MOLECULAR AND CELLULAR BIOLOGY, Oct. 1985, p. 2514–2520
0270-7306/85/102514-07$02.00/0

Vol. 5, No. 10

Cell-Specific Expression of Secreted Versus Membrane Forms of Immunoglobulin Gamma 2b mRNA Involves Selective Use of Alternate Polyadenylation Sites

CHRISTINE MILCAREK* AND BRENDA HALL

Department of Microbiology, University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania 15261

Received 18 March 1985/Accepted 27 June 1985

Immunoglobulin heavy chain genes encode at least two forms of mRNA, secretory- and membrane-specific. In less mature B cells and tumors arising from them, lymphomas, the membrane form of the protein and mRNA are in high abundance, while in more mature stages, plasma cells, and myeloma tumor cells, the secreted forms of protein and mRNA predominate. In myeloma cells producing ~8:1 ratios of secretory- to membrane-encoding forms of γ-heavy chain mRNA, we observed equimolar transcription of the secretory- and membrane-encoding exons of the gene. In isolated nuclei from 4T001 (γ2b) and K23 (γ2a) myeloma cells, the secretory-encoding mRNA polyadenylation site was used at least three times as often as the membrane-encoding mRNA polyadenylation site. In the A20 (γ2a) lymphoma, which produces equal amounts of mature secretory- and membrane-encoding heavy chain mRNAs, results of experiments with isolated nuclei showed that the membrane mRNA polyadenylation site was used about two times as often as the secretory mRNA polyadenylation site. Selective use of alternate polyadenylation and cleavage sites, therefore, can modulate the production of the two mRNAs from a single gene during B cell differentiation.

Cells in the B lineage can produce two kinds of immunoglobulin (Ig) heavy chain mRNA from a single, productively rearranged gene. One mRNA encodes a secretory form of the protein while the other mRNA includes different 3′ terminal exons that allow the resulting protein to be anchored in the cell membrane, where it may serve as antigen receptor. All of the immunoglobulin heavy chain classes have been shown to encode these two forms of mRNA from genes in which the secretory-encoding exons are 5′ of the membrane-encoding exons (1, 7, 12, 20, 22, 23, 30).

Two mechanisms have been proposed to explain how the two forms of Ig mRNA, membrane- and secretory-encoding, may arise from one gene. In the differential termination of transcription model, the production of mRNA for secreted protein would result from transcripts which end in the region 3′ to the last secretory-encoding exon but before the membrane-encoding exons. The production of mRNA for membrane protein would result from transcripts which proceed through the membrane exons and end in the region 3′ of them. This model is similar to the cessation of immunoglobulin D (IgD) production in cells previously cotranscribing both IgM and IgD genes (16). In the second model, differential polyadenylation or other RNA-processing events would occur on a single nascent transcript which always terminates beyond the last membrane exon. This would be analogous to the production of calcitonin or calcitonin gene-related peptide from a single nascent transcript by differential polyadenylation (3, 4, 24) in different tissues.

The murine cell line A20 is lymphomalike, produces a transmembrane Ig of the γ2a class but very little secreted Ig (13, 31) and therefore represents a less-differentiated state of B-cell development. The murine myeloma cell line 4T001 produces large amounts of secretory-encoding γ2b mRNA and secreted Ig protein but a small amount of membrane-encoding mRNA (15, 22) and therefore resembles a mature plasma cell, the final differentiation stage in B-cell development. The myeloma cell line K23 was derived from 4T001 (19) and produces a large amount of γ2a-secreted Ig with the same heavy-chain variable region (V_H) as 4T001 and a small amount of membrane-encoding mRNA.

We attempted to distinguish between the transcription termination and differential processing models of Ig mRNA production by studying the rates of transcription across the expressed γ2a and γ2b heavy chain genes in the myeloma- and lymphomalike cell lines. We observed equimolar transcription of the secretory- and membrane-encoding exons, implicating differential processing as the primary mechanism of regulation. In isolated nuclei, the secretory exon polyadenylation site was preferentially used in cells producing primarily secretory-encoding mRNA, while the membrane exon polyadenylation site was preferentially used in cells producing more membrane-encoding mRNA. Selective use of alternate polyadenylation sites, therefore, can modulate the production of the two mRNAs from one gene.

MATERIALS AND METHODS

Cells. Myeloma cells were maintained in suspension or spinner culture in Iscoves modified Dulbecco medium (GIBCO Laboratories) supplemented with 5% heat-inactivated (56°C, 30 min) horse serum, nonessential amino acids, glutamine, penicillin, and streptomycin. Cell line A20 was maintained in the same medium as a weakly adherent monolayer culture in tissue culture flasks (Corning Glass Works) and removed from the flasks with a rubber policeman for harvesting.

Steady-state RNA isolation and S1 nuclease mapping. Unlabeled myeloma cells were isolated and fractionated into cytoplasmic and nuclear fractions as described for nascent transcript experiments (see below). Nuclear RNA was isolated by hot phenol extraction at pH 4.8, and cytoplasmic RNA was isolated by phenol extraction at neutral pH; both were subjected to oligodeoxythymidylate-cellulose (dT-

* Corresponding author.

cellulose) chromatography to isolate the poly(A)-containing fraction. RNA was quantitated by the binding of [^{3}H]poly(U).

Denaturing RNA gels were run in formamide-formaldehyde as described previously (27) and blotted to nitrocellulose. The membrane-specific probe used was nick translated, ^{32}P-labeled pCV$_1$ cloned DNA. The mRNA for secreted Ig was detected with a nick-translated probe corresponding to CH$_1$ through CH$_3$ of γ2b, clone pγ2b-11 (26). X-ray films of gels were scanned with a densitometer (Helena Laboratories).

A 4.8-kilobase (kb) *Sma*I fragment of the γ2a gene, from the λ phage clone 2a 11 (28), encompassing the membrane region was isolated from an agarose gel, digested with *Pst*I, and labeled at the 3′ ends by an extensive T4 DNA polymerase-mediated digestion and fill-in reaction with [^{32}P]dCTP as described previously (4). The probe was hybridized with total cytoplasmic (40 μg) or poly(A)-selected (3 μg) RNA from the indicated cells and subjected to S1 nuclease protection analysis as described previously (5).

Nascent transcript labeling. Actively growing cells (about 3 × 10^7) were harvested, washed once with cold buffer (0.15 M KCl, 4 mM magnesium acetate, 10 mM Tris [pH 7.4]), and suspended in 2 ml of the same buffer with a 0.5% final concentration of Nonidet P-40. After a 5-min incubation on ice, the cells were layered over 2 volumes of 0.6 M sucrose in 100 mM Tris (pH 7.4)–5 mM magnesium chloride, and the nuclei were pelleted at 2,000 rpm in a CRU-5000 centrifuge (Damon/IEC) for 10 min. The nuclear pellet was suspended at 3 × 10^8 nuclei per ml in 50 mM Tris (pH 7.9)–25% glycerol–0.1 mM EDTA–10 mM mercaptoethanol–5 mM magnesium chloride. About 3 × 10^6 nuclei were added to a transcription mix with a final volume of 0.15 ml. The transcription mix (2) was composed of 50 mM Tris (pH 7.9)–150 mM KCl–1 mM $MnCl_2$–6 mM $MgCl_2$–0.6 mM dithiothreitol–1 mM ATP–0.06 mM CTP–0.06 mM GTP–300 μCi of [α-^{32}P]UTP (specific activity, ~3,000 Ci/mmol; New England Nuclear Corp.) so that the final UTP concentration was 0.75 μM. The nuclei plus transcription mix was incubated at room temperature for the indicated times. After 30 min, 1 to 10 cpm per nucleus was incorporated in a typical experiment.

DNase I (200 U; Worthington Diagnostics) was then added, and the reaction was incubated at 37°C for 5 min. The reaction was terminated by adding 0.15 ml of 0.3 M sodium acetate (pH 4.8)–1 mM EDTA–0.2% sodium dodecyl sulfate [SDS] and by extracting two to three times with phenol at 55°C. The reaction was extracted with $CHCl_3$ twice and ethanol precipitated with 2 volumes of ethanol. The pelleted RNA was dissolved in 10 mM Tris (pH 7.4)–0.1% SDS and passed over a Sephadex G-50 column. RNA in the void volume was ethanol precipitated and suspended in 10 mM Tris (pH 7.4)–10 mM EDTA–0.1% SDS, and NaOH (0.1 N final concentration) was added. The mix was left on ice for 30 min to break the RNA into pieces of 300 to 400 nucleotides (2) and then neutralized with Tris hydrochloride. The RNA was ethanol precipitated, suspended in 10 mM Tris (pH 7.4)–0.1% SDS, heated to 90°C for 10 min, quenched on ice, and applied in RNA hybridization buffer to DNA dots on filters.

Poly(A)$^+$ transcript labeling. Nuclei were isolated and allowed to transcribe in vitro for 10 min. The RNA was extracted with 55°C phenol, followed by $CHCl_3$. The ethanol-precipitated RNA was suspended in buffer (0.4 M NaCl, 10 mM Tris [pH 7.4], 0.2% SDS) and subjected to poly(A)-selection on dT-cellulose as previously described (4). The polyadenylated fraction represented approximately 5% of the total counts incorporated.

DNA filter hybridizations. Plasmid DNAs were cleaved with restriction enzymes and deproteinated. M13 phage DNAs were purified by phenol extraction from infected bacterial culture supernatants. Nitrocellulose filters were presoaked in 10× SSC (1 × SSC is 0.15 M sodium chloride, 0.015 M sodium citrate) and allowed to air dry. The DNA samples were suspended in 10 mM Tris (pH 7.4)–1 mM EDTA, adjusted to 0.2 M with NaOH, and heated at 80°C for 10 min. The solution was neutralized with 0.5 M Tris hydrochloride (pH 3.5), and the salt was adjusted to 5× SSC. Duplicate or triplicate samples were spotted onto nitrocellulose set up in a dot blotter (Schleicher & Schuell, Inc.) with 5 or 10 μg of DNA per dot. After baking at 80°C in vacuo for 2 h, the filters were blocked with RNA blocking buffer (50% formamide; 5× SSC; 25 mM sodium phosphate [pH 7.0]; 500 μg of sheared, denatured salmon sperm DNA per ml; 10 μg of poly(A) per ml; and 0.2% each of bovine serum albumin, Ficoll, and polyvinylpyrolidol) overnight at 45°C. Heat denatured ^{32}P-labeled RNA (1.4 × 10^7 to 4 × 10^7 cpm per 120-cm^2 filter) was applied to the filter in RNA blocking buffer and hybridized at 45°C for 72 h. The filters were washed four times in 2× SSC at 45°C, 30 min per wash, once at 37°C in 2× SSC plus 1 U of pancreatic RNase A per ml for 60 min, and twice at room temperature in 2× SSC plus 25 mM sodium phosphate–0.1% SDS for 30 min per wash. After washing, the filters were subjected to autoradiography, and the x-ray films were scanned in a densitometer (Helena Laboratories). All scans were adjusted so that the most intense hybridization on the film was set at the same gain level, i.e., relative intensity of hybridization of [^{32}P]RNA. Hybridization intensities were normalized for the insert size in the cloned DNA. Results shown are averages of at least five determinations per cloned fragment.

Derivation of clones used. Plasmid clones pMK5, pMK3, and pMK1 are *Eco*RI fragments of the rearranged, expressed γ2b gene of MPC11 (14) inserted into the *Eco*RI site of pBR325 (see Fig. 3 for relative locations). Clone pCV$_1$ was prepared by Barry Kobrin from a *Kpn*I to *Sac*I fragment of the γ2b membrane regions and the membrane 3′ untranslated region. DNA was cloned into a modified pBR322 plasmid was generously supplied by R. V. Guntaka. Clone pKG19b contains the 3.6-kb *Eco*RI fragment 3′ of the 6.8-kb embryonic *Eco*RI fragment of γ2b constant region inserted into pBR325 at the *Eco*RI site by Kevin Glass.

Clones in M13, mp8, or mp9 were made by inserting γ2b DNA fragments into the viral-encoded, double-stranded replicative form. Coding or noncoding orientation of the resulting phage DNAs (single-stranded) was deduced either by DNA sequencing of phage DNA or restriction mapping of the new double-stranded replicative forms. B13:8 and B13:9 were derived from a 1.3-kb *Bam*HI fragment of most of the CH$_1$ and 5′-flanking DNA. PB4 was derived from a *Pst*I to *Bgl*II fragment of γ2b cloned into the *Pst* and *Bam* sites of M13 mp8. It encompasses hinge, CH$_2$, CH$_3$, and the DNA 3′ of CH$_3$ but terminates just 5′ of the M1 membrane exon. M1M2:4 and M1M2:3 are *Bgl*II fragments containing the membrane exons. MUT:1 and MUT:15 are *Bgl*II fragments of the 3′ untranslated region following M2. *Bam* 3′:4 and *Bam* 3′:1 are from a 1.2-kb *Bam*HI fragment located well to the 3′ end of the mature mRNA, spanning the 3′ end of the γ2b constant-region *Eco*RI fragment and the 5′, or beginning, of pKG19b.

All the clones were tested to determine if they carried repetitive DNA by either of two criteria. First, myeloma cell

genomic DNA was nick-translated with [^{32}P]dCTP and was used as a probe against Southern blotted cloned DNA. A λ phage clone containing the γ2a gene and ~20 kb of additional DNA, λ2a 9 (28), was used as a positive control. Only highly repetitive DNA elements should be capable of hybridizing and giving a signal. Second, the individual clones were nick-translated with [^{32}P]dCTP and used as probes against Southern blotted, *Eco*RI-cleaved myeloma DNA. Clone pKG19b hybridized more with a 5-kb *Eco*RI fragment than with the expected 3.6-kb fragment, indicating that it must contain some repetitive elements. All the other clones used in our studies lacked repetitive elements by one or both of these two criteria.

RESULTS

Steady-state RNA. Myeloma cell lines produce and secrete large quantities of Ig protein, while they continue to produce a small amount of the membrane-encoding form of mRNA (22). The lymphomalike cell line A20 has been shown previously to produce more transmembrane forms of Ig than of the secretory protein (13, 31). We determined the amounts of steady-state membrane- and secretory-encoding mRNA in the myeloma cell lines 4T001 and K23 as compared with the lymphoma cell line A20. Cytoplasmic RNA was size-fractionated on denaturing agarose gels, blotted to nitrocellulose, and hybridized with Ig γ2b cDNA which detects both the membrane- (3.6 kb) and secretory- (1.88 kb) encoding forms of γ2a and γ2b mRNA. The myeloma cell lines 4T001 and K23 accumulate from 10- to 6-fold more of the secretory-encoding mRNA than do membrane forms (Table 1). In contrast, A20 accumulates about equal amounts of the 3.6- and 1.88-kb γ2a mRNAs. These results show that cells arrested at different developmental stages have different ratios of secretory to membrane-specific mRNAs in the cytoplasm, implying that a different RNA regulatory mechanism is operating in the myeloma than that in the lymphoma. We did not determine the relative levels of protein expression in these experiments. Differential translation and posttranslational processing have been observed in various B-cell stages for μ heavy chain expression (29) and could apply here to the γ gene products as well.

Kemp et al. (11) have proposed that Ig μ heavy chain gene transcripts in T cells are first polyadenylated at the 3′-most polyadenylation site (3′ of M2 exon) and are then cleaved and finally repolyadenylated at the secreted specific mRNA terminus in the production of secretory specific μ heavy chains. They have also detected the cleaved-off portion of the molecule, containing M1 and M2 plus a poly(A) tail, as a stable nuclear species. We examined steady-state nuclear RNA from the myeloma cells for both a large (>11 kb) γ2b γ transcript containing V_H through M1 and M2 and for the putative small discarded M1M2-only fragment. Only one stable membrane exon-containing nuclear species, a 3.6-kb RNA indistinguishable in size from cytoplasmic membrane-encoding mRNA, was detected (Fig. 1B, lane 1). No larger or smaller species were detectable in Northern blots of nuclear or cytoplasmic RNA with a probe corresponding to the M1 and M2 plus 3′-untranslated region. When a probe was used for the entire 6.8-kb γ2b constant region, only the previously described 11-kb putative primary transcript (26) and mature mRNA-sized molecules were observed, even with much longer exposures (data not shown). Therefore, we conclude that a polyadenylated primary transcript containing the membrane exons either does not exist or is so rapidly processed once it is polyadenylated that we cannot detect it. Similarly, a polyadenylated RNA containing only the membrane exons was undetectable.

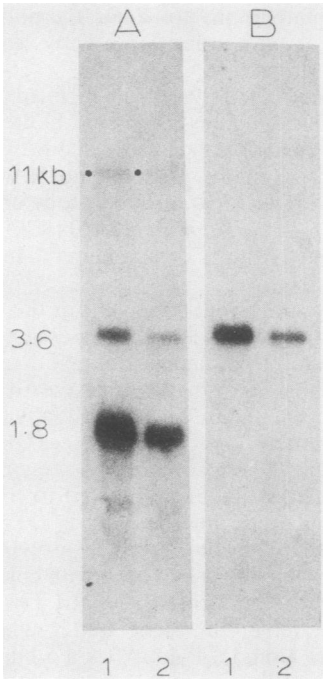

FIG. 1. Northern gel analysis of 4T001 RNA. RNA was electrophoresed on formamide-formaldehyde agarose gels (27), blotted to nitrocellulose, and hybridized with ^{32}P-labeled, nick-translated DNA from the entire γ2b constant region (A) or the M1 plus M2 plus UT region (pCV_1) (B). The 11-kb species indicated is the putative primary nuclear transcript described by Schibler et al. (26) which hybridizes with the CH_1 through CH_3 probe. The 3.6- and 1.8-kb RNAs are the membrane- and secretory-encoding forms of γ2b mRNA, respectively (22). (Lanes 1) 4T001 poly(A) plus nuclear RNA (150 ng); (lanes 2) 4T001 cytoplasmic RNA (10 μg).

TABLE 1. Steady-state levels of secretory- and membrane-encoding mRNAs

Cell line	Relative amt of the following RNAs in cytoplasm[a]		Secretory- to membrane-encoding ratio
	1.88 kb (secreted)	3.6 kb (membrane)	
4T001	10	1	10
K23	6	1	6
A20	4	4	1

[a] Determined by scanning films of Northern blots of cytoplasmic RNA. The probe used (pγ2b11) detects the CH_1 to CH_3 region (26). Identity of the membrane-encoding RNA was confirmed by using pCV_1 as a clone specific for M1 and M2 sequences (Fig. 3D).

Transcription rates across the secreted and membrane exons. Short-term nuclear labeling, the nuclear run-on assay, allows the detection of a transcript proceeding through a gene, even if the transcript is not present in high abundance in the steady-state RNA population. We sought, therefore, to examine the transcription rate across the entire γ2b heavy chain gene locus.

First, the 3′ end of the mature membrane-specific mRNA had to be located to define the minimal transcription unit. The location of the polyadenylation site 3′ of M2 has been broadly suggested (23, 30) but had not been mapped. A DNA

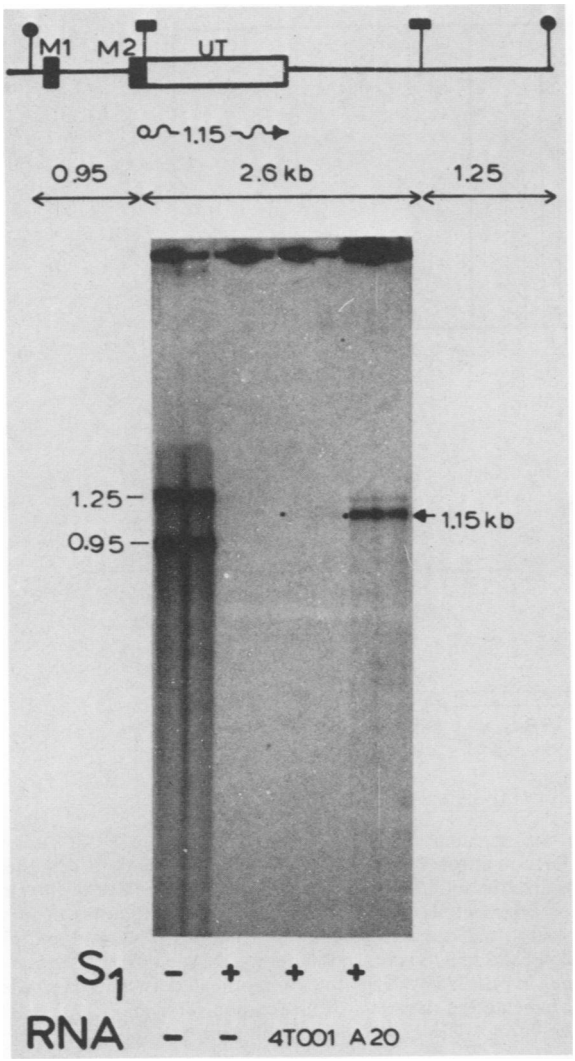

FIG. 2. S1 nuclease mapping of the membrane terminus of γ2a and γ2b mRNA. Cloned γ2a genomic DNA was digested with *Sma*I and the 4.8-kb fragment corresponding to the membrane exons isolated from agarose gels. The fragment was digested with *Pst*I, and the 3′ ends were labeled with [α-^{32}P]dCTP. The 1.25- and 0.95-kb fragments are indicated in the no S1, no RNA lane, while the 2.6-kb fragment (unmarked) is near the origin in that lane. The size of the fragment which was rendered S1 nuclease resistant by hybridization to poly(A)-containing cytoplasmic RNA from A20 or 4T001 cells was determined on an 8 M urea–5% polyacrylamide gel. Symbols: ●, *Sma*I sites in γ2a genomic DNA; ■, *Pst*I sites in γ2a genomic DNA.

clone containing the embryonic γ2a gene was digested with *Sma*I, and a 4.8-kb fragment corresponding to M1, M2, and 3.85 kb beyond M2 was isolated. The fragment was digested with *Pst*I which cuts it twice, producing a 2.6-kb fragment which begins 50 nucleotides to the 3′ side of M2 and two smaller fragments of 1.25 and 0.95 kb (Fig. 2). The 3′ ends of the *Pst*I-generated fragments were end labeled (see above). The 3′-labeled DNA was denatured, hybridized with poly(A)-selected or total cytoplasmic RNA from A20 or 4T001 cells, and subjected to S1 nuclease digestion and gel analysis. Both mRNAs protect a fragment of 1.15 kb, mapping the 3′ end of the membrane-specific mRNA 1,150 ± 50 nucleotides downstream from the *Pst*I site following M2 (or approximately 1,200 ± 50 nucleotides downstream from M2) (Fig. 2). The extensive homology between γ2b and γ2a genes (21) must extend through the 3′-untranslated regions since the γ2a probe is protected by both A20 (γ2a) and to a lesser extent by 4T001 (γ2b) heavy chain mRNA. A minor band at 1.2 kb is always observed. This may indicate the utilization of an alternate poly(A) site approximately 50 nucleotides downstream from the predominant one; however, because of the similarity in size between the 1.2-kb band and the 1.25-kb probe fragment, we cannot eliminate probe rehybridization as artifactually generating the 1.2-kb band. Sequencing of this area of DNA and S1 nuclease analysis with smaller probes will ultimately resolve the issue.

Having established the end of the mRNA-encoding region, we examined the transcription rate across the gene to determine if transcription termination or RNA processing controlled the expression of the two forms of γ heavy chain mRNA. Nuclei from the myelomas 4T001 (γ2b) and K23 (γ2a) which accumulate 10- to 6-fold more secretory- than membrane-specific mRNA were labeled in vitro and hybridized to DNA clones corresponding to various portions of the rearranged, expressed Ig gene of 4T001 (Fig. 3D). The γ2a and γ2b constant regions are ~86% homologous, even into the 3′ flanking regions (21), so one set of clones was used for all the cell lines tested.

The relative hybridization across the γ gene in 4T001 and K23 was constant, indicating that the RNA polymerase loading across the entire expressed gene is uniform (Fig. 3A). Therefore, transcription does not terminate after the CH_3 exon but proceeds through and beyond the M_2 exon into the 3′-untranslated region. The lymphoma A20 which accumulates equal amounts of membrane- and secretory-specific γ2a mRNA was also examined. RNA polymerase loading across the gene in A20 is also uniform (Fig. 3B). Presumably, A20 expresses a V_H different from that expressed in 4T001 and K23, which could explain the lower hybridization to clone pMK3. Transcription proceeds in all three lines, from the coding strand only, to at least 1 kb beyond the M2 exon into the 3′-untranslated region we had mapped. Probes corresponding to the nontemplate strand (Fig. 3C) showed no detectable hybridization. The exact site of termination has not been mapped since the clone pKG19b appears to contain some repeated elements (see above). Because of the compact spacing of the γ genes, transcription beyond pKG19b was not studied. Immediately 3′ of pKG19b is the switch region of γ2a which should cross-hybridize with the γ2b transcript of 4T001 (see below).

The transcription run-on data suggests that a precursor, common to the membrane- and secretory-encoding mRNAs, is produced which is processed differently in the lymphoma and the myeloma. The different processing might involve the rate of splicing of M1 to CH_3 or polyadenylation site selection after CH_3 versus M2. To distinguish between these two possibilities, the polyadenylation of nascent RNA transcripts in isolated nuclei was investigated. Nuclei of 4T001 (myeloma) or A20 (lymphoma) were labeled in vitro for 10 min. The nuclear polyadenylated fraction was isolated on dT-cellulose and hybridized to cloned portions of DNA containing either the CH_3 polyadenylation site (clone PB4; Fig. 3D) or the polyadenylation site after M2 (clone pCV_1). About three times more hybridization was observed with the PB4 DNA than with the pCV_1 DNA with poly(A)-selected transcripts from 4T001 nuclei (Fig. 4). The cytoplasmic ratio of secretory- to membrane-encoding steady-state mRNA is 10:1 in 4T001 cells. The poly(A)-selected transcripts from A20 nuclei hybridized about twice as well with pCV_1 as with

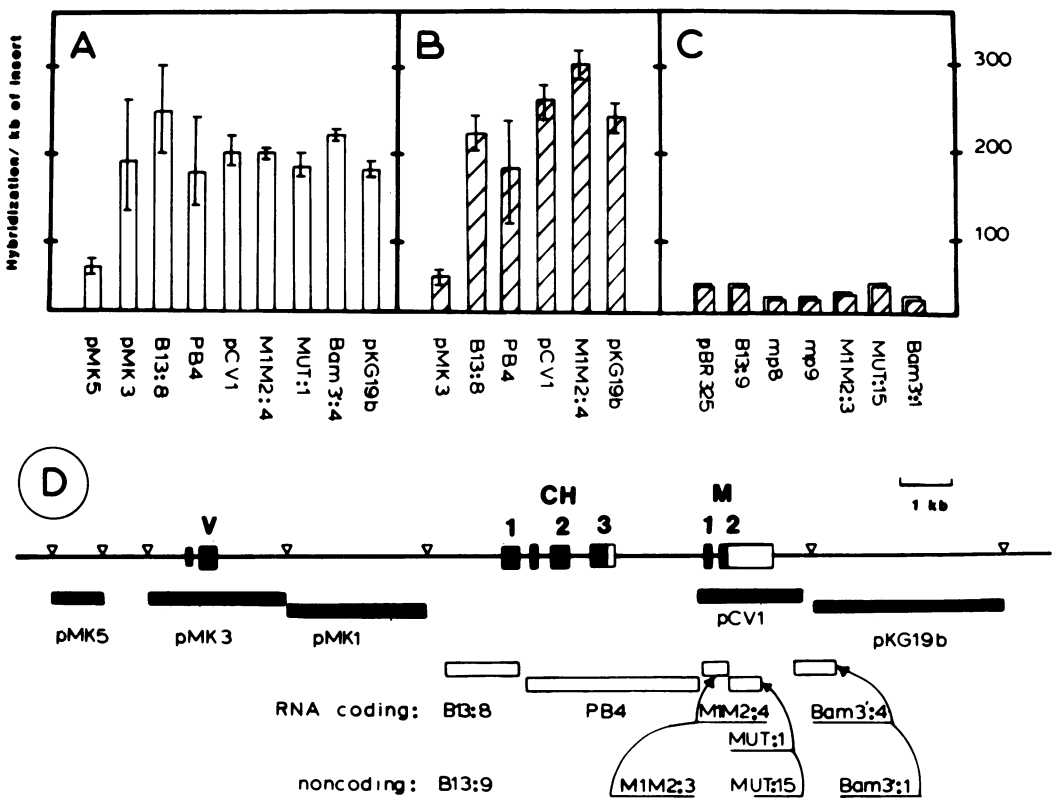

FIG. 3. Analysis of nascent transcripts across the Ig locus. Nuclei from the indicated cells were isolated and incubated in vitro with [α-^{32}P]UTP. The [^{32}P]RNA was hybridized with cloned DNA immobilized as dots on nitrocellulose. After washing, the filters were subjected to autoradiography, and the x-ray films were scanned in a densitometer. Hybridization intensity was normalized for insert size of the cloned DNA. Results shown are averages of at least five determinations per cloned fragment. Hybridization to the indicated clones of in vitro ^{32}P-labeled nuclear RNA from 4T001 and K23 cells (A); A20 cells (B); and 4T001, K23, and A20 cells (C). Clones are plasmid and phage vectors as well as the noncoding strand DNA clones in M13 phage DNA. Map of the Ig γ2b gene and flanking DNA in 4T001 (D). The area of DNA in each clone is indicated below the map. Double-stranded DNA clones in pBR325 or derivatives are indicated as solid bars. Cloned DNA fragments in M13 (mp8 or mp9) used as single-stranded phage DNA are indicated as open bars. RNA coding versus noncoding strands were determined either by sequencing or restriction mapping of the replicative forms. The *Eco*RI sites in the gene are indicated (▽).

PB4 DNA, while the cytoplasmic steady-state mRNA showed a 1:1 membrane to secretory ratio. The cell-specific expression of the two forms of mRNA in the two cell types must therefore involve preferential use of one polyadenylation site over another. Of course, other factors such as cytoplasmic stability and nuclear transport may also play a role in determining the steady-state amounts of the two mRNAs. However, nuclear polyadenylation patterns are consistent with the observed steady-state amounts of the two forms of mRNA and may contribute significantly to the overall levels.

DISCUSSION

Our transcription run-on data show equimolar transcription across the γ heavy chain gene, regardless of whether the genes are generating primarily the secretory-encoding form of the mRNA or equal steady-state levels of membrane and secretory forms of mRNA. Therefore, differential utilization of two or more transcription termination sites within the gene can be eliminated as the mechanism controlling the different ratios of the two forms of mRNA. A common precursor must exist, even though it could not be detected in experiments with steady-state nuclear RNA in which we had selected only poly(A)-containing RNA. From the results of the experiments determining the relative levels of poly(A)-containing nascent transcripts, we conclude that regulated polyadenylation site selection on this common precursor influences developmental expression of the two forms of mRNA. This is in contrast to the polyadenylation of many μ heavy chain transcripts after M2 and their repolyadenylation after the last secretory-specific exon, as previously reported (11). In those experiments the Cμ (μ constant region), without an authentic V_H to the 5′ side and its authentic promoter, was transcribed in a T-cell tumor line. Such a portion of a normal B-cell gene transcribed in cells of the T-lineage cells may not be subject to the same regulation as the intact gene when observed in B-lineage cells, thus leading to the discrepancies in our results and those reported previously (11). Results of our experiments are consistent with the observations of Nelson et al. (20), who have shown equal amounts of two large polyadenylated molecules (membrane and secretory precursor molecules) only in steady-state nuclear RNA from cells producing equal amounts of membrane and secretory forms of μ heavy chain. The observation of much more secretory precursor than membrane precursor molecules in hybridomas (plasma cell tumors expressing more secreted than membrane protein) led the authors to speculate that plasma cells must produce a *trans*-acting factor which enhances cleavage and polyadenylation at the proximal (secreted) site. We have directly shown such enhanced cleavage and adenylation at the secreted site in the γ genes by the experiments on

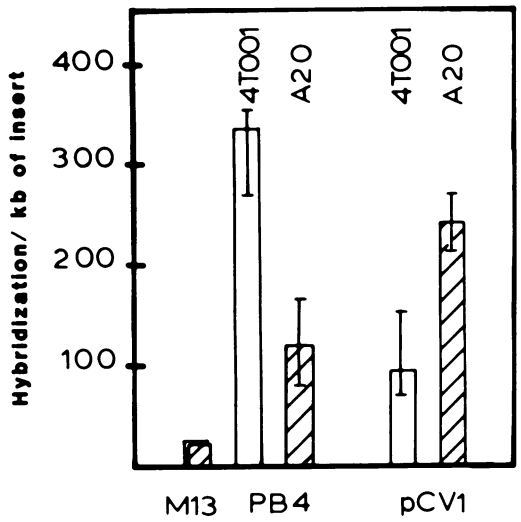

FIG. 4. Polyadenylation of nascent transcripts in isolated nuclei. Nuclei from the indicated cells were isolated and incubated in vitro for 10 min at 25°C with [α-^{32}P]UTP. RNA was isolated by hot phenol extraction and subjected to poly(A) selection on dT-cellulose as previously described (4). The polyadenylated nuclear RNA hybridized with the indicated DNAs immobilized as dots on nitrocellulose filters. The filters were washed and exposed to x-ray film; the relative hybridization intensity was determined as indicated in the legend to Fig. 3. The M13 clone PB4 encompasses hinge, CH_2, CH_3, and sequences 3′ of CH_3 but terminates just 5′ of the M1 membrane exon while pCV_1, a pBR322 derivative, represents the membrane-encoding regions and the membrane 3′ untranslated region (see the legend to Fig. 3 and the text).

nascent transcript labeling and selection of poly(A)-containing nuclear RNAs. The regulation of the γ genes also closely resembles the tissue-specific expression of the calcitonin and calcitonin gene-related peptides seen by Amara et al. (4).

Like other eucaryotic genes which have been analyzed (6, 9, 10), transcription of the expressed γ2b gene proceeds well beyond the 3′ end of the mature (membrane-encoding) mRNA. We can be certain that transcription proceeds into the DNA represented by clone *Bam* 3′:4 which is at least 1 kb beyond the membrane-specific poly(A) site. Accurate mapping of the end of the transcript was not possible in our experiments because the DNA sequences represented in clone pKG19b contain some repeated elements which could be hybridizing with RNA transcripts from other genes besides those for Ig. Analysis of sequences further downstream with the transcription run-on assays would be fruitless since immediately 3′ of the sequences in pKG19b is the switch region of the Cγ2a gene in germline DNA (27). The distance between the end of the Cγ2b gene and the γ2a switch site is even shorter in the rearranged DNA of the 4T001 genome (8). The switch region is a repeated tandem array of DNA sequences present in front of seven of the heavy chain genes. Many aberrant transcripts have been demonstrated from the heavy chain genes in other myelomas. If such transcripts are present in 4T001, K23, or A20, they would cross-hybridize with the cloned DNA containing the γ2a switch. Even more significantly, the authentic γ heavy chain gene transcript in the cells we have studied would cross-hybridize with the cloned DNA containing the γ2a switch. If the end point of transcription can be mapped, it may require S1 nuclease protection experiments as used by Hagenbuchle et al. (10) to locate the transcription end points of the α-amylase gene.

Within the 3′ untranslated regions of polyadenylated mRNAs are the poly(A) signal, AAUAAA, followed by the actual site of poly(A) addition, generally 20 to 30 nucleotides downstream of the poly(A) signal. Beyond the poly(A) addition site, sequences have been described which are important for correct and abundant polyadenylation in both viral (17, 18, 25) and eucaryotic genes (18, 32). By analogy, beyond the secretion-specific poly(A) site in the Ig γ genes, there may be sequences important for directing polyadenylation to that portion of the transcript. Experiments with Ig γ genes transfected into lymphoma and myeloma cells are in progress to assess the importance of sequences 3′ of the secretion-specific poly(A) site.

A *trans*-acting factor which enhances cleavage and polyadenylation at the secreted site is implied from experiments by Word and Kuehl (31) in which the lymphoma A20, which produced primarily membrane γ2a protein, was fused with the myeloma 4T001, which produced primarily secreted γ2b protein. Following such a fusion, the expression of large amounts of secreted γ2a protein was induced. This suggests that a *trans*-acting regulator may be supplied by the myeloma which, if it acts in a positive way, may cause preferential cleavage and polyadenylation at the secretory-specific site. If the *trans*-acting regulator acts in a negative way, it may preferentially block cleavage and polyadenylation at the membrane-specific site. The gene transfection experiments in progress may also help to distinguish between these two modes of action of the hypothetical regulator.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants CA36606 and CA00915 (Research Career Development Award) from the National Cancer Institute.

We thank Barry Kobrin for clone pCV_1 and B. Kobrin and S. L. Morrison for useful discussions. Kevin Glass provided pKG19b and expert assistance.

LITERATURE CITED

1. **Alt, F. W., A. L. Bothwell, M. Knapp, E. Siden, E. Mather, M. Koshland, and D. Baltimore.** 1980. Synthesis of secreted and membrane-bound immunoglobulin mu heavy chains is directed by mRNAs that differ at their 3′ ends. Cell **20**:293–301.
2. **Alterman, R. M., S. Ganguly, D. H. Schulze, W. F. Marzluff, C. L. Schildkraut, and A. I. Skoultchi.** 1984. Cell cycle regulation of mouse H3 histone mRNA metabolism. Mol. Cell. Biol. **4**:123–132.
3. **Amara, S., V. Jonas, M. G. Rosenfeld, E. S. Ong, and R. M. Evans.** 1982. Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. Nature (London) **298**:240–244.
4. **Amara, S. G., R. M. Evans, and M. G. Rosenfeld.** 1984. Calcitonin/calcitonin gene-related peptide transcription unit: tissue-specific expression involves selective use of alternative polyadenylation sites. Mol. Cell. Biol. **4**:2151–2160.
5. **Brandt, C. R., S. L. Morrison, B. K. Birshtein, and C. Milcarek.** 1984. Loss of a consensus splice signal in a mutant immunoglobulin gene eliminates the CH_1 domain exon from the mRNA. Mol. Cell. Biol. **4**:1270–1277.
6. **Citron, B., E. Falck-Pederson, M. Salditt-Georgieff, and J. E. Darnell, Jr.** 1984. Transcription termination occurs within a 1000 base pair region downstream from the poly(A) site of the mouse β-globin (major) gene. Nucleic Acids Res. **12**:8723–8731.
7. **Cushley, W., B. E. H. Coupar, C. A. Michelson, and A. R. Williamson.** 1982. A common mechanism for the synthesis of membrane and secreted immunoglobulin α, γ, and μ chains. Nature (London) **298**:77–79.

8. **Eckhardt, L. A., and B. K. Birshtein.** 1985. Independent immunoglobulin class-switch events occurring in a single myeloma cell line. Mol. Cell. Biol. **5**:856–868.
9. **Frayne, E. G., E. J. Leys, G. F. Crouse, A. G. Hook, and R. E. Kellems.** 1984. Transcription of the mouse dihydrofolate reductase gene proceeds unabated through seven polyadenylation sites and terminates near a region of repeated DNA. Mol. Cell. Biol. **4**:2921–2924.
10. **Hagenbuchle, O., P. K. Wellauer, D. L. Cribbs, and U. Schibler.** 1984. Termination of transcription in the mouse α-amylase gene amy-2^a occurs at multiple sites downstream of the polyadenylation site. Cell **38**:737–744.
11. **Kemp, D. J., G. Morahan, A. F. Cowman, and A. W. Harris.** 1983. Production of RNA for secreted immunoglobulin μ chains does not require transcriptional termination 5′ to the μ_m exons. Nature (London) **301**:84–86.
12. **Kikutani, H., R. Sitia, R. A. Good, and J. Stavnezer.** 1981. Synthesis and processing of the α heavy chains of secreted and membrane-bound IgA. Proc. Natl. Acad. Sci. U.S.A. **78**:6436–6440.
13. **Kim, K. J., C. Kanellopoulos-Langevin, R. M. Merwin, D. H. Sachs, and R. Asofsky.** 1979. Establishment and characterization of Balb/c lymphoma lines with B cell properties. J. Immunol. **122**:549–553.
14. **Lang, R. B., L. N. Stanton, and K. B. Marcu.** 1982. On immunoglobulin heavy chain gene switching: two IgG2b genes are rearranged via switch sequences in MPC-11 cells but only one is expressed. Nucleic Acids. Res. **19**:611–638.
15. **Laskov, R., and M. D. Scharff.** 1970. Synthesis, assembly, and secretion of gamma globulin by mouse myeloma cells. I. Adaptation of the merwin plasma cell tumor-11 to culture, cloning and characterization of gamma globulin subunits. J. Exp. Med. **131**:515–541.
16. **Mather, E. L., K. J. Nelson, J. Haimovich, and R. P. Perry.** 1984. Mode of regulation of immunoglobulin μ- and δ-chain expression varies during B-lymphocyte maturation. Cell **36**:329–338.
17. **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.
18. **McLauchlan, J., D. Gaffney, J. L. Witton, 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.
19. **Morrison, S. L.** 1979. Sequentially derived mutants of the heavy chain of murine immunoglobulins. J. Immunol. **123**:793–800.
20. **Nelson, K. J., J. Haimovich, and R. P. Perry.** 1983. Characterization of productive and sterile transcripts from the immunoglobulin heavy-chain locus: processing of μ_m and μ_s RNA. Mol. Cell. Biol. **3**:1317–1332.
21. **Ollo, R., C. Auffray, C. Morchamps, and F. Rougeon.** 1981. Comparison of mouse immunoglobulin γ2a and γ2b chain genes suggests that exons can be exchanged between genes in a multigenic family. Proc. Natl. Acad. Sci. U.S.A. **78**:2442–2446.
22. **Rogers, J., E. Choi, L. 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.
23. **Rogers, J., 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 chain. Cell **20**:303–312.
24. **Rosenfeld, M. G., J.-J. Mermod, S. G. Amara, L. W. Swanson, P. E. Sawchenko, J. Rivier, W. W. Vale, and R. M. Evans.** 1983. Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing. Nature (London) **304**:129–135.
25. **Sadofsky, M., and J. V. Alwine.** 1984. Sequences on the 3′ side of hexanucleotide AAUAAA affect efficiency of cleavage at the polyadenylation site. Mol. Cell. Biol. **4**:1460–1468.
26. **Schibler, U., K. B. Marcu, and R. P. Perry.** 1978. The synthesis and processing of the messenger RNAs specifying heavy and light chain immunoglobulins in MPC-11 cells. Cell **15**:1495–1509.
27. **Schwinghamer, M. W., and R. J. Shepherd.** 1980. Formaldehyde-containing slab gels for analysis of denatured, tritium-labeled RNA. Anal. Biochem. **103**:426–434.
28. **Shimizu, A., N. Takahashi, Y. Yamawaki-Kataoka, Y. Nishida, T. Iataoka, and T. Honjo.** 1981. Ordering of mouse immunoglobulin heavy chain genes by molecular cloning. Nature (London) **289**:149–153.
29. **Sidman, C.** 1981. B lymphocyte differentiation and the control of IgM μ chain expression. Cell **23**:379–389.
30. **Tyler, B. M., A. F. Cowman, J. M. Adams, and A. W. Harris.** 1981. Generation of long mRNA for membrane immunoglobulin γ2a chains by differential splicing. Nature (London) **293**:406–408.
31. **Word, C. J., and W. M. Kuehl.** 1981. Expression of surface and secreted IgG_{2a} by a murine B-lymphoma before and after hybridization to myeloma cells. Mol. Immunol. **18**:311–322.
32. **Woychik, R. P., R. H. Lyons, L. Post, and F. M. Rottman.** 1984. Requirement for 3′ flanking region of the bovine growth hormone gene for accurate polyadenylation. Proc. Natl. Acad. Sci. USA **81**:3944–3948.