

INHIBITION OF IMMUNOGLOBULIN GENE  
REARRANGEMENT BY THE EXPRESSION  
OF A  $\lambda 2$  TRANSGENE

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The rearrangement of Ig genes is regulated by feedback inhibition from Ig molecules encoded by productively rearranged H and  $\kappa$  genes (1-3). H and L genes are activated sequentially (1). Apparently, VDJh rearrangement is halted after H chain production (4), while the Ig gene recombinase activity persists and promotes  $\kappa$  and  $\lambda$  gene rearrangement. The final shutoff of the recombinase seems to occur after  $\kappa$  rearrangement, when a complete Ig molecule can be synthesized (1, 5). Combination of membrane  $\mu$  ( $\mu m$ )<sup>1</sup> with  $\kappa$  is required; secreted H chains do not promote feedback inhibition (6-8).

The regulation of this feedback by  $\kappa$  seems to indicate that a  $\mu m/\kappa$  combination acts differently than  $\mu m$  alone. In the process of defining the role of the L chain in this complex, it was necessary to determine whether  $\lambda$  L chains would have the same effect. In overall structure  $\lambda$  chains are very similar to  $\kappa$  (9). However, in the mouse,  $\lambda$  represents only ~5% of the total serum L chains (10). Furthermore, the regulation of  $\lambda$  gene expression appears to be different from  $\kappa$ . In general,  $\kappa$ -producing B cells have rearranged  $\kappa$ , but maintain germline  $\lambda$  genes. In contrast,  $\lambda$ -producing cells almost always have both  $\lambda$  and  $\kappa$  genes rearranged (11-13), and it has been suggested that  $\lambda$  genes may be activated only after failing to rearrange  $\kappa$  genes productively.

We report here the analysis of Ig gene expression in transgenic mice that harbor a stably integrated mouse  $\lambda 2$  gene. Since the transcriptional enhancer for  $\lambda$  has not been found (14), we replaced a portion of the J $\lambda$ 2 intron with the H chain enhancer. It was expected that this enhancer would promote transcription of the  $\lambda 2$  transgene in early pre-B cells when H chain genes are being activated.

We chose the gene encoding  $\lambda 2$  rather than  $\lambda 1$  so that transgene expression could be distinguished from endogenous  $\lambda$  production. Normally,  $\lambda 2$  is produced at a very

This work was supported by National Institutes of Health grants HD-23089, AI-24780, and GM-38649 to U. Storb; and AI-25366 to R. Brinster. Support to L. T. Doglio was from N. I. H. training grant HD-07136; and support to C. M. Rudin was from N. I. H. training grant GM-07281. J. Hackett, Jr. is a Leukemia Society of America Fellow. We also acknowledge support from the Coleman/Fannie May Foundation.

<sup>1</sup> Abbreviations used in this paper: GAM, goat anti-mouse;  $\mu m$ , membrane  $\mu$ ; PE, phycoerythrin.

low level. It represents  $\sim 10$ – $20\%$  of the total  $\lambda$  and only  $\sim 1\%$  of total L chains (10). Furthermore, the  $\lambda$ 2 transgene carries several mutations that would, if necessary, allow its distinction from endogenous  $\lambda$ 2.

The transgenic mice show that the  $\lambda$ 2 transgenic transcripts are found specifically in lymphoid tissues. The presence of the  $\lambda$  transgene decreases the expression of endogenous L and H chain genes.

### Materials and Methods

**Preparation of Plasmid  $\lambda$ 2A2H4.** A 6.6-kb Eco RI genomic fragment containing the functionally rearranged  $\lambda$ 2 gene of the plasmacytoma MOPC 315 (15), a gift of H. Murialdo, University of Toronto, was subcloned into the Eco RI site of pUC19 ( $\lambda$ 2A2). The Hind III site 14 bp 3' of J $\lambda$ 2 was converted to an Nhe I site by ligation after partial cleavage of plasmid  $\lambda$ 2A2 with Hind III and blunting cohesive ends with the large fragment of *Escherichia coli* polymerase I (Klenow; New England Nuclear, Boston, MA). The resulting plasmid,  $\lambda$ 2A2N, was digested partially with Xba I, completely with Nhe I, and ligated with an excess of a 997-bp Xba I genomic fragment of the  $\mu$  J-C intron (H chain enhancer; reference 16). Clone  $\lambda$ 2A2H4 contains the enhancer segment in the normal orientation relative to promoter sequences in place of 731 bp of the  $\lambda$ 2 J-C intron.

**Preparation of DNA for Microinjection.** Plasmid  $\lambda$ 2A2H4 was cleaved with enzymes Pvu I and Sal I. The 7.0-kb fragment for microinjection was isolated from 0.8% Sea Plaque (FMC Corp., Rockland, ME) low-melting agarose gels (Fig. 1 A).

**Transgenic Mice.** Transgenic mice were produced as described (17, 18), except that the injected zygotes were from C57BL/6 mice. One transgenic line was obtained that has 8–10 copies of the transgene presumably integrated in tandem at a single site (Fig. 1 B). The 13.4- and 3.0-kb bands are the germline C $\lambda$ 2 and C $\lambda$ 3 bands, respectively. The 2.8-kb band represents integrated transgene copies in which the Bam HI site at the 3' end of the  $\lambda$ 2 gene has been preserved; the 7.0-kb band is unit length transgene spanning two neighboring copies where the 3' Bam HI site has been deleted. There is no band that would correspond to a junction fragment with the host DNA, thus, apparently the 3' flanking end of the transgenes has the Bam HI site preserved.

**Hybridomas.** One 4-wk-old male transgenic mouse, 1275-4-8, was injected with 20  $\mu$ g of LPS intraperitoneally 3 d before splenectomy and fusion. This mouse was kept alive for breeding purposes. Hybridomas were made as previously described (7). Supernatants were tested for Ig secretion in a modified ELISA as described by Gollahon et al. (19). L chain secretion was detected using goat anti-mouse (GAM) L chain-specific antisera (1:400; Southern Biotechnology Assoc., Birmingham, AL) as the solid-phase antibody and the assay was developed with either peroxidase-conjugated GAM $\lambda$  or GAM $\kappa$  (1:1,000; Southern Biotechnology Assoc.). To determine H chain secretion, supernatants were added to plates coated with a 1:200 dilution of GAM $\mu$  (Kirkegaard & Perry Laboratories, Gaithersburg, MD) or GAM $\gamma$ Fc (Jackson ImmunoResearch Laboratories, West Grove, PA) and developed with peroxidase-conjugated GAM Ig (1:2,000; Kirkegaard & Perry Laboratories).

**Nucleic Acid Procedures.** DNA from hybridomas and tissues was prepared as described (20). After digestion with restriction enzymes (New England Biolabs, Beverly, MA) the DNAs were run on 1.0% agarose gels and blotted to Gene Screen Plus (New England Nuclear, Boston, MA) (21). Hybridization and reprobing procedures were as described (7). RNA preparation and Northern blotting were as described (16).

**Probes.** Probes for C $\mu$  and C $\kappa$  (22), pX2.1, Jh4, 5' of Jh1, and pDFL-2.7 (7) have been described previously. The C $\lambda$ 1 probe is a 158-bp Bst NI fragment isolated from pC $\lambda$ 1 (23). The C $\lambda$ 2 probe is a 313-bp Avr II/Pst I fragment of p $\lambda$ II-1 (24). The mouse tubulin  $\beta$ 5 probe is a 0.4-kb Bam HI fragment of pm  $\beta$ 5 (the gift of D. Cleveland, Princeton University; reference 25). The histone H4 probe is a 0.7-kb Bam HI/Hind III fragment of pBRH4, a gift of R. Grosschedl, University of California, San Francisco (26).

**ELISA.** The wells of 96-well half-area microtiter plates (Costar, Cambridge, MA) were coated with 50  $\mu$ l GAM $\mu$  (Kirkegaard & Perry Laboratories) or GAM $\gamma$ 2b (Southern Biotechnology Assoc.) diluted 1:200 in 0.05 M carbonate buffer, pH 9.6. The plates were incubated

overnight at 37°C and 1% BSA was used as a blocking agent. 25  $\mu$ l of mouse serum samples or purified mouse myeloma protein diluted in PBS containing 0.05% (vol/vol) Tween 20 (Sigma Chemical Co., St. Louis, MO) was added and the assay then developed with 25  $\mu$ l of the appropriate anti-mouse L chain-specific reagent. Each step required a 30-min incubation at 37°C and the plates were washed three times with PBS-Tween 20 between steps.

For IgM/ $\lambda$  quantitation, biotinylated monoclonal rat anti-mouse  $\lambda$ 1 (clone R11-153.9; PharMingen, San Diego, CA) or biotinylated monoclonal rat anti-mouse  $\lambda$ 1,2 (clone R26-46; PharMingen) diluted 1:500 and 1:400, respectively, in PBS-Tween 20 was added, followed by 25  $\mu$ l/well peroxidase-labeled avidin (1:1,000; PharMingen). Substrate (2 mM phenylenediamine and 0.012% hydrogen peroxide in 24.3 mM citric acid, 51.4 mM sodium phosphate) was then added for 5–20 min. The reaction was stopped with 5% sulfuric acid and absorbance detected at 490 nm. To determine Ig/ $\kappa$  levels, the assay was developed with peroxidase-conjugated GAM $\kappa$  (1:1,000; Southern Biotechnology Assoc.) and the TMB Microwell Peroxidase Substrate System (Kirkegaard & Perry Laboratories) was used as substrate. The reaction was stopped with 1 M phosphoric acid and the absorbance was detected at 450 nm. Affinity-purified IgM/ $\lambda$ 2 (PMG11-185), IgM/ $\lambda$ 1 (MOPC-104E), and IgM/ $\kappa$  (CH6-46.1), purchased from PharMingen, and  $\gamma$ 2b/ $\kappa$  (MOPC 195/MOPC 141), purchased from ICN Biomedicals (Naperville, IL), were used to generate the standard curves.

**Flow Cytometry.** Single cell suspensions of spleen cells were made and erythrocytes were lysed with Tris-ammonium chloride. Cells were washed two times with PBS containing 1% FCS and 0.01% sodium azide (staining buffer). Approximately  $10^6$  cells were stained with phycoerythrin (PE)-conjugated monoclonal rat anti-mouse  $\kappa$  (1:50; Becton Dickinson & Co., Mountain View, CA), PE-conjugated GAM $\mu$  (1:100; Southern Biotechnology Assoc.), or Fluorescein isothiocyanate-conjugated GAM $\lambda$  (1:80; Southern Biotechnology Assoc.) in 100  $\mu$ l of staining buffer at 4°C. Cells were washed two times and resuspended in staining buffer to  $10^6$  cells/ml for analysis.

Fluorescence analysis was performed on an Epics V flow cytometer (Coulter Diagnostics, Hialeah, FL) with a 5-W argon laser (Innova). Unless otherwise indicated, gating parameters were set based on forward angle and 90° light scatter properties to exclude myeloid cells. Two-color analysis was performed as previously described (19). Data were analyzed on an MDADS computer interfaced with the flow cytometer.

**Protein Gel Electrophoresis and Western Blotting.** Mouse sera (1  $\mu$ l) were fractionated by SDS-PAGE (12% acrylamide) under reducing conditions and electroblotted to nitrocellulose as described (7). The nitrocellulose was sequentially incubated with rabbit anti-mouse  $\lambda$  (1:200; ICN Biomedicals, Costa Mesa, CA) and peroxidase-conjugated goat anti-rabbit Ig (1:2,000; Bio-Rad Laboratories, Richmond, CA).

## Results

**Tissue-specific Expression of the  $\lambda$ 2 Transgene.** In normal mice the level of  $\lambda$ 2 expression in B cells is very low (10). Accordingly,  $\lambda$ 2 mRNA is barely detectable in the spleens of normal mice (Fig. 2). The  $\lambda$ 2 transgenic mouse line, however, strongly expresses  $\lambda$ 2 in the spleen and, as expected from a transgene with the H chain enhancer (27), in the thymus (Fig. 2). The levels of  $\lambda$ 2 in kidney and liver of the transgenic mice are higher than in normal littermates, but much lower than in spleen and thymus. Most likely, the  $\lambda$ 2 found in these tissues derives from blood-borne B and T cells, similar to the findings in other transgenic mice with Ig genes (22, 28). Thus, the  $\lambda$ 2 transgene appears to be expressed in tissue-specific fashion and these transgenic mice should be suitable as a model to study the effects of  $\lambda$ 2 expression on Ig gene rearrangement.

**Altered  $\kappa/\lambda$  Ratios on the Surface of B Cells of  $\lambda$ 2 Transgenic Mice.** To determine the numbers of B cells producing  $\lambda$ 2 and other Igs, spleen cells were analyzed by FACS during the development of the mice (Table I). At all time points, the transgenic mice had a large percentage of B cells that produced  $\lambda$ , presumably mainly  $\lambda$ 2, ranging

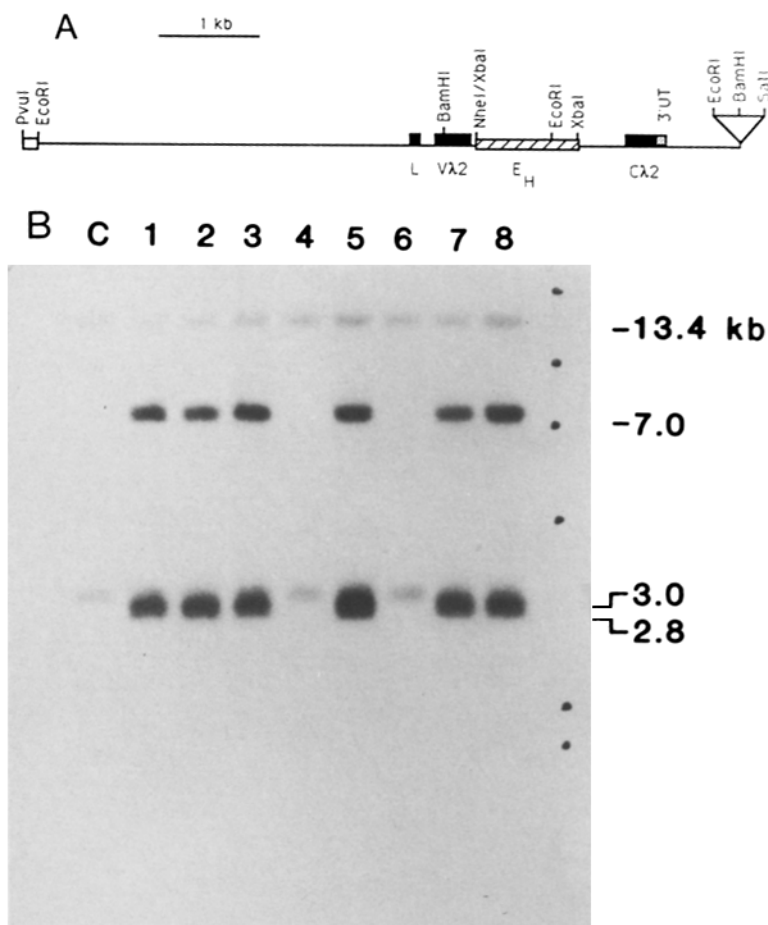


FIGURE 1. The transgene. (A) Map of  $\lambda 2$  transgene. Completely filled boxes are exons; slashed box is J-C $\mu$  intron segment; open box is 120 bp of pUC19. (B) Southern blot of kidney DNAs. Digestion with Bam HI, probed with C $\lambda 2$ . (Lane C) C57BL/6; (lanes 1, 2, 3, 5, and 7)  $\lambda 1$  transgenic mice (F<sub>2</sub> of founder mouse); (lanes 4 and 6) normal littermates; (lane 8) transgenic mouse (F<sub>1</sub> of founder).

from 65 to 79% of  $\mu^+$  B cells in 4-d-old mice and decreasing to 48–56% by 70 d of age. On the other hand,  $\kappa^+$  cells, which constitute at least 90% of the B cells in normal mice, were reduced to 33–39% in 4-d-old transgenic mice. By 16 d the proportion of  $\kappa^+$  B cells had nearly doubled in transgenic mice and had increased still further by 10 wk. Thus, it appears that the presence of the  $\lambda 2$  transgene reduces the proportion of B cells expressing endogenous  $\kappa$  genes, but that  $\kappa^+$  B cells accumulate with age.

We next investigated whether the  $\kappa^+$  B cells in transgenic mice produce  $\lambda 2$  protein. The level of  $\lambda$  expression in  $\kappa^+$  cells was found to be quite low, nearly undetectable by surface staining (Table I). That the majority of  $\kappa$  cells may coexpress  $\lambda$  became evident when we examined spleen cells from mice that had been treated with LPS intraperitoneally 3 d before the FACS analysis (Fig. 3). As compared with

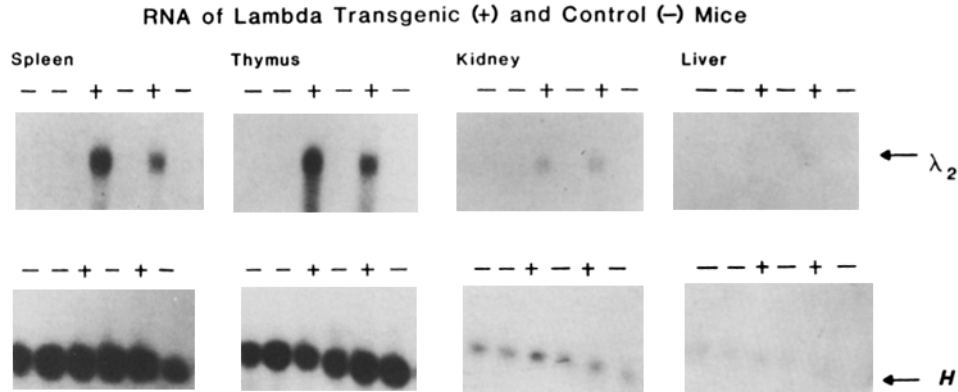


FIGURE 2. Northern blot of organ RNAs of  $\lambda_2$  transgenic mice (+) and normal littermates (-). Each lane contained 10  $\mu\text{g}$  of total cellular RNA. The blot was hybridized with a  $\text{C}\lambda_2$  probe (*top*) or a histone H4 probe (to normalize mRNA within but not between tissues) (*bottom*). The positions of mature  $\lambda_2$  RNA and of histone RNA are indicated.

TABLE I  
FACS Analysis

Age	Mouse	Transgenic*	Percent of lymphoid spleen cells:			
			$\mu$	$\kappa$	$\lambda$	$\kappa + \lambda$
4 d	1	+	35.5 <sup>‡</sup> [16.5] <sup>§</sup>	11.6 <sup>‡</sup> (33) <sup>  </sup>	24.9 <sup>‡</sup> (70)	1.9 <sup>‡</sup>
	4	+	40.7 [16.5]	13.3 (33)	28.6 (70)	1.7
	6	+	29.1 [14.3]	11.3 (39)	23.1 (79)	1.7
	8	+	31.0 [14.4]	10.1 (33)	23.5 (76)	1.9
	9	+	36.9 [15.1]	14.0 (38)	24.1 (65)	2.5
	2	-	39.6 [18.8]	35.5 (90)	4.8 (12)	<1
	3	-	38.7 [19.8]	36.9 (95)	5.7 (15)	<1
16 d	5	-	36.4 [18]	35.8 (98)	4.8 (13)	<1
	7	-	36.6 [21.3]	35.4 (97)	5.6 (15)	1.1
	1	+	42.2 [34.4]	21.7 (51)	22.1 (52)	2.6
	2	+	37.8 [29.6]	23.5 (62)	22.6 (60)	1.9
	3	+	41.7 [33.4]	25.5 (61)	23.3 (56)	2.2
	5	+	39.3 [33.4]	26.4 (67)	22.4 (57)	2.1
	7	+	36.0 [26.7]	16.5 (46)	25.1 (70)	2.2
70 d	4	-	46.1 [40.0]	50.1 (109)	6.6 (14)	<1
	6	-	56.0 [48.1]	53.1 (95)	8.2 (15)	<1
	18	+	44.6 [44]	26.1 (59)	24.8 (56)	5.5
	19	+	45.4 [44.7]	31.7 (70)	22.1 (49)	5.2
	22	+	47.4 [45.1]	31.9 (67)	22.7 (48)	6.8
	23	-	58.4 [56]	54.4 (93)	5.5 (9.4)	<1
	N1	-	52.9 [51]	50.1 (95)	4.5 (9)	<1
N2	-	56.1 [53.3]	54.9 (98)	5.2 (9)	<1	
N3	-	56.3 [54.4]	51.3 (91)	5.3 (10)	<1	

\* +, transgenic mouse; -, normal littermate (or normal age-matched C57BL/6 mouse -N1, etc.).

<sup>‡</sup> The  $\mu$ ,  $\kappa$ , and  $\lambda$  values were obtained from fluorescence histograms; the  $\kappa + \lambda$  values were obtained from two-color quadrant plots.

<sup>§</sup> In brackets are the percent of total nucleated spleen cells.

<sup>||</sup> In parentheses are the percent of  $\mu^+$  B cells.

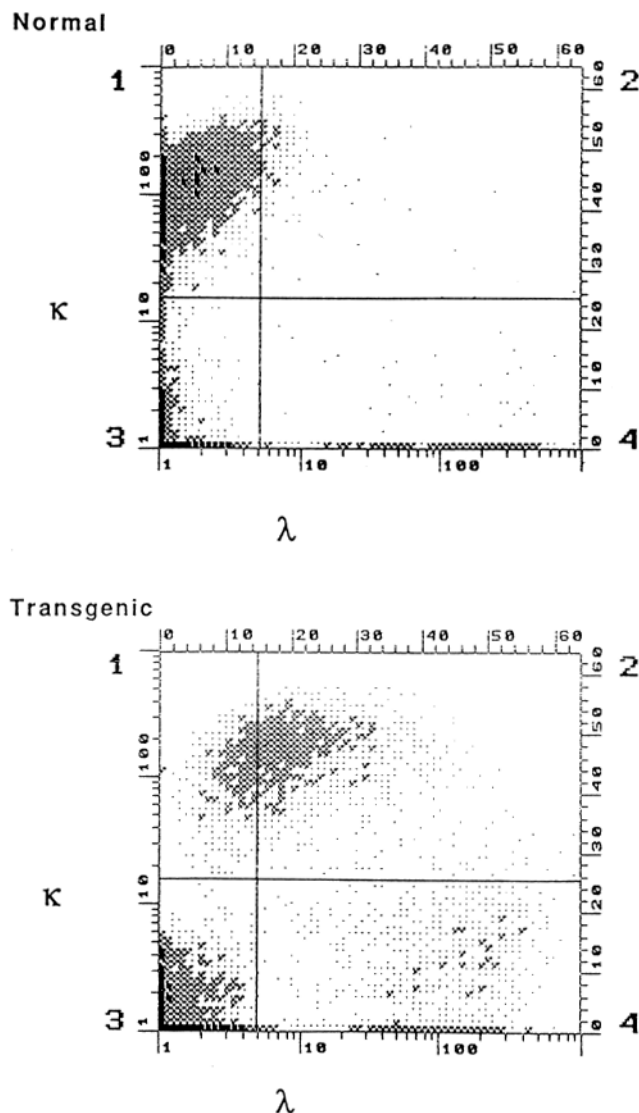


FIGURE 3. FACS profile of 4-wk-old transgenic mouse and normal littermate. The mice had been treated with LPS (20  $\mu$ g/mouse, intraperitoneally) 3 d before analysis. (Ordinate) red fluorescence PE anti- $\kappa$ ; (abscissa) green fluorescence FITC anti- $\lambda$ .

$\kappa^+$  cells from normal spleen cells, there is a significant shift in the fluorescence profile of  $\lambda$  expression in  $\kappa^+$  cells from the transgenic mice. However, the relative fluorescence intensity of  $\lambda$  staining on  $\kappa/\lambda$ -coexpressing cells is approximately a log lower than that seen for cells bearing  $\lambda$  alone. The low level of  $\lambda$  expression on  $\kappa^+$  cells could reflect transgenic  $\lambda 2$  expression by the respective cells or, alternatively, binding of cytophilic  $\lambda$ . We favor the former interpretation, because of observations made in hybridomas (see below).

Overall, there is a slight decrease in the number of IgM<sup>+</sup> B cells in the spleens of the transgenic mice of all ages (Table I).

*Suppression of Serum L Chain Levels in  $\lambda 2$  Transgenic Mice.* Since  $\lambda 2$  levels are low in normal mice, it was possible to readily observe the increased expression of the

transgenic  $\lambda 2$  isotype in serum Ig. In addition,  $\lambda 1$  and  $\lambda 2$  isotypes may be separated by size fractionation on SDS-PAGE. Western blots of the sera of 5-wk-old mice show that the transgenic mice produce large quantities of  $\lambda 2$  (Fig. 4 B). Notably, the level of  $\lambda 1$  is considerably lower than in their normal littermates, suggesting that expression of the  $\lambda 2$  transgene suppresses the production of endogenous  $\lambda$  chains. In 10-wk-old mice the amount of  $\lambda 2$  is still higher than in normal mice, and  $\lambda 1$  still appears to be suppressed (Fig. 4 C). In very young mice of 4 and 16 d of age, the serum L chains appear to be almost entirely of maternal origin. Offspring of a normal female show both  $\lambda 1$  and  $\lambda 2$ , while offspring of a  $\lambda 2$  transgenic female show mainly  $\lambda 2$  (Fig. 4 A).

To obtain a more quantitative evaluation of the serum L chain levels, ELISAs were performed (Table II). 16-d-old mice were analyzed for IgM only, due to the preponderance of maternal IgGs. In very young mice,  $\lambda^+$  IgM is many fold higher in transgenic mice due to  $\lambda 2$ . At 10 wk, however, the  $\lambda$  levels are on average only 1.5 times higher than in normal mice. A striking difference is seen when comparing the effect of the transgene on endogenous  $\lambda 1$  and  $\kappa$  production. While  $\kappa$  is reduced to 26% of normal in newborn mice, by 10 wk the quantities of  $\kappa$  recover to reach 56% of normal. In contrast, the level of  $\lambda 1$  in the newborn is only reduced by one third and becomes more depressed with age relative to normal mice, until at 10 wk it represents as little as 3% of the normal  $\lambda 1$  level. The quantities of total IgM are slightly decreased in transgenic mice of all ages, in accordance with the small reduction in surface IgM<sup>+</sup> B cells (Table I).

*Effect of  $\lambda 2$  Transgene on Splenic Ig mRNAs.* To assess the total level of stable expression of Ig genes in the transgenic mice relative to their normal littermates, Northern blots of spleen RNA were hybridized with various Ig probes (Fig. 5). In 5-d-old transgenic mice there is a high level of  $\lambda 2$  mRNA, whereas in normal littermates  $\lambda 2$  mRNA is barely detectable (Fig. 5 A). In all mice the quantities of  $\lambda 2$  mRNA increase many fold with age, but the relative levels are consistently more than five times higher in the transgenic than in the normal mice. Presumably, the dramatic overall increase with age is mainly derived from plasma cells, since the proportion of surface  $\mu^+$  cells in the spleen increases only about two- to threefold (Table I).

The amounts of  $\kappa$  and  $\lambda 1$  mRNA are suppressed by  $\sim 50\%$  in both newborn and adult transgenic mice (Fig. 5, B and C), while  $\mu$  mRNA appears to be only slightly diminished in the 16-d and adult transgenic spleens and unaffected in the 5-d-old spleens (Fig. 5 D). Interestingly, the newborn spleens of both the transgenic and the normal mice show mainly the membrane form of  $\mu$ RNA, whereas in the adults the secreted form predominates. This further supports the idea that the overall increase of Ig mRNA in the adult mice is derived from plasma cells.

Tubulin mRNA was used to normalize RNA quantities in the spleen. Unexpectedly, tubulin mRNA in spleen decreased with age, presumably reflecting a decrease in proliferative activity (Fig. 5 E). The relative levels are approximately the same in transgenic mice as in their normal littermates, suggesting that the overall cellular development is not affected by the transgene.

*Hybridomas from  $\lambda 2$  Transgenic Mice.* To determine whether the suppression of endogenous  $\kappa$  and  $\lambda$  synthesis was due to inhibition of rearrangement of the respective genes, hybridomas were produced from 4-wk-old transgenic mice. We obtained a total of 83 hybridomas representing single cell clones. Of these, 34 (41%) secreted

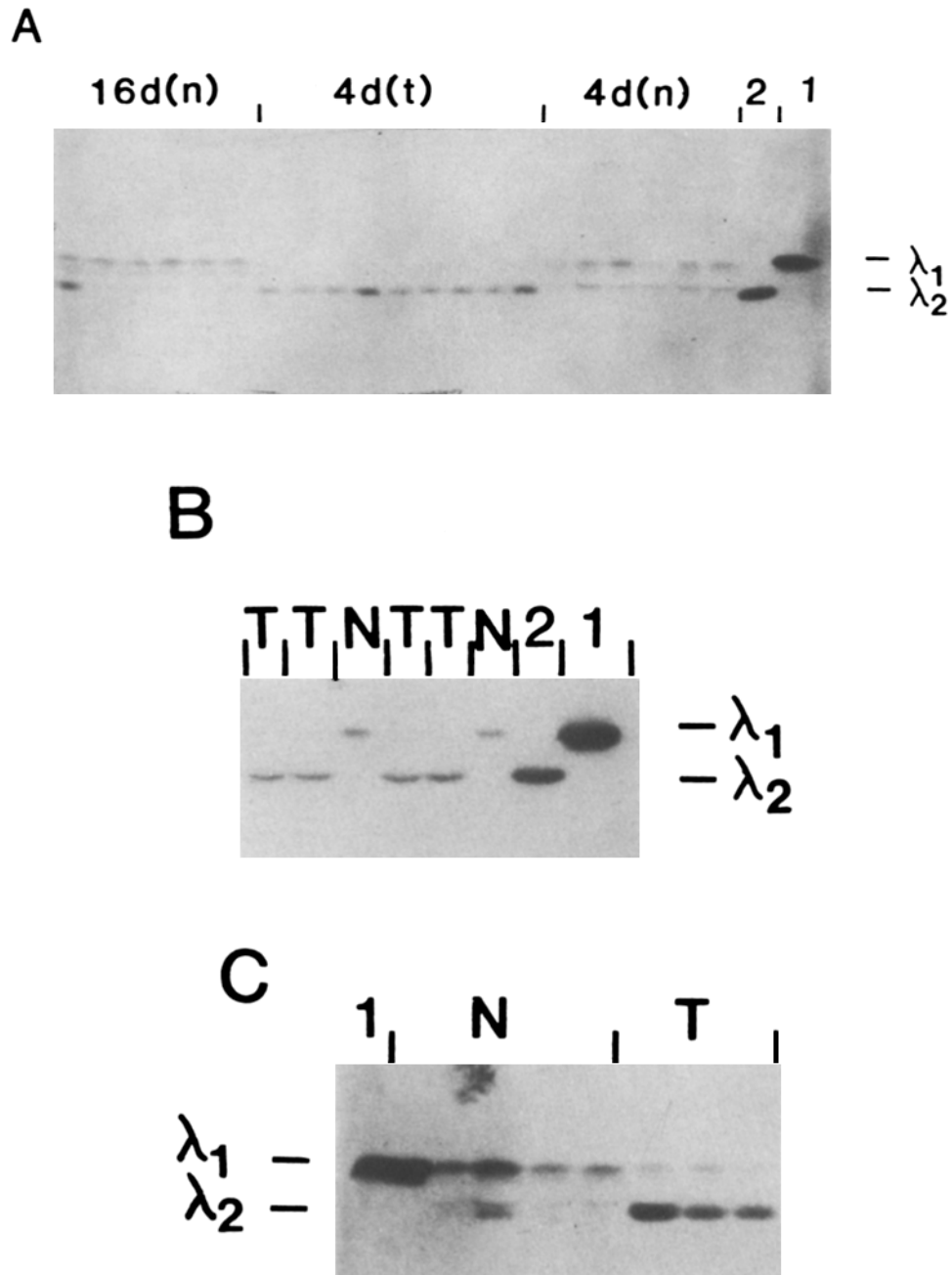


FIGURE 4. Western blot analysis of serum  $\lambda$  chains. (A) Sera from a litter of 16-d and two litters of 4-d-old mice are shown. The status of the mother is shown in parenthesis: *n*, normal C57BL/6 female; *t*,  $\lambda$ 2 transgenic female. Within the litters the following mice were  $\lambda$ 2 transgenic (from left to right): 16d(*n*) lanes 1, 3, 5, and 6; 4d(*t*) lanes 1, 2, 4, 6, and 9; and 4d(*n*) lanes 2, 4, 5, and 6. (B) Sera from a litter of 5-wk-old mice and (C) sera from a litter of 10-wk-old mice. *T*, transgenic; *N*, normal littermate. Lanes 1 and 2 contain 0.5  $\mu$ g of purified myeloma protein IgM/ $\lambda$ 1 (MOPC 104E) and IgM: $\lambda$ 2 (pmG11-185), respectively.



TABLE II  
Quantitation of Serum Igs

Age	Mouse	IgM						IgG2b
		$\lambda 1 + \lambda 2 + \lambda 3$	$\lambda 1$	Percent $\lambda 1^*$	$\kappa$	Total IgM	Percent $\lambda^\dagger$	$\kappa$
		$\mu\text{g/ml}$	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$	$\mu\text{g/ml}$	%	$\mu\text{g/ml}$
16 d	T 2	5.4	0.19	3.5	4.9	10.3	52	ND
	T 3	9.6	0.18	1.9	5.2	14.8	65	ND
	T 5	3.4	0.16	4.7	5.8	9.2	37	ND
	T 7	13.1	0.18	1.4	6.0	19.1	69	ND
	NLM 4	<0.4	0.22	~100	16.8	17.0	1	ND
	NLM 6	<0.4	0.33	~100	25	25.3	1	ND
	T/N	>20	0.64		0.26	0.63		
4 wk	T56 <sup>§</sup>	135	5.2	4	106	241	56	168
	T57 <sup>§</sup>	50	3.5	7	55	105	48	62
	NLM 60 <sup>§</sup>	13.1	9.9	75	109	122.1	11	218
	NLM 58 <sup>§</sup>	26.5	15.1	57	353	379.5	7	179
	NLM 55	14.8	8.8	59	74	88.8	17	104
	T/N	3.9	0.39		0.45	0.88		0.69
10 wk	T 18	30.4	0.55	2	80	110	28	176
	T 19	38.8	0.57	2	102	141	28	310
	T 22	38.8	0.57	2	79	118	33	392
	NLM 23	14.2	10.2	72	97	111	13	713
	N 5	27.6	15.9	58	210	238	12	254
	N 6	28.7	21.2	74	164	193	15	618
	N 7	25.9	19	73	149	175	15	296
	T/N	1.5	0.03		0.56	0.69		0.62

Serum levels were quantitated in an ELISA using goat anti-mouse H chain-specific antisera as the solid phase antibody. The assay was then developed with the appropriate anti-mouse L chain reagents as described in Materials and Methods. T, transgenic mice; NLM, normal littermate; N, age-matched normal C57 BL/6 mouse; T/N, average of transgenic sample/average of normal sample.

\* Percent  $\lambda 1 = [\lambda 1/(\lambda 1 + \lambda 2 + \lambda 3)] \times 100$ .

† Percent IgM $\lambda = [(\lambda 1 + \lambda 2 + \lambda 3)/\text{total IgM}] \times 100$ .

§ Mice T56, T57, NLM 60, and NLM 58 were LPS treated 3 d before bleeding.

$\lambda$  only; 34 (41%) secreted both  $\lambda$  and  $\kappa$ ; 9 (11%) secreted  $\kappa$  only; and 6 (7%) secreted no Ig. A subset of these was analyzed in greater detail (Table III). 39 of the 51 hybridomas examined secreted  $\lambda$  protein that was apparently derived from the transgenes in all but one case. The hybridoma No. 69 had deleted the transgene before the first assay, but the cells secreted  $\lambda$  chains due to rearrangement of endogenous  $\lambda$  genes. Three other lines (Nos. 2, 5, and 70) lost the transgenes and ceased production of  $\lambda$  chains during culture. The 14 hybridomas that did not secrete  $\lambda$  all had lost the transgenes at the time of the first testing. Thus, of the hybridomas that retained the transgenes, there is not a single one in which the transgene is inactive, in striking contrast to the observations by Weaver et al. (29), that ~75% of their transgenic hybridomas did not express the  $\mu$  transgene.

More than half of the hybridomas (26:51; 52%) do not show any endogenous  $\kappa$  or  $\lambda$  gene rearrangements. This is in contrast to normal B cells in which at least one  $\kappa$  allele is rearranged (11-13), suggesting that the  $\lambda 2$  transgene has a feedback effect on the Ig-specific recombinase activity. In previous work with  $\kappa$  transgenic

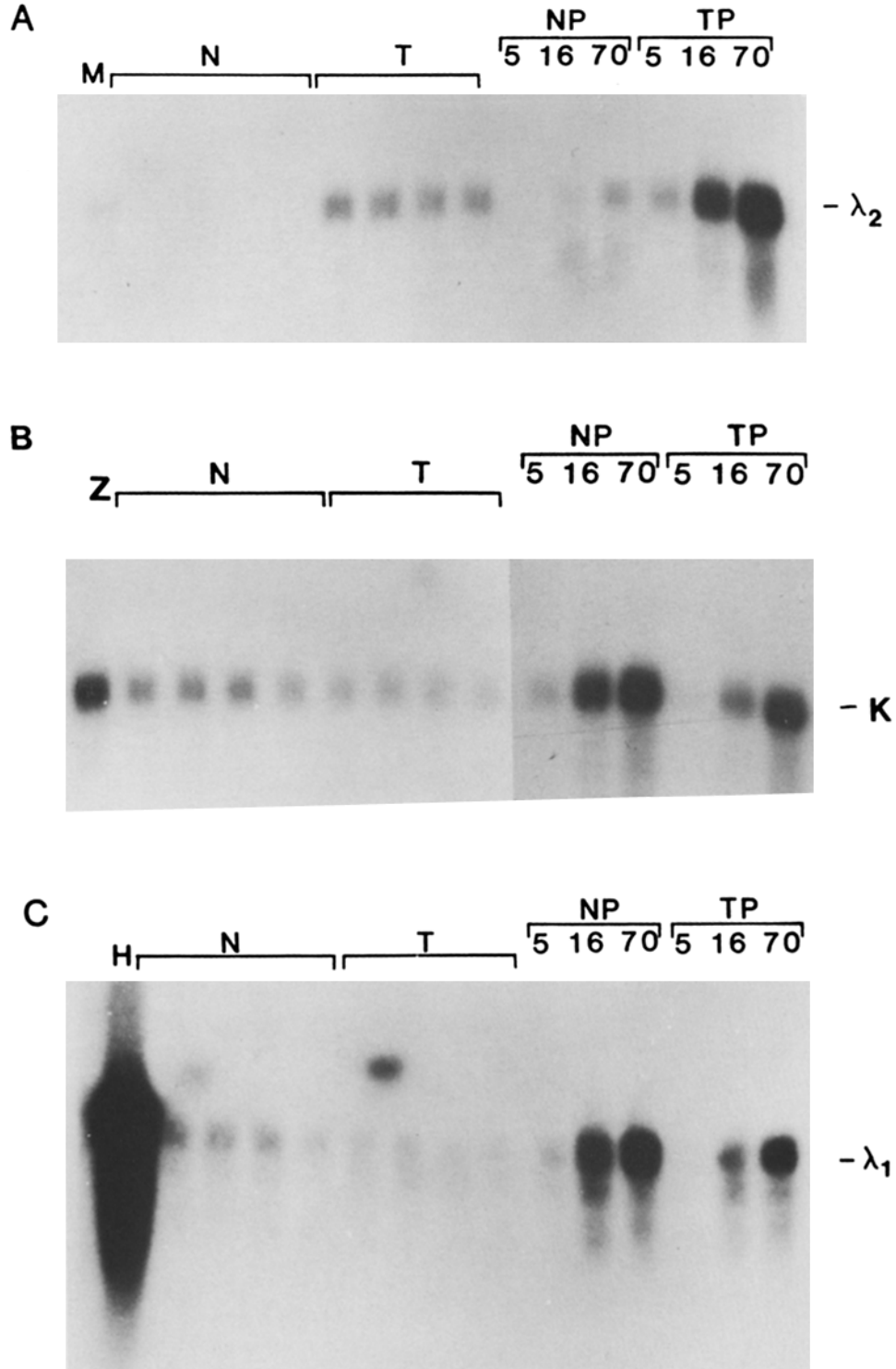


FIGURE 5. Continued on following page.

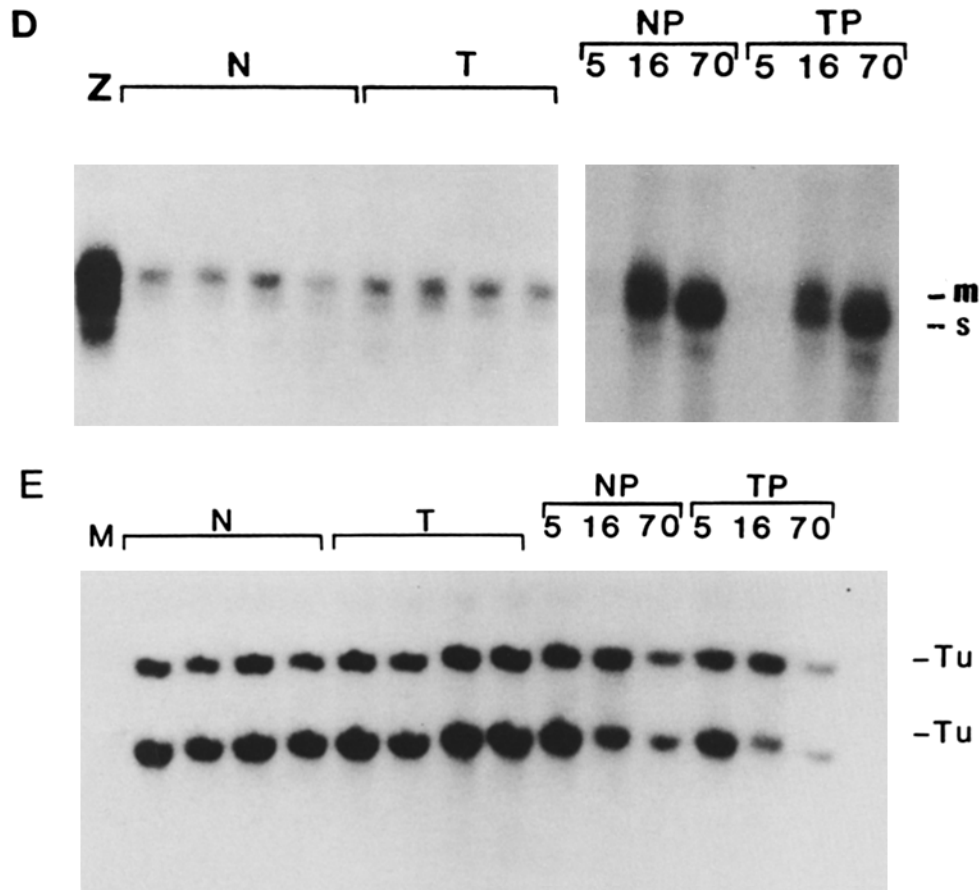


FIGURE 5. Northern blots of spleen RNAs. Each lane contained 5  $\mu$ g spleen RNA except the left lane, which contained 1  $\mu$ g of RNA of myeloma MOPC315 (American Type Culture Collection, Rockville, MD) (*M*), or H12.1.1a, a  $\lambda$ 1-producing IgM transfectant of J558L (*H*), or 3  $\mu$ g of RNA from LPS-induced pre-B cell line 70Z-3.12 (American Type Culture Collection) (*Z*). Lanes 2-9 are from spleens of 5-d-old transgenic mice (*T*) or their normal littermates (*N*). The six lanes on the right are pools of equal quantities of RNA from two to eight spleens of transgenic mice (*TP*) or normal littermates (*NP*) of 5, 16, or 70 d of age. The position of the mature form of the respective mRNAs is indicated on the right. In the case of  $\mu$ , the membrane (*m*) and secreted (*s*) forms are indicated. The blots were probed with (*A*) C $\lambda$ 2; (*B*) C $\kappa$ ; (*C*) C $\lambda$ 1; (*D*) C $\mu$ ; and (*E*) murine  $\beta$  5 tubulin.

mice, it was found that feedback inhibition of endogenous  $\kappa$  gene rearrangement only occurred in cells that produced an endogenous H chain in addition to the transgenic  $\kappa$  chain (5). In the  $\lambda$ 2 hybridomas investigated here, 11:26 of those that do not show rearrangement of endogenous L chain genes do not secrete an H chain. We presume that they lack the productive H gene allele due to chromosome loss during or after fusion, because none show evidence of more than one H gene. 12 of the 26 hybridomas (46%) without endogenous L gene rearrangement also have retained a germline H allele, suggesting perhaps that a complete shutoff of the Ig-specific recombinase had occurred. Curiously, 5:20 (25%) of the hybridomas with

TABLE III  
*Hybridomas of Spleen of 4-wk-old Transgenic Mice*

Hybridoma	ELISA*	Transgene <sup>†</sup>	κ rearr <sup>§</sup>	λ rearr <sup>  </sup>	H rearr <sup>¶</sup>
<b>λ secretors</b>					
1	i	+	-	-	gi
2	i,**M	**	-	-	VDJ
5	i**	+	-	-	gi
7	i	+	-	-	gi
12	i,[M]	+	-	-	gi
13	i	+	-	-	gi
15	i,M	+	-	-	DJ,VDJ
17	i,[G]	+	-	-	gi
27	i,G	+	-	-	gi
34	i,[G]	+	re	V <sub>1</sub> J <sub>1</sub> ,V <sub>1</sub> J <sub>3</sub> ([λ1])	VDJ
37	i	+	-	-	del
40	i	+	-	-	gi
42	i	+	re <sup>††</sup>	V <sub>2</sub> J <sub>2</sub>	gi,VDJ
43	i,M	+	-	-	DJ,VDJ
45	i	+	del	-	del
48	i,M	+	-	-	DJ,VDJ
51	i	+	-	-	del
53	i,M	+	-	-	VDJ
55	i,[G]	+	-	-	gi
57	i	+	-	-	gi
59	i,M	+	-	-	DJ,VDJ
61	i,M	+	-	-	VDJ,VDJ
67	i,[G]	+	-	-	del
69	i,M	-	re	V <sub>2</sub> J <sub>2</sub>	VDJ,VDJ
70	i**	**	-	-	gi
73	i,[G]	+	-	-	DJ
80	i,[G]	+	-	-	del
82	i	+	del	-	DJ
86	i,M	+	re <sup>††</sup>	V <sub>2</sub> J <sub>2</sub>	VDJ,VDJ
87	i,M	+	-	-	VDJ
<b>Double secretors</b>					
4	d,G	+	re	-	gi,DJ
6	d,G	+	re,gi	-	gi,VDJ
33	d,G	+	re,gi	-	gi,VDJ
38	d,M	+	re	-	VDJ
56	d,M	+	re	-	VDJ
60	d,M	+	re	-	DJ,VDJ
65	d,M	+	re,gi	-	DJ,VDJ
79	d,M	+	re,re	-	gi,VDJ

*continued*

κ or λ rearrangements also have retained a germline gene, which may suggest that normally H gene rearrangement is ongoing at the time of κ and/or λ rearrangement.

Approximately one third (19:51; 37%) of the hybridomas have rearrangements of endogenous κ genes. Five of these also rearranged their λ genes. Many of the transgene-containing hybridomas with endogenous L gene rearrangement do produce H chains,

TABLE III (continued)  
Hybridomas of Spleen of 4-wk-old Transgenic Mice

Hybridoma	ELISA*	Transgene†	$\kappa$ rearr <sup>§</sup>	$\lambda$ rearr <sup>  </sup>	H rearr <sup>¶</sup>
$\kappa$ secretors					
8	k,M	-	re,gi	-	VDJ
26	k	-	re,gi	-	del
44	k,G	-	re	-	del
49	k,M	-	re,gi	V <sub>1</sub> J <sub>3</sub>	VDJ
58	k,M	-	re,gi	-	VDJ
64	k,G	-	re	-	DJ
88	k,M	-	re,re	-	VDJ,VDJ
No Ig					
19	-	-	del	V <sub>2</sub> J <sub>2</sub>	del
21	-	-	-	-	del
24	-	-	-	-	gi
39	-	-	del	-	DJ
62	-	-	del	-	del
75	-	-	del	-	del
51					

Hybridomas were made from the spleen of a transgenic sibling of the mice whose FACS profile is shown in Figure 3; this mouse is T57 in Table II. After fusion the cells were plated very dilute and only wells that microscopically showed a single clone were further analyzed. [G],[M], low level  $\gamma,\mu$ ; ([ $\lambda$ 1]),  $\lambda$ 1 protein secreted.

\* ELISA of the hybridoma secretions showed  $\lambda$ (l),  $\kappa$  and  $\lambda$ (d),  $\kappa$ (k),  $\mu$ (M), and  $\gamma$ (G) proteins. These were assayed as soon as cell clones were visible in the wells.

† Presence of the  $\gamma$ 2 transgene was determined by Southern blot.

§ Rearrangement of endogenous  $\kappa$  genes was determined from Southern blots of Bam H1-digested DNA probed first with a C $\kappa$  probe and after stripping reprobed with a 5' of J $\kappa$ 1 probe ( $\times 2.1$ ; reference 7). -, Only germline  $\kappa$  genes were seen; del,  $\kappa$  genes (or chromosome deleted); re, rearranged; gi, germline.

|| Rearrangement of endogenous  $\lambda$  genes was determined from Southern blots of Eco R1-digested DNA probed with a V $\lambda$ 2 probe that crosshybridizes with V $\lambda$ 1 (40). V<sub>1</sub>J<sub>1</sub>, V<sub>1</sub>J<sub>3</sub>, and V<sub>2</sub>J<sub>2</sub> rearrangements result in 7.8-, 3.1-, and 6.5-kb fragments, respectively; -, endogenous  $\lambda$  genes are in germline configuration.

¶ Rearrangement of endogenous H genes was determined from Southern blots of DNA digested with Bam H1 and separately with Eco R1 and probed sequentially with 5'DH and JH4. gi, germline (unrearranged) H genes are present. In cases in which no rearrangements are seen, we do not know if both alleles were unrearranged, or the other allele was lost in the hybridoma, or the other allele was a VDJ rearrangement that overlaps with the restriction fragments of the fusing line, Ag8.653. DJ, rearrangement seen with the JH4 probe, and one to five fragments present that hybridize with 5'DH. The fusing line does not retain DNA hybridizing with 5'DH. VDJ, rearranged band seen with J4 probe, but no hybridization with the 5'D probe. Hybridomas indicated to be VDJ/VDJ show two rearranged JH4 bands. DJ/VDJ, two rearranged bands with JH4, one to five bands hybridizing with 5'DH and production of H chains. del, deletion, no evidence of H genes except in most cases the genes of the fusing line are present.

\*\* These wells were originally secreting  $\lambda$  and later became negative, apparently because of loss of the transgenes.

\*\* No rearrangement visible in Southern blot, but  $\kappa$  mRNA seen in Northern blot.

raising the possibility that they have escaped feedback inhibition despite the presence of a complete Ig molecule.

The  $\lambda$ 2 chains encoded by the transgene can be secreted in the absence of H chains as demonstrated by those lines (Nos. 37, 45, and 51) that have deleted the H genes, but still secrete  $\lambda$ .

## Discussion

In accordance with the previously demonstrated lymphoid cell specificity of the H chain enhancer (27), the  $\lambda$ 2 transgene appears to be expressed in a tissue-specific manner. Presumably, expression in the B cell lineage begins early in B cell development when endogenous H genes become active and begin to rearrange and before endogenous L chain loci are similarly activated. The presence of the H enhancer is essential for proper expression, because another transgenic mouse line with the same  $\lambda$ 2 gene without the H enhancer, but with an additional 4.0 kb 3', containing C $\lambda$ 4 and 1 kb 3' of C $\lambda$ 4, did not produce significant transgenic  $\lambda$ 2 mRNA in the spleen (unpublished observations).

The presence of the  $\lambda$ 2 transgene results in a reduction of Ig gene rearrangement at both H and L chain loci in most cells. The effect on  $\kappa$  genes is very similar to that of a  $\kappa$  transgene (5) and, presumably, results from a complete shutoff of the Ig-specific recombinase when a productive H chain combines with the transgenic  $\lambda$ 2. Thus, the  $\lambda$  protein can replace  $\kappa$  in the membrane Ig complex, which results in feedback.

Inhibition of endogenous  $\lambda$  production may suggest that  $\lambda$ -producing cells are subject to feedback inhibition of Ig gene rearrangement. This is in contrast to the finding in  $\kappa$  transgenic mice of normal numbers of  $\lambda$  cells and normal quantities of serum  $\lambda$  protein (19). The suppression of  $\lambda$ 1 synthesis is more pronounced in the  $\lambda$ 2 transgenic mice than in the  $\kappa$  transgenic mice; it is likely that the  $\lambda$ 2 protein can replace  $\lambda$ 1 in the response to many antigens where  $\lambda$  is utilized, and thus,  $\lambda$ 1 cells are not antigenically selected for expansion in the  $\lambda$ 2 transgenic mice. The protein sequences of the V regions of  $\lambda$ 1 and  $\lambda$ 2 are 93% homologous (10). In  $\kappa$  transgenic mice, on the other hand,  $\lambda$  production from an endogenous gene may be essential. It is noteworthy that of the hybridomas examined, 12% had rearranged endogenous  $\lambda$  genes (Table III). This proportion is higher than expected, based on the very reduced number of hybridomas with  $\kappa$  gene rearrangement. The proportion of cells with  $\lambda$  rearrangement is at least as high as in normal mice; there, only 3-5% of B cells produce  $\lambda$  (10), and since  $\kappa$ -producing B cell lines almost never have  $\lambda$  rearrangements (11-13), only ~3-5% of B cells would have  $\lambda$  genes rearranged. Furthermore, while the serum  $\lambda$ 1, reflecting plasma cell numbers, remains greatly depressed in adult mice compared with  $\kappa$ ,  $\lambda$ 1 mRNA levels recover similarly to  $\kappa$ . Presumably, B cells with nonproductive  $\lambda$  rearrangements produce the mRNA.

Normally, spleen B cells and hybridomas derived from them have both H gene alleles rearranged (30, 31). However, 35% of the hybridomas from the  $\lambda$ 2 transgenic mice have a germline H allele (Table III). This presumably is due to shutoff of the recombination process as soon as a productive H gene rearrangement results in a  $\lambda$ 2-containing H/ $\lambda$  Ig molecule. Similarly, germline H genes were found in hybridomas of  $\kappa$  transgenic mice, although in a smaller proportion of the clones analyzed (5, 7), presumably because the  $\kappa$  transgenes under the control of the  $\kappa$  enhancer were expressed later in B cell development than the  $\lambda$ 2 transgenes under the control of the H chain enhancer. Based on these observations, the postulated feedback by H chain alone on H gene rearrangement (4) must be much slower or less efficient than the shutoff of rearrangement induced by the complete Ig molecule. The results are compatible with the idea that H gene feedback may reduce the accessibility of V<sub>H</sub> genes for rearrangement (32), but that DJ joining would not be affected. Thus, in

pre-B cells from nontransgenic mice, DJ rearrangement may continue during L chain gene rearrangement, resulting in B cells in which the second allele is in the DJ state. The  $\lambda 2$  transgenic hybridomas suggest that frequently the rearrangement activity of one H chain allele greatly lags behind that of the other, since a large proportion have one germline H allele, while the other allele is a VDJ rearrangement (present or presumed to be lost after cell fusion). This lagging of rearrangement of one allele may also occur in B cells from nontransgenic mice, but perhaps because DJ rearrangement continues during L chain gene rearrangement, and because the chance for a functional L chain gene rearrangement is smaller than that for DJ joining, almost all B cells retain no germline H genes.

Selection of cells expressing endogenous  $\kappa$  genes is apparently a strong modulator of B cell distribution in the older  $\lambda 2$  transgenic mice. Presumably, very rare cells can be amplified by antigen-driven selection. It is apparent that the outgrowth of  $\kappa$ -expressing cells has occurred by as early as 2 wk after birth (Table I), at a time when maternal Ig is still present in the pup's serum in a high concentration (Fig. 4 A). The relative roles of antigen, antiidiotype responses, and other factors needs to be determined.

There may be several ways in which cells can escape the suppression of L chain gene rearrangement. First, the feedback by a complete Ig molecule after productive H gene rearrangement may not be instantaneous. Thus, during a certain time window, sufficient recombinase may persist to allow L chain gene rearrangement. This period may be very brief in the  $\lambda 2$  transgenic mice; if H chains are required for the activation of the L chain loci (33), then in  $\lambda 2$  transgenic cells, the signal for L chain gene rearrangement (H chain) and for inactivation of the recombinase (H chain plus  $\lambda 2$ ) would appear simultaneously. If all B cells that coexpress  $\kappa$  and transgenic  $\lambda$  arise during this interval, it would require that the signal for L chain gene activation is more efficient than that for termination of recombinase activity.

Second,  $\kappa$  and  $\lambda$  coexpressing cells may be members of a B cell population that is not strictly feedback inhibited by membrane Ig molecules during pre-B cell development ("unregulated" lineage), such as postulated previously for  $\lambda$ -producing cells and Ly1 B cells (19, 34, 35). A priori, those  $\lambda 2$  transgenic hybridomas that have germline H genes should not be derived from a feedback uninhibited B cell population. However, all B cells seem to be recombinase negative in the mature stage (36), and selection by antigen or antiidiotype may be the signal that inhibits the recombinase in the postulated unregulated B cell lineage. This signal may be generated early in transgenic B cell development, if the particular cell is selected by antigen or other stimuli.

Third, in certain pre-B cells the levels of  $\lambda 2$  protein may be too low for a feedback effect. Certainly, at the B cell stage there seems to be a broad range in surface  $\lambda 2$  expression. Most of the weakly  $\lambda 2^+$  cells are also  $\kappa^+$  (Fig. 3). It remains to be determined whether this relationship is the cause or a consequence of coexpression.

Finally, one has to consider the possibility that some pre-B cells lose the transgenes in the mouse due to intrachromosomal recombination (37), e.g., between sister chromatids. This could lead to an almost complete elimination of all copies of the transgene, e.g., if at one end of the tandem repeats a transgene fragment was integrated that could pair with a transgene copy at the other end of the complex. The absence of cells with a germline H gene among the transgene-negative hybridomas

may suggest that this event can occur sometimes (although most often the transgenes are probably lost by chromosome loss in the hybridomas). Such pre-B cells would rearrange endogenous H and L genes, like cells from normal mice.

The data presented here do not allow us to make a distinction for a given B cell between escape from feedback inhibition during a narrow window, an unregulated pre-B cell pool, low subthreshold transgene expression, or transgene deletion. The finding of relatively large numbers of cells producing endogenous L chains as early as 4 d after birth suggests that evasion of suppression can occur independently of antigen selection; at this early age in normal mice the expressed V gene repertoire does not reflect selection (38).

Incomplete feedback inhibition of Ig genes in transgenic mice continues to be a puzzle to investigators (35). The observation that  $\kappa$  and  $\lambda$  coexpressing B cells increase with the age of  $\lambda$  transgenic mice (this report, and M. Neuberger, personal communication) suggests that the apparent lack of transgene effect in many cells of transgenic mice may be due to selection and amplification of cells that have escaped the feedback. It would be interesting to re-examine  $\kappa$  and H chain transgenic mice at an earlier age.

Isotypic exclusion of  $\kappa$  and  $\lambda$  genes is still unexplained. The inactivation of  $\kappa$  genes is not (always) required for  $\lambda$  gene rearrangement since  $\lambda$ -positive cells in  $\kappa$  transgenic mice produce the transgenic  $\kappa$  and sometimes, in addition, endogenous  $\kappa$  protein (19). As discussed above, a B cell population that is not regulated by feedback inhibition at the pre-B stage may exist. Many of the endogenous  $\lambda$  gene rearranging cells in the  $\lambda 2$  (and  $\kappa$ ) transgenic mice may be recruited from such cells. It is possible that  $\lambda$  gene rearrangement can also occur in the strictly regulated cell population;  $\kappa$  genes would have to have a much greater chance than  $\lambda$  to be rearranged in these cells, as originally postulated based on the observation that virtually all  $\lambda$ -expressing cells have both  $\kappa$  loci rearranged (11-13). Since the  $\lambda 2$  transgene is under the control of the H chain enhancer, it will be expressed very early in B cell development. In a strictly feedback-regulated population, the time window may be too short to allow  $\lambda$  gene rearrangement. It will be interesting to investigate the  $\lambda 2$  mice with respect to the cell lineages (conventional or Ly1 B cells; reference 39) from which endogenous  $\lambda$ -producing cells arise.

### Summary

The rearrangement of Ig genes is known to be regulated by the production of H and  $\kappa$  L chains. To determine whether  $\lambda$  L chains have a similar effect, transgenic mice were produced with a  $\lambda 2$  gene. It was necessary to include the H chain enhancer, since a  $\lambda$  gene without the added enhancer did not result in transgene expression.

The  $\lambda 2$  transgene with the H enhancer was expressed in lymphoid cells only. The majority of the B cells of newborn transgenic mice produced  $\lambda$ , whereas  $\kappa^+$  cells were reduced. Concomitantly, serum levels of  $\kappa$  and  $\kappa$  mRNA were diminished. By 2 wk after birth the proportion of  $\kappa$ -expressing cells was dramatically increased. Adults had reduced proportions of B cells that produced  $\lambda$  only, but the levels of  $\lambda$  were still higher than in normal littermates. Also,  $\kappa^+$  cells were still lower than in normal mice. Analysis of hybridomas revealed that reduction of  $\kappa$  gene rearrangement was the basis for the decreased frequency of  $\kappa^+$  cells. Furthermore, many cells also con-



tained an unrearranged H chain allele. It was concluded that feedback inhibition by the  $\lambda 2$  together with endogenous H protein may have inhibited recombinase activity in early pre-B cells, leading to inhibition of both H chain and  $\kappa$  gene rearrangement. Thus,  $\lambda 2$  can replace  $\kappa$  in a feedback complex.

The levels of serum  $\lambda 1$  and, to a lesser degree, of spleen  $\lambda 1$  mRNA were reduced in the  $\lambda 2$  transgenic mice. However, the proportion of hybridomas with endogenous  $\lambda$  gene rearrangement was at least as high as in normal mice. It was therefore concluded that the suppression of functional  $\lambda 1$  may be a consequence of decreased selection of endogenous  $\lambda$ -producing cells because of the excess of transgenic  $\lambda$ .

The escape of  $\kappa$ -producing cells from feedback inhibition may be the result of several mechanisms that operate to varying degrees, among them: (a)  $\kappa$  rearrangement during a period in which the recombinase is still active after appearance of a  $\lambda 2/\mu$  stop signal; (b) a B cell lineage that is not feedback inhibited at the pre-B cell stage; (c) subthreshold levels of transgenic  $\lambda 2$  in some pre-B cells; and (d) loss of the  $\lambda 2$  transgenes in rare pre-B cells.

We are very grateful to Karen Hagen for excellent technical assistance with flow cytometry and to our colleagues cited in the text who have provided cloned genes.

*Received for publication 18 January 1989 and in revised form 14 February 1989.*

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