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Immunology

Coexpression of μ and γ1 heavy chains can occur by a discontinuous transcription mechanism from the same unrearranged chromosome

(immunoglobulin gene regulation/class switching/RNA processing)

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ABSTRACT **We previously documented that a single BCL_1 leukemia cell can produce μ and γ1 immunoglobulin heavy chains with identical variable segments in an allelically excluded fashion without heavy chain constant region gene rearrangement. To understand the mechanism of dual μ/γ1 synthesis in BCL_1 subclones, we have analyzed mature and pre-RNA at the nascent and steady-state levels. We find μ and γ1 sequences linked in pre-RNA. However, the primary μ and γ1 transcription units are about the same length ($\approx$15 kilobases). Initiation of γ1 pre-RNA occurs upstream of Cγ1 at sites identical to those seen in lipopolysaccharide/interleukin-4-induced normal B cells. We propose that dual μ/γ1 RNA synthesis occurs by a discontinuous transcription mechanism involving either trans-splicing or ligation of μ pre-RNA initiated 5′ of the variable–diversity–joining region to γ1 pre-RNA initiated 5′ of Cγ1.**

Class switching generally occurs by deletion of intervening heavy chain constant region (C_H) DNA by recombination of 5′-residing switch sequences (for reviews, see refs. 1 and 2). In addition to the well-established dual expression of μ and δ constant regions (Cμ and Cδ) (for review, see ref. 3), cosynthesis of μ and downstream classes can occur without apparent switch-mediated recombination in normal (4–6) and transformed (7–10) B cells. A hypothesis first put forth by Rabbitts (11) and later refined by Yaoita *et al.* (4) called for a "long transcript" initiating at the variable–diversity–joining region (VDJ) and extending unabated through the C_H locus (or at least 3′ to the C_H gene to be chosen). Alternative splicing of this theoretical precursor would allow expression of the same VDJ with multiple heavy chains without genotypic commitment (as with DNA deletion). This idea was popular among proponents of multiple-isotype-expressing memory cells and suggested the possibility of reversibility of class upon secondary response and ultimate terminal differentiation. Along these lines, Shimizu and Honjo (2) proposed that the long transcript is an intermediate to switch deletion. The downstream C_H chosen for splicing is targeted as the C_H to be deleted in the subsequent terminal step. This has led to the use of the terms "transitional" or "precommitted" to describe double (or multiple)-isotype-producing B cells. Although the long-transcript model has been invoked subsequently (4–6), there is no direct experimental evidence. In fact, the concept is at odds with the favored model of C_H deletion targeted through enhanced accessibility induced by germ-line transcription (12, 13). Acceptance of double-isotype production models has been further slowed by experimental inconsistencies involving cytophilic absorption (e.g., ref. 4 vs. ref. 14) and cell sorting (ref. 5 vs. ref. 6).

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BCL_1B_1 is an *in vitro*-adapted (15) line of a spontaneously arising murine B-cell leukemia (16). Its surface phenotype (Ia^+,IgM^+), genotypic normal ploidy, and lymphokine inducibility have provided a model for a mature B cell (for review, see ref. 17). Approximately 1 in 800 BCL_1 cells spontaneously undergoes a switch from IgM^+ to IgM^+IgG1^+ in culture (7). This laboratory has proved conclusively (7) that both μ and γ1 chains in the double-isotype-producing subclones have the same idiotype and use the identical VDJ and thus has eliminated (8) the possibility of switch recombination. A difference in the two chromosomes 12 allowed the substantiation of allelic exclusion (8). Here we show that pre-RNA containing linked μ and γ1 sequences accumulates in the nucleus. Instead of a long pre-RNA of some 150 kilobase pairs (kbp), the primary transcription unit length for γ1 is very similar ($\approx$15 kbp) to that of μ and is initiated upstream of Cγ1. Therefore, synthesis of μ and γ1 in these double-isotype-producing cells must occur by a discontinuous transcription mechanism.

MATERIALS AND METHODS

Total RNA Preparation and Northern Blot Analysis. Total RNA was extracted by the guanidine thiocyanate/CsCl spin technique (18), fractionated by formaldehyde/agarose gel electrophoresis, transferred to GeneScreen (NEN), and hybridized to gel-purified ^{32}P-labeled DNA fragments as described (19).

Nuclear RNA Preparation and Sandwich Hybridization. BCL_1 cells grown in suspension were rinsed with phosphate-buffered saline and homogenized in 10 mM NaCl/3 mM $MgCl_2$/10 mM Tris, pH 8.3 supplemented with 0.5% Nonidet P-40. Nuclei were pelleted and purified, and nuclear RNA was isolated by the method of Penman *et al.* (20). After ethanol precipitation, RNA was purified by proteinase K treatment, extracted with phenol/chloroform/isoamyl alcohol, and again ethanol-precipitated. Because of the probable DNA contamination, nuclear RNA concentration was determined by orcinol reaction. Pre-RNA integrity was confirmed by gel electrophoresis and hybridization to a U1 RNA probe (21) (data not shown). Double-stranded DNA (10 μg) from plasmids shown to be specific for Cμ and the Cγ subclasses (22) and for Cδ (23) was denatured and dot-blotted to GeneScreen as described (19). Sandwich hybridization (24) was carried out essentially as modified by Perlmutter and Gilbert (5). Baked prehybridized membranes were each hybridized to $\approx$10 μg of pre-RNA for 72 hr. Heteroduplexes were washed and, without denaturation, were hybridized to single-

Abbreviations: C_H, Cμ, Cδ, etc., heavy chain, μ chain, δ chain, etc., constant region; VDJ, variable–diversity–joining region; nt, nucleotide(s).

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stranded isotype-specific (22, 25) ^{32}P-labeled (26) inserts for 48 hr. Hybrids were washed at high stringency (0.1× standard saline citrate, 65°C) and, when indicated, treated with RNase A (40 μg/ml) and RNase T1 (2 μg/ml) at 37°C for 1 hr.

Nuclear Run-On Experiments and Probes. Nuclear run-on experiments were performed as described (23), labeling 5 × 10^7 nuclei for 12–15 min with [α-^{32}P]UTP (specific activity, 3000 Ci/mmol; 1 Ci = 37 GBq; ICN). Plasmids shown (8) to be isotype-specific and free of repetitive DNA are as follows: A, 1.1-kbp *Bam*HI fragment containing BCL_1 VDJ_H2 from genomic phage CH-28.289.1 (27); B, 0.8-kbp *Pvu* II–*Pst* I fragment containing Cμ2–Cμ4 from pμ3741 cDNA (28); C, 0.9-kbp *Kpn* I fragment containing μs–μm region from genomic phage CH-28.257.3 (29); D, 1.0-kbp *Hin*dIII–*Eco*RI fragment containing the Cμ–Cδ intervening sequence from CH-28.257.3 (29); E, 1.1-kbp *Pst* I fragment containing complete Cδ from cDNA pAG485 (30); F, 1.2-kbp *Eco*RI fragment containing the Cδ–Cγ3 intervening sequence from genomic phage γ3-13 (31); G, 2.0-kbp *Hin*dIII fragment containing the Cδ–Cγ3 intervening sequence from genomic phage γ3-25 (31); H, 0.5-kbp *Bst*EII–*Sph* I fragment containing C_H3 and the 3′ untranslated region of Cγ3 from genomic phage DRI (32); I, 1.0-kbp *Sac* I–*Hin*dIII fragment containing the Cγ3–Cγ1 intervening sequence from genomic phage γ1-6 (31); J, 1.7-kbp *Xba* I–*Eco*RI fragment containing Sγ1 from γ1-6 (31); K, 2.1-kbp *Sac* I–*Eco*RI fragment containing C_H3, 3′ untranslated region, and γ1s–γ1m region of Cγ1 from genomic phage γ1-3 (31); L, 2.0-kbp *Eco*RI–*Sac* I fragment containing C_H2 and C_H3 of Cγ2b from pγ2b(11)(7) cDNA (33); N, 4-kbp *Bam*HI fragment containing complete Cε gene (34); O, 0.9-kbp *Bst*EII fragment containing most of Cα from pα(J558) cDNA (35); P, pBR322 alone. Control experiments (results not shown) ensured that all plasmid loads (5–10 μg) were in DNA excess for the 48-hr hybridizations. Intensities of resulting autoradiograms (at linear exposures of 30% of that shown) were quantified by densitometry, pBR322 backgrounds were subtracted, and data were normalized to the length of hybridizing sequence in each plasmid. The most 5′ signals (VDJ, probe A) for μ and μ/γ1 were arbitrarily set at 5.0 to allow relative loading comparison across the locus.

UV Transcriptional Mapping. Cells cultured at 5 × 10^6 cells per ml in RPMI 1640 medium/10% (vol/vol) fetal calf serum were pelleted, resuspended in 40 ml of serum-free medium, then distributed into 10-cm dishes, and kept dark from this point until the RNA preparation. A General Electric germicidal UV lamp was mounted ≈120 cm above the cells, and cells were irradiated for various lengths of time. Irradiated and control cells were resuspended in culture medium and incubated at 37°C for 40 min to allow elongating polymerases to terminate. Nuclei were isolated and RNA was labeled as described (23) for run-on analysis. Hybridization intensities were quantified by densitometry and normalized to probe size (see above). To allow comparison among filters (time points), small differences (<15%) among normalized values for VDJ (promoter-proximal probe whose intensity is essentially UV-dose-independent) were eliminated, and the resulting correction factors were applied to the remaining promoter-distal values. Residual hybridization is computed as $R(d)/R(0)$, where $R(d)$ is normalized hybridization at dosage d_1 and $R(0)$ is normalized hybridization with no irradiation (36). As predicted by the relationship $R(d)/R(0) = (1 - e^d)/d$, pseudo-first-order kinetics are observed.

RNase Protection. The RNase protection protocol (and its formal controls) for VDJ-initiated BCL_1 transcripts (37) and for germ-line γ1 transcripts (38, 39) is detailed elsewhere. Protected products of Sp6-generated [^{32}P]UTP-labeled probes were fractionated on 15% or 6% polyacrylamide/8 M urea sequencing gels.

RESULTS

Steady-State Levels of Mature C_H mRNA in Single- and Double-Isotype-Producing BCL_1 Subclones. Northern blot analysis of representative subclones is shown in Fig. 1. No mature γ1 message is detectable in most BCL_1 subclones (such as 2.62), but rare μ/γ1 double-isotype producers have a μ/γ1 steady-state ratio of 5:1 to 2:1. As anticipated from their low-secreting phenotype (ref. 7 and data not shown), membrane forms of both isotypes predominate. As noted (13), BCL_1 expresses a 3.3-kilobase (kb) γ3 transcript that is somewhat down-regulated in the double-isotype producers. We know from analysis of cloned cDNAs that these γ3 transcripts are initiated upstream of Cγ3 (data not shown) and probably correspond to Cγ3 germ-line transcripts characterized in spleen (40).

μ and γ1 Sequences Are Linked on the Same pre-RNA. We performed sandwich hybridizations of nuclear RNA to determine whether μ, γ1, or other C_H sequences were linked in pre-RNA (Fig. 2). Double-stranded plasmids containing Cμ, Cδ, Cγ3, Cγ1, and Cγ2b inserts, previously shown to be isotype specific (22), were denatured and then spotted in DNA excess (data not shown). Nuclear RNAs from μ-only or μ/γ1 subclones were annealed to the filters, and the filter-immobilized duplexes were hybridized to ^{32}P-labeled sense (+) and antisense (−) probes (Fig. 2 *A–D*) or denatured double-stranded probes (Fig. 2*E*). As anticipated, μ and γ1 probes were specific (Fig. 2*A*), and immobilized μ sequences selected only μ-containing nuclear precursors from the μ-only producer (Fig. 2*B*). However, μ/γ1 double-isotype-producer pre-RNA selected by μ or γ1 hybridized specifically to antisense μ (Fig. 2*C*) or reciprocally to antisense γ1 (Fig. 2*D*). Treatment with a single-strand-specific RNase mixture

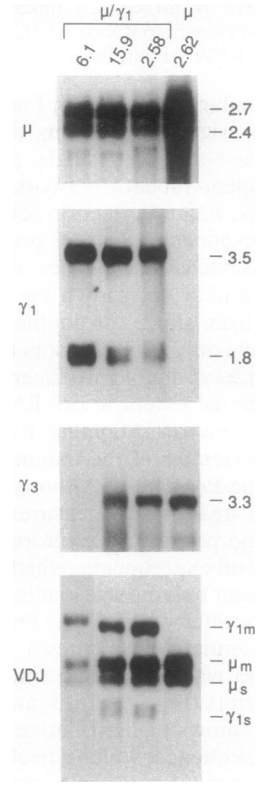

FIG. 1. Steady-state mRNA levels in BCL_1 μ and μ/γ1 producers. Isotype-specific probes (described in ref. 19 and Fig. 3) are indicated on the left, RNA cell sources are above, and RNA sizes (in kilobases) and species (m, membrane form; s, secreted form) are on the right. Phenotype and genotype of the BCL_1 clones used have been described (7, 8). Each panel is a separate blot.

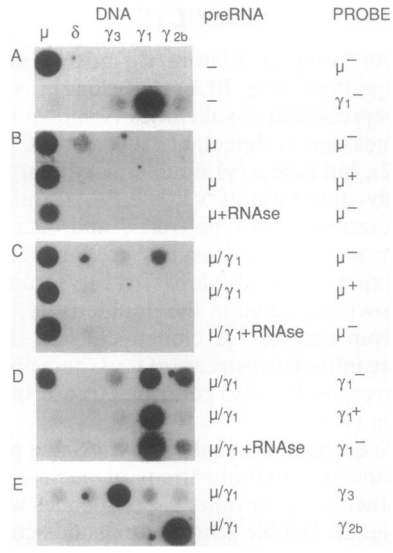

FIG. 2. Detection of linked Cμ and Cγ1 sequences in nuclear RNA from a μ/γ1 double-isotype-producing BCL_1 subclone by sandwich hybridization. (*A*) No-RNA control showing specificity of antisense Cμ and Cγ1 probes (μ^- and $\gamma1^-$). (*B*) Cμ-hybridizing sequences contained in nuclear RNA (pre-RNA) from μ-producing BCL_1 subclone 2.62(8) are not linked to Cγ sequences. (*C*) Cμ-hybridizing sequences contained in pre-RNA from μ/γ1-producing BCL_1 subclone 2.58(8) are linked to Cγ1 sequences. Linkage is on the sense strand since the sense-strand probe (μ^+) does not hybridize. Loss of the Cγ1 signal by treatment of the heteroduplexes with single-strand-specific nucleases demonstrates that the linkage is RNA and single-stranded. (*D*) Cγ1-hybridizing sequences contained in 2.58 pre-RNA are linked to sense-strand Cμ and the linkage is RNase-sensitive. The $\gamma1^-$ probe shows low cross-hybridization to Cγ3 and Cγ2b. There is no Cγ–Cγ linkage since the cross-hybridization signal is resistant to RNase. (*E*) Cγ3 or Cγ2b sequences contained in 2.58 pre-RNA are not linked to other C_H sequences.

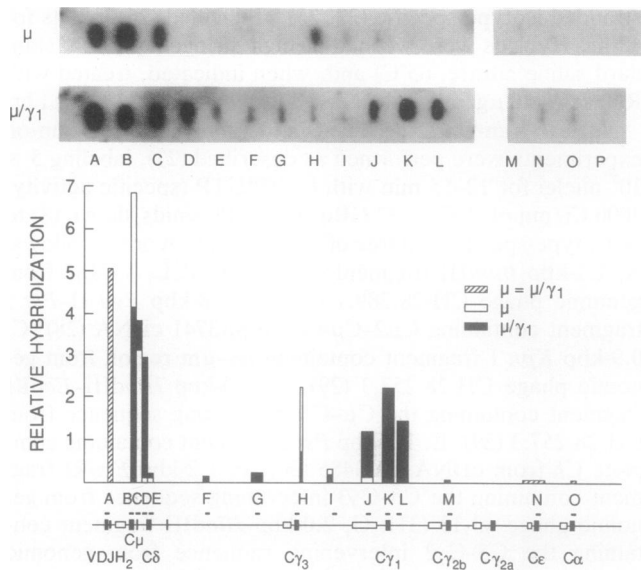

FIG. 3. Polymerases are loaded unevenly across the C_H locus of BCL_1 μ/γ1-double-isotype producers. (*Upper*) Nascent transcripts labeled in μ-only (clone 2.62) or μ/γ1-double-isotype-producing (clone 2.58) BCL_1 nuclei were hybridized to a series of plasmids, plasmids A–P, slot-blotted on nitrocellulose. (*Lower*) Normalized slot-blot intensities vs. the probe positions are plotted.

eliminated the secondary signal (γ1 in Fig. 2*C*; μ in Fig. 2*D*) but not the primary signal, confirming that μ and γ1 were linked on a nuclease-sensitive single pre-RNA and that artifacts such as repeat–repeat "networking" could not account for the signals. Faint δ selection achieved with μ (Fig. 2*C*) probably corresponds to Cμ–Cδ precursors, since the signal was nuclease-sensitive. Genes downstream of γ1 (Cγ2b in Fig. 2 and data not shown for Cε and Cα) never selected pre-RNA from single- or double-isotype producers (Fig. 2*E*). Cγ3 selects only γ3 precursors (Fig. 2*E*). Thus the data indicate that in μ/γ1 double producers only μ and γ1 are linked to any significant extent in pre-RNA.

Nuclear Run-On and UV Mapping Experiments Suggest Discontinuous Transcription of the Immunoglobulin Locus in BCL_1 Double-Isotype-Producers. Although demonstrating a μ/γ1 precursor, the steady-state measurements only identify stable pre-RNAs and provide little information as to mechanism. Nuclear run-on experiments, which measure to a first approximation nascent polymerase loading, provided further evidence for discontinuity (Fig. 3). Transcription in the μ-only producer essentially terminates 5′ to Cδ. The peak of hybridization at Cγ3 was anticipated since BCL_1 generates germ-line γ3 transcripts (Fig. 1, ref. 13, and data not shown). The μ/γ1 producer shows higher relative loading across the Cμ–Cδ intervening sequence, which correlates with its higher μm/μs steady-state ratio. Sparse-to-background polymerase loading is observed across probes 5′ (with the exception of Cγ3) of Cγ1. The long-transcript model would predict, for μ/γ1 clone 2.58 (where the steady-state ratios of μ/γ1 are ≈2:1), a 50% polymerase density across all Cμ–Cγ1 intervening probes. Clearly this is not the case. However, the loading calculation assumes no effect of RNA splicing, which conceivably could occur during the 12-min pulse and reduce apparent molarity by destabilization of spliced intermediates. Significant transcription across Sγ1 and Cγ1 terminates after γ1m. Thus with the data of Fig. 2, it is probable that the C_H gene targeted for subsequent productive splicing to VDJ in these cells is the 3′ most of the transcription unit.

To formally distinguish between a long transcript of at least 150 kb and alternative discontinuous mechanisms suggested by the above experiments, we sized the γ1 transcription unit by a modification of the classical UV mapping technique (for review, see ref. 36). UV-irradiated DNA templates are correctly bound and initiated by RNA polymerase. During chain elongation, the transcribing polymerase is likely to encounter a UV-damaged site. The enzyme terminates without resuming transcription beyond the UV damage. As a consequence, UV photoproducts exert a strong polar effect; i.e., promoter-distal portions of a monocistronic transcription unit undergo more rapid inactivation than promoter-proximal regions. Thus according to the long-transcript model, elongation through Cγ1, putatively 150 kb from the promoter, should be UV-inactivated at a considerably faster rate than elongation through Cμ. The data of Fig. 4 contradict this hypothesis. BCL_1 double-isotype-producing cells were irradiated for various times, reincubated at 37°C for 40 min to allow elongating polymerases to terminate, and then analyzed by standard nuclear run-on experiments (Fig. 4*A*). Fig. 4*B* shows the normalized hybridization to μ and γ1 probes after various doses of UV as a fraction of hybridizing RNA in unirradiated cells. As the length of UV exposure increases, the amount of residual hybridization decreases exponentially (36). Hybridization of μ and γ1 nascent RNAs is inactivated with essentially identical first-order kinetics. The transcription initiation sites 5′ to VDJ are indistinguishable in single- and double-isotype-producing BCL_1 cells (Fig. 4*C*). Therefore, we conclude that μ and γ1 derive from primary transcripts of similar length. The length of VDJ–Cμ membrane-form pre-RNA has been estimated to be ≈15 kb (23). By using a clone containing the mouse 28S rRNA gene, we observed very similar inactivation curves (data not shown). The 45S rRNA precursor is 14.5 kb (41), consistent with the calibration of its UV-inactivation with μ and γ1 in BCL_1.

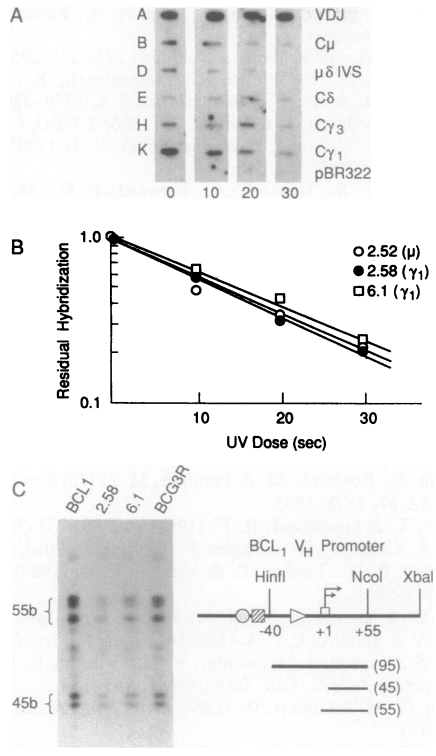

FIG. 4. Transcription units μ and γ1 in μ/γ1 double-isotype producers are of equivalent lengths. (*A*) Slot blots of ^{32}P-labeled nascent transcripts from nuclei of μ/γ1-double-isotype producer 2.58 isolated from cells irradiated at the indicated times with UV. Probes indicated on the right correspond in letters (on the left) to those in Fig. 3. (*B*) UV-inactivation kinetics of μ and γ1 transcription. The 2.58 hybridization data shown in *A* for μ (○) and γ1 (●) and the γ1 hybridization data (not shown) for a second μ/γ1 producer, 6.1 (□), were converted to residual hybridization and plotted as a function of UV dose. Equivalent slopes for μ and γ1 indicate near equivalent lengths of their transcription units. (*C*) Transcription start sites for VDJ-initiated mature μ and γ1 mRNAs are indistinguishable. Total RNA (1–5 μg) from the parental BCL_1 line, two double-isotype-producing clones (2.58 and 6.1), and a μ-only clone (BCG3R) protect two major bands of 55 and 45 bases (55b and 45b, respectively) of a 95-base uniformly labeled RNA probe. The diagram to the right illustrates template, probe, and products. Arrows, start sites; triangle, TATA box; square and circle, conserved upstream elements.

μ/γ1-Double-Isotype-Producing BCL_1 Clone (2.58) Constitutively Expresses Germ-Line γ1 Transcripts. One explanation for the peak of γ1 hybridization in the nuclear run-on experiments and for the similarity in the sizes of the μ and γ1 transcription units might be that γ1 transcription is initiated upstream of the unrearranged Cγ1 gene to produce germ-line γ1 RNAs similar to those induced by interleukin-4 in normal B cells (38, 42–44). To test this possibility, an RNase protection assay was performed using as a probe a labeled antisense RNA prepared *in vitro* from a germ-line γ1 cDNA clone. This probe contains 285 nucleotides (nt) of a 5′ Iγ1 exon spliced to the C_H1 exon of Cγ1 (39). Mature germ-line γ1 transcripts protect a 419-nt fragment of this probe whereas mature functional VDJ-containing γ1 mRNAs protect only 134 nt of the probe (Fig. 5*A*). As can be seen in Fig. 5*B*, RNA from the μ/γ1 double-isotype-producing line protected a 419-nt fragment of the probe, indicating the presence of germ-line γ1 transcripts. As expected, a protected fragment of 134 nt was also observed due to the presence of functional γ1 mRNA and perhaps also to the presence of some unprocessed germ-line γ1 pre-RNA. A prominent protected fragment of 285 nt further indicated the presence of unprocessed germ-line γ1 pre-RNA. Neither germ-line γ1 transcripts nor

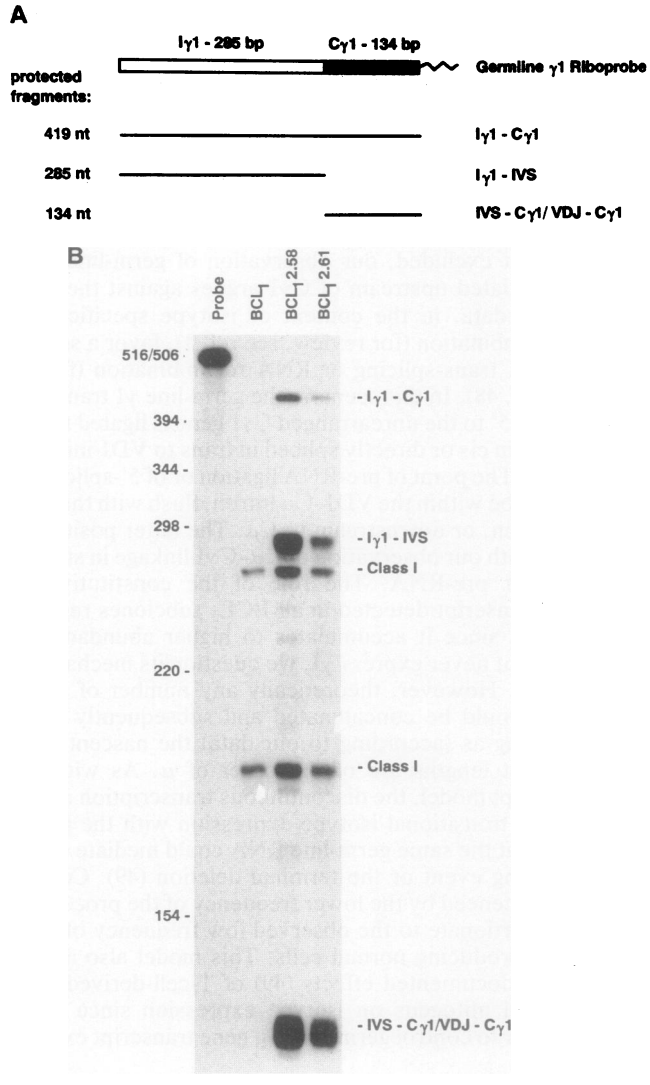

FIG. 5. Detection of germ-line γ1 RNAs in μ/γ1-producing BCL_1 subclones by RNase protection. (*A*) Schematic diagram showing the germ-line γ1 single-stranded RNA probe and the size of the expected fragments of the probe protected by the various Iγ1- and Cγ1-containing transcripts. (*B*) RNase protection analysis of 5 μg of total cellular RNA from the indicated single- and double-isotype-producing BCL_1 clones. Inclusion of a class I (H-2D^d specific) RNA probe (pG3DB/P) (44) and the protection of 270-nt and 190-nt fragments of this probe by class I mRNA served as an RNA-loading control. *Hin*fI-digested pBR322 size markers, undigested germ-line γ1 probe, and protected fragments are indicated.

functional γ1 mRNAs were detectable in the μ-only BCL_1 line. We confirmed that these germ-line γ1 transcripts were initiated upstream of the Iγ1 exon by RNase protection of an antisense probe corresponding to the *Bgl* II–*Nco* I fragment upstream of Sγ1 (data not shown). RNA from the double-isotype-producing BCL_1 line protected numerous fragments of the probe, suggesting the presence of multiple initiation sites clustered 100- to 200-bp upstream of Sγ1. These transcription initiation sites are similar to those identified for germ-line γ1 transcripts induced in normal B cells by interleukin-4 (44). Initiation at these sites generates an Iγ1–Cγ1m transcription unit of ≈15 kb, consistent with the UV mapping measurements of Fig. 4.

DISCUSSION

This laboratory has shown (7, 8) that μ/γ1 double-isotype-producing clones of BCL_1 expressed functional Cμ and Cγ1 from the same chromosome by a mechanism that does not

involve switch recombination (7, 8). In this report, we have provided evidence that this coexpression occurs by discontinuous transcription and suggests a model for multiple-isotype expression observed in normal B cells. Two mechanisms, each with precedents in other systems, are plausible. First, a given polymerase II may initiate transcription upstream of VDJ but skip over Cμ–Cγ1 intervening sequences before reinitiating at a position 5′ to Cγ1. Polymerase hopping has been documented (46, 47) for two viral genes. Although not excluded, our observation of germ-line transcription initiated upstream of Cγ1 argues against the idea. Instead our data, in the context of isotype specificity of switch recombination (for review, see ref. 1), favor a second model: RNA trans-splicing or RNA recombination (for review, see ref. 48). In this scenario the germ-line γ1 transcript that initiates 5′ to the unrearranged Cγ1 gene is ligated to and then spliced in cis or directly spliced in trans to VDJ-initiated μ pre-RNA. The point of pre-RNA ligation or of 5′-splice-site attack could be within the VDJ–Cμ intron, flush with the VDJ splice junction, or downstream to Cμ. The latter position is consistent with our observation of Cμ–Cγ1 linkage in steady-state nuclear pre-RNA. The role of the constitutive γ3 germ-line transcript detected in all BCL_1 subclones remains unclear. But since it accumulates to higher abundancy in subclones that never express γ1, we question its mechanistic involvement. However, theoretically any number of germ-line RNAs could be concatenated and subsequently processed, as long as (according to our data) the nascent transcription unit lengths are of the order of μ. As with the long-transcript model, the discontinuous transcription model provides for transitional isotype expression with the added attraction that the same germ-line RNA could mediate either the processing event or the terminal deletion (49). Control might be influenced by the lower frequency of the processing event, proportionate to the observed low frequency of double-isotype-producing normal cells. This model also allows for the well-documented effects (49) of T-cell-derived lymphokines and mitogens on isotype expression since these agents appear to control germ-line C_H gene transcript expression.

Note. During revision, Shimizu *et al.* (51) have provided evidence for discontinuous transcription in double-isotype-producing transgenic B cells. Although the mechanism remains unclear, mature mRNAs in which μ transgene–VDJ associated with endogenous Cγ were found in the absence of switch recombination. This adds further significance to our results in BCL_1 and strongly implies that normal B cells have at least two routes to achieve a class switch.

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