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Sequences near the 3′ Secretion-Specific Polyadenylation Site Influence Levels of Secretion-Specific and Membrane-Specific IgG2b mRNA in Myeloma Cells

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The expressed immunoglobulin γ_{2b} (IgG2b) heavy-chain gene of 4T001 was cloned into the shuttle vector pSV2-gpt and transfected into myeloma J558L and lymphoma A20.2J. Northern blots indicated that the transfected γ_{2b} gene was processed in a manner similar to the endogenous heavy chain in both lymphoma and myeloma cells. To identify sequences important for immunoglobulin mRNA processing, we constructed deletions around the secretion-specific polyadenylation site and introduced the deleted genes into J558L cells. The BAL deletion lacked 670 base pairs of intervening sequence between secreted and membrane regions; the Kpn deletion lacked 830 base pairs in this region. J558L cells transfected with either the entire γ_{2b} gene or the ΔBAL vector produced predominantly secretion-specific γ_{2b} mRNA and protein. J558L cells transfected with the ΔKpn vector produced approximately equimolar amounts of secretion-specific and membrane-specific γ_{2b} mRNA. Both 55,000-dalton secreted and 62,000-dalton putative surface IgG2b proteins were detected in the ΔKpn transfectants. We conclude that sequences absent in the Kpn deletion but present in the BAL deletion exert an important role in the production of secretion-specific mRNA. The Kpn deletion removes the normal site of cleavage and poly(A) addition, and it is possible that it is the absence of this site which changes the processing pattern. Alternatively, it is possible that sequences absent in the Kpn deletion but present in the BAL deletion function in regulating the production of predominantly secretion-specific mRNA in myeloma cells. The possible role of a highly conserved sequence found in this region is discussed.

During B-lymphocyte ontogeny, qualitative and quantitative changes in the levels of immunoglobulin message and protein occur. The first event in B-lymphocyte ontogeny is heavy-chain rearrangement, in which the precise joining of variable, diversity, and joining sequences produces an immunoglobulin M (IgM) heavy-chain gene (reviewed in reference 1). In the murine system, precursors of B lymphocytes synthesize intracellular μ chains (36) and generally contain approximately equal amounts of membrane-specific (mb) and secretion-specific (sec) cytoplasmic μ messages (2). The sec and mb forms of the immunoglobulin mRNA differ at their 3′ termini. Steady-state cytoplasmic μ mRNA levels are low, with approximately 10^2 molecules of μ message per cell (35).

As B cells mature, they rearrange and express their light-chain genes. Light and heavy chains assemble into IgM monomers H_2L_2, which are displayed on the surface of the cell membrane as antigen receptors (48, 49).

At 10 to 15 days after birth, a second class of immunoglobulin, IgD, begins to appear on the surface of cells expressing IgM (47). Different B-cell subpopulations express different levels of IgM and IgD on their surfaces with increased levels of IgD correlated with a more mature state of a virgin B lymphocyte (40). Analysis of lymphomas representative of this stage of B-cell differentiation has indicated that the total amount of cytoplasmic μ plus δ message is approximately 10^2 molecules per cell. The ratio of μ_{sec} to μ_{mb} ranges from 0.5:1 to 1:1 (35).

Maturation beyond the virgin B-cell stage requires activation by either mitogens or an appropriate combination of antigen, T helper cells (or soluble factors isolated from T-helper-cell supernatants), and macrophages (12) (for details see reviews in references 23 and 24).

The activated B cell can mature into one of two cell types: memory B cells or plasma cells, each of which may express another heavy-chain gene instead of μ.

(i) Memory B cells. Memory B cells transfer immunological memory and contain both sec and surface immunoglobulin mRNAs and proteins (32). A20.2J is an IgG2a, κ-producing lymphoma which contains a moderate excess of sec over mb IgG2a message and protein; it is representative of the memory stage of B-lymphocyte differentiation (38, 53). The overall level of γ_{2a} message in A20.2J is slightly greater than 10^3 molecules per cell, a value that is intermediate between those of a virgin B lymphocyte and a plasma cell (38; this work).

(ii) Plasma cells. Plasma cells secrete large amounts of antibody and have little or no surface immunoglobulin (29). From 5×10^3 to as many as 3×10^4 molecules of heavy-chain mRNA are present per cell (34, 41). Therefore, the overall levels of accumulated cytoplasmic heavy-chain message in a plasma cell are 50- to 300-fold greater than in a virgin B lymphocyte. The ratio of sec to mb heavy-chain message in plasma cells (or their in vitro models, myelomas) is altered in favor of the sec form, ranging from 10:1 to 100:1 (45; P. Gregor and S. L. Morrison, unpublished data). This dramatic increase in the level of sec mRNA is reflected in the amount of protein that is synthesized and secreted (16).

The objective in the present studies was to investigate the molecular mechanism accounting for the formation of pre-

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dominantly sec message in myeloma cells and to define DNA sequences important in this regulation. Two hypotheses have been proposed for this regulation: differential transcription termination or differential nuclear RNA processing. In differential termination of transcription, myelomas would terminate most primary transcripts before the membrane exons, resulting in mainly sec mRNA. Alternatively, differential polyadenylation or splicing of a common primary transcript or both could control the relative abundance of the two messages. Recently, the transcription rate across the sec- and mb-encoding exons of myeloma 4T001 was measured (25), and equimolar transcription across the entire heavy-chain gene was found. Since the cytoplasmic sec/mb γ_{2b} mRNA ratio was 25:1, the authors concluded that the mechanism accounting for predominantly sec mRNA in myeloma cells was not termination of transcription and suggested that alternate utilization of polyadenylation sites was a primary determinant in regulating sec versus mb immunoglobulin mRNA.

The capability to transfer immunoglobulin genes into lymphoid cells provided us with an alternative approach to this problem. We transfected an immunoglobulin heavy-chain gene into myeloma and lymphoma cells and determined the quantity and quality of message produced. The differentiation state of the recipient cell was found to determine the pattern in which the primary heterogeneous nuclear RNA transcript was processed. By creating deletions in the heavy-chain gene and introducing the deleted genes into myeloma cells, we identified sequences important for the processing to sec heavy-chain mRNA in myelomas. The possible role of a 65-nucleotide sequence near the 3′ sec mRNA poly(A) addition site is discussed.

(This work is submitted in partial fulfillment of the requirements for the Ph.D. degree [B. J. Kobrin].)

MATERIALS AND METHODS

Plasmids. pSV2-MPC11H was produced by cloning the entire heavy-chain gene from MPC-11 into pSV2-gpt (27). After partial digestion with *Eco*RI, a 12.6-kilobase (kb) fragment carrying the variable and constant regions, including the membrane exons and 3′ untranslated region, was isolated from the Charon 4A recombinant RBL216 (15; a gift of K. Marcu) and was inserted into the *Eco*RI site of pSV2-gpt. A 2.8-kb *Eco*RI fragment containing the 5′ flanking sequence, N-terminal leader, variable-region exon, and 3′ flanking sequence was subcloned from the bacteriophage into the *Eco*RI site of pBR325 and designated pMK-3. A 2.6-kb *Sac*I-*Kpn*I fragment from the γ_{2b} gene which included the membrane exons, intron, and 5′ and 3′ flanking sequence was subcloned and designated pCVK-1. A schematic of these plasmids is shown in Fig. 1. pSV2-MPC11H ΔKpn was produced by partially digesting pSV2-MPC11H with *Kpn*I, excising from agarose gels the 16.9-kb fragment which lacked the 830-base-pair (bp) *Kpn*I fragment, and ligating. pSV2-MPC11H ΔBAL was created by inserting a 4.8-kb *Xho*I-*Eco*RI fragment containing sequences from the CH_2 exon to the 3′ end of the γ_{2b} gene into a pSV2-gpt plasmid which had been modified to contain an unique *Xho*I site. The resultant plasmid was digested to completion with Bal I, and a 8.6-kb fragment lacking the 670-bp Bal I fragment was excised and ligated to itself. This plasmid lacking the sequences between the Bal I sites was digested with *Xho*I and *Sma*I, and the 1.4-kb *Xho*I-ΔBAL-*Sma*I fragment was inserted into the *Xho*I-*Sma*I sites of the 15.6-kb pSV2-MPC11H fragment. The plasmid was designated pSV2-MPC11H ΔBAL.

Tissue culture. J558L (λ_1), A20.2J (γ_{2a},κ), and 4T001, a thioguanine-resistant, oubain-resistant tissue culture line derived from the MPC-11 myeloma (γ_{2b},κ) cells, were maintained in Iscove modified Dulbecco medium supplemented with heat-inactivated (56°C for 30 min) horse serum, nonessential amino acids, glutamine, penicillin, and streptomycin. The protoplast fusion transfection procedure used to transfect J558L and A20.2J was essentially as described previously (31) with the following modifications. Bacteria were incubated for 8.5 min instead of 10 min at 37°C after the addition of 0.05 M Tris hydrochloride (pH 8.0). Fusion of protoplasts with suspension cells was effected in 35% polyethylene glycol in Dulbecco modified Eagle medium, because 50% polyethylene glycol proved toxic to the cells. Mycophenolic acid at a concentration of 1 to 3 μg/ml was used to select for cells transfected with pSV2-gpt vectors (31). Clones of transformants were visible in 10 to 24 days.

Proteins. Metabolic labeling of transfectants was done as detailed previously (20). Cytoplasmic lysates were prepared as described previously (20) and reacted with either a polyclonal rabbit anti-mouse IgG2b antiserum or with a monoclonal anti-IgG2a antibody, 20-83 (30), coupled to Sepharose. Immunoprecipitates were reacted with Ig-Sorb (The Enzyme Center) at 4°C for 1 h, and the labeled IgG was removed from the Ig-Sorb by boiling for 3 min in 2% sodium dodecyl sulfate (SDS). All samples were reduced with 0.3 M 2-mercaptoethanol for 30 to 60 min at 37°C and resolved on 12% Tris glycine-polyacrylamide gels (17).

RNA. Cytoplasmic RNA was prepared from cells either by the phenol extraction method (20) or by pelleting through CsCl gradients (6). RNA samples were fractionated on formaldehyde-containing agarose gels (20) and transferred to nitrocellulose as detailed previously (42). ^{32}P-labeled probes were prepared essentially as described in reference 18. Blots were hybridized for 10 to 20 h at 42°C by the method of Thomas (42). The 28S (5.1-kb) and 18S (2.0-kb) rRNA bands were used as molecular weight markers in determining the apparent molecular weight of the different immunoglobulin mRNA species. Quantitation of relative amounts of RNA species was determined by densitometer scanning of the Northern blot. To verify that the quantity of RNA loaded was correct, the heavy-chain probe was washed from the blot, and the blot was rehybridized to the appropriate light-chain probe. The level of light-chain production should not vary in the different transfected cells.

S1 nuclease analysis. DNA restriction fragments were 3′ end labeled with T4 DNA polymerase in a replacement reaction (18). Probes were used without further manipulation such as strand separation. S1 protection was determined essentially as described by Berk and Sharp (5).

Genomic DNA preparation and Southern blotting. Preparation of genomic DNA was essentially as described in reference 52. Southern blotting and hybridization were as detailed previously (20).

RESULTS

The expressed IgG2b heavy chain of MPC-11 containing the membrane exons was cloned into the eucaryotic shuttle vector pSV2-gpt (27) and designated pSV2-MPC11H (Fig. 1).

pSV2-MPC11H transfected into J558L and A20.2J cells. pSV2-MPC11H was introduced into myeloma J558L (a heavy-chain loss variant of IgA, λ_1-producing myeloma J558) by protoplast fusion, and stable transfectants were isolated at a frequency of 10^{-4}. The transfectants produced and secreted an IgG2b heavy chain which assembled with the

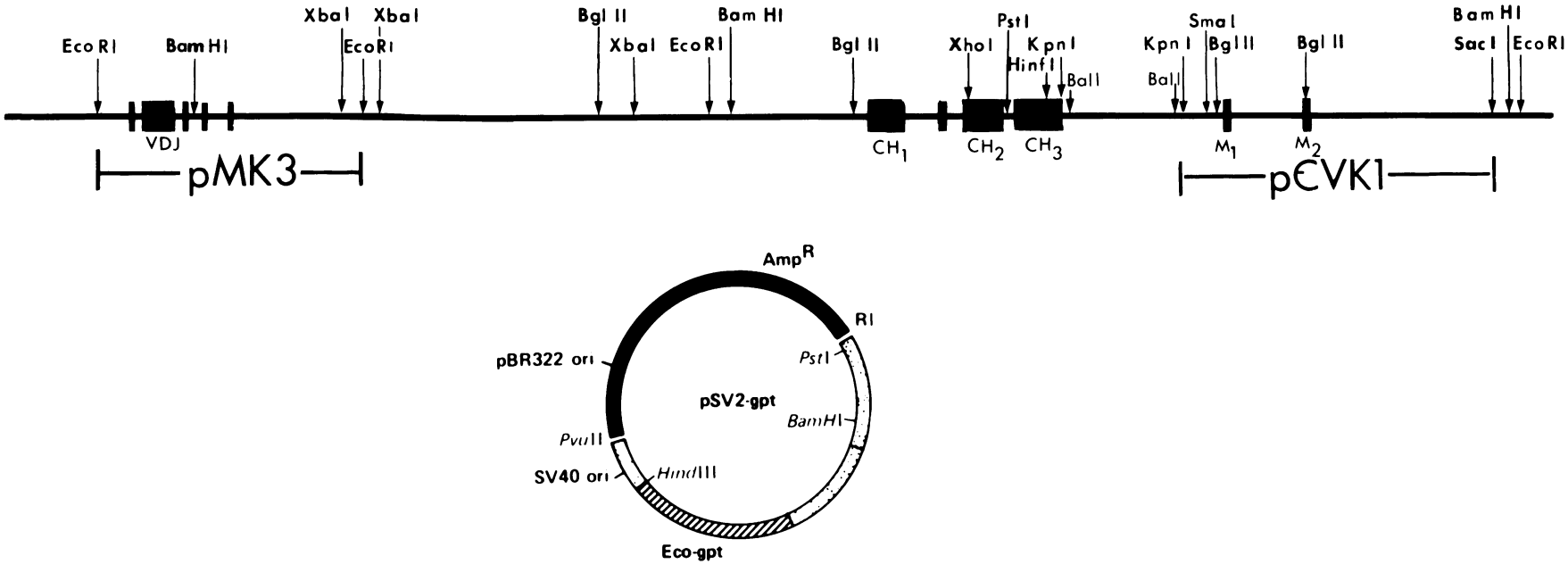

FIG. 1. pSV2-MPC11H was produced by cloning the entire heavy-chain gene from MPC-11 into pSV2-gpt. Exons are denoted by boxed areas. The locations of pMK-3 and pCVK-1 are noted. The MPC-11 heavy-chain gene was oriented so its transcription was in the opposite direction to that from the Eco-gpt gene. Only the *Hin*fI and *Pst*I sites used for cloning and S1 analysis are indicated. ori, Origin.

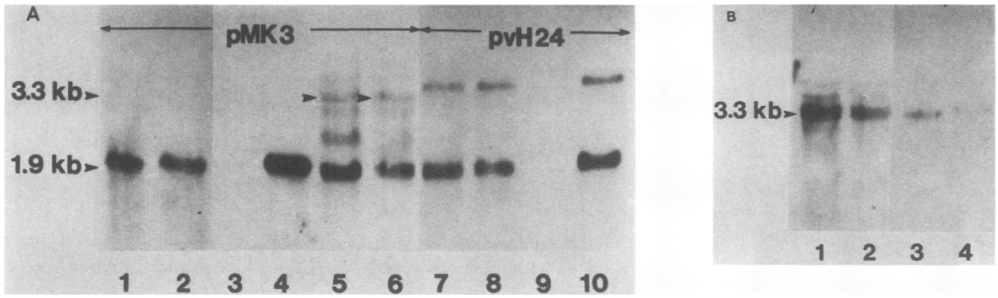

FIG. 2. Northern blot analysis of γ_{2b} messages in J558L and A20.2J cell lines transfected with pSV2-MPC11H. (A) Total cytoplasmic RNA was fractionated on agarose gels and transferred to nitrocellulose (42). The fractionated cytoplasmic RNAs were hybridized to pMK-3 to detect specific transcripts from the MPC-11 gene (lanes 1 to 6). pVH24, a *Sac*I fragment containing CH_3 sequences from γ_{2a} cloned into pBR322, was used to detect the endogenous γ_{2a} transcripts (lanes 7 to 10) (S. Tilley and B. Birshtein, personal communication). Lanes: 1, 4T001, 5 μg; 2, transfectant of pSV2-MPC11H into J558L, 15 μg; 3, A20.2J, 5 μg; 4, 4T001, 5 μg; 5 and 6, two independent transfectants of pSV2-MPC11H into A20.2J, 30 μg; 7 and 8, two independent transfectants of pSV2-MPC11H into A20.2J, 5 μg; 9, 4T001, 5 μg; 10, A20.2J, 5 μg. Arrows indicate a 3.3-kb MPC-11 variable-region-positive species in the A20.2J transfectants which has the same apparent molecular weight as the 3.3-kb mb mRNA in 4T001. (B) The probes used in the hybridization shown in panel A were removed, and the same filter was hybridized to pCVK-1. Representative lanes are shown. Lanes 1 and 2 correspond to lanes 5 and 6 in panel A; lanes 3 and 4 correspond to lanes 9 and 10. Lanes: 1 and 2, two independent transfectants of pSV2-MPC11H into A20.2J, 30 μg; 3, 4T001, 5 μg; 4, A20.2J, 5 μg.

resident λ_1 light chain and was secreted (data not shown). The overall quantity of IgG2b protein was similar to that seen when a chimeric mouse variable-, human constant-region gene was transfected in this cell line (26).

Northern blot analysis indicated that cytoplasmic γ_{2b} mRNA was present in one of these transfectants at about 25% of the level of γ_{2b} mRNA in myeloma 4T001 (Fig. 2A, lanes 1 and 2). Upon hybridization to mb probe pCVK-1 (Fig. 1), a 3.3-kb species was detected in both parent myeloma and transfectant (data not shown). The sec/mb ratio was 25:1 in myeloma 4T001 and 20:1 for this transfectant (see Materials and Methods and Table 1). Two other γ_{2b}-producing J558L transfectants had sec/mb ratios of 10:1 and >25:1 (Table 1). The total amount of cytoplasmic γ_{2b} mRNA in the three J558L transfectants was approximately 20, 25, and 55%, respectively, of the levels of γ_{2b} mRNA seen in 4T001.

pSV2-MPC11H was then introduced into the γ_{2a},κ-producing lymphoma A20.2J, and stable transfectants were isolated at a frequency of 10^{-6}. Five of nine A20.2J transfectants produced detectable levels of MPC-11 mRNA by Northern blot analysis, and results for two of these five are shown (Fig. 2). Three probes were used for hybridization: pMK-3 (Fig. 2A, lanes 1 to 6) containing the variable region of MPC-11 detects message specific for the transfected gene; pVH24 (Fig. 2A, lanes 7 to 10) contains the CH_3 region from γ_{2a} and does not cross-hybridize with γ_{2b} under stringent conditions; and pCVK-1 (Fig. 2B) which hybridizes to both γ_{2a} and γ_{2b} membrane sequences. Analysis of the γ_{2a} messages in A20.2J and in the transfectants showed that the sec/mb ratio for the endogenous γ_{2a} mRNA was approximately 3:1 (Fig. 2A, lanes 7, 8, and 10). Hybridization with the MPC-11 variable-region sequence showed that the A20.2J transfectants produce correctly sized γ_{2b} mRNAs of 1.9 and 3.3 kb (Fig. 2A, lanes 5 and 6); in addition, three aberrant species of 2.4, 2.9, and 3.9 kb were present. Densitometer scanning of these bands in lanes 5 and 6 revealed that the MPC-11 γ_{2b}-specific message level in these transfectants was one-third the level of the γ_{2a} message in A20.2J and that the ratio of the 1.9- to 3.3-kb species was 3:1.

TABLE 1. Summary of phenotypes of J558L cells transfected with wild-type, ΔKpn, and ΔBAL vectors

Cell line	sec/mb γ_{2b} mRNA[a]	Total γ_{2b} mRNA as % 4T001	Mol wt of IgG2b (daltons)
4T001	25:1	100	55,000
Wild-type pSV2-MPC11H transfected into J558L (TAL lines)	9.5:1, 20:1, 25:1	20–55	55,000
ΔKpn transfected into J558L (TBE lines)	0.32:1, 0.78:1, 0.79:1, and 1.52:1	5–25	55,000 and 62,000
ΔBAL transfected into J558L (TDT lines)	2:1, 4:1, 8:1, >25:1, ∞[b]	10–23	55,000

[a] Ratios were determined by scanning the Northern blots with a Joyce-Loebl scanning densitometer and comparing the areas under the peaks.
[b] No mb immunoglobulin detected.

To identify mb messages, the probes were eluted, and the blot was hybridized to the γ_{2b} mb probe pCVK-1. mb messages were seen in both 4T001 and A20.2J (Fig. 2B, lanes 3 and 4). The mb message in A20.2J was slightly longer than the mb message in 4T001 (38). In A20.2J cells transfected with pSV2-MPC11H, we noted a broad, membrane-specific band which was consistent with the presence of both a γ_{2a}- and a γ_{2b}-specific membrane message (Fig. 2B, lanes 1 and 2). The mb message migrated to the same position as the 3.3-kb mRNA detected with a MPC-11 variable-region-specific probe (Fig. 2A, lanes 5 and 6, indicated by arrowheads). The aberrant mRNA species did not hybridize to the mb probe.

All the γ_{2b} messages detected in the A20.2J transfectants were poly(A)$^+$ (data not shown). S1 nuclease protection experiments indicated that the 3′ terminus of the sec γ_{2b} mRNA in both J558L and A20.2J transfectants was identical to the 3′ terminus of the γ_{2b} sec mRNA in 4T001. Southern blot analysis of genomic DNAs from the two MPC-11 mRNA-positive A20 transfectants (lanes 7 and 8) showed them to have one to three copies integrated (B. Kobrin, Ph.D. thesis, Columbia University, New York, 1985).

The IgG2b protein encoded by one of the A20.2J transfectants was analyzed on SDS-polyacrylamide gels after immunoprecipitation from cytoplasmic lysates as described in the legend to Fig. 3. Because of the extensive cross-reactivity

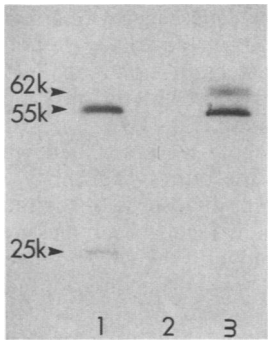

FIG. 3. SDS-polyacrylamide gel electrophoresis of radiolabeled cytoplasmic IgG2b immunoglobulins in A20.2J cells transfected with pSV2-MPC11H. Cells were labeled by growth for 3 h in medium containing [^{14}C]valine, [^{14}C]threonine, and [^{14}C]leucine. Cells were pelleted, and cytoplasmic lysates were prepared and reacted with monoclonal antibody 20-83 specific for γ_{2a} (30; a gift of V. T. Oi) coupled to cyanogen bromide-activated Sepharose to remove γ_{2a}-specific proteins. Rabbit anti-mouse IgG2b antiserum bound to Ig-Sorb was then used to precipitate IgG2b protein. The IgG2b was removed by boiling for 3 min in 2% SDS. All samples were reduced with 0.3 M 2-mercaptoethanol for 30 min at 37°C and analyzed on 12% Tris glycine gels. Lanes: 1, myeloma 4T001; 2, lymphoma A20.2J; 3, transfectant of pSV2-MPC11H into A20.2J. To prevent overexposure of the 55,000-dalton (55k) IgG2b protein in the myeloma, lane 1 contained only one-fifth the amount of immunoprecipitate relative to lanes 2 and 3.

between γ_{2a} and γ_{2b}, it was necessary to use a monoclonal antibody to remove all γ_{2a} proteins from the transfectant before immunoprecipitation with anti-γ_{2b}. Myeloma 4T001 produced a 55,000-dalton γ_{2b} heavy chain and a 25,000-dalton κ chain (Fig. 3, lane 1) (26). The 62,000-dalton sec and 68,000-dalton surface IgG2a proteins of A20.2J were completely removed by the monoclonal anti-γ_{2a} antibody so that no protein remained to react with the rabbit anti-γ_{2b} antiserum (Fig. 3, lane 2). The A20.2J transfectant produced both a 55,000- and a 62,000-dalton IgG2b protein (Fig. 3, lane 3). While additional experimentation is needed to unequivocally determine that the 62,000-dalton protein is the membrane form of the heavy chain, it is clear that a second IgG2b protein, with the molecular weight expected of membrane IgG2b and at one-third the level of the 55,000-dalton protein, is present in the transfected lymphoma cells.

From the data presented so far we conclude that the state of differentiation of a recipient B-cell line determines whether the primary transcript is processed to form mb or sec γ_{2b} mRNA in the appropriate ratio. In myeloma J558L, the transfected heavy-chain gene was processed mostly to sec mRNA (Fig. 2A, lane 2), similar to the processing of the γ_{2b} heavy-chain gene in myeloma 4T001 (Fig. 2A, lane 1). In lymphoma A20.2J, both transfected (Fig. 2A, lanes 5 to 8) and endogenous (Fig. 2A, lane 10) heavy-chain genes were processed in a similar manner, producing sec/mb γ_{2b} mRNA ratios of 3:1.

Once we had demonstrated that transfected heavy-chain genes are processed in a manner similar to that of the endogenous heavy-chain gene, we could begin to use gene transfection to identify sequences important in regulating differential processing.

Two deletions in pSV2-MPC11H were constructed which removed sequences around the sec poly(A) addition site and a large portion of the intervening sequence (IVS) between the sec terminus and the membrane exons (Fig. 4).

The ΔKpn deletion had its 5′ endpoint 6 bp 5′ of the myeloma wild-type sec poly(A) addition site and extended approximately 820 bp into the IVS located between the sec and mb coding region. The BAL 1 deletion had its 5′ endpoint 59 bp 3′ of the wild-type 3′ sec mRNA poly(A)

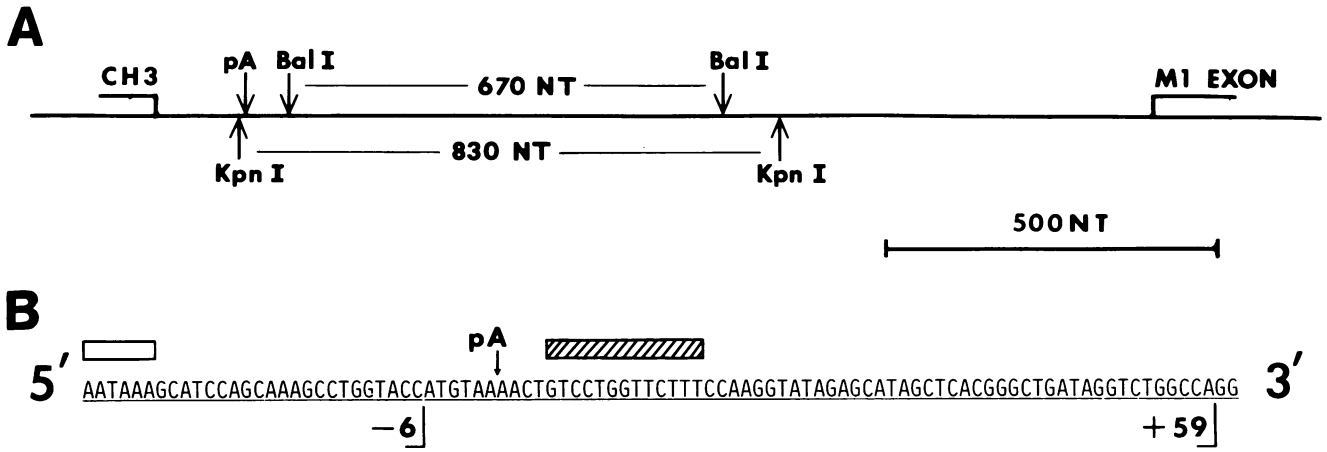

FIG. 4. pSV2-MPC11H deletions ΔKpn and ΔBAL and nucleotide sequence of part of the γ_{2b} sec mRNA 3′ untranslated region and 3′ flanking region and a consensus sequence located near the sec poly(A). (A) A diagram of the Kpn and BAL deletions is shown. The poly(A) addition site for the sec mRNA, located 110 bp downstream of the 3′ end of the CH_3 exon, is indicated as pA. The IVS between the pA site and the M_1 exon is approximately 1.35 kb. The Kpn deletion has its 5′ terminus 6 bp 5′ of the poly(A) site and extends 824 bp into the IVS. The BAL deletion has its 5′ terminus 59 nucleotides 3′ of the poly(A) site and extends 670 nucleotides into the IVS. The relative position of the 5′ endpoints of the deletions was known from the published sequence (43). The relative position of the 3′ endpoints was determined by linearizing the ΔBAL plasmid with Bal I, 3′ end labeling with T4 DNA polymerase, and truncating the labeled fragment with *KpnI*. Polyacrylamide gel electrophoresis indicated that the Kpn deletion extended 95 ± 3 bp further 3′ into the IVS than the Bal I deletion. By using different enzymes and a similar strategy, the 5′ endpoints of the Kpn and BAL deletions were confirmed. (B) Nucleotide sequence was from reference 43. The poly(A) addition site is designated within nucleotides 0 and +1. The open box above nucleotides −29 to −34 denotes the AATAAA signal important in the cleavage and polyadenylation of eucaryotic messages (11). The Kpn deletion has its 5′ terminus at −6. The BAL deletion has its 5′ terminus at +59. The hatched box above nucleotides +5 to +17 denotes a conserved sequence.

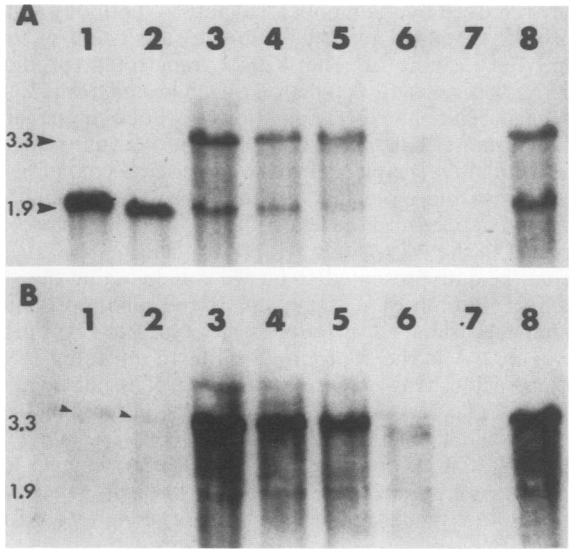

FIG. 5. Northern blot analysis of γ_{2b} mesages in J558L cells transfected with pSV2-MPC11H ΔKpn. (A) Total cytoplasmic RNAs were prepared from cells, fractionated on formaldehyde-containing agarose gels, transferred to nitrocellulose, and hybridized to ^{32}P-labeled pCM13, a plasmid consisting of the 6.8-kb *Eco*RI fragment containing the entire γ_{2b} constant region cloned into pBR325. The letters TBE are used to designate clones of J558L cells transfected with pSV2-MPC11H ΔKpn. Lanes: 1, 4T001, 2 μg; 2, wild-type transfectant into J558L, 8 μg; 3, TBE-3, 20 μg; 4, TBE-6, 20 μg; 5, TBE-12, 20 μg; 6, TBE-9, 30 μg; 7, TBE-10, 30 μg; 8, TBE-11, 30 μg. (B) The probe from the blot shown in panel A was eluted, and the blot was rehybridized to ^{32}P-labeled pCVK-1. Lanes are as described in the legend to panel A. The position of the mb mRNA in 4T001 and J558L transfected with pSV2-MPC11H is indicated by arrowheads.

addition site and extended 670 bp into the IVS between the sec and mb coding regions (Fig. 4).

pSV2-MPC11H ΔKpn transfected into J558L cells. pSV2-MPC11H ΔKpn was introduced into J558L cells by protoplast fusion, and stable transfectants were isolated at a frequency of 1.5×10^{-5} to 2.0×10^{-5}. These transfectants were designated by the letters TBE. Of 12 stable transfectants, 8 appeared to contain γ_{2b} message by slot-blot assay, and total cytoplasmic mRNA prepared from 6 of these transfectants was subjected to Northern blot analysis with the entire γ_{2b} constant region used as a hybridization probe (Fig. 5A).

The ratio of sec/mb γ_{2b} mRNA was significantly different in J558L cells transfected with the ΔKpn vector from that in cells transfected with the wild-type vector. Both wild type-transfected cells and myeloma 4T001 accumulated predominantly sec γ_{2b} mRNA (Fig. 5A, lanes 1 and 2). The cells transfected with the ΔKpn vector had elevated amounts of membrane-sized message and reduced amounts of sec γ_{2b} mRNA (Fig. 5A, lanes 3 to 5 and 8). The γ_{2b} constant-region probe was eluted, and the blot was rehybridized to the mb probe pCVK-1 to verify that the 3.3-kb species in the cells transfected with the ΔKpn vector was mb (Fig. 5B, lanes 3, 5, and 8). This blot was overexposed to show the low level of mb RNA in 4T001 and in J558L transfected with the intact MPC-11 heavy-chain gene (lanes 1 and 2, indicated by arrowheads).

One transfectant (Fig. 5A, lane 7) contained no cytoplasmic γ_{2b} mRNA, while another (Fig. 5A, lane 6) accumulated very low levels of γ_{2b} message, with an aberrantly sized mb mRNA. These two transfectants were not analyzed further. The other four transfectants contained mRNA species of 1.9 and 3.3 kb. Hybridization with the mb probe verified that the 3.3-kb species was mb (Fig. 5B).

The bands in Fig. 5 were scanned with a Joyce-Loebl densitometer, and the ratio of sec/mb γ_{2b} RNA was determined. Two different exposures of the autoradiogram were used to detect the low levels of mb message in the myeloma and cells transfected with the wild-type vector. The ratio of sec/mb γ_{2b} mRNA was significantly different in J558L cells transfected with the ΔKpn vector from that in cells transfected with the wild-type vector. Both 4T001 (lane 1) and the cells transfected with wild-type vector (lane 2) had a sec/mb ratio of 25:1. TBE-3, -6, -12, and -11 had sec/mb γ_{2b} mRNA ratios of 0.8:1, 0.8:1, 0.3:1, and 1.5:1, respectively (lanes 3 to 5 and 8, respectively).

The total amount of cytoplasmic γ_{2b} mRNA in the cells transfected with the ΔKpn vector ranged from 5% (Fig. 5A, lane 5) to 25% (Fig. 5A, lane 3) of the levels of γ_{2b} in myeloma 4T001. By comparison, the total amount of cytoplasmic γ_{2b} mRNA in the cells transfected with the wild-type vector ranged from 20% (Fig. 5A, lane 2) to 55% (Table 1) of the levels seen in 4T001.

To map the 3′ terminus of the sec mRNA in the ΔKpn transfectants, a 2.0-kb *Pst*I fragment from pSV2-MPC11H ΔKpn (encompassing the IVS between CH_2 and CH_3, the CH_3 exon, the 3′ untranslated region of the sec mRNA, the Kpn deletion, the remaining portion of the IVS between sec and mb regions and the M_1 and M_2 exons [Fig. 1]) was digested with *Hin*fI and 3′ end labeled with T4 DNA polymerase and [^{32}P]dCTP; this *Pst*I fragment was designated pCVK-3. The labeled *Hin*fI site that can hybridize to the 3′ terminus of sec mRNA is located 184 bp 5′ of this terminus (43). Since this probe contained the Kpn deletion which removed six bp 5′ of the wild-type poly(A) addition site, one would expect wild-type γ_{2b} message to protect a fragment from S1 digestion which is six bp shorter than is protected by the intact gene (shown schematically in Fig. 6).

The data shown in Fig. 6 indicate that mRNA from myeloma 4T001 protected the expected fragment of 178 ± 2 bp from S1 digestion (Fig. 6, lane 4). The mRNAs from both TBE-3 and TBE-11 protected species of approximately 178 ± 2 and 167 ± 2 bp corresponding to +22 and +11 from the AATAAA sequence (Fig. 6, lanes 5 and 6).

In addition, heterogeneous species of approximately 197 to 215 bp corresponding to termination +41 to +59 nucleotides downstream of the AATAAA sequence were protected in the TBE lines (lanes 5, 6, 8, and 9). TBE-3 contained additional species of 195 and approximately 255 nucleotides (+39 and +98 from AATAAA) (lanes 5 and 8). The extended transcripts terminate in the IVS 3′ of the ΔKpn vector in a region which has not been sequenced. All species seen in the total RNA were present in the poly(A)$^+$ RNA, demonstrating that the RNAs are both cleaved and poly(A)$^+$. We conclude that the sec mRNAs of two cell lines transfected with ΔKpn vector, which lacks the normal site for cleavage and polyadenylation, have multiple termini; some termini extend into the IVS.

Two IgG2b proteins were encoded by the ΔKpn transfectants: a 55,000- and a 62,000-dalton protein (Fig. 7A, lanes 4 and 5). Only the 55,000-dalton protein, which comigrated with the 4T001 γ_{2b} protein, was secreted from these cells (data not shown). The 62,000-dalton protein had the expected size for surface IgG2b.

pSV2-MPC11H ΔBAL transfected into J558L cells. pSV2-

MPC11H ΔBAL was introduced into J558L cells by protoplast fusion, and stable transfectants were isolated at a frequency of 1×10^{-4} to 2×10^{-4}, similar to the frequency obtained with pSV2-MPC11H in this cell line. ΔBAL transfectants are designated by the letters TDT.

Cytoplasmic mRNAs were prepared from five ΔBAL transfectants and analyzed by Northern blots, with the entire γ_{2b} constant region used as a probe. Four of the five ΔBAL transfectants produced predominantly sec mRNA (Fig. 8, lanes 2 to 5). One ΔBAL transfectant produced unusually high levels of both sec and mb γ_{2b} mRNA (Fig. 8, lane 6). Upon hybridization to the mb probe pCVK-1, a 3.3-kb mb message was detected in all ΔBAL transfectants except TDT-1. Transfectant TDT-1 produced very low levels of sec γ_{2b} mRNA and no detectable mb mRNA (data not shown). Northern blot analysis with poly(A)$^+$ mRNA indicated that all γ_{2b}-positive species shown in Fig. 8 were poly(A)$^+$ (data not shown). The γ_{2b} mRNA sec/mb ratios (Table 1) in the ΔBAL transfectants were determined by densitometric scanning of the bands in Fig. 8, using two different exposures of the autoradiogram. The sec/mb ratios were 4:1 (Fig. 8, lane 3), 8:1 (lane 2), >25:1 (lane 5), undefined (no mb species detected, lane 4), and 2:1 (lane 6). TAL-4, J558L transfected with wild-type pSV2-MPC11H, had a sec/mb γ_{2b} ratio of 10:1 (Fig. 8, lane 7; Fig. 2, lane 2). The overall levels of γ_{2b} message in three of five ΔBAL transfectants were 10 to 23% of the levels of γ_{2b} mRNA in myeloma 4T001. TDT-2 (lane 6), which had a sec/mb ratio of 1.8:1, accumulated more cytoplasmic γ_{2b} mRNA than any wild-type transfectant. Metabolic labeling and immunoprecipitation with anti-γ_{2b} antiserum showed that all of the ΔBAL transfectants produced and secreted various levels of a 55,000-dalton γ_{2b} protein. A protein species of the size expected for mb immunoglobulin was not detected in any transfectants (data not shown) including TDT-2 which has an abundant mRNA species of the size expected for mb RNA. Apparently, TDT-2 exhibits aberrant mRNA synthesis and utilization.

We conclude that cells transfected with the ΔBAL vector are similar to cells transfected with the wild-type vector, both in terms of their sec/mb γ_{2b} mRNA ratios and their production and secretion of a 55,000-dalton IgG2b protein. Cells transfected with the ΔKpn vector have altered sec/mb γ_{2b} mRNA ratios and produce an additional 62,000-dalton IgG2b protein.

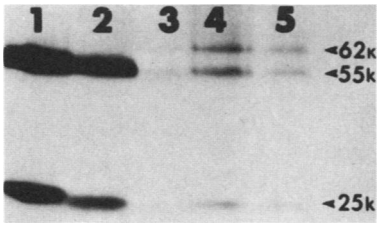

FIG. 7. SDS-polyacrylamide gel electrophoresis of radiolabeled cytoplasmic and secreted IgG2b immunoglobulin produced by J558L cells transfected with pSV2-MPC11H ΔKpn. Cells were labeled for 3 h in medium containing [^{14}C]valine, [^{14}C]threonine, and [^{14}C]leucine. Cytoplasmic lysates were prepared and immunoglobulin was immunoprecipitated as described in Materials and Methods. TBE designates J558L cells transfected with pSV2-MPC11H ΔKpn. Lanes: 1, 4T001; 2, wild-type pSV2-MPC11H into J558L; 3, TBE-1; 4, TBE-3; 5, TBE-12. Unequal amounts of protein samples were loaded in an attempt to equalize the intensities of the bands in the J558L transfectants. k, Kilodaltons.

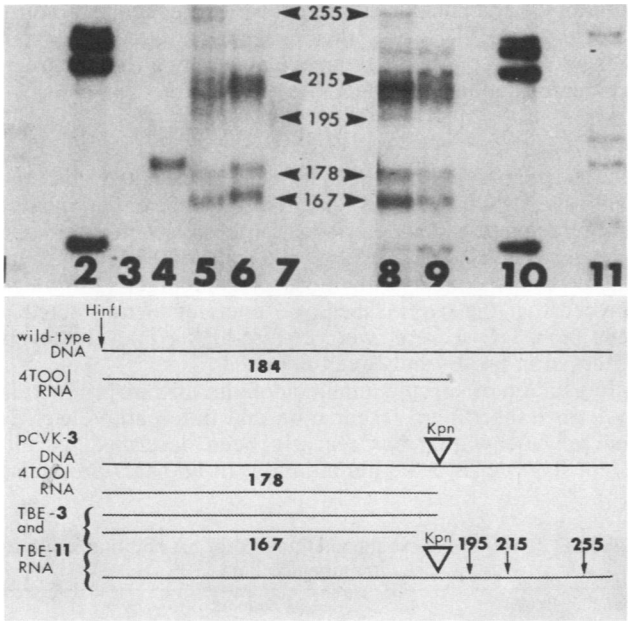

FIG. 6. S1 nuclease analysis of 3′ sec mRNA terminus of TBE-3 and TBE-11. The *Pst*I fragment from ΔKpn containing CH_3 (designated pCVK-3) was digested with *Hin*fI, 3′ end labeled with T4 DNA polymerase, hybridized to myeloma or transfectant RNAs, and digested with S1 nuclease as described in Materials and Methods. S1-protected DNA fragments were resolved on 8% acrylamide–7 M urea gels. TBE designates J558L cells transfected with pSV2-MPC11H ΔKpn. Lanes: 1, molecular weight marker pBR322 digested with *Hpa*II; 2, probe digested with *Hin*fI; 3, probe plus tRNA plus S1 nuclease; 4, probe plus 4T001, 1 μg of total cytoplasmic RNA; 5, probe plus TBE-3, 16 μg of total cytoplasmic RNA; 6, probe plus TBE-11, 25 μg of total cytoplasmic RNA; 7, probe plus J558L, 25 μg of total cytoplasmic RNA; 8, probe plus TBE-3, 0.33 μg of cytoplasmic poly(A)$^+$ mRNA; 9, probe plus TBE-11, 0.5 μg of cytoplasmic poly(A)$^+$ mRNA; 10, probe digested with *Hin*fI; 11, molecular weight marker pBR322 digested with *Hpa*II. Lanes 8 to 10 containing the poly(A)$^+$ mRNAs were from a separate gel. The protected fragments are shown schematically below the gel.

Integration pattern of wild-type, ΔKpn, and ΔBAL pSV2-

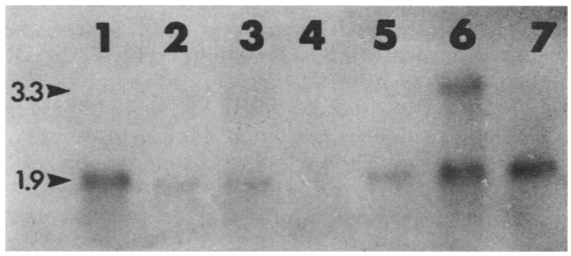

FIG. 8. Northern blot analysis of γ_{2b} message in J558L cells transfected with pSV2-MPC11H ΔBAL. Total cytoplasmic RNA was fractionated on agarose gels (46), transferred to nitrocellulose, and hybridized to ^{32}P-labeled pCM13, the 6.8-kb *Eco*RI γ_{2b} constant-region fragment cloned in pBR325. Lanes: 1, 4T001, 3 μg; 2, TDT-8, J558L transfected with pSV2-MPC11H ΔBAL, 6 μg; 3, TDT-1, J558L transfected with pSV2-MPC11H ΔBAL, 12 μg; 4, TDT-3, J558L transfected with pSV2-MPC11H ΔBAL, 12 μg; 5, TDT-4, J558L transfected with pSV2-MPC11H ΔBAL, 12 μg; 6, TDT-2, J558L transfected with pSV2-MPC11H ΔBAL, 12 μg; 7, TAL-4, J558L transfected with pSV2-MPC11H, 6 μg.

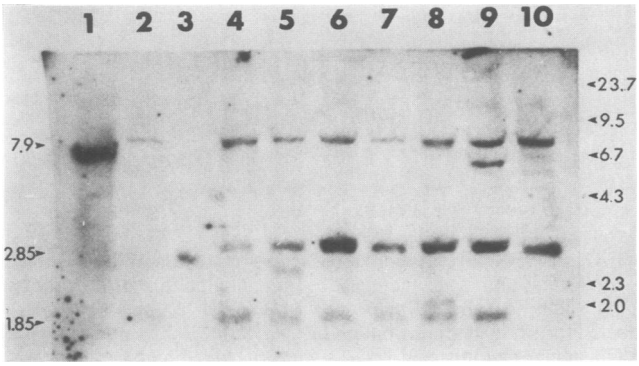

FIG. 9. Southern blot analysis of integrated JH_3-JH_4 sequences in J558L cells transfected with wild-type, ΔKpn, and ΔBAL vectors. Procedure was as described in Materials and Methods. Hybridization of blot was to a J_{H3}-J_{H4} probe derived from pMK3. Lanes: 1, BALB/c liver DNA, 10 μg; 2, myeloma J558L, 10 μg; 3, blank; 4, TAL-2, J558L transfected with pSV2-MPC11H, 10 μg; 5, TAL-4, J558L transfected with pSV2-MPC11H, 10 μg; 6, TBE-3, J558L transfected with pSV2-MPC11H ΔKpn, 10 μg; 7, TBE-11, J558L transfected with pSV2-MPC11H ΔKpn, 10 μg; 8, TDT-4, J558L transfected with pSV2-MPC11H ΔBAL, 10 μg; 9, TDT-2, J558L transfected with pSV2-MPC11H ΔBAL, 10 μg; 10, TDT-5, J558L transfected with pSV2-MPC11H ΔBAL, 10 μg. λ *c*I857 DNA digested with *Hin*dIII was used as a molecular weight marker. Numbers are in kilobases.

MPC11H vectors. To estimate the number of integrated gene copies, genomic DNA from selected transfectants was digested with *Eco*RI, fractionated on agarose gels, and transferred to nitrocellulose. Using the J_{H3}-J_{H4} fragment derived from pMK-3 as a probe (*Bam*HI-*Eco*RI from pMK-3; Fig. 1), a 2.85-kb *Eco*RI J_{H3}-J_{H4}-positive and presumably MPC-11 variable-region species was present in one to three copies in all cell lines transfected with wild-type (Fig. 9, lanes 4 and 5), ΔKpn (Fig. 9, lanes 6 and 7), and ΔBAL (Fig. 9, lanes 8 to 10) vectors. Myeloma J558L had J_{H3}-J_{H4}-positive species of 7.9 and 1.85 kb (Fig. 9, lane 2). Aberrantly integrated sequences were noted in one of two wild-type transfectants (Fig. 9, lane 5). Both wild-type gene transfectants produced high levels of predominantly sec mRNA and a 55,000-dalton sec protein. No aberrantly integrated fragments were noted in the ΔKpn transfectants TBE-3 and TBE-11 (Fig. 9, lanes 6 and 7). TDT-4, a ΔBAL transfectant that produced moderate levels of γ_{2b} mRNA with a sec/mb ratio of >25:1, and TDT-2, which contained unusually high levels of γ_{2b} mRNA with a sec/mb ratio of 1.8:1, both contained unique aberrant J_{H3}-J_{H4}-region fragments (lanes 8 and 9). TDT-5, a ΔBAL transfectant that did not accumulate any cytoplasmic γ_{2b} mRNA, contained two copies of the 2.85-kb variable-region gene but had apparently lost a 1.85-kb J_{H3}-J_{H4}-hybridizing species that was contributed by the parent myeloma J558L (Fig. 9, lane 10).

Subsequent hybridization of this blot to γ_{2b} constant-region and pSV2-gpt probes indicated that all transfectants apparently contained one to three copies of the transfected gene. However, no clear correlation between integration pattern and gene expression was noted.

Consensus sequence 3′ of sec poly(A) addition site. A summary of the phenotypes of the wild-type, ΔKpn, and ΔBAL transfectants is given in Table 1. On average, the ΔBAL transfectants produced predominantly sec mRNA, with sec/mb ratios similar to those found in the wild-type transfectants. Both ΔBAL and wild-type transfectants produced and secreted a 55,000-dalton IgG2b protein. The ΔKpn transfectants, which contained approximately the same overall steady-state level of γ_{2b} message as the ΔBAL transfectants (Table 1), produced altered ratios of sec and mb mRNA and protein.

The nucleotide sequence of part of the terminus of sec γ_{2b} mRNA and its 3′ flanking sequence is shown in Fig. 4. If one defines the myeloma poly(A) addition site as zero, the Kpn deletion commences with nucleotide −6, and the BAL deletion commences with nucleotide +59. Cleavage and polyadenylation of the heterogeneous nuclear RNA to produce sec mRNA in myeloma 4T001, wild-type, and ΔBAL transfectants occurred within the stretch of A's from −1 to +2. The Kpn transfectants cleaved and polyadenylated some of their sec mRNAs at position −6 and within the A stretch from −17 to −15.

Sequences between −6 and +59 apparently are important in determining the levels of sec message in myeloma cells. The sequence indicated by the hatched box in Fig. 4 is conserved both in nucleotide sequence and in position in the 3′ secretory terminus of immunoglobulin genes. It exhibits 90% overall homology to mouse μ, γ_3, γ_{2a}, γ_{2b}, and γ_1 genes and human γ_1, γ_2, and γ_4 genes (9, 10) (Table 2). It is not present in the mouse α gene (44). It is exactly 33 bp 3′ of the AATAAA signal in mouse and human IgGs and 16 bp 3′ of this signal in mouse IgM. In the two cases in which the mb 3′ terminus and flanking region have been sequenced, mouse IgM and IgG3 (37, 50), this consensus sequence is not present. This consensus sequence may play a role in directing the formation of sec mRNA.

DISCUSSION

Data from J558L and A20.2J cells transfected with the wild-type MPC-11 gene indicate that the differentiation state of the recipient B cell determines both the sec/mb ratio and the overall levels of γ_{2b} mRNA of a transfected IgG2b gene. These results concur with those of Neuberger (28) which showed that the overall message levels of a transfected μ gene in an IgG secretor were 20-fold higher than the levels observed in pre-B- and B-cell lines.

Regulation of specific immunoglobulin mRNA production by both transcription termination and differential cleavage and polyadenylation has recently been described for different stages of B-cell differentiation. In IgM-IgD-producing

TABLE 2. Consensus sequence found 3′ of pA addition site for sec immunoglobulin[a]

Immunoglobulin	Sequence												
Mouse	G	T	C	C	T	G	G	T	T	C	T	T	T
μ	–	–	^	–	–	–	–	–	–			–	–
γ_1	–	–	–	–	–	–	–	–	G	A	–	–	–
γ_{2a}	–	–	–	–	–	–	–	–	–	–	–	–	–
γ_{2b}	–	–	–	–	–	–	–	–	–	^	–	–	–
γ_3	A	–	–	T	–	–	–	–	–	–	–	–	–
Human													
γ_1	–	–	G	A	–	–	–	–	–	–	–	–	–
γ_2	–	–	G	A	–	–	–	–	–	–	–	–	–
γ_4	–	–	G	A	–	–	–	–	–	–	–	–	–
Frequency (%)	89	100	67	56	100	100	100	100	89	78	89	100	100

[a] The consensus sequence was compared with sequences near the sec poly(A) addition site in other immunoglobulin genes. –, Identity with the γ_{2b} sequence. A blank space denotes the lack of any nucleotide in that position; ^ denotes an insertion of a nucleotide. References are: mouse μ, 14; mouse γ_1, 13; mouse γ_{2a}, 33; mouse γ_{2b}, 43; mouse γ_3, 50; human γ_1, 10; and human $\gamma 2$ and γ_4, 9.

lymphomas, both μ and δ loci are actively transcribed (19). However, in plasmacytomas that secrete large quantities of IgM, there is no detectable transcription across the δ locus. Transcription termination occurred somewhere between the μ and δ regions. However, the mechanism by which myelomas produce sec as compared with mRNA does not involve transcription termination. The data of Milcarek and Hall (25) suggest that a single precursor common to both sec and mb mRNA is differentially processed. The authors suggest that the developmental expression of sec versus mb mRNA involves the preferential utilization of one polyadenylation site over another, similar to the regulation that mediates the expression of calcitonin and calcitonin-related gene products (3). However, the possibility cannot be excluded that differential processing is the result of differential splicing. By creating specific deletions around the 3′ sec poly(A) site and reintroducing the deleted genes into myeloma cells, we showed that a 65-nucleotide sequence near this site influences the processing to sec mRNA in myeloma cells. This sequence contains both the site of cleavage and poly(A) addition and a sequence which is highly conserved at the 3′ terminus of sec immunoglobulin.

The hexamer AAUAAA has been shown to form part of the recognition site for polyadenylation (11, 51). Removal of the normal site of polyadenylation, with retention of the AAUAAA, resulted in polyadenylation at a new site, suggesting flexibility in the site at which poly(A) is added. Similarly, in the ΔKpn transfectants, in which the AAUAAA was retained but the normal site of polyadenylation was removed from the gene, some cleavage and polyadenylation occurred at new sites.

Some investigators have suggested that small nuclear RNAs may be involved in the 3′ processing of eucaryotic messages. A comparison of sequences flanking the poly(A) site of 67 eucaryotic messages revealed a set of loosely defined consensus sequences of either AAUAAA . . . . .CAYUG. . . .poly(A) addition site or AAUAAA. . . .poly(A) addition site. . . .AAA. . .CAYUG (4). These sequences in theory may be base paired to the small nuclear RNA U4. The format AAUAAA . . . C-CUG . . . . poly(A) addition site, which may base pair with U4 RNA, is present in the 3′ sec terminus of IgG2b mRNA at nucleotides −11 to −15. This sequence probably does not account for the differential processing in ΔKpn compared with that in wild-type and ΔBAL transfectants as it is present, intact, in all three constructs.

Recently, a second element downstream of the AAUAAA has been described by a number of investigators. McDevitt and colleagues (21) determined that sequences located 20 to 35 bp 3′ of the poly(A) site of the adenovirus E2A message were essential for the formation of mature E2A mRNA. Deletion of these sequences resulted in extended transcripts that read through the poly(A) site. As the sequence between +21 and +32 was complementary to the sequence preceding and including the AAUAAA, the authors postulated that a potential stem-loop may form in the heterogeneous nuclear RNA in which the cleavage site would be located in an exposed single-stranded loop. In an analogous fashion, Cole and Stacy (7) found that a G-T-rich region located 20 to 38 bp downstream from the AAUAAA was critical for efficient processing and polyadenylation of herpes simplex thymidine kinase mRNA. This region, as RNA, could form a stem-loop structure with the AAUAAA.

In a different viral system, Sadofsky and Alwine (39) determined that deletion of sequences 3 to 60 nucleotides 3′ of the AAUAAA resulted in increased amounts of extended transcripts of simian virus 40 (SV40) late mRNA. Similarly, in the ΔKpn transfectants, deletion of nucleotides 3′ of the AAUAAA resulted in many extended transcripts. However, in both the SV40 and the γ_{2b} genes, these sequences were not complementary to the sequences 5′ of the AAUAAA hexamer. Therefore, the stem-loop mechanism suggested by McDevitt et al. (21) is not apparent in these systems.

In an analogous study, Conway and Wickens (8) determined that sequences from +4 to +26 relative to the SV40 late message poly(A) addition site were necessary for the formation of mature late SV40 message. Additional fine mapping revealed that deletions of the wild-type polyadenylation site and 7 bp 5′ and 4 bp 3′ of that site (−7 to +4) reduced cleavage to 40% of wild-type levels for SV40 late message. Deleting an additional 4 bp 3′ into the flanking region −7 to +8 reduced cleavage to 3% of wild-type levels. The authors propose that the downstream element (+4 to +26) is important for efficient cleavage of SV40 late transcripts but that another element located in the immediate vicinity of the polyadenylation site may also modulate cleavage efficiency. Thus, it remains uncertain whether the poly(A) site itself or sequences downstream of the poly(A) site are essential for proper cleavage and polyadenylation in the γ_{2b} mRNA.

In an attempt to find a common denominator for 3′-end message formation, McLauchlan and co-workers (22) examined the nucleotide sequence 3′ of the poly(A) addition site in more than 75 eucaryotic genes. The resulting consensus sequence, YGTGTTYY, was present in 67% of all genes 20 to 30 bp 3′ of the poly(A) site. Generally, the trinucleotide TGT, occasionally repeated and in conjunction with oligo(T) stretches, was located in a region about 30 bp 3′ of the poly(A) site. The mechanism by which this consensus downstream element mediates cleavage and polyadenylation is not known.

The sequence GTCCTGGTTCTTT was found beyond most immunoglobulin heavy-chain 3′ sec mRNA termini (Table 2) but not in the 3′ mb termini sequenced to date. This sequence is located 13 to 33 bp 3′ of the AAUAAA. The absence of this sequence in mouse IgA may be related to the unusually short 3′ untranslated region in the IgA message (44) and suggests that this isotype uses a different mechanism for 3′ sec mRNA formation. This consensus sequence is not homologous to the YGTGTTYY sequence of McLauchlan but does contains a TGT (a T is located immediately 5′ of this sequence) with oligo(T)s. There are no other sequences homologous to either the YGTGTTYY sequence of McLauchlan or a TGT with a stretch of oligo(T) in the region downstream of the 3′ sec poly(A) addition site in the γ_{2b} gene. If the GTCCTGGTTCTTT sequence is important for regulation, it remains to be demonstrated whether it functions in all eucaryotic cells or whether it is important in the tissue-specific regulation of immunoglobulin expression.

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