

TRANSCRIPTIONAL REGULATION OF
THE μ - δ HEAVY CHAIN LOCUS IN NORMAL
MURINE B LYMPHOCYTES

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B cell differentiation is accompanied by distinct nucleic acid rearrangements at the immunoglobulin gene locus. The first is a recombination of V_H , D , and J_H gene segments (reviewed in 1) as hematopoietic stem cells differentiate into pre-B cells. Expression of this functional variable (V) region with the most 5' constant (C) region gene, $C\mu$, produces cytoplasmic μ chains in the absence of light (L) chains. Subsequent recombination of V_L and J_L segments accompanies the maturation of pre-B cells into immature B cells that express only IgM on their surface. At some point in these differentiative steps, $C\delta$, tightly linked to $C\mu$ ~2 kilobasepairs (kbp) downstream (2-4), is cotranscribed with VDJ and $C\mu$ without further DNA rearrangement. Although not formally proven, simultaneous expression of membrane IgM and IgD (mIgM, mIgD)¹ must result from alternative processing of the primary $C\mu$ and/or $C\mu$ - $C\delta$ transcript (4, 5). Upon stimulation by antigen or mitogen, B cells differentiate into plasma cells in which the production of IgM is dramatically increased.

The relative expression of IgD and IgM on murine B lymphocytes changes during B cell development and differentiation (see Table I). The density of IgD in neonatal B lymphocytes (2-10-d-old) is low but increases during development (6). Although adult, resting B lymphocytes are heterogeneous with respect to mIgD (7, 8), the levels of mIgD on the predominant subpopulation present in spleen and lymph nodes exceed that of mIgM. Upon activation by mitogen, the relative density of the two isotypes changes again such that the total amount of mIgD decreases to <1/10 that of mIgM (9, 10). Heterogeneity among normal B cells presents major difficulties in the analysis of the molecular regulation of μ and δ gene expression unless the cell surface phenotype and secretory status of the cells used in the experiments are well defined. Although the use of neoplastic B cells (e.g., lymphomas and plasmacytomas) can bypass the heterogeneity

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¹ *Abbreviations used in this paper:* b-GA δ , biotinylated goat anti-delta; b-GA μ , biotinylated goat anti-mu; b-GA α , biotinylated goat anti-ovalbumin; BSA, bovine serum albumin; BSS, balanced salt solution; $C\delta$, δ constant region; $C\mu$, μ constant region; δm , δ chain of membrane IgD; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; FI-AV, fluorescein isothiocyanate-conjugated avidin; IVS, intervening sequence; LPS, lipopolysaccharide; 2-ME, 2-mercaptoethanol; mIgD, membrane IgD; mIgM, membrane IgM; μm , μ chain of membrane IgM; NP-40, Nonidet P-40; PBS, phosphate-buffered saline; SB, staining buffer; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SSC, 0.15 M NaCl, 0.015 M Na citrate; TCA, trichloroacetic acid.

problem to a certain extent, a number of drawbacks should be noted. First, the normal counterpart of each tumor cell line is difficult to convincingly ascertain. For example, there is as yet no murine B cell tumor that can be classified as representative of a typical resting B lymphocyte, i.e., one bearing high densities of IgD. Second, with rare exceptions (11, 12), neoplastic B cells are capable of only a limited degree of *in vitro* differentiation. Therefore we feel it is particularly relevant to study the control of regulated changes displayed by normal differentiating B cells.

Our recent studies (10, 13; see Table I) using normal splenic B cells have shown that the steady state concentration of mRNA for δ m chains is 10 times lower than that for μ m chains, and that this difference in mRNA concentration is reflected in the synthesis rate of the two polypeptide chains. However, post-translational events further modulate the expression of the two isotypes, resulting finally in higher expression of IgD than IgM on the cell surface (10). In addition, we have shown that the decrease in IgD expression after mitogen activation of B cells can be directly attributed to a decrease in the steady state concentration of mRNA for δ chains (13). To understand the molecular basis for the changes in steady state concentration of mRNA for μ and δ chains during B cell differentiation, we have now analyzed the transcriptional activity across the 26 kbp region that encodes J_H , $C\mu$, and $C\delta$ by means of *in vitro* nascent RNA chain elongation. Our results show, first, that in adult cells expressing both IgD and IgM, the transcriptional level of RNA encoding μ chains is two to three times greater than that for δ chains. Second, despite the low levels of expression of IgD in neonatal cells, the relative transcription of $C\delta$ and $C\mu$ in these cells is not significantly different from that of adult cells. Finally, although B cell activation results in an increased transcription of the $C\mu$ gene, the transcriptional level of the $C\delta$ gene does not decrease to levels below that of normal cells. We conclude from the analysis of these three major cell populations, which differ dramatically in their expression of mIgD, that the basal level of $C\delta$ transcription remains constant and that posttranscriptional processing must play an important role in the changes in expression of mIgD during B cell differentiation and activation.

Materials and Methods

Cells. Spleen cells from 6–8-wk-old BALB/c female mice (Cumberland Farms, Clinton, TN) were teased and washed in balanced salt solution (BSS). T lymphocytes were eliminated by complement-mediated cytolysis as described previously (14) using the monoclonal anti-Thy-1.2 antibody, HO-13.4 (15). Spleen cells from 10–12-d BALB/c mice were prepared as above but not treated with anti-Thy-1.2 and complement.

Plate Separation. IgD-bearing cells were isolated by the method described by Wysocki and Sato (16). Briefly, 3×10^7 spleen cells were incubated for 1 h in each 150×15 mm plastic petri dish (Fisher Scientific Co., Pittsburgh, PA), precoated with $75 \mu\text{g}/\text{plate}$ of monoclonal anti- δ antibody, H10.4.22 (17), in phosphate-buffered saline (PBS). After removal of unbound cells, bound cells were eluted by flooding the plates with PBS containing 5% fetal calf serum (FCS). Usually, 30% of the input cells were recovered in the bound fraction.

Percoll Density Gradient Sedimentation. Small, dense lymphocytes were separated from larger, light plasma cells by means of Percoll density gradient sedimentation as described previously (10).

Immunofluorescence Analysis and Cell Sorting. Cells were washed with staining buffer (SB), consisting of BSS containing 0.2% NaN_3 and 0.1% bovine serum albumin (BSA).

Aliquots of 5×10^5 cells in 50 μ l of SB were incubated with optimal amounts of affinity-purified, biotinylated goat anti- δ (b-GA δ), goat anti- μ (b-GA μ), or goat antiovalbumin (b-GA α) for 30 min at 4°C. All affinity-purified goat antibodies were prepared as described for rabbit antibodies in Isakson et al. (18). After washing, the cells were stained with the secondary reagent, fluorescein isothiocyanate-conjugated avidin (FI-AV) (Beckton, Dickinson & Co., Sunnyvale, CA) by incubation for 20 min at 4°C. Cells were analyzed with an Ortho 50HH Cytofluorograph (Ortho Diagnostic Systems, Westwood, MA) coupled to a Data General 2150 computer system. Fluorescein was excited with 500 mW of the 488 line of an argon ion laser (Coherent Inc., Palo Alto, CA). Dead cells that stained red with propidium iodide (Sigma Chemical Co., St. Louis, MO) were excluded from the analysis by detection of red fluorescence with a filter (7).

For sorting, cells were stained with b-GA δ and FI-AV as above and analyzed with a fluorescence-activated cell sorter (FACS III; B-D FACS Systems, Sunnyvale, CA) using a laser light of 488 nm at an intensity of 300 mW. Sorting was accomplished by deflecting and collecting brightly staining cells. Dead and large cells were distinguished from live cells by near forward-angle light scatter and excluded.

Cell Culture. T-depleted splenocytes were resuspended at 5×10^5 /ml in RPMI medium supplemented as described previously (19) with penicillin-streptomycin, 10% FCS, glutamine, sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol (2-ME), and lipopolysaccharide (LPS) (Difco Laboratories, Detroit, MI) at 50 μ g/ml. All media and supplements were purchased from Gibco Laboratories, Grand Island, NY. Cells were incubated at 37°C in 150-cm² flasks (Corning Glass Works, Corning Medical and Scientific, Corning, NY), gassed with a mixture of 7% O₂ and 10% CO₂. Three times the initial cell number was usually recovered at the end of the 4–5-d culture period.

Biosynthetic Labeling, Immunoprecipitation, and SDS-PAGE. The procedure for biosynthetic labeling of lymphocytes as well as immunoprecipitation and subsequent sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis (SDS-PAGE) of the immunoprecipitates is described in detail elsewhere (10).

Nuclear Run-on Transcription. The method used for nuclear transcription was adapted from that described by Hofer and Darnell (20). Cells were lysed at 4°C in a high pH buffer (0.01 M Tris, pH 8.4, 1.5 mM MgCl₂, and 0.14 M NaCl) containing 0.05% Nonidet P-40 (NP-40). The nuclei were separated by sedimentation at 1,200 g for 10 min and washed once with cold buffer containing 0.14 M KCl, 0.01 M MgCl₂, 1 mM MnCl₂, 14 mM 2-ME, and 20% glycerol and buffered with 0.20 M Tris, pH 8.0. Labeling was carried out at 30°C in 0.1–0.2 ml of the same buffer containing in addition, 0.1 mg/ml creatine kinase, 0.01 M phosphocreatine, 1 mM each of ATP, GTP, CTP, 0.3 μ M UTP, and 500 μ Ci [³²P]UTP (3,000 Ci/mmol; New England Nuclear, Boston, MA). Alpha-amanitin (Sigma Chemical Co.) when used was added to nuclei immediately before in vitro labeling.

RNA Extraction. Labeled nuclei were treated with 100 μ g/ml of RNase-free pancreatic DNase (Worthington Biochemical Corp., Freehold, NJ), treated to remove RNase activity as described by Zimmerman and Sandeen (21), in buffer containing 0.5 M NaCl, 0.05 M MgCl₂, 2 mM CaCl₂, and 0.01 M Tris, pH 7.4. The reaction was stopped by the addition of a 2 \times volume of 0.05 M EDTA and 1% SDS in 0.05 M Tris, pH 8.0. RNA was extracted with hot (60°C) phenol/chloroform/isoamyl alcohol (3:1:0.01, vol/vol) (22, 23) and concentrated by ethanol precipitation. The precipitate was dissolved, reprecipitated once, and finally dissolved in 0.01 M Tris (pH 7.4), 0.01 M EDTA, 0.2 M NaCl and incubated with 0.2 N NaOH for 10 min at 4°C. This partially hydrolyzed RNA was used for all the hybridizations.

Hybridization. Labeled RNA was diluted into Denhardt's solution containing 50% formamide, 0.1 mg/ml herring sperm DNA, 30 μ g/ml poly(A) and poly(C) (Sigma Chemical Co.) in 5 \times SSC (24) and hybridized to DNA immobilized on nitrocellulose filters at 40°C for 4–5 d. Filters were briefly washed with 3 \times SSC (0.15 M NaCl, 0.015 M Na citrate), 0.1% SDS, then washed with 0.1 \times SSC for 2–3 h at 42°C, dried, and exposed to x ray film for 2–14 d.

Relative hybridization to each probe was evaluated by densitometer tracings of the autoradiographs and quantitation of the area under each peak. The absolute amount

hybridized was determined by excision of the band containing probe No. 5 (usually the highest hybridization) and counting by liquid scintillation spectrophotometry. Background counts, as determined by counting the band containing pBR322 DNA alone, were subtracted.

5 μ g of plasmid DNA containing each of the inserts described below was established to be in excess for nuclear RNA from 2×10^8 unstimulated B cells and 5×10^7 LPS-stimulated lymphocytes by rehybridizing the supernatant from the first incubation with a second filter also containing 5 μ g of DNA. Less than 50% additional hybridization was found for each probe (corrected for loss in radioactive decay). In addition, when 5×10^7 cell equivalents of nuclear RNA from nonstimulated B cells were hybridized to either 2 or 5 μ g of a panel of DNA probes (as shown in Fig. 3), no difference was found in the amount hybridized per DNA probe (data not shown).

DNA Clones. To avoid potential artifacts due to fragment contamination, all DNA segments used for hybridization analysis were individually cloned. Purified DNA from these plasmids (described below) were denatured by boiling for 10 min in 0.3 N NaOH, neutralized with HCl, and spotted by hand or loaded onto nitrocellulose (Schleicher and Schuell, Keene, NH) using a slot blot apparatus (Schleicher and Schuell). The filters were subsequently dried at 70°C in a vacuum oven and presoaked in the hybridization buffer for 4 h before incubation with the labeled RNA preparations.

A map of the probes is shown in the bottom of Fig. 3 and each probe is described in detail below. The sequence of all of the probes has been determined (25–27; Blattner and Tucker, unpublished data).

Probe No. 1: A 1,983 bp BamHI-EcoRI genomic fragment from a BALB/c mouse containing coding sequences for J_H3 and J_H4 , subcloned from genomic clone CH4A-142.7 (27) into pBR322. Probe No. 2: A 674 bp EcoRI-HindIII genomic fragment from a BALB/c mouse containing sequences immediately 3' to probe No. 1, subcloned from genomic clone CH-28.289.1 (5) into pBR322. Probe No. 3: A 760 bp HindIII-HindIII genomic fragment from a BALB/c mouse containing sequences immediately 3' to probe No. 2, subcloned from CH 28-289.1 into pBR322. Probe No. 4: A 700 bp HindIII-HindIII genomic μ -switch region (S_μ) fragment subcloned from genomic clone CH4A-142.7 (27) into pBR322. S_μ typically deletes during cloning (3 kbp in germline, 1.5 kbp in CH4A-142.7). Therefore, we partially mapped the deleted portion (represented by a wavy line in Fig. 3) by double digests with Sac I and Pvu II that cut 230 and 100 bp, respectively, within the 5' and 3' HindIII sites (Yuan and Tucker, unpublished data). Probe No. 5: A 950 bp cDNA constructed in pMB9 by A-T tailing into the EcoRI site. This plasmid, p μ (3741)⁹ contains most of the constant region sequences of the μ chain (Tucker and Marcu, unpublished data). Probe No. 6: A 917 bp Kpn-HincII genomic fragment from BALB/c that contains sequences just within (includes μ m) the repetitive sequences designated *a* and *b* in Fig. 3, subcloned from CH 28-257.3 (2) into pUC 8. Probe No. 7: A 1,084 bp HindII-Eco RI genomic fragment composed of the intervening sequences between C_μ and C_δ genes, subcloned from CH 28-257.3 into pBR322. Probe No. 8: A 447 bp EcoRI-Bgl II genomic fragment immediately 3' to probe No. 7, subcloned from CH 28-257.3 into pAT 153. Probe No. 9: A 861 bp cDNA p δ 54J (28) tailed into the Pst I site of pBR322. This plasmid contains most of the constant region sequence of C_δ , including the carboxyl terminus of the secreted form (δ S in Fig. 3) and 3' untranslated region. Probe No. 10: A 1,288 kb BamHI-MboI genomic fragment, containing the carboxyl terminal δ membrane exons, δ M1 and δ M2, subcloned from CH 28-257.3 into pBR322. Probe No. 11: A 1,750 bp BamI-Sph I genomic fragment, including the entire $C_\gamma 3$ region, subcloned from genomic clone CH-318 (29) into pUC 13. It will cross-hybridize with other γ subclass RNAs. Probe No. 12: pBR322 vector with no insert. Probe No. 13: C_μ -negative (noncoding) strand clone M μ -8. The 720 bp Pvu II-Pst I fragment of probe No. 5, containing $C_\mu 2$, $C_\mu 3$, and half of $C_\mu 4$, was subcloned into the Sma I and Pst I site of M13-mp11. μ^+ clones were selected by plaque hybridization to ³²P end-labeled poly(A)⁺ cytoplasmic RNA from LPS-stimulated cells followed by sequence characterization using the chain termination procedure (30). Probe No. 14: C_μ -positive (coding) strand clone M μ -4. The same insert as probe No. 13 was subcloned into the Pst

I-Sma I (opposite orientation) site of M13-mp13. Selection of white plaques was by annealing to clone M-8, followed by sequence characterization.

It should be noted that all repetitive sequences designated in Fig. 3 are not contained in any of the probes. Probe No. 7 contains an inverted repeat sequence that allows formation of a stem loop structure (2) but does not hybridize to repetitive sequence in RNA (D. Yuan, unpublished observations).

Results

Kinetics of In Vitro Nascent RNA Chain Elongation in B Cells. The method we have adopted to measure transcriptional activity of the $C\mu$ and $C\delta$ genes is that of in vitro nascent chain elongation in isolated nuclei (31, 32). This technique has been used by a number of workers to analyze the transcriptional activity of genes whose activity is too low for detection by in vivo pulse labeling (33–35).

To establish appropriate parameters for in vitro labeling of nuclei from B lymphocytes with [^{32}P]UTP, nuclei from both unstimulated cells and cells polyclonally stimulated for 5 d with LPS were isolated by NP-40 lysis and pulse labeled as described in Materials and Methods. Aliquots of the reaction mixture were removed at various time intervals and centrifuged, and the RNA was prepared from the nuclei by phenol extraction. The kinetics of incorporation of [^{32}P]UTP into trichloroacetic (TCA)-precipitable material at each time point is shown in Fig. 1. Although the rate was not linear, total incorporation into nuclear RNA continued to increase for at least the first 10 min of incubation. For the same number of cells, the incorporation rate into RNA of nuclei from LPS-stimulated cells was approximately twofold higher. Size fractionation by agarose gel electrophoresis (data not shown) revealed that the labeled RNA is highly heterogeneous but consists mainly of molecules larger than the 28s ribosomal RNA marker. These results are consistent with published reports in other systems (31) that the label is added to nascent RNA chains.

In addition, we determined that the transcription of mRNA under these conditions is sensitive to alpha-amanitin, which selectively inhibits RNA polymerase II. Hybridization of RNA, labeled in the presence of 250 $\mu\text{g}/\text{ml}$ of alpha-amanitin, to plasmids containing $C\mu$ and $C\delta$ inserts (see below) was reduced to <10 percent of control, whereas total RNA synthesis (which includes ribosomal RNA synthesis by RNA polymerase I) was reduced by only 50% (data not shown).

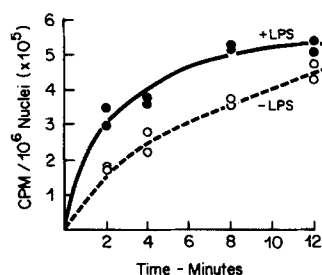


FIGURE 1. Kinetics of [^{32}P]UTP incorporation into nuclear RNA of unstimulated and LPS-stimulated B lymphocytes. 2×10^7 nuclei from nonstimulated and day 5 LPS blasts were each incubated at 30°C in 100 μl of reaction mixture containing 500 μC [^{32}P]UTP as described in Materials and Methods. 20- μl aliquots were removed into cold buffer, spun, and treated with RNase-free pancreatic DNase. Duplicate aliquots were precipitated with TCA and counted by liquid scintillation spectrophotometry.

Relative Transcription of μ and δ Genes in Nonactivated Small Lymphocytes. Recent data obtained by two-color immunofluorescence analysis on the FACS of doubly stained splenic B lymphocytes has shown that while the majority of these cells express high levels of mIgD and lower levels of mIgM, there is a minor population (~6–10%) that display high densities of mIgM only (7, 8). To remove this population and to ascertain that in our analysis we were measuring RNA transcription of the μ and δ genes in B cells that express both IgM and IgD, we selected IgD⁺ cells by binding splenocytes to anti- δ -coated plastic petri dishes (see Materials and Methods). Characterization of the eluted cells by staining with specific antibodies and FACS analysis showed that while only 40% of the starting population was mIgD⁺, >85% of the cells selected in this manner bore mIgD (Fig. 2A). Further, the percent of the cells was very similar to those which are mIgM⁺. On the other hand, IgD-bearing cells were virtually undetectable in the nonbound fraction (Fig. 2B) while ~15% of this population were still mIgM⁺. These results suggest that plate selection effectively removed the cells that express only mIgM. Nuclei were prepared from $2-3 \times 10^7$ such anti- δ -selected lymphocytes and labeled in vitro with [³²P]UTP for 12 min. RNA extracted from the nuclei was treated with mild alkali (0.2 M) and hybridized for 4 d to a panel of DNA probes (as depicted in the bottom of Fig. 3). The detailed description of each of these probes is presented in Materials and Methods. Sequence determination of the entire 26 kbp region spanning the μ - δ transcriptional unit (25–27, 36, 37, and Blattner and Tucker, unpublished results) insured that none of the probes contained repetitive sequences that would hybridize to RNA nonspecifically. Fig. 3, lanes 1 and 1', shows that there is significant hybridization to all of the probes with the exception of DNA No. 11, which contains the C γ -specific probe, and No. 12, the control, which consists of plasmid DNA with no insert. The extent of hybridization to the C δ probes (Nos. 9 and 10) was approximately one-third that of the C μ region probes (Nos. 5 and 6), suggesting that not all of the initiated transcripts continue into the C δ region.

If the anti- δ -selected cells contain plasma cells that synthesize μ chains of secreted IgM, and if these transcripts terminate 5' to C δ (see results in next section), then the relative level of μ - δ transcription observed may be skewed towards μ . The extent of plasma cell contamination was determined by labeling the anti- δ -selected cells biosynthetically with [³⁵S]methionine and assaying the amount of Ig secretion by immunoprecipitation of the culture medium. SDS-PAGE analysis of the immunoprecipitates (not shown) revealed that this population indeed contains cells actively secreting both IgM and IgG. In an attempt to remove these plasma cells (which must have copurified with the IgD⁺ cells by binding nonspecifically to the anti- δ plates), we further fractionated the selected cells by Percoll gradient sedimentation. The small cells derived from this fractionation were found to be depleted of Ig-secreting cells by ~50% (not shown). Analysis of the nascent RNA synthesized by these cells is shown in Fig. 3, lane 2. For each experiment, the amount of labeled RNA hybridized to each DNA probe was quantitatively determined as described in Materials and Methods, normalized to the number of bases contained in the insert, and plotted in terms of the fraction hybridized per input counts per minute for each experiment.

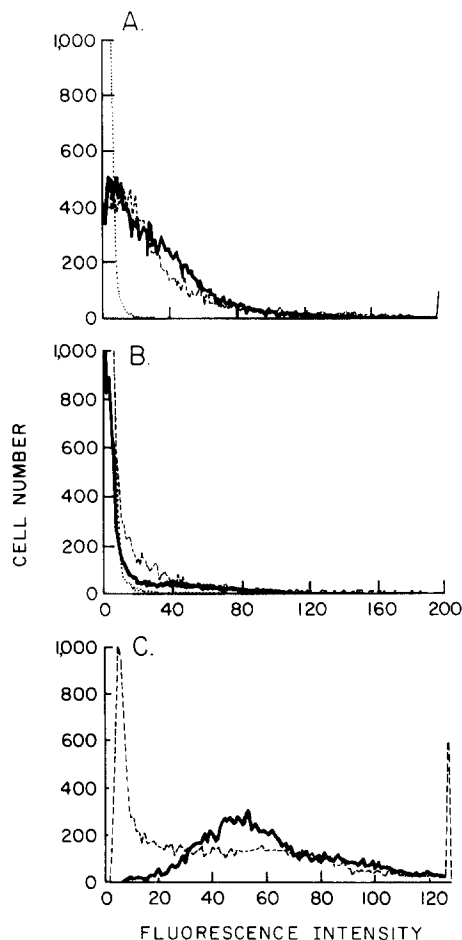


FIGURE 2. FACS analysis of cells separated by means of plating on antibody-coated plates or by cell sorting. Both the bound (A) and nonbound (B) fraction from splenocytes that had been separated by binding to anti- δ -coated plates were incubated overnight in culture medium to allow regeneration of Ig receptors which may have been stripped by the panning procedure. Aliquots from each were then stained with either b-GA δ (—), b-GA μ (---), or b-GA ν (· · ·), followed by FI-AV and analyzed on the Ortho 50HH cytofluorograph (C). T-depleted splenocytes were stained with b-GA δ followed by FI-AV and analyzed on the FACS III before (---) and after (—) sorting for the bright-staining population.

From comparing the two plots, it is apparent that even after removal of 50% of the plasma cells, hybridization to $C\mu$ or $C\delta$ sequences was not significantly affected.

In a further effort to prepare a population of cells bearing both IgD and IgM only, we stained B lymphocytes with goat anti- δ and sorted the positive cells on the FACS. At the same time the cells were gated by scatter to exclude any large cells. Analysis of the sorted cells showed that >99% of the population were surface IgD positive (Fig. 2C). Analysis of [35 S]methionine-labeled cell lysates showed an 80% decrease in intracellular μ chains as compared with nonsorted cells (data not shown). Nuclei were prepared from 5×10^6 cells and labeled with

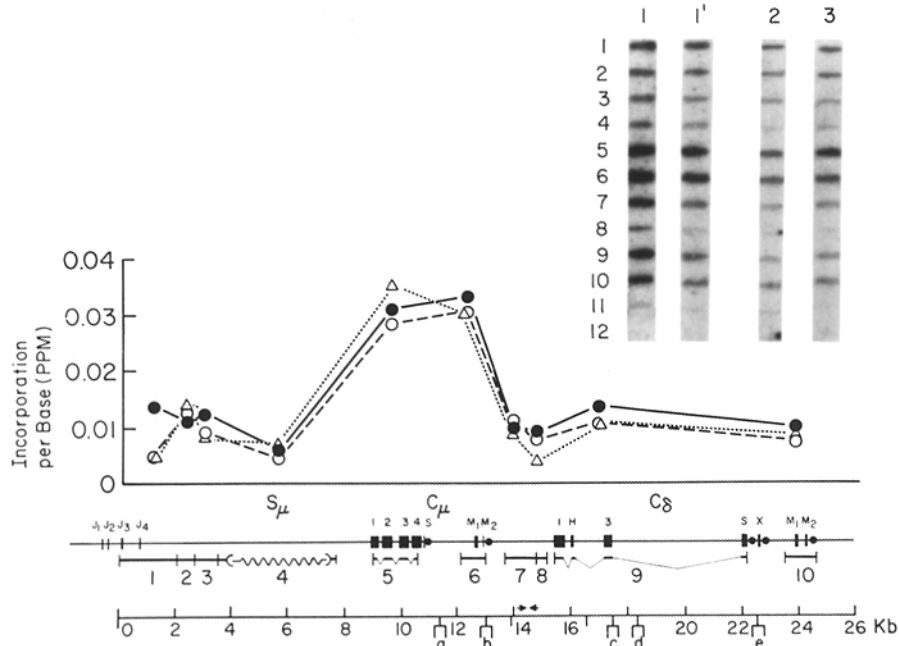


FIGURE 3. Transcription of the μ - δ gene complex in nonactivated small lymphocytes. Nuclei from 2×10^7 lymphocytes eluted from anti- δ -coated plate (lanes 1 and 1'; ●), 2×10^7 plate-purified cells further separated from large cells by Percoll density gradient centrifugation (lane 2; ○), or 0.5×10^7 FACS-sorted IgD⁺ cells (lane 3; △) were labeled with 500 μ Ci of [³²P]UTP for 12 min. RNA extracted from each preparation was hybridized to the panel of DNA probes depicted at the bottom of the figure. Filters were exposed to film for 2–8 d. Relative hybridization to each probe was measured by densitometer scanning of the autoradiograms. To determine the fraction of total input cpm hybridized for each experiment, the probe with the highest signal (No. 5) and control probe (No. 12) were excised and counted by liquid scintillation. The net counts hybridized (cpm in No. 5 after subtraction of cpm in No. 12) were divided by the number of bases in the insert to normalize for size. The extent of hybridization to other probes was determined by their relative density as compared with probe No. 5 and normalized for the number of bases represented in the insert. The values thus obtained for each probe were arbitrarily plotted in the center of the coding sequence within each probe. Since lanes 1 and 1' represent two independent experiments performed under similar conditions, their average values were graphed (●). Nos. 1–10 indicate the DNA segment contained in each probe. For probe 4, the wavy line represents a partial DNA deletion generated within the germline sequence during cloning (see Materials and Methods). Noncontinuous lines in probe Nos. 5 and 9 indicate the absence of intronic sequences in the cDNA insert. (a–e) Location of repetitive sequences previously characterized (37). (a) (CA)₃₃; (b) (GGGAGA)₁₂(GA)₂₈; (c) (CT)₃₀(CA)₃₀; (d) (GAAA)₁₁; and (e) (GA)₁₆. (→←) The position of a 160 bp stem–180 bp loop inverted repeat of unique sequence composition and almost perfect complementarity (37).

[³²P]UTP as before. The hybridization profile is shown in Fig. 3, lane 3. Densitometer scan of the autoradiograph showed the fraction of total radioactivity incorporated per base per DNA probe to be virtually identical to experiment 2.

From these enrichment experiments, we conclude that the plasma cell component may contain a high abundance of μ s mRNA and that it synthesizes secreted Ig. But, because of their low number, their transcription profile does

not contribute significantly to that of the majority of the cells. Therefore, in resting B cells expressing both IgM and IgD, the transcription of the $C\mu$ gene is two- to threefold that of the $C\delta$ gene.

Nuclear Transcription of Neonatal Lymphocytes. FACS analysis of splenocytes obtained from 10-d-old neonatal mice showed that <20% of the cells were mIg⁺ and that the mean fluorescence intensity of cell surface IgD was one-third that of adult cells (data not shown). Biosynthetic labeling of neonatal cells showed that on a per cell basis the IgM secretory rate was approximately fivefold lower than for adult splenocytes (data not shown). Nuclei were prepared from 0.5 – 1×10^8 splenocytes from similar aged animals and labeled for 12 min with [³²P]-UTP as described in the preceding section. Fig. 4 shows the normalized hybridization of RNA to each of the probes. Due possibly to the highly variable percent of Ig-bearing cells in neonatal splenocytes (which depends on age and litter size), the fraction of total labeled RNA that hybridizes to the Ig genes was more variable than in adult cells. However, the relative level of $C\delta$ transcription in these cells was always 20–40% that of $C\mu$ and not greatly different from that in adult animals despite the fact that a much lower percent of cells express mIgD. The relatively strong hybridization to the μ - δ intronic probe (No. 7) in both neonatal and adult B cells suggests that the $C\delta$ transcription is an extension of the $C\mu$ transcription, and not an independent initiation event.

Nuclear Transcription of LPS-stimulated B Lymphocytes. To determine the effect of mitogen activation on the transcriptional activity of $C\mu$ and $C\delta$, B lymphocytes were cultured for 4–5 d with LPS. FACS analysis of the cells at the end of this culture period indicated that 80% of the cells were blasts (Fig. 5, insert), while the mean fluorescence intensity of the cells staining with anti- δ had decreased by at least 64% (data not shown). Some 30% of the small cells were blasts that had reverted to small lymphocytes because they bore mIgG (data not shown). Therefore, the maximum number of unstimulated cells remaining in the culture should not exceed 16%.

Nuclei from such LPS-stimulated cells were labeled in vitro with [³²P]UTP and the RNA hybridized with the same series of DNA probes used for resting B cells. As shown in Fig. 5, the profile of hybridization differs significantly from

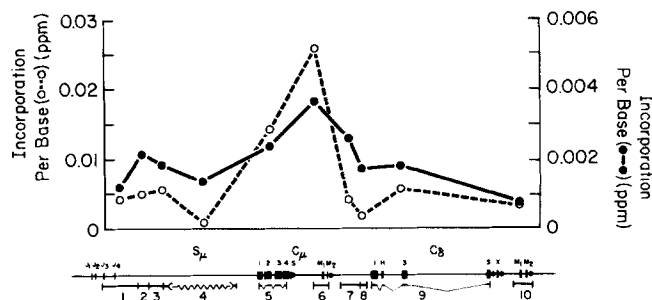


FIGURE 4. Transcription of the μ - δ complex in neonatal lymphocytes. Nuclei from 5×10^7 (○) or from 1×10^8 (●) splenocytes of 10–12-d-old mice were labeled with 500 μ Ci of [³²P]UTP for 12 min. RNA extracted from the nuclei was hybridized to the panel of DNA probes depicted at the bottom of the figure. Fraction of total input RNA hybridized per base per probe was determined as in Fig. 3.

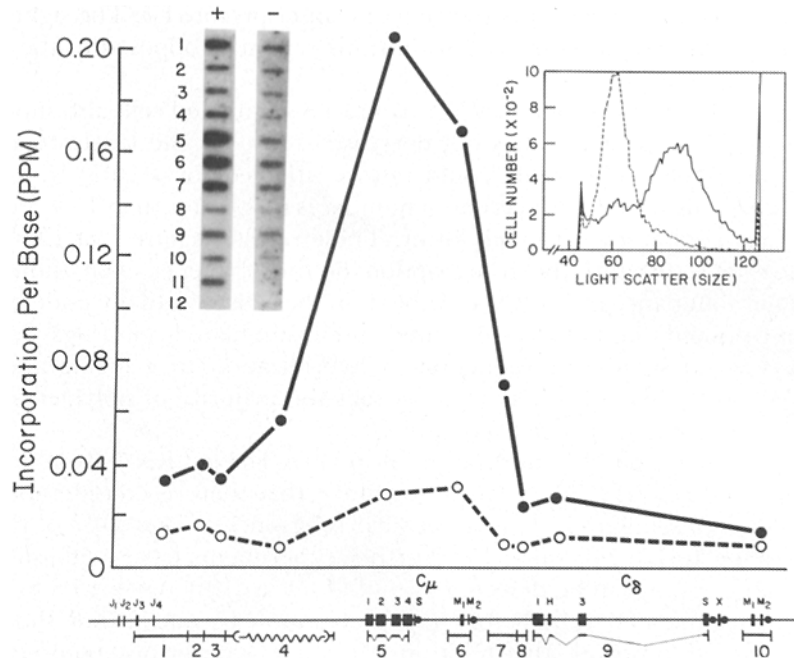


FIGURE 5. Transcription of the μ - δ complex in LPS-stimulated lymphocytes. Nuclei from 5×10^7 lymphocytes stimulated with LPS for 5 d were labeled with $500 \mu\text{Ci}$ of $[^{32}\text{P}]\text{UTP}$ for 12 min. RNA extracted from the nuclei was hybridized to the panel of DNA probes depicted at the bottom of the figure. Fraction of total input RNA hybridized per base per probe (●) was determined from densitometer tracing of the autoradiogram (+ lane) and by liquid scintillation counting as in Fig. 3 and compared with the same profile obtained from nonstimulated cells (- lane; ○). Inset shows the light scatter (size) profile of unstimulated B cells (---) compared with cells that had been cultured in the presence of LPS for 5 d (—) as analyzed by the FACS III.

that observed for nonstimulated cells. First, the incorporation per base is three-fold higher across the J_H - $C\mu$ intronic region and approximately eightfold higher across the $C\mu$ complex. This increased run-on of transcripts during the labeling period could have resulted from a greater RNA elongation rate in activated cells. Therefore, we repeated the experiment but reduced the labeling period from 12 to 2 min. A comparison of profiles obtained from the two time points (not shown) shows that although at 2 min of labeling there was lower total incorporation of radioactivity, the relative hybridization to each DNA probe was very similar to that obtained from the 12-min pulse period. Although difficult to formally prove, it is more probable that the overall increase in transcription is due to an increase in the rate of initiation.

The increase in transcription of the μ gene region must account for the increased abundance of μ s mRNA in the cytoplasm (13) and, concomitantly, the high IgM secretory state of these cells. It is apparent that the increased hybridization extends into probe No. 7 (the 5' μ - δ intervening sequence [IVS] probe), suggesting that the termination site for the μ s mRNA may be within this region. The abrupt drop in the hybridization to the intronic region just downstream (probe 8) suggests that, within the region defined by probe No. 7, a marked

polymerase unloading prevents extension of transcripts into C δ . The significance of a termination site for μ s mRNA within this region as opposed to the region 5' to μ m is discussed below.

The profile of hybridization of RNA from LPS-stimulated cells also shows that the level of C δ transcription has not decreased to below the level observed in nonstimulated cells. This value could not be affected (by >10%) by residual nonactivated cells since their maximum number is no greater than 15% and their overall transcription level is much lower. These results suggest that LPS stimulation does not turn off the transcription of the C δ gene, even though the cytoplasmic abundance of δ mRNA (Table I) in the cells is virtually undetectable. It should be noted that in both stimulated and unstimulated cells (Figs. 3, 4, and 5) there was no significant reduction in hybridization to δ membrane exon sequences (probe No. 10). This suggests that the majority of polymerases that reach C δ transverse the entire C δ region.

The autoradiograph of hybridization of in vitro-labeled RNA from nuclei of LPS-stimulated cells (Fig. 5, + lane) also shows that there is considerable transcription of C γ 3 sequences. This is not surprising since at least 20% of the cells are expressing IgG at this time (19). Further experiments have established that the C γ transcription can be detected even if as few as 10% day 4 LPS blasts are added to unstimulated cells before the preparation of nuclei (not shown). It should be noted, however, that C γ transcription was never observed in any of the experiments performed with nuclei from unstimulated cells in spite of the fact that some of the preparations contained plasma cells secreting IgG as well as IgM. This further reinforces our contention that despite their high secretory activity, the number of contaminating plasma cells must fall below the sensitivity

TABLE I
*Changes in Relative Expression of IgM vs. IgD During B Cell
Maturation*

		Neonatal B cells	Adult resting B cells	Activated B cells
Transcriptional activity*	μ	1.2	3	10
	δ	0.4	1	2
Newly synthesized cytoplasmic RNA [‡]	μ m	ND	2.5	2
	δ m		1	0.16
Steady state cyto- plasmic mRNA [§]	μ m	ND	10	20
	δ m		1	0.1
Translation rate (10)	μ m	ND	7	ND
	δ m		1	
Cell surface half-life (10)		ND	mIgM, 10 h	ND
		ND	mIgD, >24 h	
Cell surface expres- sion [¶]	μ m	5	0.5-1	10
	μ δ	1	1	1

* Transcriptional level of δ (calculated as fraction of total input cpm hybridized) in resting B cells was set as 1.

[‡] Fraction of total input cpm hybridized to δ cDNA was set as 1 (13).

[§] Relative concentration of mRNA for δ m in resting B cells was set as 1 (13).

[¶] Relative 1^{125} cpm in δ band of each population was set as 1 (6, 10).

of detection of the *in vitro* transcription system, and therefore should not significantly influence our conclusions regarding the extent of $C\mu$ transcription in resting B cells.

We have summarized in Table II the relative transcriptional activity of the $C\mu$ gene in various B cell populations expressed as the fraction of the total transcription. It is apparent that LPS-stimulated B cells represent the only cell population that displays a significant increase in $C\mu$ transcription over and above the general overall increase in RNA synthesis. Therefore, hybridoma cells such as BCL₁ × SP2/0 may secrete as much Ig as LPS-stimulated cells, but the transcription of the $C\mu$ gene is not selectively enhanced. Rather, the increased secretory rate is due to the higher metabolic activity of the transformed cells, in that more total RNA is made per cell. LPS stimulation of B lymphocytes, on the other hand, results in a selective enhancement in the transcription of the Ig gene complex over and above the increase in total RNA synthesis, and is a reflection of regulatory events not apparently observable in tumor cells.

Reduced Apparent Transcription of 5' Sequences. It is clear from Fig. 5 that hybridization to upstream sequences is not equimolar to that of the $C\mu$ gene. The inequality is more pronounced in the hybridization profile from activated cells. There are two alternative interpretations for this observation. Either hybridization to the $C\mu$ region probes is greater than the actual transcriptional activity of the $C\mu$ gene, or hybridization to the 5' probes is artifactually low.

TABLE II
Relative Transcriptional Level of $C\mu$ Gene in Different Cell Populations

Cell type	Experiment	Fraction of total input hybridized to μ probe (No. 5)	Average
Unstimulated B lymphocytes	1	0.03	0.026
	2	0.02	
	3	0.03	
	4	0.03	
	5	0.02	
LPS-stimulated B lymphocytes	1	0.21	0.24
	2	0.36	
	3	0.17	
Neonatal B cells	1	0.002	0.008
	2	0.014	
BCL ₁ cells*	1	0.04	0.03
	2	0.01	
BCL ₁ × SP2/0 [‡] hybridoma	1	0.03	0.03
	2	0.03	

* B lymphoma (48) that expresses surface IgM and IgD at ~4:1 ratio.

[‡] Fusion line derived as indicated; secretes IgM at 50% of the level of day 5 LPS-stimulated cells.

A greater hybridization to the $C\mu$ probes may be due to transcription of both strands of DNA in this region. To address this possibility, we constructed (see Materials and Methods) single-stranded probes containing each of the two strands of the $C\mu$ DNA insert in probe No. 5. Fig. 6 shows that although approximately equivalent amounts of DNA from each strand were spotted, the labeled RNA hybridized only to the noncoding strand. Therefore, the relatively enhanced transcription in the $C\mu$ region does not result from RNA precursors generated within $C\mu$ on the opposite strand. We cannot formally rule out the possibility that "sterile" or nonproductive precursors generated by independent initiation events within $C\mu$ on the coding strand give rise to the enhanced exonic transcription. However, the above events are improbable since no transcripts fitting these

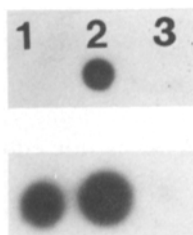


FIGURE 6. Hybridization of in vitro-labeled nuclear RNA from LPS-stimulated cells to the coding and noncoding strands of $C\mu$ gene. Approximately 2 μ g of DNA from probe No. 14, containing the coding strand of probe No. 5, $p\mu(3741)^{\delta}$, was spotted in position 1, while 2 μ g of DNA from probe No. 13, containing the noncoding strand, was spotted in position 2. Position 3 contains DNA from the vector M13-mp11. The filter was hybridized to nuclear RNA from LPS-stimulated cells labeled in vitro with [32 P]UTP as in Fig. 7. The blot depicted at the bottom is the autoradiogram of the same filter subsequently hybridized to [32 P]dATP-labeled probe No. 5, which contains DNA from both strands. Exposure time was 16 h.

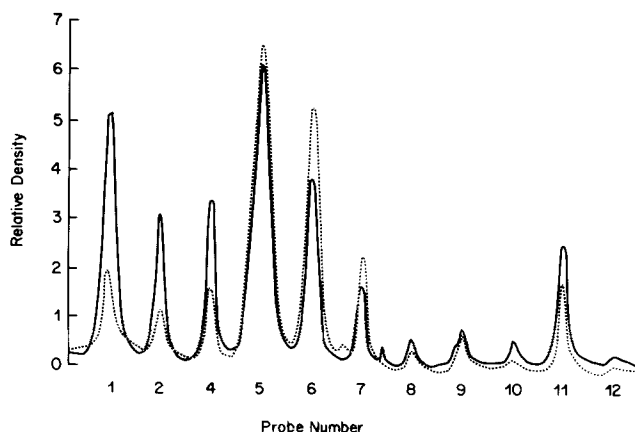


FIGURE 7. Effect of cell preparation time on RNA chain initiation vs. elongation in LPS-stimulated lymphocytes. One aliquot (· · ·) of a culture of day 4 LPS-stimulated cells was harvested at 4°C and incubated for 3 h at 4°C before cell lysis; another aliquot (—) was harvested at 24°C and the cells lysed immediately. Both nuclear preparations were labeled and hybridized to the panel of DNA probes as described in Fig. 3. Direct densitometric tracings of the autoradiogram of the hybridization profile of each RNA preparation are shown without normalization for probe size. The total label incorporation into RNA as well as the percent hybridization to the $C\mu$ probe (No. 5) in the two samples were similar.

criteria have been observed in Northern blot analyses of mRNA from normal B cells (13) or from B and T cell tumor nuclear (22, 38) or mRNA (39).

A possible explanation for the artifactually low hybridization in the $J_H-C\mu$ region, observed particularly in LPS-stimulated nuclei, is a pronounced disparity between initiation and elongation during experimental manipulations. Assume that during cell harvest and nuclei preparation at 4°C before RNA labeling no template reinitiation occurs (upstream to J_H) but elongation continues, albeit at a reduced rate. The net effect would then be a depletion of polymerases immediately 3' to the promoter ($J_H-C\mu$ intron) by virtue of their downstream movement in the absence of concomitant replenishment from the reinitiation pool. When we processed the cells at 24°C instead of 4°C, a two- to threefold increase in hybridization was seen for probes 1-4 relative to the $C\mu$ probe (No. 5) while the relative hybridization to $C\delta$ was not changed (Fig. 7). Therefore, in this system initiation may be significantly more temperature dependent than is elongation.

Discussion

Differential Transcription of μ and δ Genes During B Cell Maturation. In the experiments presented here, we have demonstrated the feasibility of using nuclear transcription for the analysis of the relative transcriptional activity of μ and δ genes in normal B lymphocytes. Selection of IgD⁺ cells by either plate purification or FACS sorting followed by analysis of the cell surface phenotype showed that >85-99% of the cells in the population used in our analysis expressed both surface IgD and IgM. Although some plasma cells were present, we have presented evidence to show that their numbers were insufficient to significantly alter the conclusions. Our results indicate that in normal resting B cells expressing both IgM and IgD, the $C\mu$ gene is transcribed at two to three times the level of the $C\delta$ gene. This suggests that, while 50% of the transcripts initiated upstream of the V region are terminated 5' to $C\delta$, the remaining primary transcripts contain sequences for both genes. Since in previous experiments (13) we have shown that the steady state level of μ m mRNA is some 10-fold higher than that of δ mRNA (see Table II), posttranscriptional events, such as RNA processing and/or turnover rates, must be different for the two transcripts.

It is interesting that the ratio of μ to δ transcription is not significantly different in neonatal cells, which express much lower densities of IgD on their membrane (6, 8). Therefore, similar posttranscriptional regulation may also play a role in determining the final amount of mRNA available for translation. Furthermore, the detection of a relatively high $C\delta$ transcriptional activity in a population of cells that express much higher densities of IgM than adult cells (8) suggests that even in cells expressing only IgM, $C\delta$ is transcribed.

Activation of B lymphocytes by LPS stimulation results in an overall increase in the general transcriptional rate, as shown by the kinetics of label incorporation into nuclear RNA. In addition, the rate of initiation of transcription of the μ - δ complex must also be enhanced since the extent of hybridization to all of the DNA sequences is increased, resulting in a greater percent of initial counts hybridized. This specific increase in transcription, which we have observed only in activated normal B cells and not in transformed IgM-secreting cells, is the

most probable source of the increased steady state abundance of μ m and μ s mRNA found in the cytoplasm after LPS stimulation (13).

A rather surprising finding is that although a large fraction of the transcripts in activated cells appears to be terminated 3' to the $C\mu$ gene, the transcription of the $C\delta$ gene does not decrease to levels below that in nonstimulated cells. Therefore, the dramatic decrease in steady state concentration of δ mRNA after LPS stimulation is not a direct result of changes in transcriptional activity of the δ gene. Again, posttranscriptional events such as differential processing and/or turnover rates must further regulate the expression of the δ mRNA.

Alternative Cleavage Mediates μ s vs. μ m Expression. The developmental switch from the membrane bound to the secreted form of heavy chain mRNA has been shown to result from alternative 3' processing of mRNA precursors (40–42). In the case of μ s, this leads to incorporation of the secreted carboxyl-terminal segment (S in Fig. 3) in place of the membrane exons (M1 and M2). However, it is not clear whether a single precursor can be processed in two ways or if there is some primary difference (e.g., length) between the precursor of membrane and secreted mRNA. Analysis of nascent transcripts cannot distinguish directly between these alternatives since a continuous RNA precursor terminated by polymerase exiting at the end of the transcriptional unit would yield the same hybridization pattern as a transcript that had been endonucleolytically cleaved before polymerase unloading, assuming that the "downstream fragment" so generated was not degraded. However, we have observed in both resting and actively secreting B cells that termination of μ transcription, as defined by polymerase unloading, must occur 3' to the μ m exons, within the region of DNA defined by probe No. 7. The level of hybridization to this probe is always lower than that of the μ probe (No. 6) but higher than the IVS probe, which is further downstream (No. 8). Furthermore, run-on analysis of nuclear RNA from hybridoma cells secreting IgM but not synthesizing any detectable membrane IgM also displays significant hybridization to probe No. 7 (D. Yuan, unpublished observations). This strongly suggests that polymerase does not disengage the $C\mu$ template, regardless of the activation state of the cell, until it has progressed several kilobases beyond both μ s and μ m exons. Therefore, differential expression of secreted and membrane μ chains is most probably determined by differential RNA cleavage. Consistent with this interpretation, Kemp et al. (43) have detected a small polyadenylated fragment that, by hybridization criteria, contains μ m but not μ s exonic sequences. A common μ s- μ m precursor mechanism could accommodate such a downstream fragment in a μ s-producing cell, perhaps as an endonucleolytic and/or splicing by-product. The alternative model, polymerase unloading 5' to the μ m exons, could not.

The relative role of cleavage vs. termination in selection between δ m and δ s 3' termini cannot be addressed by the data presented here since no secreted-form δ message is detectable in normal cells (44, 13). However, in a plasmacytoma (TEPC-1033) that has deleted the $C\mu$ gene and expresses large quantities of δ s mRNA and protein (44), we have observed essentially molar hybridization to the downstream δ m sequences (D. Yuan, unpublished observations), consistent only with the cleavage alternative.

Detailed studies in prokaryotic systems have shown that termination of tran-

scription is accompanied by a pause in chain elongation (45). This pause often occurs during synthesis of a region in the RNA that can form a hairpin structure. A large, 160 bp stem-180 bp loop inverted repeat ($\rightarrow\leftarrow$ in Fig. 3) occurs in the μ - δ intervening sequence, within the region (probe 7) just preceding polymerase unloading (37). A potential role for this inverted repeat in structure-induced pausing or in dissociation of the primary polymerase-DNA-RNA complex is further supported by recent studies (46) in sea urchin histone genes in which an RNA stem-loop structure near the 3' terminus is absolutely essential for polymerase III termination. In this regard, we have not defined the termination site for the δ transcript due to the lack of probes extending further downstream. It would be of great interest to determine if a similar stem-loop structure to that present in the region covered by probe No. 7 may correlate with the termination of the δ transcript.

Developmental Regulation of μ - δ Transcriptional Termination. The 26 kbp μ - δ transcriptional unit is extremely complex. For example, it contains 16 AATAAA hexanucleotides (generally referred to as poly(A) addition sites), 6 of which are normally used (dots in Fig. 3) to define the endpoints of two μ and four δ mRNAs (26, 42). Since it is not clear what structural features of the precursor RNA determine 3'-end selection (e.g., polyadenylation and/or cleavage), we have denoted functional AATAAA sequences that might be part of a complex recognition element "end sites". A simple way to regulate this 3'-end selection is by developmental alteration in the levels (or activities) of a putative endonuclease ("endase") that cleaves the precursor RNA just 3' to end sites (47). When endase is high (e.g., in plasma cells), cleavage occurs predominantly at the first end site (μ_s). Under limiting endase concentrations (resting cells), downstream end sites are cleaved at a rate proportional to their distance from the promoter ($\mu_m > \delta_m$). Choices of RNA splicing, in the case of μ , would then follow automatically from choices of end sites.

Our data generally support the major tenet of this model with regard to μ expression: that cleavage of the growing transcript at a particular end site (μ_s or μ_m) principally determines what μ mRNA is produced. The model would also predict our finding of significant δ transcription in neonatal B cells (downstream end-site usage) even though these cells express lower amounts of IgD. But perhaps of greater consequence than these cleavage decisions is the observation that a given percentage of polymerases disengages the template upstream from C δ . The frequency of this termination event must also be developmentally regulated since, in actively secreting cells, in which transcriptional initiation is markedly increased, the δ hybridization remains at the resting cell baseline, consistent with an increased exiting of upstream polymerases. Therefore, in addition to endase regulation, other trans-acting factors must be acting in activated B cells to facilitate both enhanced initiation and termination.

Multiple Regulatory Levels of μ - δ Expression. Gene expression, defined ultimately in terms of the amounts and rates of production of various proteins, can be controlled at several levels. We have documented in this paper, as well as in previous communications (10, 13) (see Table I), a series of steps at which the synthesis of IgM and IgD in normal cells can be regulated.

In resting B cells, the ratio of newly synthesized cytoplasmic mRNA for μ and

δ chains (as determined by [^3H]uridine pulse labeling [Table I]) reflects the relative nuclear transcription of the two genes. Therefore, the simplest hypothesis would be that all of the μm mRNA is derived from transcripts that have been terminated 5' to the δ gene whereas the long μ - δ transcripts are spliced preferentially to yield δm mRNA, discarding the μ portion in the process. In the cytoplasm, functional full-length δm mRNA must have a shorter half-life than μm mRNA since the steady state concentration of the latter is 10-fold higher. Although the rate of polypeptide chain synthesis mirrors the mRNA concentration, the final expression of IgM and IgD is a function of further posttranslational regulatory events. Relative to δ , these include a decreased stability of both μm precursor chains in the cytoplasm and IgM monomers on the cell surface. The increase in IgM production after B cell activation is clearly a result of increased transcriptional initiation through the $\text{C}\mu$ gene complex, accompanied by increased transcriptional termination of most of the polymerases before they can continue into the $\text{C}\delta$ gene. However, a further regulatory signal must be activated in the stimulated cells so that the primary μ transcript is cleaved at the appropriate end site for the generation of large amounts of μs mRNA. The putative endase (47) may be operative at this level. Furthermore, in LPS-stimulated cells, the basal level of $\text{C}\delta$ transcription is maintained. Since relatively low amounts of newly synthesized δm mRNA can be detected in the cytoplasm, it is possible that posttranscriptional events, also mediated by the endase, further regulate the formation of mature δ mRNA.

Finally, our studies of neonatal B lymphocytes are still incomplete. Although the relative transcription of the μ and δ genes is similar to that of adult cells, until we have examined the fate of newly synthesized RNA we cannot predict the regulatory events that result ultimately in a lower expression of IgD than IgM on the cell surface.

Summary

The heavy chain genes for IgM ($\text{C}\mu$) and IgD ($\text{C}\delta$) are expressed differentially during B cell maturation and activation. We have determined the role that transcription plays in the regulation of these changes by using the method of *in vitro* nascent RNA chain elongation. In neonatal cells that express much lower densities of IgD than IgM on their surface, transcription of $\text{C}\delta$ is observed at half the level of $\text{C}\mu$. This 3:1 transcriptional ratio of μ to δ is preserved in mature resting cells, which express higher densities of IgD on the surface than IgM. When activated by the mitogen, lipopolysaccharide (LPS), transcription of $\text{C}\mu$ is preferentially enhanced. However, $\text{C}\delta$ transcription is not shut off even though the expression of IgD in the stimulated cells is greatly decreased. In all three differentiative stages, polymerase unloading occurs in the vicinity of a large inverted repeat sequence, 5' to $\text{C}\delta$ and 3' to the μ membrane exons. This suggests that the developmental selection of secreted vs. membrane-bound carboxyl-terminal exons is controlled by RNA cleavage. The data presented here, together with our previous analysis of mRNA and protein synthesis, show that the differential expression of IgM and IgD in normal B lymphocytes is regulated at the transcriptional, translational, and posttranslational levels.

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Note added in proof: Mather et al. (*Cell*, 36:329, 1984) have recently found similar μ/δ transcriptional levels using tumors representing various stages of B cell differentiation.

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