FEEDBACK INHIBITION OF IMMUNOGLOBULIN GENE REARRANGEMENT BY MEMBRANE μ, BUT NOT BY SECRETED μ HEAVY CHAINS

By J. MANZ,*‡ K. DENIS,§ O. WITTE,§ R. BRINSTER,‖ and U. STORB*

*From the *Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, Illnois 60637; the ‡Department of Microbiology, University of Washington, Seattle, Washington 98195; the §Howard Hughes Institute, University of California, Los Angeles, California 90024; and the ‖School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104*

During the development of B lymphocytes, genes rearrange in sequential order, first heavy (H), then light (L) chain genes (1). Since the recombinase for all types of Ig gene rearrangements appears to be the same (1, 2), the locus specificity of rearrangement must be a property of the particular targets. It has been postulated that accessibility of H genes to the recombinase is associated with transcription of germline V_H genes (1). So far, no experimental evidence for a similar mechanism in L chain genes has been obtained. However, a regulating role for turning on κ gene rearrangement has been found in the expression of μ genes; when functional μ genes were transfected into cells of a pre-B cell line, rearrangement of κ genes was observed (3).

While the control of turning on Ig gene rearrangement is important for the tissue and developmental specificity of Ig gene activation, the controlled turning off of rearrangement is essential for allelic exclusion (4, 5) of Ig gene expression. That is, any one B cell will produce an Ig molecule consisting of H and L chains each produced from only one of two possible alleles. Previous work with transgenic mice has implicated μ, δ, and κ proteins in the feedback inhibition of H and κ gene expression (6–10). Endogenous κ gene arrangements were inhibited by intact Ig molecules consisting of a transgenic κ chain plus an endogenous H chain (6). H gene rearrangements appear to be influenced by the presence of μ or δ transgenes (7–10).

In this report we have analyzed the abilities of membrane bound μ (μm)[1] compared with secreted μ (μs) to promote feedback inhibition of H and κ gene rearrangement. The experiments were carried out with transgenic mice that harbor a κ transgene and either a μ transgene (μ × κ) or a μ transgene whose transmembrane and intracytoplasmic portion had been deleted (μΔm × κ) (11). As a control, mice with the κ transgene only were used. Hybridomas were produced from the spleen and the status of their endogenous Ig genes was determined. It was found

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[1] *Abbreviations used in this paper:* AMuLV, Abelson murine leukemia virus; μm, membrane bound μ; μs, secreted μ; μΔm, μ transgene whose transmembrane and intracytoplasmic portion has been deleted.

that μm, but not μs was capable of inhibiting rearrangement of endogenous Ig genes in the transgenic B cells. The inability of μs to cause feedback inhibition was confirmed in Abelson murine leukemia virus (AMuLV)-transformed bone marrow cells.

Materials and Methods

Transgenes. The transgenes were constructed as described (11). Briefly, three constructs were made (see Fig. 1); a rearranged κ gene, μ gene, or μΔm gene with the variable regions from the productively rearranged κ or heavy (H) chain genes of the myeloma MOPC 167. The μΔm construct lacks the μ membrane exons.

Transgenic Mice. Transgenic mice were produced as described (12, 13) from (C57BL/6 × SJL) F_2 zygotes. The specific lines used in this study have been characterized (11). The mice used in this analysis were produced by mating transgenic κ mice from the 234-4 line to either μ mice from the 243-4 line or to μΔm mice from the 254-3 line. The specific mice used in this study were 2-5 (μ × κ) and 12-9 and 13-3 (μΔm × κ). The κ line 234-4 and two other κ lines (233-4 and 233-8) were also used without the μ transgene. Pre-B cell lines were made from mice of a μΔm × κ strain (217-4). The transgenic mice had been kept on a (SJL × C57BL/6) background by mating the founder lines with F_1 mice. In this way, in several of the mice used for this study the endogenous, unrearranged κ genes could be distinguished from one another by a Bam HI polymorphism. Thus the following κ alleles were seen in the hybridomas (letters refer to hybridoma designations in Figs. 2-4 and Table I): A(κ 233-8), B (κ 233-4), D (κ 234-4), and F (κ × μΔm [234-4 × 254-3]) had C57 and SJL κ alleles; C (κ × μ [234-4 × 243-4]) and E (κ × μΔm [234-4 × 254-3]) had only SJL κ alleles.

Hybridomas. Mice were injected intraperitoneally with 20 μg of LPS (Gibco Laboratories, Grand Island, NY) 3-4 d before spleen removal and subsequent fusion (14) with X63-Ag8.653 (15). Cloning was done either by limiting dilution and by micromanipulation of a single cell into a 96-well dish or by micromanipulation alone. Hybridomas that had retained expression of both μ and κ transgenes were selected on the basis of secretion of PC binding antibody in a PC binding ELISA (16). Hybridomas from κ only transgenic mice were screened for the presence of κ MOPC-167 mRNA and/or the transgene.

Antisera and Monoclonal Antibodies. Rabbit antisera specific for mouse IgA, IgG, or IgM were purchased from ICN Biomedicals (Naperville, IL). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit Ig antiserum was purchased from Bio-Rad Laboratories (Richmond, CA). HRP-conjugated anti-mouse Ig antiserum was purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD).

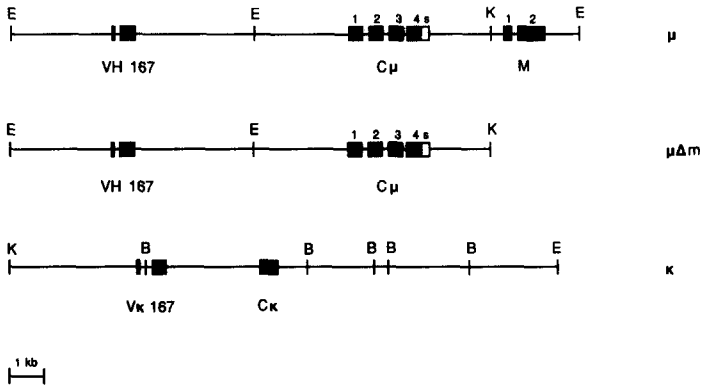

FIGURE 1. Restriction maps of the MOPC 167 μ, μΔm, and κ transgenes (see Materials and Methods). B, Bam HI; E, Eco RI; K, Kpn I.

The rat mAb 331.12 recognizes both the allelic Igh6a and Igh6b μ proteins when they assemble with light chain into an IgM molecule (17). AF6-78.25 is a mouse mAb that recognizes the Igh6b μ protein (18). DS-1 is a mouse mAb specific for the Igh6a μ protein (D. Sieckman, Naval Medical Research Institute, Bethesda, MD). Biotinylated or unconjugated 331.12, AF6-78.25, and DS-1 were kindly provided by A. M. Stall and L. A. Herzenberg (Stanford, CA), and D. Sieckman.

Pre-B Cell Lines. Bone marrow from five 4-wk-old transgenic mice (217-4, μΔm [17 copies] + κ [8 copies], reference 11) and from two normal littermates was transformed by AMuLV (19, 20). Production of μ and κ was determined by cytoplasmic staining of fixed cells or two-dimensional gels of cytoplasmic extracts.

Detection of the μ Allotype Produced in Hybridomas. Hybridoma supernatants were tested in an ELISA to determine if either the μ^a protein was produced from the transgene (BALB/c origin) and/or μ^b was produced from the endogenous μ genes (C57BL/6, SJL). Supernatants were placed into microtiter wells coated with the 331.12 antibody and bound IgM was detected with either the biotin-AF6-78.25 or biotin-DC-1 antibody followed by incubation with a streptavidin-biotin-peroxidase complex (SABC kit; Zymed Laboratories, San Francisco, CA).

Nucleic Acid Procedures. DNA from hybridomas and mouse kidney was prepared as described (21). The DNA was digested with either Eco RI or Bam HI restriction enzymes (New England Biolabs, Beverly, MA), run on a 0.8% agarose gel, and blotted to nitrocellulose filters by the method of Southern (22). Filters were then hybridized with either [^{32}P]RNA or DNA probes. Filters that were to be reprobed had bound labeled probe removed by either soaking in a solution of 100 mM NaOH, 100 mM NaCl for 10 min at 20°C (RNA probes) or by soaking in 10 mM Tris-HCl (pH 7.5), 1 mM Na_2EDTA for 5 min at 95°C (DNA probes).

Probes. C_κ sequences were detected using either labeled RNA or DNA from pGEM2C_κ, which contains ~500 bp of exon-specific BALB/c C_κ sequences (11). The plasmid, pX2.1, contains a 2.1-kb Xba I fragment from the region 5′ of J_κ (23) cloned into pBSC, a chloramphenicol-resistant version (Engler, P., and U. Storb, unpublished observations) of pBS (Stratagene, La Jolla, CA). J_H sequences were detected using labeled RNA produced from pGEM1JH34, which contains a 2.2-kb Bam HI-Eco RI insert from the BALB/c J_H 3 and 4 region (24) cloned into PGEM1 (Promega Biotec, Madison, WI). Sequences 5′ of J_H and 3′ of D_H were detected using labeled RNA from p4-11 (a gift of D. Weaver and D. Baltimore, MIT, Cambridge, MA), which contains a 1.85-kb Eco RI-Xba I insert from a region 5′ of the J_H cluster (8) (5′ J_H). Sequences 5′ of the D_H cluster but 3′ of V_H were detected using labeled RNA from pDFL-2.7 (5′ of D_H). pDFL-2.7 was constructed by subcloning the Eco RI-Bgl II fragment from pJ38B9-7.1 (a gift of F. Alt, Columbia University, New York, NY) (25) into pBSC.

Protein Gel Electrophoresis and Western Blotting. Hybridoma supernatants and lysates were examined by immunoblot analysis. 40 μl of supernatants or 40 μl of cell lysate were electrophoresed on a one dimensional gradient (8-15.5%) SDS-polyacrylamide according to the method of Laemmli (26). Proteins were electroblotted to nitrocellulose for 4 h in 25 mM Tris-base, 192 mM glycine, and 20% methanol (27). Blots were blocked with 5% nonfat dry milk solution in PBS (28) for 60 min at 20°C and were sequentially probed with rabbit antisera specific for mouse IgA, IgG, IgM, and HRP-goat anti-rabbit Ig antisera (29). The blots were developed with HRP Color Developing Reagent (Bio-Rad Laboratories) and H_2O_2.

Results

Rearrangement Profile of Endogenous H Chain Genes in Hybridomas. The μ gene encodes two different forms of μ message, either μs or μm (30). B cells contain both forms of μ RNA, while mature plasma cells produce predominately μs (31, 32); the choice between μs and μm appears to be regulated at the cleavage/poly(A) addition step (32, 33). To examine the possible regulatory role of the μs vs. μm or the IgM molecule on both H and L chain gene rearrangements, hybridomas were made from LPS stimulated transgenic B cells. The transgenic mice were the positive offspring

from a κ 167 transgenic mouse bred to either a μ or to a μΔm transgenic mouse. Pure κ mice were used as controls.

As described previously, the 234-4, 233-4, and 233-8 κ lines contain ~18, 41, and 13 copies, the μ and μΔm lines contain ~6 and 4 copies of the transgene, respectively (11). Each line expresses high amounts of the transgenic message in spleen cells, as detected by RNA hybridization analysis (11), and the transgenic μ and κ proteins are present at high levels in serum as detected by ELISA (Pinkert, C., J. Manz, R. Brinster, and U. Storb, manuscript in preparation). The μ line contains a complete, rearranged μ transgene that encodes both the μm and the μs proteins. The μΔm line, however, contains a transgene that is lacking the exons necessary for membrane insertion (Fig. 1). Two combinations of transgenic mice were examined in this analysis; (*a*) μ × κ and (*b*) μΔm × κ. It should be noted that the same κ line (234-4) was used for these crosses and that the transgenes are located on two different chromosomes since the genes were injected separately.

To examine the effect of the transgenes on endogenous heavy chain gene rearrangements, Southern blot analysis was performed on DNA from 34 μ × κ, 23 μΔm × κ, and 29 κ hybridoma clones. Several of the hybridomas had lost the transgenes at the time when subclones were expanded. We presume that they lost the respective chromosme during culture, because evidence of transgene expression had been there early after cell fusion. Because H chain genes rearrange by two deletional events, we were able to examine the extent of H chain gene rearrangement by using probes that hybridize either to regions upstream of J_H or to regions upstream of D_H (see Fig. 2 *A*). The absence of hybridizing bands is indicative of rearrangements. By using the 5′ of J_H probe, we found that hybridomas C6, 14, 23, 25, 27, and 41 (all from the μ × κ mouse 2–5) have retained at least one H chain allele in germline configuration (Fig. 2 *B*, *left panel*; Table I), and evidence presented later suggests that all, except C6, have both alleles completely unrearranged. These six hybridomas represent 18% of the μ × κ hybridomas examined. In contrast, no hybridizing sequences were detected with this probe in the μΔm × κ hybridomas (Fig. 2 *B*, *right panel*), which indicates that minimally there was a D-J rearrangement on both alleles. A second probe, pDFL.7, hybridizes to sequences just 5′ of a family of D_H segments that includes the most 5′ gene in the D_H gene cluster (see Fig. 2 *A*). This probe will hybridize only if no V-DJ rearrangements have occurred. Therefore, it will hybridize to germline alleles and to alleles that have only undergone D-J rearrangements. When the two sets of hybridomas were analyzed using this probe, again a significant difference in the extent of rearrangements was seen (Table I). 65% (22/34) of the μ × κ hybridomas retained at least one allele that had not undergone V-DJ rearrangements (Fig. 2 *C*), while only 17% (4 of 23) of the μΔm × κ hybridomas had this profile (Fig. 2 *D*). These blots were also probed with a J_H probe that detects J_H sequences in both rearranged and germline configurations (data not shown). Results using this probe were consistent with the results obtained with the 5′ of D and 5′ of J probes. Additionally, no further rearrangements could be detected with the J_H probe in hybridomas C6, 14, 23, 25, 27, and 41, which suggests that both endogenous alleles were completely unrearranged in these cells. However, we concluded that C6 has one H allele in germline configuration and the other allele has undergone a productive VDJ rearrangement because despite being unable to detect rearranged sequences in C6, this hybridoma produced transgenic as well as endogenous

μ protein (as discussed below and shown in Table I). The presence of rearranged VDJ sequences was potentially obscured by J_H sequences hybridizing to the transgene as well as the genes of the fusion partner. None of the other five hybridomas with germline H genes had any endogenous protein (Table I). Therefore, it appears that the presence of the transgenic μ protein is able to inhibit DNA rearrangements of the endogenous Ig heavy chain alleles and that the inhibition is mediated solely by the membrane form of the molecule since there appears to be no inhibition by the secreted form.

As controls, hybridomas from κ only mice were also analyzed (Table I). The rearrangement profiles of endogenous H genes in these cells were found to be very similar to the μ × κ hybridomas, i. e., 14% had retained an unrearranged J_H gene, and 48% had retained at least one set of D_H genes not rearranged to V. We presume that all four lines that retain an unrearranged J_H gene and in which we can detect no rearranged H gene (A3, A10, D6, D29) at one time had a functional VDJ rearrangement on the other chromosome and produced μ protein. Normally, in hybridomas from mice without Ig transgenes, no unrearranged J_H genes are found and the proportion of cells that retain 5′ DH sequences is much lower (10). It appears, therefore, that the κ transgene has an effect on endogenous H gene rearrangement. A similar effect had been observed previously with a κ transgene encoding the L chain of the myeloma MOPC 21 (34, 35). 5 of 25 hybridomas in that study retained one unrearranged J_H gene. It can be assumed that in such cells the first H gene rearrangement was productive and that in combination with the transgenic κ protein a complete shutoff of the rearrangement process occurred (see Discussion).

Rearrangemnt Profile of Endogenous L Chain Genes in Hybridomas. As shown previously, endogenous κ genes were prevented from rearranging in the presence of a transgenic κ molecule associated with an endogenous H chain molecule (6). This inhibition was not detected in cells that produced the transgenic κ protein but failed to produce a functional H chain, implying that the κ gene rearrangements are regulated by the complete Ig protein and not by the κ protein alone. By analyzing hybridomas from the μ × κ mice versus the μΔm × κ mice we were able to determine whether the membrane form or the secreted form of Ig, or perhaps both, could cause inhibition of κ gene rearrangements.

The rearrangement status of the hybridomas from the two transgenic combinations was examined by Southern blotting, hybridizing first with a C_κ probe. After stripping, the blots were rehybridized with a 5′ of J_κ probe, X2.1 (23). The C_κ probe will detect all rearranged or germline κ sequences of endogenous, transgenic, or fusing line origin, while the X2.1 probe will hybridize only to germline sequences or to those sequences that are remnant fragments retained after an inversional rearrangement (23). Unrearranged κ genes were detected in 59% of μ × κ cells and 21% of the μ × κ cells appear to have both alleles in germline configuration (Fig. 3 *B*, Table I). By contrast, while 52% of the μΔm × κ cells retained one germline κ gene, there were no cells of this type that had both κ alleles unrearranged. These results indicate that the membrane form of an IgM molecule, but not the secreted form, is able to cause a cessation of κ gene rearrangement. It is significant to note that the five μ × κ hybridomas that show no evidence of endogenous heavy chain gene rearrangements also show no evidence of endogenous light chain gene rearrangements. λ rearrangements were also searched for by doing Southern blots. Lines C30 and

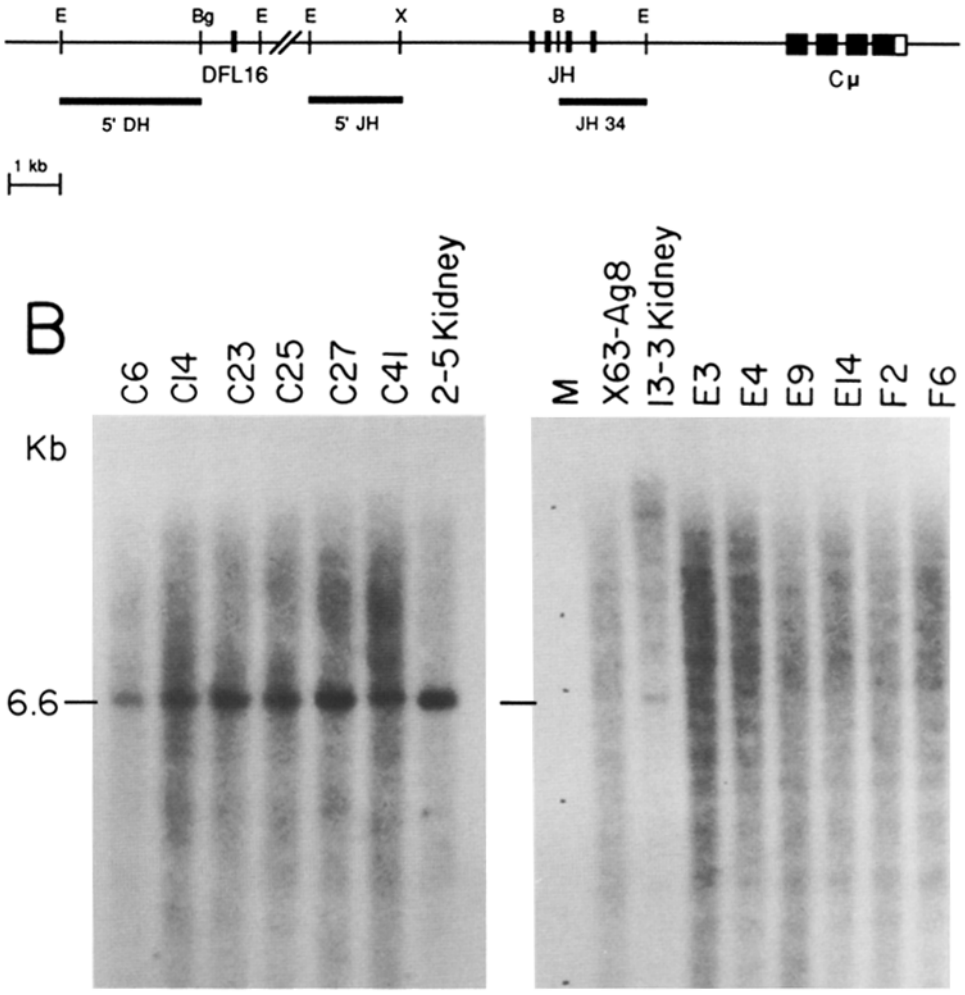

FIGURE 2. Southern blot analysis of DNA rearrangements of the endogenous Ig H chain alleles. (*A*) Diagram of the endogenous H gene. The 5′ of D_H probe (pDFL-2.7) will detect only H chain alleles that have not undergone V_H to D_H rearrangement. The 5′ of J_H probe will detect only H chain alleles that are in germline configuration. Blots described in *C* and *D* were also probed with the JH34 probe, but data are not shown. B, Bam HI; Bg, Bgl II; E, Eco RI; X, Xba I. (*B*) DNA from transgenic hybridomas was digested with Bam HI, electrophoresed, blotted, and hybridized with the 5′ of J_H probe. Hybridoma DNA samples from mouse 2-5 (μ × κ) are designated C, and samples from mice 12-5 and 13-3 (μΔm × κ) are designated E and F. Southern blot of Bam HI-digested hybridoma DNA from mouse 2-5 (μ × κ) hybridized with the 5′ of D_H probe. The 5′ D_H probe crosshybridizes with the 5′ region of multiple D genes, which results in five hybridization bands. The band seen at ~8.1 kb with kidney DNA is due to incomplete digestion. Hybridoma DNA samples from mice 12-9 and 13-3 (μΔm × κ) were treated as in *C*.

C48 appear to have Vλ1 to Cλ1 rearrangements and F7 has a Vλ1 to Cλ3 rearrangement. These lines also had endogenous κ gene rearrangements and may be members of the postulated κ/λ lineage (29, 36).

Examples of cells without any endogenous κ gene rearrangement were also found among the hybridomas from κ only transgenic mice (Table I). 3 of 29 (10%) had

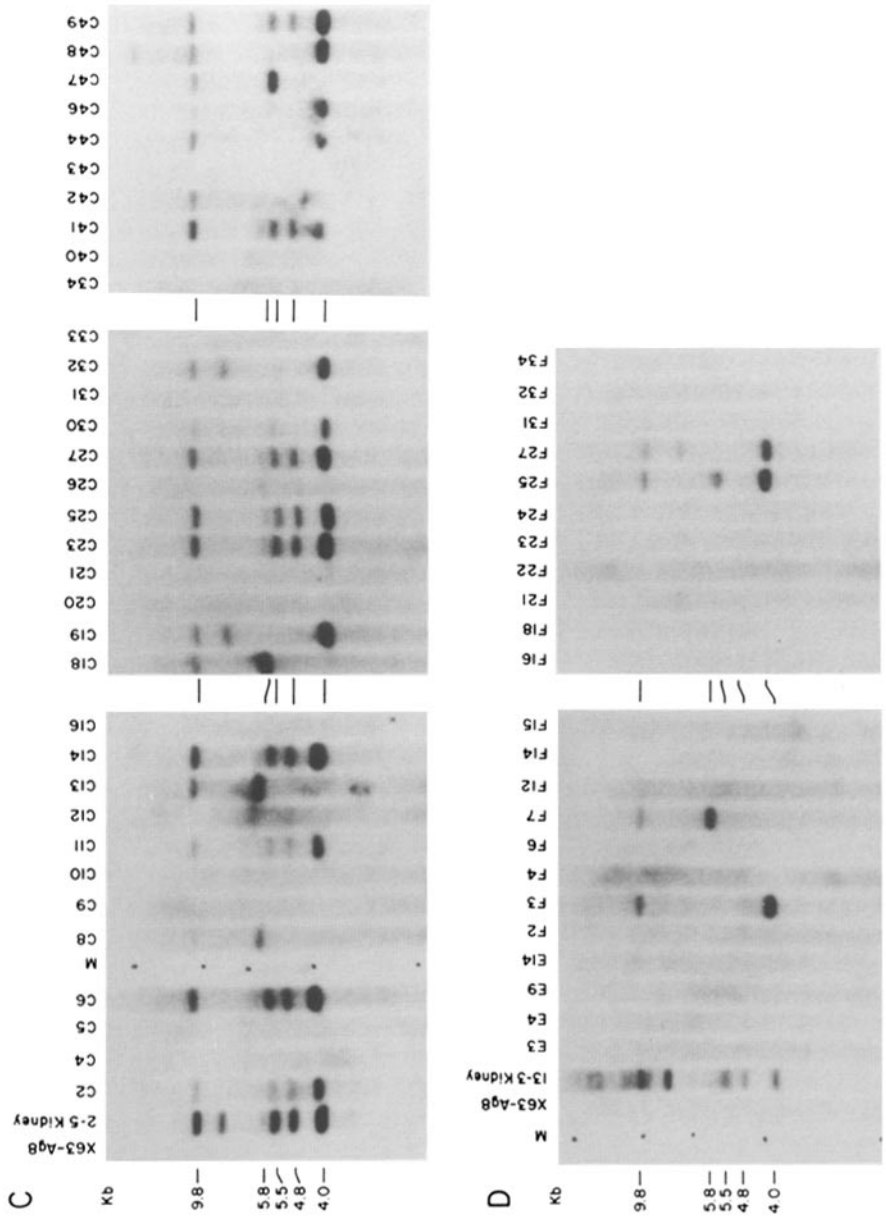

FIGURE 2. (*Cont'd.*) See legend on facing page.

TABLE I

Summary of Hybridomas

	μ × κ						μΔm × κ						κ				
	DNA			Protein			DNA			Protein			DNA			Protein	
Hybridoma	5′JH	5′DH	C_κ	μ*	L	Hybridoma	5′JH	5′DH	C_κ	μ*	L	Hybridoma	5′JH	5′DH	C_κ	H	L
C6§	+	+	GR	ab	−E							A3§	+	+	G−	−§§ or γ	−−
C14	+	+	GG	a−	T−							A10§	+	+	G−	−§§	−−
C23	+	+	GG	a−	T−							D6	+	+	GG	−§§	T−
C25	+	+	GG	a−	T−							D29	+	+	GG	−§§	T−
C27	+	+	GG	a−	T−												
C41	+	+	GG	a−	T−												
C2	−	+	GR	a−	TE	F3	−	+	RR	ab	TE	A1	−	+	GR	γ	TE
C8	−	+	GR	a−	T−	F7	−	+	RR‖	ab	TE	A2	−	+	GR	−	TE
C11	−	+	RR	ab	TE	F25	−	+	GR	ab	TE	A15	−	+	GR	γ	TE
C12	−	+	GR	ab	TE	F27	−	+	GR	ab	−E	A16§	−	+	GR	μ	−E
C13	−	+	GR	−−	ND							A22	−	+	GR	μ	TE
C18	−	+	R−	ab	T−							A28§	−	+	RR	γ	−E
C19	−	+	GR	ab	TE							B33§	−	+	GR	−	−E
C26	−	+	GG	a−	T−							D13	−	+	GR	γ	TE
C30	−	+	RR‖	ab	ND							D15	−	+	RR	−	TE
C32	−	+	GR	ab	−E							D25§	−	+	RR	μ	−E
C42	−	+	RR	a−	TE												
C44	−	+	GR	a−	TE												
C46	−	+	RR	a−	TE												
C47‡	−	+	R−	−b	TE												
C48	−	+	−−‖	ab	TE¶												
C49	−	+	GR	ab	TE												
C4	−	−	RR	−b	TE	E3	−	−	GR	ab	TE	A4	−	−	GR	μ	TE
C5	−	−	R−	a−	TE	E4§	−	−	RR	ab	−E	A6	−	−	GG	μ	T−

C9	–	–	GR	a–*	TE	E9	–	–	GR	ab	TE	A8	–	–	GR	μ	TE
C10	–	–	R–	a–	TE	E14	–	–	GR	ab	TE	A9	–	–	GR	μ	TE
C16	–	–	RR	ab	TE	F2‡§	–	–	RR	––**	–E	A20	–	–	G–	γ	T–
C20	–	–	GR	ab	TE	F4	–	–	GR	ab	TE	A23	–	–	GR	γ	TE
C21§	–	–	RR	a–	–E	F6	–	–	RR	ab	TE	A24	–	–	RR	γ	TE
C31	–	–	GR	ab	TE	F12	–	–	GR	ab	TE	A25	–	–	G–	μ	T–
C33	–	–	GG	a–	T–	F14	–	–	GR	a–‡‡	TE	A26	–	–	R–‖	μ	TE
C34§	–	–	GR	ab	–E	F15	–	–	RR	a–**	TE	A27	–	–	GR	–	TE
C40	–	–	RR	ab	TE	F16	–	–	R–	ab	T–	D4	–	–	––	μ	T–
C43	–	–	GR	ab	TE	F18	–	–	RR	ab	–T	D11	–	–	––	μ	T–
						F21	–	–	GR	a–**	TE	D17	–	–	GR	μ	TE
						F22	–	–	R–	a–**	TE	D19§	–	–	R–	μ	–E
						F23	–	–	GR	ab	TE	D24§	–	–	R–	μ	–E
						F24	–	–	RR	a–**	TE						
						F31	–	–	R–	a–**	TE						
						F32	–	–	GR	a–**	TE						
						F34	–	–	GR	a–‡‡	TE						
Total	un.JH = 6/34; DJ = 22/34; GG_κ = 7/34 (18%) (65%) (21%)					Total	un.JH = 0/23; DJ = 4/23; GG_κ = 0/23 (0%) (17%) (0%)					Total	un.JH = 4/29; DJ = 14/29; GG_κ = 3/29 to 7/29 (14%) (48%) (10%) (24%)				

The data are organized in three blocks relative to the H gene status of the hybridomas: Top, cells with germline JH genes; middle, cells with DJ rearrangements; bottom, cells that have only complete VDJ rearrangements. G, germline; R, rearranged; +, presence of DNA sequence; –, absence of DNA sequence or gene or protein; a and b, μ allotypes; T, E, transgenic, endogenous κ protein; un.JH = unrearranged JH gene.

* The μ × κ and μΔm × κ hybridomas produced only μ chains, except C9 which also produces γ.

‡ Loss of the μ transgene. These hybridomas probably had the transgenes in the early stages; also for §.

§ Loss of the κ transgene.

‖ λ Gene rearrangement.

¶ λ Protein.

** These hybridomas have two rearranged endogenous H genes; apparently neither is functional.

‡‡ Only one rearranged endogenous H gene found, the homologue may have been lost or obscured by transgene hybridization bands.

§§ The hybridomas A3, A10, D6, and D29 only show unrearranged H genes whose intensity corresponds to one or less than one copy per cell; no rearranged H genes can be detected. We presume that these cells once possessed a productive VDJ rearrangement (6) and that the chromosome was lost.

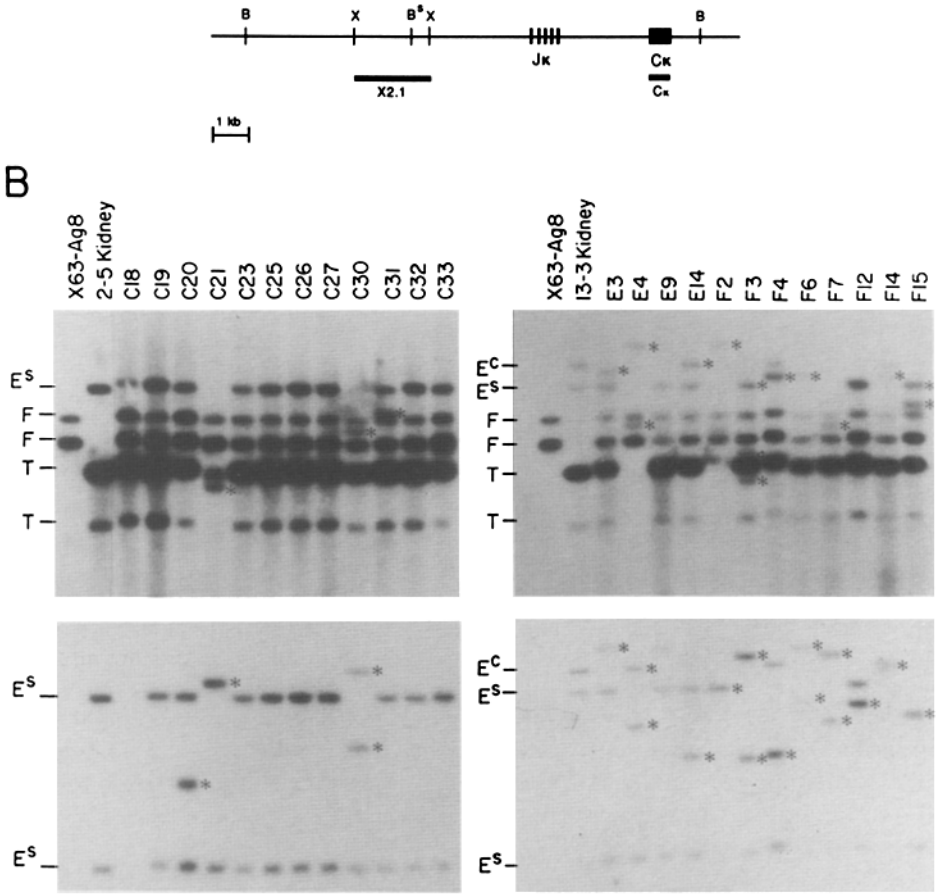

FIGURE 3. Southern blot analysis of DNA rearrangements of the endogenous Ig κ chain alleles. (*A*) Structure of the endogenous κ alleles. A polymorphism at the κ locus between C57/BL6 and SJL results in 12.5-kb and 8.0-kb Bam HI fragments, respectively. The C_κ probe will hybridize to both rearranged and germline κ genes, while the X2.1 probe will hybridize only to germline alleles or remnant fragments of rearrangement by inversion. Bam HI (B), Xba I (X). B^s indicates a Bam HI restriction site present in SJL but not in C56BL/6 κ genes. (*B*) DNA from transgenic hybridomas was digested with Bam HI, and processed as in Fig. 2. Blots were first probed with the C_κ probe (*top panels*) and then with 5′ of J_κ (*bottom panels*). The position of the endogenous germline C_κ and X2.1 bands are marked (E). Mouse 2-5 contains only the SJL alleles for both endogenous κ genes (E^s), while mice 12-9 and 13-3 each contain one C57/BL6 (E^c) and one SJL (E^s) κ allele. Fusing line bands and transgenic bands are designated F and T, respectively. (*) A rearranged endogenous κ gene; <, an aberrantly rearranged κ transgene.

both endogenous genes in germline context. All the κ mice had two distinguishable endogenous κ genes because of a Bam HI polymorphism between SJL and C57/BL. Therefore it was determined that four additional hybridomas (A3, A10, A20, and A25) retained only one homologue of germline κ genes. These four cells showed no evidence for rearrangement of the second κ allele, using C_κ or 5′ of J_κ as probes, and have presumably lost the chromosome carrying the other κ gene. This allele could have been in the germline state in some or all of the cells, which would in-

crease the proportion of cells with unrearranged κ genes to as high as 24%, the same proportion as in the μ × κ hybridomas (Table I). Thus, at the level of κ genes as well, the κ hybridomas behave similarly to the μ × κ and unlike the μΔm × κ cells. This further supports the idea that μs is incapable of feedback inhibition and also raises the possibility that it may actually prevent the feedback (see Discussion).

As an aside, a surprisingly large number of hybridomas retained a sequence upstream of J_κ in a rearranged context, indicative of gene inversion (37, 38). Of 86 rearranged κ genes in the 3 types of hybridomas, 55 (64%) apparently involved an inverted V_κ gene.

Characterization of Transgenic and Endogenous Ig proteins in Hybridomas. Secreted and cytoplasmic Ig were analyzed by immunoblotting (Fig. 4). Endogenous L chains were easily distinguished from the transgenic L chains on a gradient polyacrylamide gel since the κ 167 molecule migrates more slowly than the majority of other κ molecules. The results from these gels (as well as from IEF immunoblots; data not shown) confirm the rearrangement status of the endogenous κ genes in the three types of hybridomas (summarized in Table I). That is, cells that retain only germline κ, genes produce only the transgenic κ protein and many cells that have rearranged their endogenous κ genes produce both transgenic and endogenous κ proteins.

Endogenous H chain production was analyzed by immunoblots as well as by ELISA. We were able to distinguish endogenous and transgenic μ proteins in an ELISA by using allele-specific mAbs since the transgene is of the a allotype and the endogenous genes are of the b allotype (Table I). As expected, the five hybridomas, C14, C23, C25, C27, and C41, which show no evidence of either endogenous H or L chain gene rearrangements, only make the transgenic μ protein (μ^a) in association with the transgenic κ protein. These cells appear to have been completely inhibited from rearranging their endogenous H, κ, and λ genes. Of 34 μ × κ hybridomas

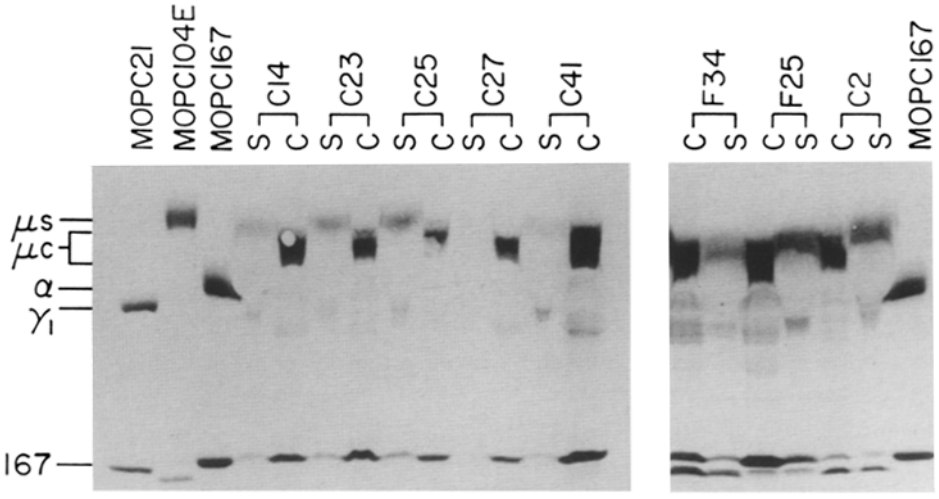

FIGURE 4. Immunoblot analysis of transgenic hybridoma Ig proteins. Hybridoma supernatants (S) and cytoplasmic proteins (C) were run on reducing SDS-polyacrylamide gels, electroblotted to nitrocellulose, and probed with antisera as described in Materials and Methods. Hybridoma designations are as in Figs. 3 and 4.

tested, 16 (47%) do not make an endogenous H chain. 9 μΔm × κ hybridomas of 23 tested (39%) do not make endogenous H chains. These numbers include four hybridomas, three μ × κ and one μΔm × κ, that do not appear to produce the transgenic protein or endogenous H chains. In these cases, the μ transgene is no longer present, presumably due to chromosome loss from the hybridoma. We are assuming, however, that the transgene was lost after fusion and that it was expressed in the B cell. The μ × κ and μΔm × κ hybridomas used in these analyses were stimulated with LPS but only those clones that secreted antibody capable of binding phosphorylcholine were chosen to be examined in detail. This was done in order to assure that each cell produced the transgenic H and L chains to permit a comparison between the effects of μm and μs on the protein level.

AMuLV Pre-B Cell Lines. To further assess the feedback potential of μs in the absence of cell selection we analyzed the pre-B cell lines produced from μΔm + κ transgenic mice (Table II). All of the cell lines had rearranged their endogenous H genes and several had also rearranged endogenous L genes. As in the pre-B cells from normal littermates, ~50% of the transgenic cells had one H allele in the immature DJ state, i. e., 8/30 alleles (27%) were not completely rearranged. This is the same as has been found previously in bone marrow pre-B cells where 23% (25) or 30% (39) of the alleles were DJ rearrangements. In pre-B cells from transgenic mice that carry a complete μ gene, however, 40% of the cells had retained a germline JH allele (8) that is normally not found in AMuLV-transformed bone marrow cells, thus demonstrating an inhibitory effect of μ. It is clear that μs does not have such a feedback effect on either H or κ gene rearrangement. All the transgenic cell lines tested produced cytoplasmic μ and κ, but no Ig secretion was observed, presumably because the cells lack J chains and an effective secretory apparatus.

Discussion

Feedback inhibition of Ig gene rearrangment has been observed previously with transgenic mice harboring μ, δ, or κ genes (6–10). There remained, however, some concern about the possible effects of the transgenes competing for limiting quantities of enzymes or factors required for recombination. The two types of μ hybridomas examined here were made from mice whose two sets of transgenes were identical except for the 3′ end (containing the membrane exons) of the μ genes. Therefore, those sequences that are known to bind regulatory factors should be shared by the two types of μ transgenes. Thus, the lack of feedback by the μs transgene largely alleviates concerns about factor competition.

We wish to consider the following conclusions derived from the data presented here: (*a*) the production of μ together with κ can terminate Ig gene rearrangement; (*b*) μs with κ does not have this feedback effect; (*c*) μs interferes with the effect of membrane μ and κ; and (*d*) the feedback shown here probably represents shutoff of the recombinase by μm + κ, but the data do not address the question of μ alone affecting the accessibility of H genes for rearrangement.

While the feedback shown in the present study is incomplete (see below) an effect on endogenous Ig gene rearrangement can be seen with the complete μ transgene in combination with κ. No such effect is observed on H gene rearrangement in pre B cells, or on H or κ gene rearrangement in hybridomas from μΔm + κ transgenic

mice. Since the complete μ gene encodes both μm and μs and since μs is incapable of feedback inhibition, by inference the μm molecule creates a feedback signal.

It could be argued that the results with the hybridomas from μΔm × κ mice may be skewed because B cells with secreted μ only would not exist in the spleen and that, therefore, cells could be selected only if they expressed endogenous membrane Ig. However, while almost all the hybridomas from the μΔm × κ mice secreted anti PC antibodies, 9 of 23 (39%) produced no endogenous H chains; of these, 7 showed two endogenous H gene rearrangements, i. e., had apparently retained both homologues of chromosome 12 (Table I). Thus, at least 7/23 (30%) of the μΔm × κ cells were not selected on the basis that they had evaded feedback of Ig gene rearrangement. Furthermore, at the level of AMuLV-transformed pre-B cells from the μΔm + κ mice, no feedback on H gene rearrangement was seen as well. Thus, even in the absence of selection, μs does not prevent Ig gene rearrangement. The question of maturation of some B cells in the absence of membrane Ig is intriguing and will need to be further investigated.

The ability of μm to inhibit rearrangement of endogenous H chain genes has also been seen using a rearranged human μ gene that could only encode μm (9); however, human μs has not been tested. Transgenic δ was also effective in inhibiting H chain gene rearrangement to a degree similar to that seen in our experiments with μ (10). Presumably, it is also the membrane form of this protein which is responsible for the effect (Kohler, G., personal communication). In contrast to these results, we have found that a complete γ2b transgene that encodes both secreted and membrane protein (40) does not inhibit endogenous H chain gene rearrangement in pre-B cells or B cells (Denis, K., K. Gollahon, J. Hackett, L. Doglio, R. Brinster, and U. Storb, unpublished observations) which indicates that the regulatory potential may not be a property of all membrane bound Ig molecules.

Reth et al. (3) observed a similar difference in the regulatory potential of μm and μs to turn on κ gene rearrangement. These authors speculated that the transmembrane and intracytoplasmic portion of the μm chain may be involved in a signaling process that would eventually generate an intranuclear signal. Thus, it appears that the membrane domain is the integral part of the μ molecule that is required for regulating both the activation of κ gene rearrangement, as well as the cessation of H and κ gene rearrangement. Further experiments will be required to elucidate the details of the role of μm and of other molecular components in the creation of transduction of the feedback signal.

There is a striking difference in the status of endogenous H and κ genes between the hybridomas from μΔm × κ and κ only transgenic mice. The former contain no cells without H gene rearrangement on both chromosomes and κ gene rearrangement on at least one chromosome, whereas in the transgenic κ hybridomas, 14% retain a germline J_H gene and 10–24% retain two germline κ genes. In fact, the Ig gene status of the κ hybridomas is similar to that of the μ × κ hybridomas. From these observations we propose that the effects observed in this study are the result of feedback inhibition by a combined μm-κ molecule leading to termination of rearrangement in both the H and κ loci due to cessation of the activity of the specific Ig gene recombinase. Absence of recombinase function has been observed previously in $\mu^+\kappa^+$ B cells, whereas pre-B cells with rearranged H genes and unrear-

ranged κ genes possess the recombinase (41–44). It has been proposed that further H-gene rearrangement is inhibited in such pre-B cells by a conformational change in V_H genes and concomitant stop in V_H gene transcription (45). Our data presumably do not permit addressing the question of a direct feedback by H genes alone on H gene recombination, since in the mice studied here a functional κ gene was present throughout B cell development. The κ transgene is apparently expressed sufficiently in pre-B cells to permit detection of the κ protein (Table II). Since pre B cells normally lack the transactivating factor NFκB required for enhanced κ gene transcription, presumably, in these transgenic pre-B cells the elevated level of κ is due to the additive effect of low level transcripts from multiple (8–41) κ transgenes. Evidence for transcription of all κ transgene copies has been obtained in a three-copy κ transgenic mouse (16).

Before further discussion of the difference in feedback between the μs + κ and the μm + κ or κ mice one has to consider the imcompleteness of the inhibition by μm +/or κ observed in this study as well as in studies by others (7–9). The leakiness of feedback by the μ gene may possibly relate to a delay between activation of the recombinase and transcription of the transgene. However, with respect to κ gene rearrangement the feedback was complete with the MOPC-21 κ transgene when it was expressed together with endogenous H chains (6). In the data presented here with the MOPC-167 κ transgene the feedback is incomplete. One possible explana-

TABLE II

AMuLV Pre-B Lines from μΔm + κ Transgenic Mice

	Ig protein		Endogenous Ig genes		
Pre-B line*	Cytoplasmic	Secreted	H	κ	λ
Transgenic Mice					
0.10	ND	ND	R (DJ/VDJ)	G	G
1.5	μ κ	-	R (VDJ/VDJ)	G	G
1.9	μ κ	-	R (DJ/VDJ)	G	G
1.10	μ κ	-	R (VDJ/VDJ)	G	ND
2.13	μ κ	-	R (DJ/VDJ)	R	G
4.2	μ κ	-	R (VDJ/VDJ)	G	G
4.4	μ κ	-	R (VDJ/VDJ)	G	G
4.5	μ κ	-	R (DJ/VDJ)	G	G
6.2	ND	ND	R (VDJ/VDJ)	G	G
6.3	μ κ	-	R (DJ/VDJ)	G	G
6.4	μ κ	-	R (DJ/VDJ)	G	G
6.5	μ κ	-	R (DJ/VDJ)	G	G
6.6	μ κ	-	R (VDJ/VDJ)	G	G
6.9	μ κ	-	R (VDJ/VDJ)	G	G
6.10	μ κ	-	R (DJ/VDJ)	R	G
Normal littermates					
3.8	ND	ND	R (VDJ/VDJ)	G	G
3.13	- -	-	R (DJ/VDJ)	G	G
7.1	- -	-	R (DJ/VDJ)	G	G
7.3	μ -	-	R (VDJ/VDJ)	G	G

The transgenic mice were from line 217-4 (11). R, rearranged; G, germline.

* Each number represents a separate pre-B line. The prefix indicates the mouse from which the cell lines were derived. 0, 1, 2, 4, and 6 were transgenic, and 3 and 7 were normal littermates.

tion for this discrepancy may lie in the relative amounts of transgenic κ protein produced. Although no attempt was made to determine the precise amount of κ protein produced by the two genes, analysis of protein gels suggests that any differences appear to be minor. Interestingly, however, several hybridomas that produced equivalent amounts of κ proteins (MOPC 167 and endogenous) intracellularly, secreted a visibly higher amount of endogenous κ than transgenic κ, suggesting a qualitative difference between the κ 167 and endogenous κ molecules (Fig. 4). This difference was also seen in preliminary experiments with transfectants containing the MOPC 167 μ gene, MOPC 167 κ gene, and the MOPC 21 κ gene; the MOPC 21 κ chain appears to be preferentially secreted with the MOPC 167 H chain (Manz, J., and U. Storb, unpublished observations). Thus, if feedback inhibition of κ gene rearrangement requires a high affinity combination of H and κ, the MOPC 167 κ chain may not be very efficient. The argument can be made, however, that in combination with the autologous MOPC 167 H chain this κ chain should cause feedback. However, since both the H and κ V regions from MOPC 167 are extensively mutated from the germline (46, 47), during the pre-B cell stage of the MOPC 167 myeloma precursor the affinity of the unmutated H and L chains for each other was most likely different and possibly higher than that of the transgenic proteins. These ideas may be checked in transgenic mice with different combinations of μ and κ V regions.

We can now consider the possible scenarios of Ig gene rearrangement in transgenic and nontransgenic pre-B cells. In hybridomas from nontransgenic mice no unrearranged H genes have been observed (8, 10). Why is there an apparent feedback on DJ and VD rearrangement in κ transgenic pre-B cells? It can be assumed that in these cells, when endogenous H chains are being produced after a correct rearrangement, they have a stronger feedback effect than normally, because in the presence of transgenic κ, at least in some cells where a sufficiently good combination of μ and κ exists, the recombinase is shut off altogether, compared with the mere inactivation of V_H gene targets by H alone (see above). Similarly, in the MOPC-21 κ transgenic hybridomas several had one unrearranged J_H allele indicating total cessation of rearrangement (34, 35). Presumably, this effect is not seen with the μΔm + κ cells, because of the excess of μs protein. Normally, pre-B cells produce extremely low levels of μs compared with μm, because μ transcripts are mainly processed to the μm form (48). Thus, transgenic mice with a complete μ gene (8) have a similar feedback as mice with a μm gene (9). However, a μΔm transgene results in high levels of μs in pre-B cells (Table II). Perhaps a sufficient level of μm + κ cannot be achieved in these cells so that most $\mu_2\kappa_2$ tetramers would have at least one μs chain. Possibly, the feedback signal requires a homogeneous $\mu m_2\kappa_2$ tetramer. Thus, without it, recombinase activity would be continuously present and both endogenous H and κ genes would rearrange.

This scenario attributes a considerable influence on the relative levels of μm versus μs. The control of these levels by the regulation of RNA splicase and endase activities are currently being investigated (49, 50, and many others). The remote possibility exists that λ gene expression may be influenced by the relative levels of μm and μs. Apparently, rearrangement is not inhibited in λ-producing pre-B cells (29, 36, 51); λ production may be a property of Ly-1 B cells, which are notorious for very high levels of μs (52).

In summary, the control of Ig gene rearrangement appears to be increasingly com-

plex. The competing effects of μm and μs in feedback control may help unravel the molecular basis of the feedback signal.

Summary

Previous work (6–10) has shown that allelic exclusion of Ig gene expression is controlled by functionally rearranged μ and κ genes. This report deals with the comparison of membrane μ (μm) and secreted μ (μs) in promoting such feedback inhibition. Splenic B cell hybridomas were analyzed from transgenic mice harboring a rearranged κ gene alone or in combination with either an intact rearranged μ gene or a truncated version of the μ gene. The intact μ gene is capable of producing both membrane and secreted forms of the protein, while the truncated version can only encode the secreted form. The role of the μs was also tested in pre-B cell lines. Analysis of the extent of endogenous Ig gene rearrangement revealed that (*a*) the production of μm together with κ can terminate Ig gene rearrangement; (*b*) μs with κ does not have this feedback effect; (*c*) μs may interfere with the effect of μm and κ; and (*d*) the feedback shown here probably represents a complete shutoff of the specific recombinase by μm + κ; the data do not address the question of μ alone affecting the accessibility of H genes for rearrangement.

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