MOLECULAR AND CELLULAR BIOLOGY, Nov. 1995, p. 6420–6429 Vol. 15, No. 11
0270-7306/95/$04.00+0

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

GRETCHEN EDWALDS-GILBERT AND CHRISTINE MILCAREK*

Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261-2072

Received 3 May 1995/Returned for modification 1 June 1995/Accepted 16 August 1995

During the development of mouse B cells there is a regulated shift from the production of membrane to the secretion-specific forms of immunoglobulin (Ig) mRNA, which predominate in the late-stage or plasma B cells. By DNA transfection experiments we have previously shown that there is an increase in polyadenylation efficiency accompanying the shift to secretion-specific forms of Ig mRNA (C. R. Lassman, S. Matis, B. L. Hall, D. L. Toppmeyer, and C. Milcarek, J. Immunol. 148:1251–1260, 1992). When we look in vitro at nuclear extracts prepared from early or memory versus late-stage or plasma B cells, we see cell stage-specific differences in the proteins which are UV cross-linked to the input RNAs. We have characterized one of these proteins as the 64-kDa subunit of the general polyadenylation factor cleavage-stimulatory factor (CstF) by immunoprecipitation of UV-cross-linked material. The amount of 64-kDa protein and its mobility on two-dimensional gels do not vary between the B-cell stages. However, the activity of binding of the protein to both Ig and non-Ig substrates increases four- to eightfold in the late-stage or plasma cell lines relative to the binding seen in the early or memory B-cell lines. Therefore, the binding activity of a constitutive factor required for polyadenylation is altered in a B-cell-specific fashion. The increased binding of the 64-kDa protein may lead to a generalized increase in polyadenylation efficiency in plasma cells versus early or memory B cells which may be responsible for the increased use of the secretory poly(A) site seen in vivo.

Immunoglobulin (Ig) heavy-chain proteins exist in two forms, differing only at their carboxyl termini. The membrane-bound antigen receptor is found on the surface of early or memory B cells, while the secreted form is produced by plasma cells. The mRNAs for the two protein forms are derived from a single pre-mRNA that is alternatively processed at the 3′ end (1, 8, 44, 45). The 3′ end of the secretion-specific (sec) mRNA is encoded by the last constant-region exon; the sec poly(A) site is about 100 nucleotides downstream of the last constant-region exon. The 3′ end of the membrane-specific (mb) mRNA is encoded by a large portion of the last constant-region exon and by two downstream exons, M1 and M2. mb mRNA is produced when splicing of the last constant-region exon to M1 takes place using a 5′ splice site within the last constant-region exon and polyadenylation occurs at the poly(A) site at the end of M2.

The ratio of sec to mb mRNA is indicative of a B cell's developmental stage (reviewed in reference 18). Early B, pre-B, and memory cells and their tumor analogs, lymphomas, make approximately equal amounts of sec and mb mRNA. Plasma cells and their tumor counterparts, myelomas, make 10- to 100-fold more sec mRNA than mb mRNA. While translational and posttranslational control mechanisms play a small role in contributing to the sec-versus-mb protein ratios of Ig-producing cells, the major contribution to the phenotype comes from differential production of sec and mb mRNAs (13, 24, 25, 41). Studies of the mouse Ig γ2b locus have shown that transcription termination occurs downstream of the mb poly(A) site in both early and late-stage B cells (11). This finding is in contrast to some studies of the Ig μ transcription unit (13, 19), in which differential transcription termination has been suggested as a possible mechanism for the switch in poly(A) site use during B-cell development. The stability of both γ sec and γ mb mRNAs increases in late-stage or plasma cells, and differential mRNA stability therefore cannot account for the major shift in the ratio of the two mRNAs (33).

The key point in understanding posttranscriptional processing of Ig heavy-chain mRNAs is that splicing of the last constant-region exon to M1 and polyadenylation at the sec poly(A) site are two mutually exclusive events. The balance between the two is reflected in the final sec-to-mb mRNA ratio and could potentially be controlled by regulation of either event. However, when the efficiency of splicing of the last constant-region exon to M1 exons was examined in either the μ or γ gene, there was no change in early versus late-stage B cells or in lymphoid versus nonlymphoid cell lines (3, 38, 40, 56), indicating that splicing is a constitutive, nonregulated step. In contrast, experiments using stable transfections have shown that regulated expression of the mouse Ig γ2b gene is influenced by poly(A) site order and strength (26).

The biochemistry of cleavage and polyadenylation and the sequences required in *cis* in the pre-mRNA for 3′-end formation have been well characterized (reviewed in reference 58). In addition to the poly(A) signal sequence, AAUAAA, and the cleavage site 10 to 30 nucleotides downstream of the AAU AAA, many poly(A) sites have a sequence, often GU/U rich, downstream (14, 20, 29, 30, 47) or upstream (4, 9, 15, 43, 46, 48, 57) of the cleavage site involved in poly(A) site efficiency. Protein factors required for accurate cleavage and polyadenylation, including cleavage and polyadenylation specificity factor (CPSF) (2, 17, 35), cleavage stimulatory factor (CstF) (17, 52),

* Corresponding author. Phone: (412) 648-9098. Fax: (412) 624-1401. Electronic mail address: chris@server1.mgen.pitt.edu.

TABLE 1. IgG-producing mouse B-cell lines representing different stages of B-cell development

Cell line	B-cell stage	IgG sec:mb RNA ratio
A20	Memory	2:1
AxJ	Hybrid of A20 and J558L	36:1
J558L	Plasma	18:1[a]
2PK3	Memory	2:1
4T001	Plasma	10:1

[a] On a transfected IgG gene; endogenous alpha heavy chain lost from these cells.

poly(A) polymerase (7, 53), and cleavage factors I and II (6, 16, 54), have been isolated from HeLa cell nuclear extracts and calf thymus (6, 16, 54); cDNAs for some of these factors have been isolated (21, 49–51). CPSF is a multisubunit factor that recognizes the poly(A) signal sequence AAUAAA (17, 21, 23) and, as the name implies, is required for both cleavage and polyadenylation (2, 17, 35). CstF is required to increase the efficiency of the cleavage reaction (27); its 64-kDa subunit can be cross-linked to poly(A) site-containing RNAs in the presence of CPSF and the other subunits of CstF (27, 61). CstF is thought to function by stabilizing the interaction of CPSF with the poly(A) site (27, 59) via protein-protein interactions with CPSF and protein-RNA interactions with the region downstream of the poly(A) site.

We have taken an in vitro approach to dissecting the mechanism of differential poly(A) site use during B-cell development. Nuclear extracts efficient for specific polyadenylation were prepared from mouse B-cell lines representing different stages of development. UV cross-linking of protein to labelled pre-mRNA was performed to examine potential differences in proteins involved in cleavage and polyadenylation between early and late-stage B cells. We find an increase in the amount of cross-linking of a 64-kDa protein to poly(A) site-containing RNAs in plasma or myeloma extracts compared with memory B or lymphoma extracts. The protein was identified as the 64-kDa subunit of the general polyadenylation factor CstF by immunoprecipitation of UV-cross-linked material. The amount and form of the 64-kDa protein do not change in the different B-cell stages, suggesting increased binding efficiency. This increase in efficiency of 64-kDa binding could permit the relatively weak, but promoter-proximal, sec poly(A) site to be used in preference to the strong, promoter-distal, mb poly(A) site and may be important in regulating the expression of other plasma cell-specific genes.

MATERIALS AND METHODS

Cell growth and nuclear extract preparation. A20, AxJ, J558L, and 4T001 cells were grown in Iscove modified Dulbecco medium containing 5% horse serum. 2PK3 cells were grown in Dulbecco minimal essential medium containing 10% fetal calf serum, and HeLa cells were grown in minimal essential medium containing 10% horse serum. The characteristics of the five mouse cell lines are shown in Table 1. A20 is a γ2a/κ producer, while J558L is a myeloma line which has lost its endogenous α but retains a myeloma phenotype on transfected IgG genes (24). AxJ is a hybrid between A20 and J558L which produces secreted γ2a, κ, and the J558L λ light chain (33). Nuclear extracts were prepared as described by Dignam et al. (10) with the following modifications. All buffers contained 0.1 mM phenylmethylsulfonyl fluoride, 1.0 mM Na_3VO_4, and 5 mM NaF. Nuclei were extracted in 350 mM KCl, and the protease inhibitors aprotinin, leupeptin, and trypsin-chymotrypsin inhibitor were added to 1 μg/ml in the extraction buffer. HeLa cell extracts were dialyzed against a mixture of 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 0.2 mM EDTA, 10% glycerol, and 150 mM KCl. B-cell extracts were dialyzed against the same buffer containing 230 mM potassium glutamate in place of KCl. Aliquots of extract were frozen and stored at −80°C. Protein concentrations were determined by a Bradford assay (Bio-Rad) and were typically in the range of 1.5 to 4 mg/ml.

In vitro transcription of RNA substrates. Plasmid pGB6 contains 167 nucleotides of the Ig γ2b heavy chain that includes the CH3 exon 3′ untranslated region and the secretory poly(A) site cloned into pGEM4. The DNA was linearized with *Hin*dIII for full-length templates or *Asp*718 for precleaved templates and transcribed with SP6 RNA polymerase to make the pre-mRNA substrate used in in vitro assays. IgG sec-dsc contains a synthetic oligonucleotide corresponding to the 31-bp downstream consensus sequence described by Lassman et al. (25) cloned into pGEM3. The Ig γ2b membrane poly(A) site is contained in a 223-bp fragment of the 3′ untranslated region of the IgG heavy chain gene. This site was used by Lassman et al. (25) for in vivo studies. Plasmids containing the simian virus 40 late poly(A) (SVL) site or the SVL site with a deletion of the AAUAAA sequence were generously supplied by Jeff Wilusz (60). The adenovirus L3 and L3/AACAAA plasmids were kindly provided by Elizabeth Weiss and Joseph Nevins (59).

Plasmids were linearized and transcribed with SP6 RNA polymerase in the presence of 500 μM cap analog $m^7G(5')ppp(5')G$; 500 μM (each) ATP, CTP, and GTP; 50 μM UTP; and 50 μCi of [α-^{32}P]UTP (New England Nuclear). RNAs were gel purified by crushing and extracting in RNA extraction buffer containing guanidinium thiocyanate and phenol (5).

In vitro cleavage and polyadenylation assays. Cleavage reaction mixtures contained 5 mM creatine phosphate, 0.5 mM 3′-dATP, 2.5% polyvinyl alcohol, 30,000 to 50,000 cpm of ^{32}P-labelled RNA substrate, and 11 μl of nuclear extract in a 20-μl reaction volume (34). Reaction mixtures were incubated at 30°C for 2 to 3 h. Proteinase K (2.5-mg/ml final concentration) was added, and tubes were incubated at 37°C for 15 min. Following extraction with phenol and chloroform, RNA was precipitated and separated on a 5% polyacrylamide–8 M urea gel. For polyadenylation assays, 12 μl of nuclear extract was added to a 20-μl (final volume) reaction mixture containing 1 mM ATP, 0.7 mM $MgCl_2$, 0.1 mM EDTA, 0.6 mM dithiothreitol, 2% polyvinyl alcohol, and 5 to 10 fmol of ^{32}P-labelled RNA substrate. Reaction mixtures were incubated at 30°C for 45 min, and RNA was extracted by following the method of Chomczynski and Sacchi (5). RNA was separated on a 4.5% polyacrylamide–8 M urea gel.

UV cross-linking and immunoprecipitation. UV cross-linking reaction mixtures contained 5 μg of nuclear extract, 10 to 20 fmol of substrate RNA, 1 mM ATP, 0.7 mM $MgCl_2$, and 40 ng of carrier tRNA, brought to a 25-μl final volume with dialysis buffer. Reaction mixtures were incubated for 5 min at 30°C and then subjected to UV cross-linking on ice for 10 min in a Stratalinker (1.8×10^6 μJ/cm^2). Samples were treated with RNase A (1 mg/ml) for 15 min at 37°C. Proteins were boiled for 5 min in cracking buffer (0.1 M dithiothreitol, 2% sodium dodecyl sulfate [SDS], 80 mM Tris [pH 6.8], 10% glycerol, 0.2% bromphenol blue) and separated by SDS–10% polyacrylamide gel electrophoresis (PAGE).

For immunoprecipitation of UV-cross-linked proteins, 25 to 50 μg of nuclear extract was incubated with 100 fmol of substrate RNA under UV cross-linking conditions in a 25-μl reaction mixture essentially as described above. Following treatment with RNase, an aliquot of the reaction mixture was removed to run directly on an SDS–10% polyacrylamide gel. One hundred microliters of IP buffer (50 mM Tris [pH 7.4], 50 mM NaCl, 0.05% Nonidet P-40) was added to the remaining sample, along with 1/10 volume rabbit anti-mouse antibody (diluted 1:100; Sigma). Samples were incubated for 5 min on ice, and then an equal volume of Pansorbin (Calbiochem) equilibrated in IP buffer was added to clear nonspecific Ig absorbing material. Incubation continued for another 15 min on ice. Cleared extracts were recovered by centrifugation at 4°C for 10 min. Supernatants were transferred to fresh tubes, and one of the following antibodies was added: mouse anti-β-galactosidase (Sigma), mouse 3A7 anti-human 64-kDa subunit of CstF (52), or mouse 2C1 anti-human 50-kDa subunit of CstF (52). The mouse IgG2 antibodies to the 64- and 50-kDa subunits of CstF were gifts from Jim Manley and Clint MacDonald. Samples were rotated at 4°C for at least 1 h. Protein G-Sepharose (5 to 10 μg; Pharmacia) equilibrated in IP buffer was added, and incubation was continued at 4°C for another hour. Immunoprecipitated material was washed three times in IP buffer and then boiled for 5 min in cracking buffer (see above) and separated by SDS–10% PAGE.

Western analysis (immunoblot) of nuclear extracts. Ten micrograms of unfractionated nuclear extract was separated by SDS–10% PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane according to the instructions of the manufacturer (New England Nuclear). Filters were blocked in a mixture of 1% bovine serum albumin, 0.1% Tween 20, 10 mM Tris (pH 7.4), and 50 mM NaCl for 1 h, and then antibody was added (anti-64-kDa subunit of CstF, 1:100). Filters were incubated with shaking at room temperature for at least 1 h. Following three washes in 0.05% Tween 20–50 mM Tris (pH 7.4)–150 mM NaCl, sheep anti-mouse alkaline phosphatase-conjugated antibody diluted 1:5,000 (Sigma) was added as the second antibody. Filters were incubated for an hour at room temperature and washed three times. Immunoreactive proteins were detected by incubation with the alkaline phosphatase substrate 5-bromo-4-chloro-3-indolylphosphate toluidinium–Nitro Blue Tetrazolium (Sigma).

For Coomassie staining of proteins, nuclear extracts were separated by SDS–10% PAGE and then the gel was stained in 0.2% Coomassie blue–50% methanol–7% acetic acid for 30 min. The gel was destained in 5% methanol–7% acetic acid and then dried.

Two-dimensional electrophoresis. Isoelectric focusing gels (15 M urea, 7% polyacrylamide) were prepared with a pH range of 3.5 to 9.5 by using ampholytes from Pharmacia. Samples were prepared by the addition of 2 volumes of lysis buffer (9 M urea, 4% Nonidet P-40, 200 μg of ampholytes of the same pH range

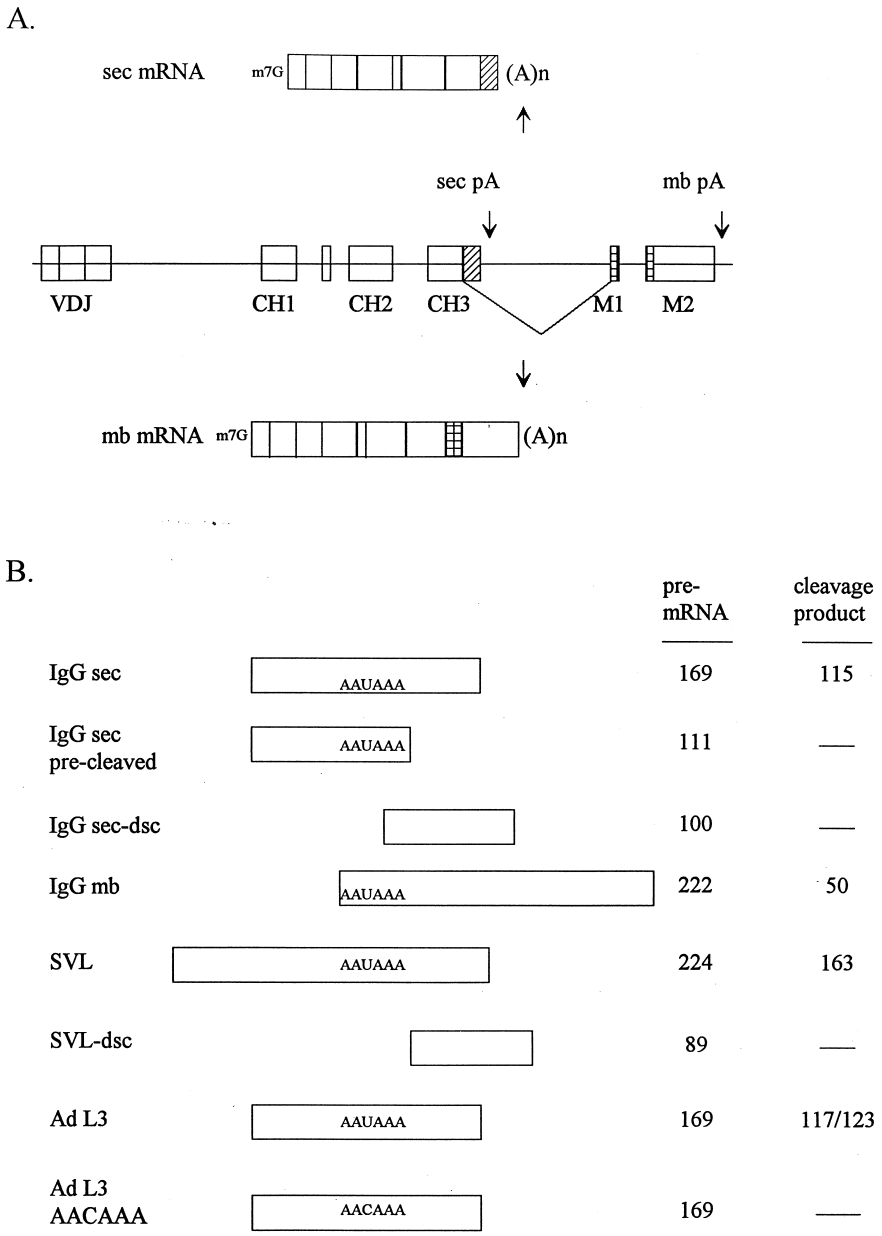

FIG. 1. (A) Schematic drawing of the mouse Ig γ2b heavy-chain transcription unit. Boxes represent exons; lines represent intron sequences. The box with diagonal lines is the sec mRNA-specific sequence. The cross-hatched boxes are mb-specific exons. The sec and mb poly(A) sites (pA) are noted. Splicing of the CH3 to M1 exon is represented by the angled line below the map. The processed sec transcript is represented above the genomic map, and the processed mb mRNA is shown below the map. (B) Substrates used for in vitro studies. Sizes of the pre-mRNAs and cleavage products are shown at right. The approximate location of the poly(A) signal sequence, AAUAAA, within the substrate is illustrated. IgG sec contains sequences both upstream and downstream of the cleavage site, whereas the precleaved substrate ends at the cleavage site. IgG sec-dsc does not have an AAUAAA sequence but contains a previously described consensus sequence downstream of the cleavage site (25). The other substrates have been described previously (see Materials and Methods). Ad, adenovirus.

as the gel per ml). Gels were run overnight at 800 V. Isoelectric focusing gels were removed from the capillary tubes and placed horizontally on an SDS–10% PAGE gel. Following electrophoresis, either the gel was dried and exposed to film or proteins were transferred to a PVDF membrane for Western analysis as described above.

RESULTS

Figure 1A schematically illustrates the mouse Ig γ2b transcription unit. sec mRNA is produced when the poly(A) site at the end of CH3 exon is used. mb mRNA is produced when a suboptimal splice donor site within the CH3 exon is spliced to the M1 exon and the poly(A) site at the end of the M2 exon is used. Although there is a change in both splicing and polyadenylation of the Ig heavy-chain pre-mRNA during B-cell development, previous work suggests that regulation of Ig γ2b mRNA production occurs predominantly at the level of polyadenylation (24, 25, 32). This indicates that the mb and sec poly(A) sites are used with approximately equal efficiencies in early or memory B cells whereas the sec poly(A) site is more efficient in late-stage or plasma B cells. Thus, when the γ2b sec poly(A) site is placed upstream of the mb site in the absence of a competing splicing event, the sec site is used preferentially in

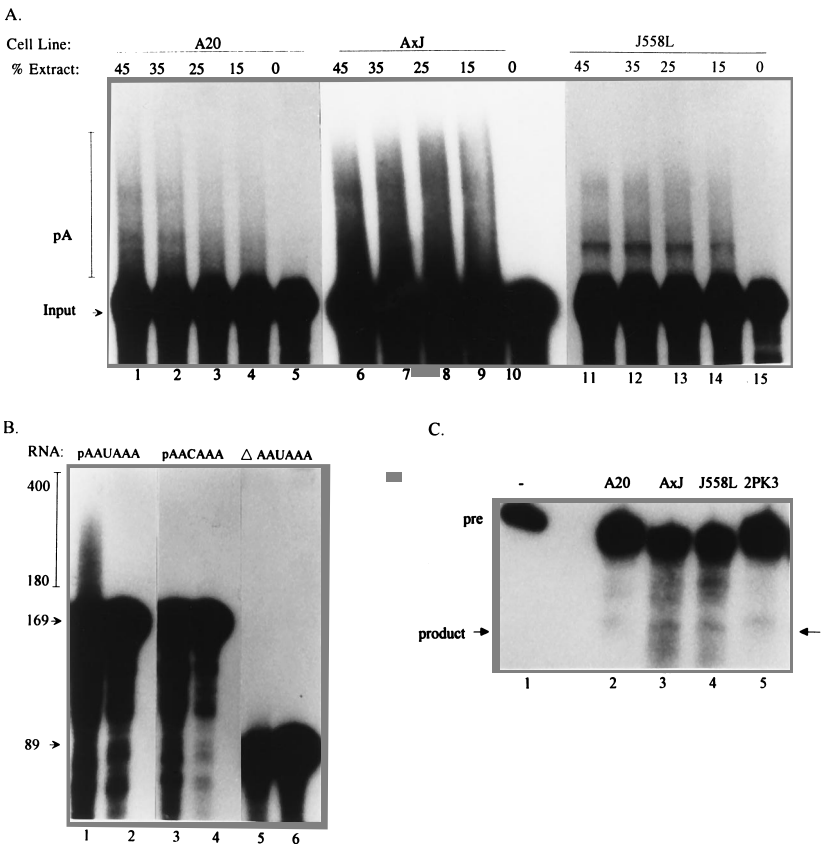

FIG. 2. Mouse B-cell nuclear extracts are competent for specific cleavage and polyadenylation. (A) The indicated amount of nuclear extract was incubated with ^{32}P-labelled pre-mRNA containing the SVL site under polyadenylation conditions as described in Materials and Methods. pA, poly(A). (B) A20 nuclear extract or buffer D incubated with the adenovirus L3 pre-mRNA (lanes 1 and 2), adenovirus L3/AACAAA (lanes 3 and 4), or SVL dsc lacking AAUAAA (lanes 5 and 6) under polyadenylation conditions (60% nuclear extract) as described in Materials and Methods. Lanes 1, 3, and 5, A20 nuclear extract; lanes 2, 4, and 6, buffer D. (C) The indicated extracts (lanes 2 to 5) or buffer D (lane 1) were incubated with adenovirus L3 pre-mRNA under cleavage conditions described in Materials and Methods. The precursor (pre) and accurately cleaved product are indicated.

myeloma but not lymphoma cells; accordingly, poly(A) site use appears to be a regulated event (26).

Mouse B-cell lines represent different stages of B-cell development. To dissect the mechanism of the poly(A) site switch during B-cell development, nuclear extracts were prepared from mouse B-cell lines representing different stages of development and tested for polyadenylation activity in vitro; phenotypic characteristics of the different cell lines are listed in Table 1. The ratio of sec to mb RNA was determined by densitometric scanning of Northern (RNA) blots probed with Ig γ2b DNA to detect both forms of RNA. A20 and 2PK3 are lymphoma cell lines that represent an early or memory stage of mouse B-cell development and have a 2:1 ratio of sec to mb RNA. J558L and 4T001 are myeloma cell lines that represent late-stage B-cell or plasma cell lines and have switched to predominantly sec mRNA. The J558L cell line has lost its endogenous heavy-chain gene, but when an IgG gene is transfected, the ratio of sec to mb mRNA is 18:1 (24). The AxJ cell line was made by fusion of the A20 and J558L cell lines (33). Notably, the plasma cell phenotype is dominant in AxJ since the sec-to-mb mRNA ratio is at least 36:1. The A20 and AxJ cell lines are a useful experimental tool for studying Ig poly(A) site use because the Ig γ genes expressed in the two cell lines are identical.

Mouse B-cell extracts are competent for cleavage and polyadenylation. Nuclear extracts were prepared as described in Materials and Methods and were tested for the ability to specifically cleave and polyadenylate the RNA substrates illustrated in Fig. 1B. Figure 2A shows the results of polyadenylation reactions in which various amounts of extract were incubated for the same time with an RNA substrate containing the SVL site. The extracts representing different B-cell stages were all competent for polyadenylation. Efficiencies observed with our extracts were comparable to those of HeLa nuclear extracts and were typically in the range of 25% conversion to polyadenylated RNA with 45% extract, depending on the RNA substrate used (data not shown). At 15% extract all the cell lines show a diminished capacity to polyadenylate (Fig. 2A, compare lanes 4, 9, and 14), again indicating equal efficiencies of the various extracts. Figure 2B demonstrates the specificity of the reaction with the A20 extract in that no polyadenylation activity is detected on a substrate that contains a mutation in the poly(A) signal sequence, AACAAA (lane 3), or on a substrate lacking a poly(A) signal (lane 5). Similar results were obtained for all other extracts. To show that cleavage at the poly(A) site was accurate, the adenovirus L3 poly(A) site was used in a cleavage reaction (Fig. 2C). The in vitro cleavage reaction was accurate, but not much of the cleaved product is seen, presumably because of nucleases in the B-cell extracts.

UV cross-linking reveals stage-specific differences in B-cell extracts. We used UV cross-linking to see if there is a difference between early and late B-cell stages with respect to the

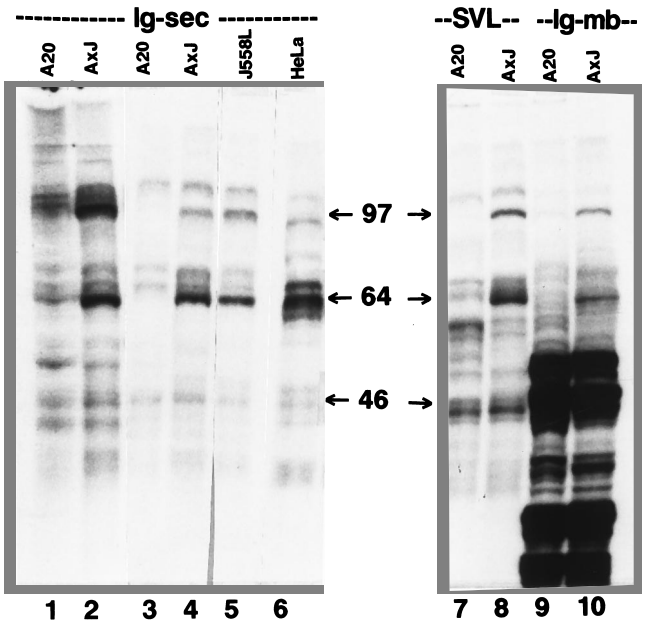

FIG. 3. UV cross-linking of proteins from lymphoma (A20), fusion of lymphoma and myeloma (AxJ), myeloma (J558L), or HeLa cells to poly(A) site-containing pre-mRNA substrates. ^{32}P-labelled sec, SVL, or mb pre-mRNAs were incubated with 5 μg of the indicated extracts under UV cross-linking conditions (see Materials and Methods) and subjected to UV irradiation for 10 min on ice. Following RNase digestion, proteins were separated by SDS–10% PAGE, and dried gels were exposed to film. Molecular weight markers are approximate.

number of proteins that contact a poly(A) site-containing RNA or their avidity for RNA. Substrate RNA for this assay was synthesized in vitro with [^{32}P]UTP, cap analog (m^7GpppG), unlabelled nucleoside triphosphates, and SP6 RNA polymerase (see Fig. 1B and Materials and Methods). After gel purification, the RNA was incubated briefly with nuclear extract under conditions that allow polyadenylation complex formation but no significant polyadenylation. The reactions were then subjected to UV cross-linking, which causes the transfer of ^{32}P label to protein that is in contact with the RNA. Following RNase digestion, the products were separated by one- or two-dimensional gel electrophoresis and visualized by autoradiography.

Nuclear extracts were incubated with a ^{32}P-labelled RNA substrate containing the IgG sec poly(A) site and subjected to UV cross-linking (Fig. 3, lanes 1 to 6). Although equal amounts of total protein (5 μg) and substrate were used in each reaction, it is clear that more of a protein of approximately 64 kDa was cross-linked in the plasma or myeloma cell extract than in the lymphoma or memory B-cell extract (Fig. 3, compare lanes 1 and 3 with lanes 2, 4, and 5). We have seen the same results with numerous extracts prepared over the past 3 years. The AxJ hybrid, which shows a dominant myeloma phenotype with respect to mRNA production (Table 1), behaved like the myeloma partner in that there was increased cross-linking of the 64-kDa protein. Therefore, there is a direct correlation between in vivo sec mRNA expression and increased 64-kDa protein cross-linking in vitro.

The sec poly(A) site, but not the mb or simian virus 40 early site, is regulated during B-cell development (25). To examine whether the difference in UV cross-linking pattern between early and late B-cell extracts is specific for the sec poly(A) site, we tested the Ig mb poly(A) and SVL sites in the same assay (Fig. 3, lanes 7 to 10). Again, the AxJ and J558L extracts (plasma cell like) show more binding of a 64-kDa protein to the RNA substrates compared with early B-cell extracts (Fig. 3). This indicates that, at least in vitro, the enhanced binding of the 64-kDa protein is B cell stage but not sequence specific.

We also see an increase in cross-linking of an approximately 100-kDa protein to input RNAs in plasma or myeloma cell extracts versus early or lymphoma extracts in this experiment. That cross-linking is somewhat variable; therefore, we have not characterized it further here.

We speculate that while we see a difference in 64-kDa protein binding, we do not see a difference in polyadenylation efficiency on these substrates between myeloma and lymphoma extracts because polyadenylation assays are done with 7- to 10-fold more extract than are UV cross-linking assays, and the incubation time for polyadenylation is 45 min as opposed to 5 min for UV cross-linking. Therefore, we believe that we have achieved saturation of the available poly(A) sites in the polyadenylation assay.

The protein showing increased cross-linking in plasma cell extracts is a subunit of the general polyadenylation factor CstF. The electrophoretic mobility of the major cross-linked protein suggested that it might be a subunit of the general polyadenylation factor CstF. CstF contains three subunits of 77, 50, and 64 kDa; its 64-kDa subunit has been shown to cross-link specifically to poly(A) site-containing RNAs in the presence of the other subunits and CPSF (27, 61). To show conclusively that the cross-linked 64-kDa protein is the 64-kDa subunit of CstF, we immunoprecipitated material cross-linked to the Ig sec poly(A) site using a monoclonal antibody to the human 64-kDa protein, kindly provided by Jim Manley and Clint MacDonald. As illustrated in Fig. 4, the anti-64-kDa protein monoclonal antibody but not a control monoclonal antibody (mouse anti-β-galactosidase, lanes 2 and 5) specifically immunoprecipitated a cross-linked protein of 64 kDa from A20 (lymphoma) and AxJ (myeloma-like) extracts (lanes 3 and 6). The immunoprecipitated protein comigrates with the dominant cross-linked protein in the total reactions (Fig. 4, lanes 1 and 4). The total UV-cross-linking reactions are both very dark (Fig. 4, lanes 1 and 4) because of the large amount of substrate and extract added to achieve a signal in the immunoprecipitation. A monoclonal antibody to the 50-kDa subunit of CstF precipitated what appears to be the same 64-kDa protein, presumably as part of a complex (data not shown),

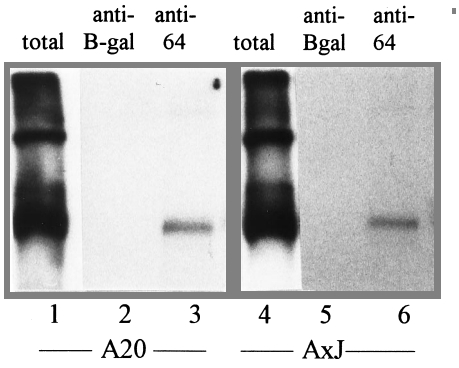

FIG. 4. Immunoprecipitation of proteins UV cross-linked to poly(A) site-containing RNAs. The indicated nuclear extracts (50 μg) were incubated with ^{32}P-labelled IgG sec poly(A) site and subjected to UV cross-linking and immunoprecipitation as described in Materials and Methods. Lanes 1 and 4, one-half of the total UV cross-linking reaction mixture prior to immunoprecipitation; lanes 2 and 5, mouse anti-β-galactosidase monoclonal antibody (Sigma); lanes 3 and 6, mouse anti-human 64-kDa subunit of CstF hybridoma supernatant.

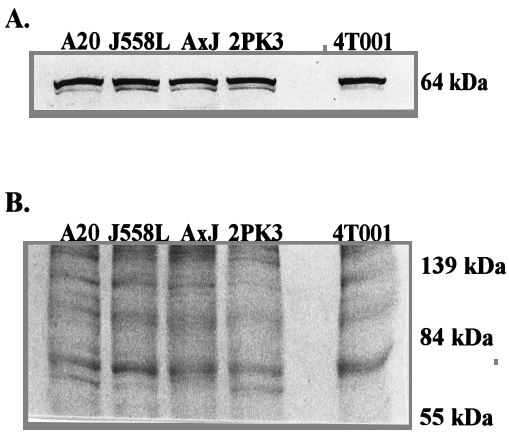

FIG. 5. Western analysis of nuclear extracts. (A) The indicated extracts (10 μg) were separated by SDS–10% PAGE. Proteins were transferred to a PVDF membrane and reacted with anti-64-kDa protein hybridoma supernatant as described in Materials and Methods. (B) Nuclear extracts (10 μg) were separated by SDS–10% PAGE. The proteins were then stained with Coomassie blue.

strengthening the suggestion that the protein showing increased binding to poly(A) sites in plasma cell extracts is the 64-kDa subunit of CstF. The 64-kDa protein monoclonal antibody also immunoprecipitates the 64-kDa protein cross-linked to the Ig sec, SVL, or Ig mb substrate by using the J558L, 2PK3, 4T001, and HeLa extracts (data not shown). The 64-kDa protein is not cross-linked to a substrate lacking either the poly(A) signal or the region downstream of the poly(A) site (see Fig. 7C and D; also data not shown).

The amount and form of the 64-kDa protein do not change in the different B-cell stages. Having demonstrated that the protein showing increased cross-linking to poly(A) site-containing RNAs in plasma or myeloma nuclear extracts is the 64-kDa subunit of the general polyadenylation factor CstF, we next wanted to examine the expression of this polypeptide in the different B-cell stages. Although the levels of mRNA encoding the 64-kDa protein do not vary between early and late-stage B cells (31), it is possible that translational and posttranslational mechanisms regulate the amount or form of 64-kDa protein. Ten micrograms of protein from unfractionated nuclear extracts was separated by SDS–10% PAGE and either transferred to a PVDF membrane for Western analysis (Fig. 5A) or stained with Coomassie blue (Fig. 5B) to confirm equal loading. There is no significant difference in the amounts of 64-kDa protein visualized by reactivity to the anti-64-kDa protein monoclonal antibody among the extracts (Fig. 5A), and so changes in the amount of 64-kDa protein therefore cannot account for the changes in UV cross-linking pattern between early and late-stage B-cell extracts.

The 64-kDa protein contains consensus sites for phosphorylation by protein kinase C and other potential modification sites (49). We therefore investigated whether the form(s) of 64-kDa protein differed between early and late-stage B-cell extracts by two-dimensional electrophoresis. Proteins were separated in the first dimension by isoelectric focusing, pH 3.5 to 9.5, and in the second dimension by SDS–10% PAGE. Proteins were transferred to a PVDF membrane and reacted with anti-64-kDa protein monoclonal antibody in a Western analysis. As seen in Fig. 6, there are four major species of 64-kDa protein which potentially represent different phosphorylation states. A comparison of the A20 (early or memory) cell extract (Fig. 6A) with the J558L (plasma) cell extract (Fig. 6B), however, shows identical patterns. The forms of the HeLa 64-kDa protein were also examined by two-dimensional electrophoresis, and four similarly migrating proteins were detected (data not shown). Therefore, we conclude that the 64-kDa protein is not modified in a B-cell-specific manner.

Quantitation of the cross-linking of the 64-kDa protein to poly(A) sites in early versus late-stage B-cell extracts. To be able to quantify the difference in binding of the 64-kDa protein to poly(A) site-containing RNAs in early versus late-stage B cells, UV cross-linking assays were performed using an internal control RNA, to compensate for loading differences, and then the products were separated in two dimensions. Our test RNAs were either the IgG sec poly(A) site or the SVL site (Fig. 1B). The 64-kDa protein binds to these RNAs in a consistent pattern of four major spots, as shown in Fig. 7A and B. The pattern of four spots is identical to that seen on the two-dimensional Western blot using anti-64-kDa protein antibody (Fig. 6) and is identical among the B-cell extracts (compare Fig. 7A and B and 8A and B). The control RNA in the reaction is the SVL site which lacks the poly(A) signal (termed SVL dsc; Fig. 1B) and so does not bind the 64-kDa subunit of CstF. However, this RNA does bind to several acidic proteins that have a characteristic pattern on a two-dimensional gel (Fig. 7C and D) and therefore can be used as our internal standard.

A 10-fold molar excess of either the Ig γ2b sec poly(A) site or the SVL site was mixed with the SVL dsc probe, incubated with nuclear extract, and subjected to UV cross-linking and two-dimensional electrophoretic analysis. An example of such an experiment using the A20 (lymphoma) and J558L (myeloma) extracts is shown in Fig. 7E and F. The same experiment performed with the 2PK3 (lymphoma) and 4T001 (myeloma) extracts is shown in Fig. 8. Densitometric scanning was used to determine the regulation index, which signifies the amount of radioactivity in the 64-kDa protein spots relative to the SVL dsc internal control from the plasma cell extract divided by the amount of 64-kDa protein spot relative to the internal control in the memory cell extract run under identical conditions.

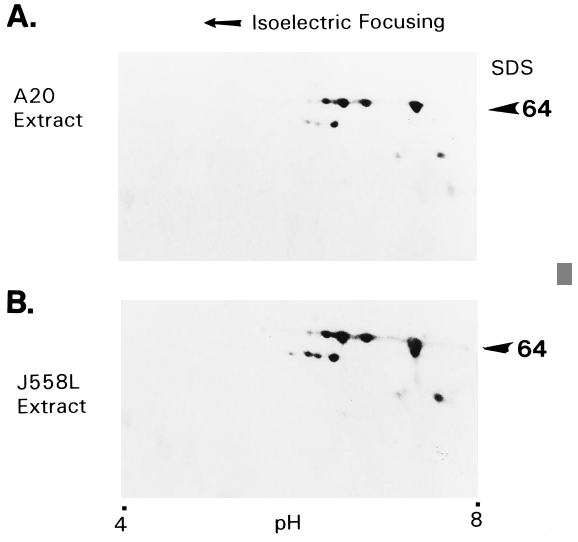

FIG. 6. Western analysis of nuclear extracts; two-dimensional gels. Unfractionated nuclear extracts (20 μg) were separated by isoelectric focusing, pH 3.5 to 9.5, in the first dimension and then by SDS–10% PAGE as described in Materials and Methods. Proteins were transferred to PVDF and reacted with antibody to the 64-kDa subunit of CstF.

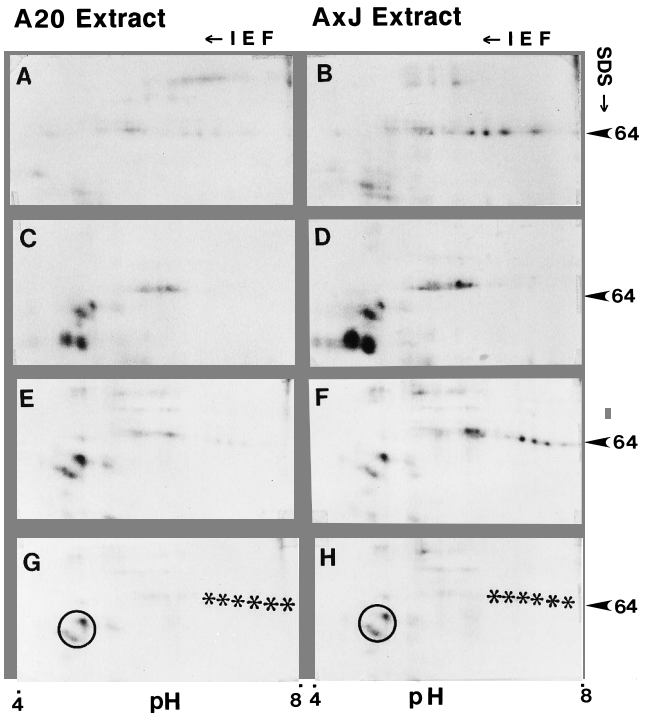

FIG. 7. Two-dimensional analysis of proteins UV cross-linked to RNA for A20 and AxJ extracts. (A and B) Proteins cross-linked to the Ig sec poly(A) site in the A20 (lymphoma) or AxJ (lymphoma × myeloma hybrid) extract; (C and D) proteins cross-linked to the SVL dsc RNA in the A20 or AxJ extract; (E and F) proteins cross-linked to RNAs when extracts were incubated with a 10-fold molar excess of Ig sec RNA mixed with the SVL dsc RNA; (G and H) circled areas denote proteins bound to the SVL dsc RNA substrate whose intensity was measured by densitometry as an internal loading control, and starred areas indicate the positions of 64-kDa subunit of CstF cross-linked to the RNA substrate which were measured by densitometry. IEF, isoelectric focusing.

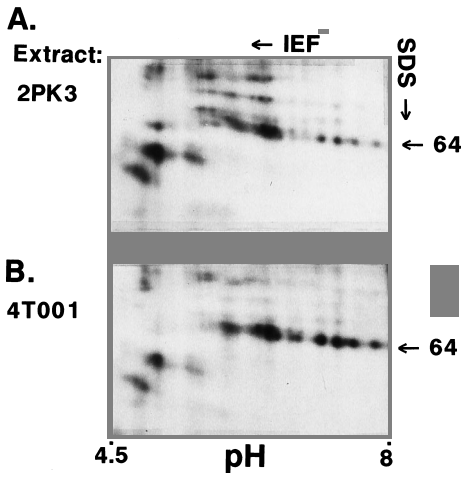

FIG. 8. Two-dimensional analysis of proteins UV cross-linked to RNA for 2PK3 and 4T001. A 10-fold molar excess of Ig sec poly(A) site substrate over the internal control SVL dsc RNA was incubated with the indicated extract and subjected to UV cross-linking and two-dimensional gel electrophoresis as described in Materials and Methods. Regions scanned for quantitation were as in Fig. 7G and H. (A) 2PK3 (lymphoma); (B) 4T001 (myeloma).

Areas used for scanning are indicated in Fig. 7G and H. Results of multiple experiments with several different representatives of the two B-cell stages are shown in Table 2. There is a four- to eightfold better binding of the 64-kDa subunit of CstF to poly(A) site-containing RNAs in plasma cell extracts compared with that in memory B-cell nuclear extracts. These results demonstrate that the binding activity of a constitutive factor required for cleavage and polyadenylation is altered in a B-cell-specific fashion but is not sequence specific in vitro.

DISCUSSION

Poly(A) site use in Ig heavy-chain transcription units is regulated during B-cell development. In early B cells or their tumor counterparts, lymphomas, the amounts of sec mRNA and mb mRNA produced are approximately equal. There is a switch to predominant use of the promoter-proximal sec poly(A) site in plasma or myeloma cells. In this study we have demonstrated that in vitro the binding activity of a general polyadenylation factor is altered in a B-cell-specific manner. There is an increase in the amount of 64-kDa subunit of CstF cross-linked to poly(A) site-containing RNAs in plasma cell extracts compared with that in early B-cell extracts (Fig. 3, 7, and 8). One-dimensional (Fig. 5) and two-dimensional (Fig. 6) Western and UV cross-linking (Fig. 7 and 8) analyses demonstrate that the amount and forms of the 64-kDa polypeptide do not change in the different B-cell stages. Although only the sec or other weak poly(A) sites (39) are regulated in vivo, all poly(A) sites tested in vitro show the increase in cross-linking of the 64-kDa protein. It is possible that in vivo the local concentration of factors is rate limiting, which would not result in an increased use of already strong sites, while in vitro there is a large excess of factors relative to the input RNAs, so the regulation is seen to act on strong sites as well as weak ones.

Polyadenylation has also been shown to regulate L1 versus L3 mRNA production in adenovirus infection (36, 37). The adenovirus major late transcription unit is a complex transcription unit that undergoes a switch in poly(A) site use late in infection. The promoter-proximal poly(A) site, L1, is weaker than the promoter-distal L3 poly(A) site (42), analogous to the Ig sec and mb poly(A) site arrangement; the switch, however, is to predominant use of the downstream L3 poly(A) site late in infection. The switch in poly(A) site use in adenovirus shows many similarities to that of the Ig γ2b transcription unit. There is a change in the binding of the 64-kDa subunit of CstF during adenovirus infection (28) and in B-cell stages with no change in the amount of 64-kDa protein in either system. Late in adenovirus infection, the activity of binding of the 64-kDa protein to poly(A) site-containing RNAs decreases, suggesting a decrease in overall polyadenylation efficiency (28); as a consequence, use of the stronger poly(A) site is favored. Meanwhile,

TABLE 2. Quantitation of UV cross-linking of the 64-kDa subunit of CstF to poly(A) site-containing RNA substrates

Extract in which:		RNA substrate[a]	Regulation index[b]
sec = mb	sec ≫ mb		
A20	AxJ	IgG sec	5.7
A20	J558L	IgG sec	4.0
2PK3	4T001	IgG sec	4.0
A20	J558L	SVL	5.3
2PK3	4T001	SVL	8.2

[a] The indicated RNA was present in a 10-fold molar excess over the internal control SVL dsc RNA.

[b] The amount of radioactivity in the 64-kDa protein spots relative to the internal control (SV dsc) from the cell line in the second column divided by the amount in 64-kDa protein spots relative to the internal control in the cell line in the first column.

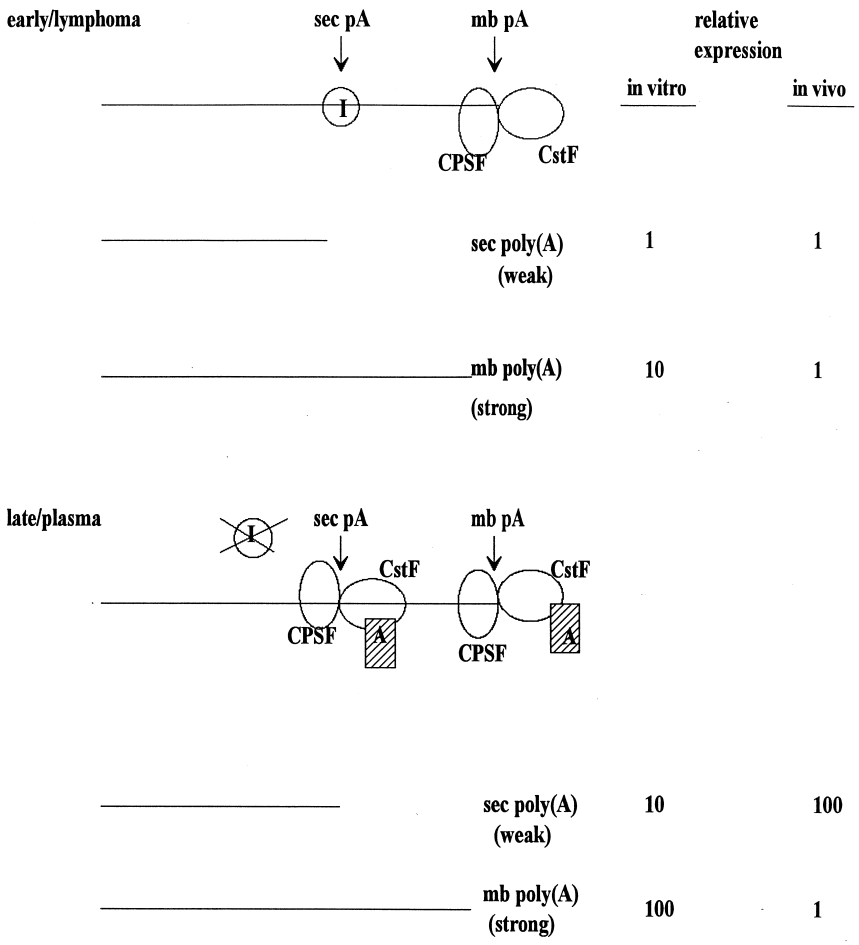

FIG. 9. Model. The presence of a sec poly(A) (pA) site inhibitor (I) in early B cells or lymphomas allows the 3′ mb site to compete for polyadenylation factors. In late-stage or plasma cells, there is an increase in binding of the 64-kDa protein to all poly(A) sites tested in vitro. Loss of the sec-specific inhibitor should result in an increase in cross-linking of the 64-kDa protein to the sec poly(A) site only. The presence of a nonspecific activator (A) of polyadenylation in addition to loss of an inhibitor could cause the increase in 64-kDa protein binding to all poly(A) sites in vitro and cause an increase in polyadenylation efficiency at the sec site seen in vivo in late-stage or plasma cells.

in late-stage or plasma cells, the activity of binding of the 64-kDa subunit of CstF increases, implying an increase in polyadenylation efficiency; consequently, increased use of the weaker, promoter-proximal poly(A) site is favored.

Previous studies using stable (25, 26) or transient (40) transfections have also noted that overall cleavage-polyadenylation efficiency increases in plasma cells compared with early B cells as measured by the relative use of tandem poly(A) sites. Recent transfection and biochemical studies have suggested that there is a negative regulator (inhibitor) of Ig sec poly(A) site use in lymphoma cells (39, 62). The AxJ cell line is a fusion between the A20 (lymphoma or early B-cell) and J558L (myeloma or plasma cell) lines, and it has the phenotype of a plasma cell line, with a greater than 36:1 ratio of sec to mb mRNA production. Whatever the myeloma contributes to the AxJ hybrid must be enough to neutralize this negative regulator by introducing a dominant, positively acting factor (activator), by repressing the negative regulator, or both. On the basis of our data we argue (Fig. 9) that an activator must be contributed by the myeloma because AxJ hybrids show an increase in 64-kDa protein binding in vitro to both weak and strong poly(A) sites. An activator which is not poly(A) site specific, perhaps with the loss of this potential inhibitor, could increase overall polyadenylation efficiency in vivo, thereby stabilizing complex formation and allowing the weaker 5′ sec poly(A) site to be used in plasma cells. This could affect a first-come first-served mechanism which operates in late-stage or plasma B cells, whereas in early or memory B cells, the distal mb poly(A) site, which is stronger than the sec poly(A) site, can effectively compete for polyadenylation factors.

A plasma cell poly(A) site activator such as the one that we implicate here could cause a heretofore unrecognized regulatory mechanism for the activation of a whole family of genes with weak poly(A) sites. One member of this family might be *CD40*, a gene important for B-cell development (reviewed in reference 12) that has two poly(A) sites whose relative use changes during B-cell development (55). Before B cells are activated, both mRNAs are produced; after activation, the shorter, potentially more stable mRNA is the predominant species (55). In light of our findings of increased 64-kDa binding in plasma cells, it will be interesting to study the regulation of poly(A) site use in *CD40* and in other genes (22) with multiple poly(A) sites.

ACKNOWLEDGMENTS

We thank Makiko Hartman and Tanima Sinha for superb technical assistance. We thank Justus Cohen for helpful comments on the manuscript and members of the Milcarek and Cohen laboratories for useful

discussions. We thank Jim Manley and Clint MacDonald for the 3A7 64-kDa protein-specific and 2C1 50-kDa protein-specific monoclonal antibodies.

This work was supported by National Institutes of Health grant GM-50145 to C.M.

REFERENCES

1. **Alt, F. W., A. L. Bothwell, M. Knapp, E. Siden, M. Koshland, and D. Baltimore.** 1980. Synthesis of secreted and membrane bound immunoglobulin μ heavy-chain is directed by mRNAs that differ at their 3′ ends. Cell **20:**293–301.
2. **Bienroth, S., E. Wahle, C. Suter-Crazzolara, and W. Keller.** 1991. Purification of the cleavage and polyadenylation factor involved in the 3′-processing of messenger RNA precursors. J. Biol. Chem. **266:**19768–19776.
3. **Brown, S. L., and S. L. Morrison.** 1989. Developmental regulation of membrane and secretory Ig γ2b mRNA. J. Immunol. **142:**2072–2080.
4. **Carswell, S., and J. C. Alwine.** 1989. Efficiency of utilization of the simian virus 40 late polyadenylation site: effects of upstream sequences. Mol. Cell. Biol. **9:**4248–4258.
5. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
6. **Christofori, G., and W. Keller.** 1988. 3′ cleavage and polyadenylation of mRNA precursors in vitro requires a poly(A) polymerase, a cleavage factor, and a snRNP. Cell **54:**875–889.
7. **Christofori, G., and W. Keller.** 1989. Poly(A) polymerase purified from HeLa cell nuclear extract is required for both cleavage and polyadenylation of pre-mRNA in vitro. Mol. Cell. Biol. **9:**193–203.
8. **Cushley, W., B. E. H. Coupar, C. A. Mickelson, and A. R. Williamson.** 1982. A common mechanism for the synthesis of membrane and secreted immunoglobulin α, γ, and μ chains. Nature (London) **298:**77.
9. **DeZazzo, J. D., and M. J. Imperiale.** 1989. Sequences upstream of AAUAAA influence poly(A) site selection in a complex transcription unit. Mol. Cell. Biol. **9:**4951–4961.
10. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.
11. **Flaspohler, J. A., and C. Milcarek.** 1990. Myelomas and lymphomas expressing the IgG2a H chain gene have similar transcription termination regions. J. Immunol. **144:**2802–2810.
12. **Fuleihan, R., N. Ramesh, and R. S. Geha.** 1993. Role of CD40-CD40-ligand interaction in Ig-isotype switching. Curr. Opin. Immunol. **5:**963–967.
13. **Galli, G., J. W. Guise, M. A. McDevitt, P. W. Tucker, and J. R. Nevins.** 1987. Relative position and strengths of poly(A) sites as well as transcription termination are critical to membrane versus secreted μ-chain expression during B-cell development. Genes Dev. **1:**471–481.
14. **Gil, A., and N. J. Proudfoot.** 1984. A sequence downstream of AAUAAA is required for rabbit β-globin mRNA 3′-end formation. Nature (London) **312:**473–474.
15. **Gilmartin, G. M., E. S. Fleming, and J. Oetjen.** 1993. Activation of HIV pre-mRNA 3′ processing in vitro requires both an upstream element and TAR. EMBO J. **12:**4419–4428.
16. **Gilmartin, G. M., and J. R. Nevins.** 1989. An ordered pathway of assembly of components required for polyadenylation site recognition and processing. Genes Dev. **3:**2180–2189.
17. **Gilmartin, G. M., and J. R. Nevins.** 1991. Molecular analyses of two poly(A) site-processing factors that determine the recognition and efficiency of cleavage of the pre-mRNA. Mol. Cell. Biol. **11:**2432–2438.
18. **Guise, J. W., G. Galli, J. R. Nevins, and P. W. Tucker.** 1989. Developmental regulation of secreted and membrane forms of immunoglobulin μ chain, p. 275–302. *In* T. Honjo, F. W. Alt, and T. H. Rabbitts (ed.), Immunoglobulin genes. Academic Press, Inc., San Diego, Calif.
19. **Guise, J. W., P. L. Lim, D. Yuan, and P. W. Tucker.** 1988. Alternative expression of secreted and membrane forms of immunoglobulin μ-chain is regulated by transcriptional termination in stable plasmocytoma transfectants. J. Immunol. **140:**3988–3994.
20. **Hart, R. P., M. A. McDevitt, H. Ali, and J. R. Nevins.** 1985. Definition of essential sequences and functional equivalence of elements downstream of the adenovirus E2A and the early simian virus 40 polyadenylation sites. Mol. Cell. Biol. **5:**2975–2983.
21. **Jenny, A., H. Hauri, and W. Keller.** 1994. Characterization of cleavage and polyadenylation specificity factor and cloning of its 100-kilodalton subunit. Mol. Cell. Biol. **14:**8183–8190.
22. **Kan, J. L. C., and R. G. Moran.** 1995. Analysis of a mouse gene encoding three steps of purine synthesis reveals use of an intronic polyadenylation signal without alternative exon usage. J. Biol. Chem. **270:**1823–1832.
23. **Keller, W., S. Bienroth, K. M. Lang, and G. Christofori.** 1991. Cleavage and polyadenylation factor CPF specifically interacts with the pre-mRNA 3′ processing signal AAUAAA. EMBO J. **10:**4241–4249.
24. **Kobrin, B. J., C. Milcarek, and S. L. Morrison.** 1986. Sequences near the 3′ secretion-specific polyadenylation site influence levels of secretion-specific and membrane-specific IgG2b mRNA in myeloma cells. Mol. Cell. Biol. **6:**1687–1697.
25. **Lassman, C. R., S. Matis, B. L. Hall, D. L. Toppmeyer, and C. Milcarek.** 1992. Plasma cell-regulated polyadenylation at the Igγ2b secretion-specific poly(A) site. J. Immunol. **148:**1251–1260.
26. **Lassman, C. R., and C. Milcarek.** 1992. Regulated expression of the mouse γ2b Ig H chain gene is influenced by poly(A) site order and strength. J. Immunol. **148:**2578–2585.
27. **MacDonald, C. C., J. Wilusz, and T. Shenk.** 1994. The 64-kilodalton subunit of the CstF polyadenylation factor binds to pre-mRNAs downstream of the cleavage site and influences cleavage site location. Mol. Cell. Biol. **14:**6647–6654.
28. **Mann, K. P., E. A. Weiss, and J. R. Nevins.** 1993. Alternative poly(A) site utilization during adenovirus infection coincides with a decrease in the activity of a poly(A) site processing factor. Mol. Cell. Biol. **13:**2411–2419.
29. **McDevitt, M. A., M. J. Imperiale, H. Ali, and J. R. Nevins.** 1984. Requirement of a downstream sequence for generation of a poly(A) addition site. Cell **37:**993–999.
30. **McLauchlan, J., D. Gaffney, J. L. Whitton, and J. B. Clements.** 1985. The consensus sequence YGTGTTYY located downstream from the AATAAA signal is required for efficient formation of mRNA 3′ termini. Nucleic Acids Res. **13:**1347–1365.
31. **Milcarek, C.** Unpublished data.
32. **Milcarek, C., and B. Hall.** 1985. Cell-specific expression of secreted versus membrane forms of immunoglobulin gamma 2b mRNA involves selective use of alternate polyadenylation sites. Mol. Cell. Biol. **5:**2514–2520.
33. **Milcarek, C., M. Hartman, and S. C. Croll.** Submitted for publication.
34. **Moore, C. L., and P. A. Sharp.** 1985. Accurate cleavage and polyadenylation of exogenous RNA substrate. Cell **41:**845–855.
35. **Murthy, K. G. K., and J. L. Manley.** 1992. Characterization of the multisubunit cleavage-polyadenylation specificity factor from calf thymus. J. Biol. Chem. **267:**14804–14811.
36. **Nevins, J. R., and J. E. Darnell.** 1978. Steps in the processing of Ad2 mRNA: poly(A)+ nuclear sequences are conserved and poly(A) addition precedes splicing. Cell **15:**1477–1493.
37. **Nevins, J. R., and M. C. Wilson.** 1981. Regulation of adenovirus-2 gene expression at the level of transcriptional termination and RNA processing. Nature (London) **290:**113–118.
38. **Nishikura, K., and G. A. Vucolo.** 1984. Synthesis of two mRNAs by utilization of alternate polyadenylation sites: expression of SV40-mouse immunoglobulin μ-chain gene recombinants in COS monkey cells. EMBO J. **3:**689–699.
39. **Peterson, M. L.** 1994. Regulated immunoglobulin (Ig) RNA processing does not require specific *cis*-acting sequences: non-Ig RNA can be alternatively processed in B cells and plasma cells. Mol. Cell. Biol. **14:**7891–7898.
40. **Peterson, M. L., E. R. Gimmi, and R. P. Perry.** 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.
41. **Peterson, M. L., and R. P. Perry.** 1989. The regulated production of μ_m and μ_s mRNA is dependent on the relative efficiencies of the μ_s poly(A) site usage and the Cμ4-to-M1 splice. Mol. Cell. Biol. **9:**726–738.
42. **Prescott, J. C., and E. Falck-Pedersen.** 1992. Varied poly(A) site efficiency in the adenovirus major late transcription unit. J. Biol. Chem. **267:**8175–8181.
43. **Prescott, J. C., and E. Falck-Pedersen.** 1994. Sequence elements upstream of the 3′ cleavage site confer substrate strength to the adenovirus L1 and L3 polyadenylation sites. Mol. Cell. Biol. **14:**4682–4693.
44. **Rogers, J. E., E. Choi, C. Souza, C. Carter, C. Word, M. Kuehl, D. Eisenberg, and R. Wall.** 1981. Gene segments encoding transmembrane carboxyl termini of immunoglobulin γ chains. Cell **26:**19–27.
45. **Rogers, J. E., P. Early, C. Carter, K. Calame, M. Bond, L. Hood, and R. Wall.** 1980. Two mRNAs with different 3′ ends encode membrane-bound and secreted forms of immunoglobulin μ chains. Cell **20:**301–312.
46. **Russnak, R., and D. Ganem.** 1990. Sequences 5′ to the polyadenylation signal mediate differential poly(A) site use in hepatitis B viruses. Genes Dev. **4:**764–776.
47. **Sadofsky, M., and J. C. Alwine.** 1984. Sequences on the 3′ side of hexanucleotide AAUAAA signals affect efficiency of cleavage at the polyadenylation site. Mol. Cell. Biol. **4:**1460–1468.
48. **Schek, N., C. Cook, and J. C. Alwine.** 1992. Definition of the upstream efficiency element of the simian virus 40 late polyadenylation signal by using in vitro analysis. Mol. Cell. Biol. **12:**5386–5393.
49. **Takagaki, Y., C. C. MacDonald, T. Shenk, and J. L. Manley.** 1992. The human 64-kDa polyadenylation factor contains a ribonucleoprotein-type RNA binding domain and unusual auxiliary motifs. Proc. Natl. Acad. Sci. USA **89:**1403–1407.
50. **Takagaki, Y., and J. L. Manley.** 1992. A human polyadenylation factor is a G protein β-subunit homologue. J. Biol. Chem. **267:**23471–23474.
51. **Takagaki, Y., and J. L. Manley.** 1994. A polyadenylation factor subunit is the human homologue of the Drosophila suppressor of forked protein. Nature (London) **372:**471–474.
52. **Takagaki, Y., J. L. Manley, C. C. MacDonald, J. Wilusz, and T. Shenk.** 1990. A multisubunit factor, CstF, is required for polyadenylation of mammalian

pre-mRNAs. Genes Dev. **4:**2112–2120.
53. **Takagaki, Y., L. C. Ryner, and J. L. Manley.** 1988. Separation and characterization of a poly(A) polymerase and a cleavage/specificity factor required for pre-mRNA polyadenylation. Cell **52:**731–742.
54. **Takagaki, Y., L. C. Ryner, and J. L. Manley.** 1989. Four factors are required for 3′-end cleavage of pre-mRNAs. Genes Dev. **3:**1711–1724.
55. **Torres, R. M., and E. A. Clark.** 1992. Differential increase of an alternatively polyadenylated mRNA species of murine CD40 upon B lymphocyte activation. J. Immunol. **148:**620–626.
56. **Tsurushita, N., and L. J. Korn.** 1987. Effects of intron length on differential processing of mouse μ heavy-chain mRNA. Mol. Cell. Biol. **7:**2602–2605.
57. **Valsamakis, A., S. Zeichner, S. Carswell, and J. C. Alwine.** 1991. The human immunodeficiency virus type 1 polyadenylation signal: a 3′ long terminal repeat element upstream of the AAUAAA necessary for efficient polyadenylation. Proc. Natl. Acad. Sci. USA **88:**2108–2112.
58. **Wahle, E., and W. Keller.** 1992. The biochemistry of 3′-end cleavage and polyadenylation of messenger RNA precursors. Annu. Rev. Biochem. **61:** 419–440.
59. **Weiss, E. A., G. M. Gilmartin, and J. R. Nevins.** 1991. Poly(A) site efficiency reflects the stability of complex formation involving the downstream element. EMBO J. **10:**215–219.
60. **Wilusz, J., and T. Shenk.** 1990. A uridylate tract mediates efficient heterogeneous nuclear ribonucleoprotein C protein-RNA cross-linking and functionally substitutes for the downstream element of the polyadenylation signal. Mol. Cell. Biol. **10:**6397–6407.
61. **Wilusz, J., T. Shenk, Y. Takagaki, and J. L. Manley.** 1990. A multicomponent complex is required for the AAUAAA-dependent cross-linking of a 64-kilodalton protein to polyadenylation substrates. Mol. Cell. Biol. **10:**1244–1248.
62. **Yan, D.-H., E. A. Weiss, and J. R. Nevins.** 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.