(A) site occurs during transcription, on a nascent RNA molecule (22). Thus, when multiple poly(A) sites are present in the pre-mRNA, the potential for a polar effect exists, with the proximal site being favored over the distal sites. In those instances where there is evidence of regulated use of poly(A) sites, it appears that such a polar effect does indeed play a role since the strength of the poly(A) sites coupled with their position in the transcription unit are critical determinants of the use of the poly(A) sites (7, 14, 26). A clear example of the relationship of transcription unit arrangement and poly(A) site function is found in the Ig heavy-chain transcription unit (11). The two poly(A) sites in the Ig μ transcription unit differ in their relative efficiency of use, with the promoter-proximal μs site being considerably less efficient than the distal μm poly(A) site. This arrangement appears to be crucial to the regulated expression since altering the relative spacing of the poly(A) sites or reversing the order of the sites dramatically changes the use of the sites (6, 26).

Obviously, these considerations are critical for developing an understanding of the underlying basis for regulation of the Ig poly(A) sites that occurs during B-cell differentiation. In considering the available data, it seems most simple to view the control as the increase in the efficiency of use of the normally inefficient μs poly(A) site. The experiments we present here suggest a mechanism that may explain the underlying inefficiency of the μs poly(A) site in a B-cell environment. We suggest that in some manner, a negative-acting component interferes with the ability of the CPSF and CF1 factors to form a stable processing complex. The consequence of this effect is a less stable μs processing complex and a reduced efficiency of processing.

Although this finding does suggest a mechanism whereby the μs poly(A) site is rendered inefficient in a B cell, it does not address the mechanism for regulation of the μs site during B-cell differentiation. That is, what is responsible for the change in μs function from the inefficient state to a very efficient state? Certainly, one possibility would be the loss of the negative-acting component as a B-cell matures to a plasma cell. As depicted by the schematic shown in Fig. 6, we suggest the possibility that the negative-acting component destabilizes the interaction of the essential processing factors on the μs poly(A) site, thus effectively reducing the efficiency of use of this site. This would then allow the utilization of the distal μm

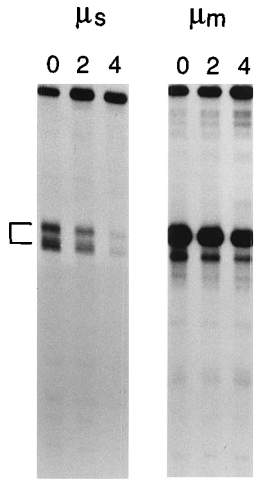

FIG. 5. Effect of the B-cell inhibitory activity on μs and μm poly(A) site processing. RNAs containing the μs and μm poly(A) sites were incubated with A20 cell nuclear extract in the present of various amounts (0, 2, and 4 μl) of A20 inhibitory fraction which was partially purified by DEAE, S-Sepharose, and heparin-Sepharose fractionation. The in vitro cleavage assays were performed as described in Materials and Methods. The processing efficiency was measured by quantitation obtained from a PhosphorImager analysis. The 5′ cleavage products from both poly(A) sites are indicated by the bracket.

poly(A) site which would otherwise be precluded from a functional interaction by the upstream μs poly(A) site. Although we depict the inhibitory factor as binding to sequences near the μs poly(A) site, this is only speculation, as we have no direct evidence for such at this time. In addition, we do not wish to imply that the inhibitor is unique in controlling the μs poly(A) site. Indeed, recent experiments suggest that the B-cell-specific inefficiency of the μs poly(A) site can be generated with other

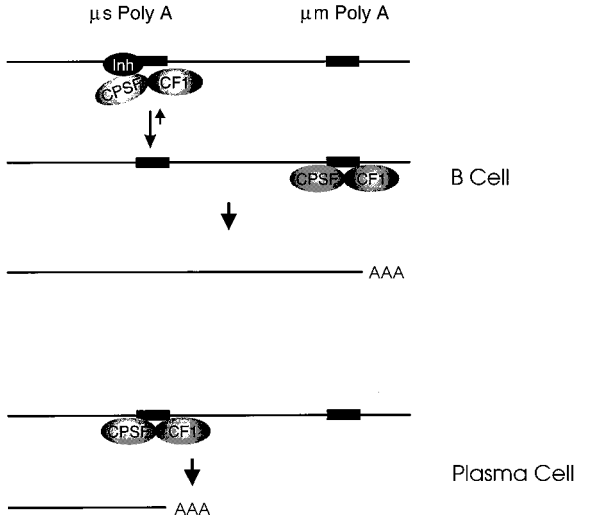

FIG. 6. A possible role for the B-cell inhibitory factor in Ig poly(A) site function. The model depicts the interaction of poly(A) site processing factors (CPSF and CF1) with either the μs or the μm poly(A) sites in the Ig heavy-chain primary transcript. The inhibitory activity (Inh) is depicted as interfering with the formation of the complex on the μs poly(A) site, thus allowing the formation of a functional complex on the μm poly(A) site and the processing of RNA to yield the μm mRNA. We speculate that when B cells mature to antibody-secreting plasma cells, the inhibitory activity is lost, thus allowing formation of a stable complex on the μs poly(A) site and processing of the RNA to yield the μs mRNA.

poly(A) sites as well (25). Regardless of the precise mechanism of action and degree of specificity, if this factor were to decline upon B-cell differentiation to plasma cells, the processing factors would no longer be impaired in their ability to form a stable complex on the μs poly(A) site, thus driving processing of the Ig μ RNAs to yield the μs RNA. Although this model is speculation based on the available data, it does provide a framework for future experiments that might address the control of this inhibitory component during B-cell maturation.

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