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Isotype Switching in Murine Pre-B Cell Lines

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Summary

Isotype switching at the pre-B cell stage was studied by employing A-MuLV-transformed cell lines. Two γ_{2b}-producing cell lines that did not have other cytoplasmic heavy chains or light chains were established from A-MuLV-transformed cell lines. One clone (SL2-1-52) arose spontaneously from a non-Ig-producing cell line (SL2-1) during in vitro culture. Another clone (AT11-2-24-6-99) underwent isotype switching from a μ-producing cell line (AT11-2-24-6), which had been derived from another non-Ig-producing line (AT11-2). Southern blot analysis of two γ_{2b}-producing clones was performed in comparison to that of their respective parent clones. The results showed that isotype switching can operate at the stage of pre-B cells by a C_H gene deletion mechanism without utilizing the switch region. In addition, the possibility is presented that deletion of the intervening C_H genes could occur prior to the formation of the functional V region-coding DNA segment. This indicates that the prior expression of μ chains is not obligatory for the expression of other isotypes and that isotype commitment could occur in pre-B cells.

Introduction

Differentiation of B cells is accompanied by two distinct types of DNA rearrangement—variable region formation and isotype switching (Sakano et al., 1980; Davis et al., 1980; Kataoka et al., 1980). Hematopoietic stem cells differentiate initially into pre-B cells that produce cytoplasmic μ chains but not light chains (Levitt and Cooper, 1980). This rearrangement is a joining of V_H, D, and J_H gene segments. Subsequently, pre-B cells mature into B cells expressing surface IgM molecules. During this process, V_L and J_L gene segments join to form a complete L chain gene. Then B cells differentiate into immunoglobulin secreting cells under the influence of antigens and T cells (Kishimoto et al., 1980). During this latter process, some of IgM-expressing B cells show isotype-switching and produce IgG, IgA, or IgE. The isotype-switching is accompanied by one or more DNA rearrangements which bring the expressed C_H gene into the vicinity of the V_H-D-J_H gene complex and delete all of the intervening C_H genes. The aforementioned series of events, which occurs at the DNA level, has been deduced primarily by comparing the structure of relevant Ig genes present in non-Ig-producing cells such as embryo and liver, and myelomas. To date, only a few investigators (Eckhardt et al., 1982; Sablitzku et al., 1982) have observed in vitro heavy chain class switches in myelomas or hybridomas and performed comparative analyses of gene structures of the precursor and its progeny. Thus it is uncertain whether the model proposed from the analysis of myelomas can truly reflect the events occurring during normal B cell development, although there is a study suggesting the deletion mechanism of isotype-switching and the rearrangement between J_H and C_μ regions in normal plasmablasts after LPS stimulation (Hurwitz and Cebra, 1982). Furthermore, it would be difficult to elucidate early events in B cell differentiation from this type of analysis.

At present, in spite of great efforts, nontransformed B cell lines (Howard et al., 1981; Sredni et al., 1981) are not available. Therefore transformed B cell lines representing early stages of differentiation provide the most suitable material available. Baltimore and his colleagues have established Abelson murine leukemia virus (A-MuLV) transformed cell lines (Siden et al., 1979, Alt et al., 1981). Some of these lines are capable of carrying out κ gene rearrangement and κ chain production during in vitro culture (Lewis et al., 1982) and one line undergoes isotype switching during in vitro culture. (Alt et al., 1982). Recently we also established a large number of A-MuLV-transformed cell lines that had characteristics of immature B precursor cells. This paper deals with two clones which produce intracytoplasmic γ_{2b} chains: one clone (SL2-1-52) arose spontaneously from a non-Ig-producing cell line during culture. The second clone (AT11-2-24-6-99) underwent isotype-switching from a μ-producing cell line (AT11-2-24-6), which had been derived from another non-Ig-producing line (AT11-2). The comparison of gene structures of the precursors and the γ_{2b} producers shows that the expression of γ_{2b} chains can occur by a C_H gene deletion mechanism at the stage of pre-B cell, and that this deletion does not involve the S (switch) region sequences used in the isotype switch that occurs in a later stage of B cell development.

Results

Two Independent A-MuLV-transformed Cell Lines Underwent Heavy Chain Class Switch during In Vitro Culture

A large number of A-MuLV-transformed cell lines were established by in vivo infection of BALB/c and CBA/N mice, as described by Sugiyama et al. (1982). These cell lines were examined for the heavy and light chain production by immunofluorescence. These analyses led to the identification of two independent cases of isotype switch ocurring during in vitro culture. One clone of a newborn CBA/N mouse origin, SL2-1, initially did not produce any detectable intracytoplasmic Ig, but 0.1% to 0.2% of the cells became stained with the FITC-conjugated anti-γ_{2b} antibody after 8 weeks of in vitro culture. A one hundred

percent γ_{2b}-positive subclone of SL2-1, referred to as SL2-1-52, was isolated by repeated subcloning on soft agar plates. When examined by immunofluorescence, SL2-1-52 cells did not express any heavy chain other than γ_{2b} and as the parental line this subclone did not express any L chain. Several subclones which were scored as Ig-negative at the time of subcloning were shown to contain a small population of γ_{2b} producers at ratio of 0.1% to 0.2% upon further expansion of the cultures. These results indicate that $\mu^- \rightarrow \gamma_{2b}$ isotype switch are ongoing events in the SL2-1 cell culture. Another clone AT11-2, previously designated as B2 (Sugiyama et al., 1982), also did not initially express any detectable intracytoplasmic Ig. However, intracellular μ positive cells (0.5% to 1%) were detected after a long-term culture (approximately 4 months). Such AT11-2 culture was subjected to recloning and an intracellular μ-positive subclone (AT11-2-24-6) was obtained. The AT11-2-24-6 culture was subjected to recloning. One of the subclones, AT11-2-24-6-99, contained intracytoplasmic γ_{2b} chains in 100% of the cells. Apparently $\mu^+ \rightarrow \gamma_{2b}$ isotype switch occurs during in vitro culturing of the clone AT11-2-24-6.

The production of γ_{2b} chains by the two clones, SL2-1-52 and AT11-2-24-6-99, was confirmed by SDS–PAGE analysis. As shown in Figure 1, bands corresponding to a γ chain were observed in SL2-1-52 and AT11-2-24-6-99 but μ chain bands were not detectable. SL2-1 did not reveal any band corresponding to a μ chain or γ chain. In addition, no L chain production was observed in any of these clones.

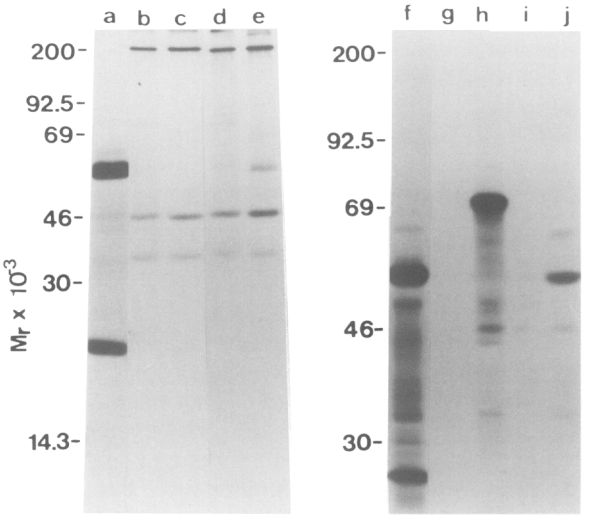


Figure 1. SDS-PAGE Analysis of Immunoglobulin Heavy or Light Chains Synthesized in Pre-B Cell Lines

Pre-B cell lines, SL2-1 (b and c), SL2-1-52 (d and e), AT11-2-24-6 (g and h), AT11-2-24-6-99 (i and j), and hybridomas secreting anti-DNP IgG_{2b} antibody, a gift from Dr. M. Ueda, Kyoto University (a and f) were biosynthetically labeled with ^{35}S-methionine and lysed. Labeled lysates were immunoprecipitated with a mixture of anti-IgG_{2b} and anti-IgM antibodies (a, c, e, f, h, and j) or with normal rabbit serum alone (b, d, g, and i). Immunoprecipitates were analyzed on 9% SDS–PAGE.

No κ Gene Rearrangement in SL2-1 and SL2-1-52 Cells

As shown in Figure 2, the Hind III/Bam HI fragment (probe a) containing C_κ was employed as a probe for the κ gene analysis. DNAs were digested with a mixture of Bam HI and Eco RI and hybridized with the C_κ probe. Analysis of the κ gene showed only the embryonic 6.5 kb C_κ-containing fragment in a non-Ig-producing parent clone (SL2-1) and a γ_{2b}-producing subclone (SL2-1-52), indicating no κ gene rearrangement in both clones (Figure 3). Bam HI digests also did not reveal any new fragments with sizes other than germline in both clones (data not shown). These results confirm that these cell lines represent pre-B cell stages. On the other hand, AT11-2-24-6 (μ-producer) and AT11-2-24-6-99 (γ_{2b}-producer) have already undergone κ gene rearrangements on both alleles (data not shown).

Parent Non-Ig-producing Clone, SL2-1, and γ_{2b}-producing Clone, SL2-1-52, Carry One Copy each of γ_1, γ_3, and δ Genes

As SL2-1-52 cells synthesized a γ_{2b} chain, DNA rearrangement in the 5′ flanking region of the γ_{2b} gene was expected. DNA from SL2-1-52 cells was digested with Eco RI or Bgl I and Southern blot analysis was carried out using the 4.0 kb Xba I/Hha I fragment (probe h in Figure 2) of the γ_{2b} gene as a probe (Figure 4A). As this probe cross-hybridized with the γ_{2a} gene, Eco RI-digested DNA of CBA/N liver showed two major hybridization bands of 6.8 and 23 kb, which corresponded to the fragments containing the γ_{2b} and γ_{2a} genes respectively (Rabbitts et al., 1980). Bgl I digests gave rise to two fragments of 7.6 and 17 kb in CBA/N liver DNA. The 7.6 kb fragment corresponded to the fragment spanning the region from the Bgl I site upstream of $S_{\gamma 2b}$ to the hinge exon Bgl I site of the $C_{\gamma 2b}$. As shown in Figure 4A, DNAs of parental nonproducer (SL2-1) and γ_{2b} producer (SL2-1-52) did not produce any new hybridization bands, suggesting that the 5′ region of the γ_{2b} gene including $S_{\gamma 2b}$ is unaltered in these DNAs.

Since the expressed gene might differ extensively upstream of the γ_{2b} gene, further analysis regarding deletion of the γ_1, γ_3, and δ genes was performed. As shown in Figure 4A, Southern blot analysis dealing with the γ_1, γ_3, and δ genes and their flanking sequences showed the germline configuration but the intensity of bands from nonproducer or γ_{2b} producer was markedly reduced as compared with that of liver DNA, suggesting that one copy each of the δ, γ_3, and γ_1 genes was deleted in both clones. The deletion of one copy of the γ_1 gene was confirmed by quantitative analysis. Using the C_κ genes as an internal control, Hind III/Eco RI-digested DNAs (2, 3, 5, 7 μg per lane) were hybridized with a mixture of the γ_1 probe and the C_κ probe. This hybridization yielded two fragments of 6.6 kb and 4.5 kb, which corresponded to a fragment containing the $C_{\gamma 1}$ gene and a fragment containing the C_κ gene respectively. The ratio of the intensity of the γ_1 gene band to the C_κ gene band as determined by

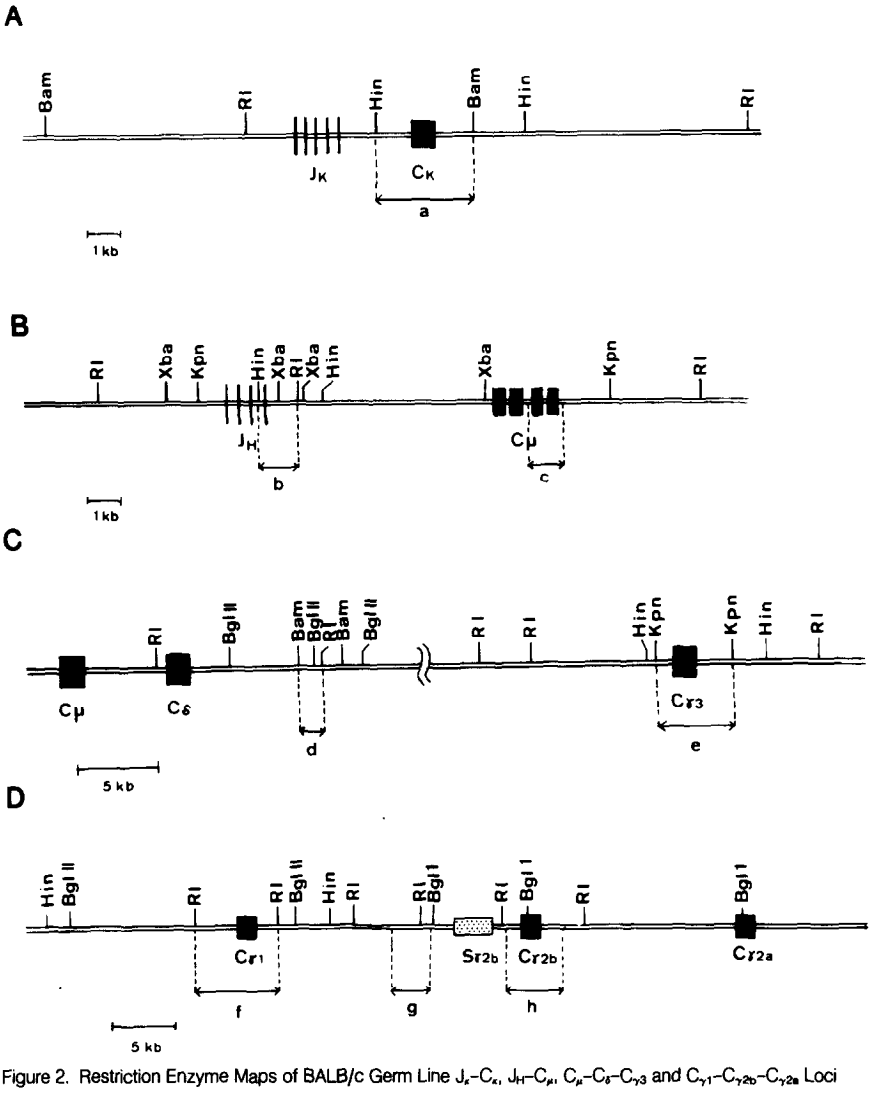


Figure 2. Restriction Enzyme Maps of BALB/c Germ Line J_κ–C_κ, J_H–C_μ, C_μ–C_δ–$C_{\gamma 3}$ and $C_{\gamma 1}$–$C_{\gamma 2b}$–$C_{\gamma 2a}$ Loci

Fragments used as probes for analyses of κ gene (a), J_H region (b), μ gene (c), δ gene (d), γ_3 gene (e), γ_1 gene (f), IVS between $C_{\gamma 1}$ and $C_{\gamma 2b}$ (g), and γ_{2b} gene (h) are indicated below the maps. Bam, Hin, Kpn, RI, Xba are Bam HI, Hind III, Kpn I, Eco RI, and Xba I respectively. Only restriction sites related to the results in this study are shown in these maps. Although SL2-1 series were of CBA/N mouse origin, maps by analysis of total genomic DNA from CBA/N liver showed that CBA/N liver germline map was almost identical to that of BALB/c.

densitometer tracings was compared among these three DNAs. The mean γ_1/C_κ ratio in SL2-1 and SL2-1-52 DNAs was 56% and 54% of that in liver DNA respectively (Figure 5A and 5C). These data indicate that one copy each of the δ, γ_3, and γ_1 genes is deleted in both clones.

The Functional V Region Formation on the C_μ-deleted Chromosome of Nonproducer Gave Rise to the γ_{2b} Producer in SL2-1 Series

To examine whether there were any changes between DNAs from nonproducer and γ_{2b} producer, we performed Southern blot analysis of the J_H and C_μ genes. J_H gene rearrangement was studied by employing the 1.5 kb J_H Hind III/Eco RI fragment (probe b in Figure 2) as a probe. Eco RI, Xba I, or Kpn I was used to digest DNAs from SL2-1, SL2-1-52, and CBA/N liver. Eco RI and Kpn I digests of liver DNA produced 6.6 kb and 14 kb fragments respectively. As Xba I cut within the J_H probe, the Xba I digest of liver DNA gave two bands, 0.9 kb and 3.8 kb and the rearrangement that utilized one of the four J_Hs would alter the size of the 3.8 kb fragment. As shown in Figure 6A, J_H rearrangements occurred in both heavy chain alleles in nonproducer (SL2-1) and γ_{2b} producer (SL2-1-52). In addition, in the cases of Eco RI and Xba I digestions, one fragment in each enzyme digest was common (3.1 kb, and 11.5 kb for Eco RI and Xba I respectively) between the two cell lines, while another differed in size. In the case of Kpn I digestion, DNA from γ_{2b} producer, SL2-1-52, showed a single 25 kb band, while DNA from nonproducer, SL2-1, showed two bands, 25 kb and 12 kb. The intensity

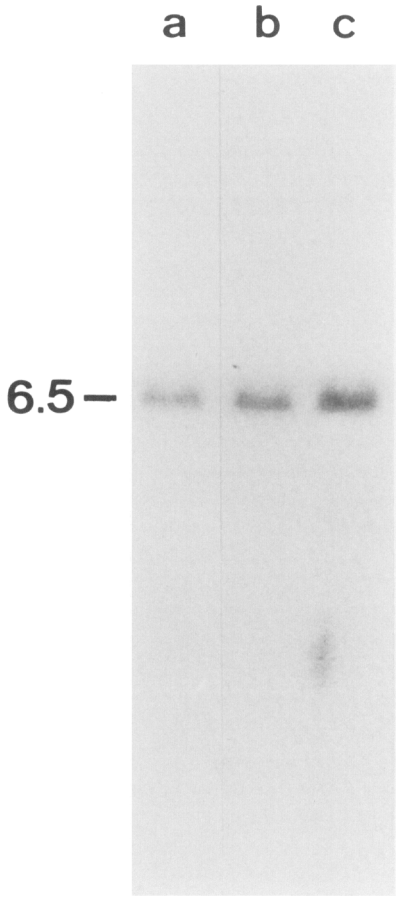


Figure 3. κ Gene Status of DNA from CBA/N Liver (lane a), SL2-1 (lane b), and SL2-1-52 (lane c)

DNAs were digested with Bam HI/Eco RI and hybridized with the C_κ probe.

of the 25 kb band in the Kpn I digest from SL2-1-52 DNA suggested that two fragments with a similar size moved to the same position. These data showed that one chromosome was shared between two cell lines, while another was different, suggesting that further rearrangement including the J_H segment was required for the expression of the γ_{2b} chain.

The status of C_μ genes was also investigated. DNAs digested with Kpn I were assayed with the C_μ probe (probe c in Figure 2). As shown in Figure 6A, the C_μ and J_H probes showed an identical pattern of hybridization of a 14 kb Kpn I fragment in embryonic DNA, as its fragment carried both C_μ and the entire J_H gene locus. These two clones, however, showed a single 25 kb fragment with the C_μ probe. The result indicated deletion of the C_μ gene from one chromosome in both subclones. In addition, the mobility of this fragment coincided with that of the fragment which had not undergone the further rearrangement including J_H segments. When taken together, all of these results suggests that the further rearrangement within the J_H gene cluster upon the Ig^- to γ_{2b}^+ transition occurred in the C_μ-deleted chromosome.

To further confirm that the expressed J_H gene segment is assigned to the chromosome accompanied with the further J_H-associated rearrangement, γ_{2b}-producing hybrid clones were obtained by fusion of SL2-1-52 with a nonsecreting myeloma cells, P_3U_1. Most of them were unstable and the percentage of γ_{2b}-producing cells in several clones decreased during in vitro culture. DNAs from several clones containing γ_{2b}-producing cells at different percentages were digested with Eco RI and hybridized to the J_H probe. As shown in Figure 7, clones containing 100% γ_{2b}-producing cells revealed three fragments of 6.6, 3.1, and 2.6 kb, of which the latter two fragments were derived from SL2-1-52 and the other from P_3U_1. The 2.6-kb fragment decreased in its intensity in proportion to the decrease of the percentage of γ_{2b}-producing cells, and γ_{2b}-negative clones lost the 2.6 kb fragment completely. The result suggests that the 2.6 kb Eco RI fragment which is absent in the non-Ig-producing parent clone SL2-1 and therefore must have arisen by a further J_H-associated rearrangement, is responsible for the expression of the γ_{2b} chain in clone SL2-1-52.

To know the nature of the secondary J_H-associated rearrangement in γ_{2b} producer, SL2-1-52, further Southern blot analysis was carried out. Since germline DNA bears a Bgl II site between J_1 and J_2, a Bam HI site between J_2 and J_3, and a Hind III site between J_3 and J_4, double digests (Bgl II/Eco RI, Bam HI/Eco RI, and Hind III/Eco RI) and Hind III digests were hybridized with the J_H probe to map the recombination sites in SL2-1 and SL2-1-52. Both alleles of the two clones retained the Hind III site between J_3 and J_4. One allele retained the Bam HI site between J_2 and J_3 but another allele eliminated this site in both SL2-1 and SL2-1-52, producing a novel fragment which differed in mobility when Bam HI–Eco RI digests were hybridized with the J_H probe (data not shown). In nonproducer, SL2-1, recombination appears to have occurred with J_3 on one allele and J_2 on another allele. Further rearrangement seems to have occurred upstream of J_3 on the former allele to produce a γ_{2b} chain in SL2-1-52. Restriction maps of the two clones deduced from these Southern blot analyses are shown in Figure 9.

Switch from μ to γ_{2b} Production Was Accompanied by Loss of the Intervening C_H Genes in AT11-2 Series

To examine whether any rearrangements or deletions were accompanied with the switch from μ to γ_{2b} production in AT11-2 series clones, DNAs were digested with Eco RI and hybridized to the C_μ probe. As shown in Figure 6B, Southern blot analysis revealed a single Eco RI fragment of 12.5 kb in BALB/c liver DNA, and two fragments of 12.5 and 11 kb in μ producer, AT11-2-24-6, indicating a rearrangement within one copy of the 12.5 kb Eco RI fragment in AT11-2-24-6. However, in γ_{2b} producer, AT11-2-24-6-99, the 11 kb fragment was missing, suggesting that the switch from μ to γ_{2b} production was accompanied with deletion of the C_μ gene.

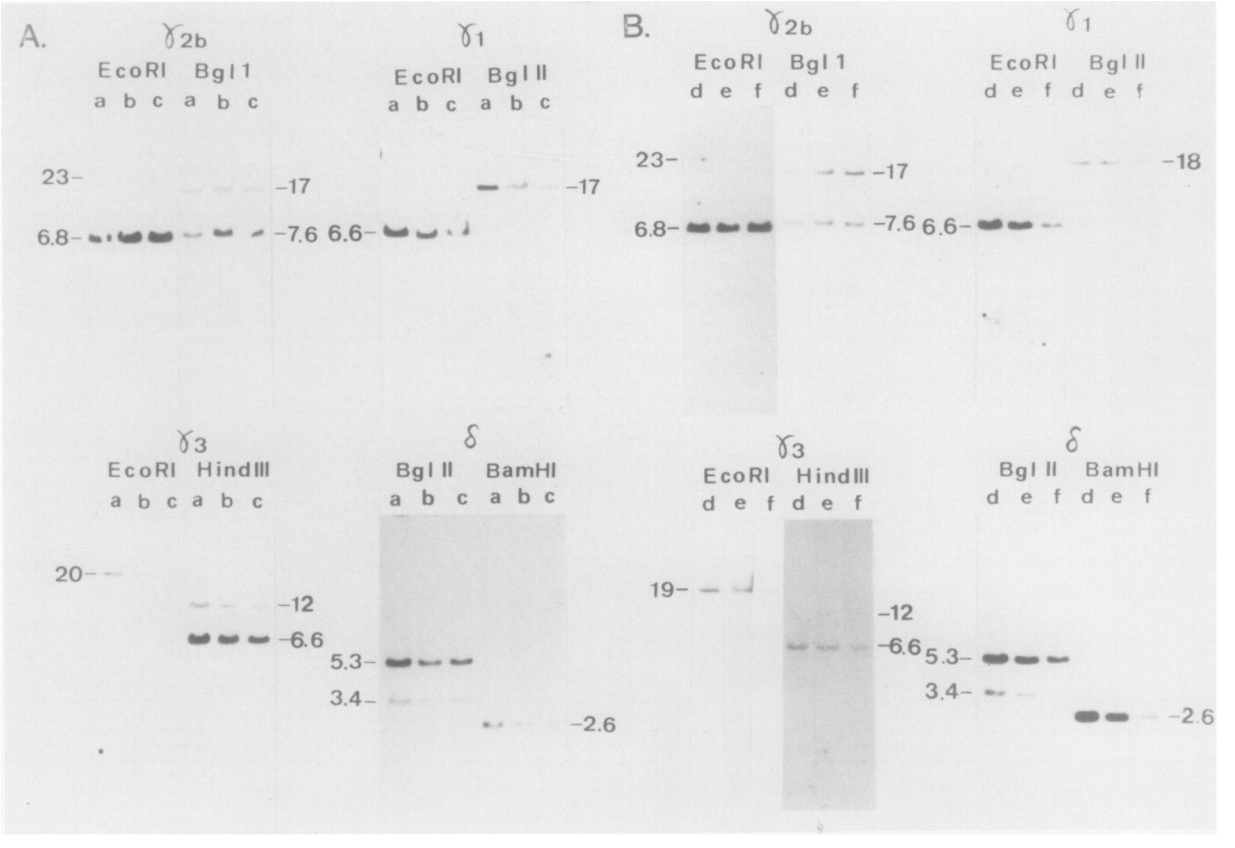


Figure 4. Comparison of the Context of the $C_{\gamma 2b}$, $C_{\gamma 1}$, $C_{\gamma 3}$, and C_{δ} Gene in DNA from SL2-1 Series (A) and AT11-2 Series (B)

DNAs were digested with the indicated restriction enzymes and hybridized with the indicated probe. DNA in each lane is as follows: (a) CBA/N liver; (b) SL2-1; (c) SL2-1-52; (d) BALB/c liver; (e) AT11-2-24-6; (f) AT11-2-24-6-99. A 12 kb fragment of Hind III-digested DNAs with the $C_{\gamma 3}$ probe seems to be produced as a result of contamination of a fragment located just 5′ of the $C_{\gamma 3}$ probe.

DNAs were also digested with Eco RI or Xba I and hybridized to the J_H probe. As shown in Figure 6B, Eco RI digest gave a single 4.5 kb fragement in both μ producer and γ_{2b} producer, indicating that one J_H segment was probably lost in both clones and that the switch to γ_{2b} production did not influence the rearrangement of the J_H genes. Xba I digest showed essentially the same result (data not shown).

To relate the J_H gene to the C_μ gene, DNA was digested with Kpn I, hybridized to the C_μ probe, and then, after autoradiography, the same filter was washed and rehybridized to the J_H probe (Figure 6B). The C_μ probe showed 18 and 12.5 kb fragments in AT11-2-24-6 and a single 18 kb fragment in AT11-2-24-6-99, indicating that the 12.5 kb C_μ-bearing Kpn I fragment was lost upon the switch to γ_{2b} production. When rehybridized to the J_H probe, AT11-2-24-6 revealed a single 12.5 kb fragment and AT11-2-24-6-99 contained a fragment of 6.7 kb, different in mobility from AT11-2-24-6. These data showed that a single copy of the J_H gene was associated with the C_μ gene which was lost in the switch from μ to γ_{2b} production.

Further analysis regarding rearrangement of the δ, γ_3, and γ_{2b} genes was performed (Figure 4B). DNA from μ producer, AT11-2-24-6, remained embryonic and did not decrease in band intensity as compared with liver DNA when tested with δ, γ_3, γ_1, and γ_{2b} as probes, suggesting that AT11-2-24-6 contained two copies of the δ, γ_3, γ_1, and γ_{2b} genes. In contrast, DNA from γ_{2b} producer, AT11-2-24-6-99, did not show any novel fragments, but decreased in the intensity of the δ, γ_3, and γ_1 genes as compared with liver DNA, suggesting deletion of each copy of the δ, γ_3, and γ_1 genes in AT11-2-24-6-99. Quantitative analysis of the γ_1 gene showed that the mean γ_1/C_κ ratio in AT11-2-24-6-99 was 46% of that in liver DNA (Figure 5B).

Switch Recombination Took Place Upstream of $S_{\gamma 2b}$ in Two γ_{2b}-producing Cell Lines

From the above results the switch recombinations were expected to have taken place upstream of $S_{\gamma 2b}$ and downstream of the $C_{\gamma 1}$ gene. To confirm this possibility, the 3.0 kb Bam HI fragment (probe g in Figure 2) of Ch·M·Ig γ_{2b}-69 that is located just upstream of $S_{\gamma 2b}$ was employed as a probe. DNAs were digested with Bst EII or Eco RI, and hybridized with the probe. Each digest, from γ_{2b} producers (SL2-1-52 and AT11-2-24-6-99) revealed an additional new

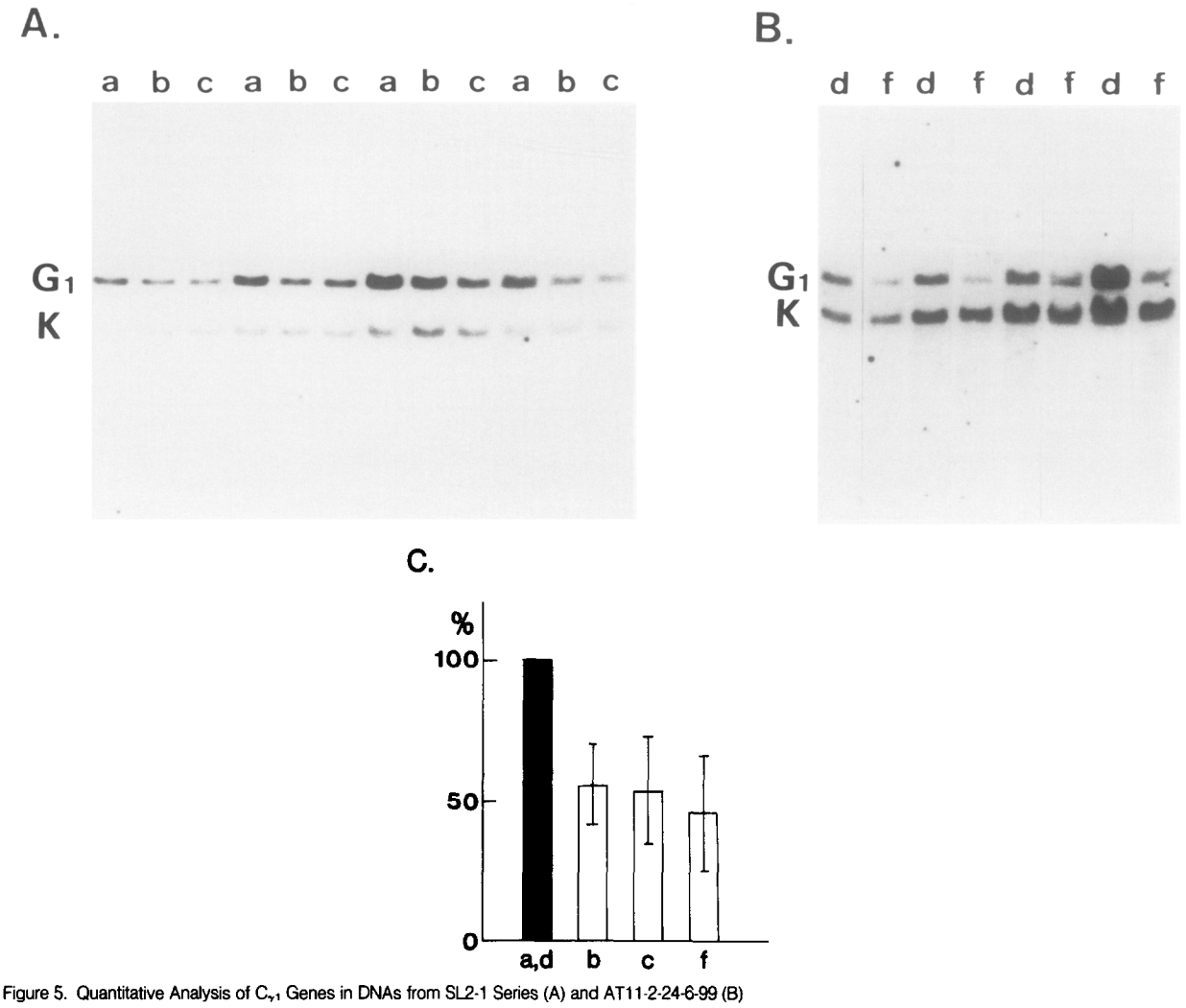


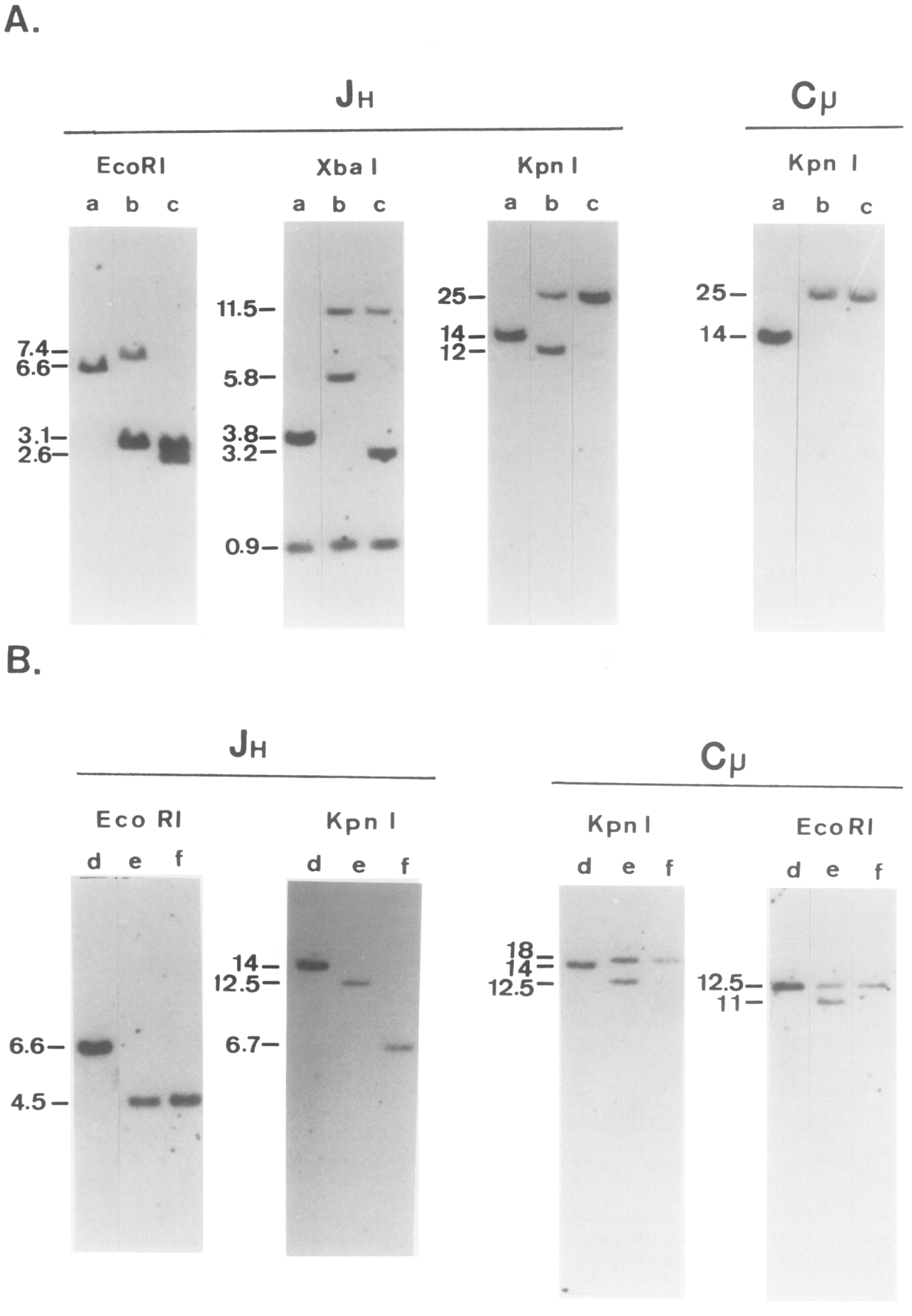
Figure 5. Quantitative Analysis of $C_{\gamma 1}$ Genes in DNAs from SL2-1 Series (A) and AT11-2-24-6-99 (B)

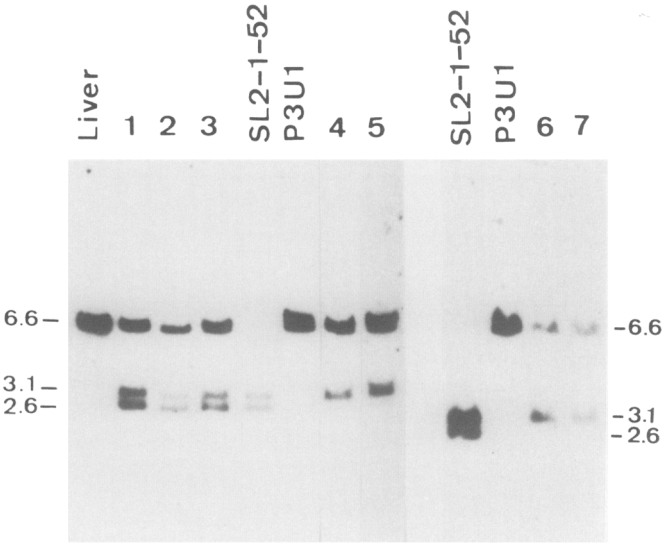
Hind III/Eco RI-digested DNAs (2, 3, 5, 7 μg per lane) were hybridized with a mixture of the γ_1 probe (probe f) and the C_κ probe (probe a). AT11-2-24-6-99 had two copies of the rearranged C_κ genes but rearrangement did not affect the 4.5 kb C_κ Hind III fragment (data not shown). Intensities of bands corresponding to $C_{\gamma 1}$-bearing fragments (G1) and C_κ-bearing fragments (K) were determined respectively by densitometer tracings. The ratio of the intensity of the γ_1 gene band to the C_κ gene band in each pre-B cell line DNA was compared with that in liver DNA (C). The results were expressed as percentage of G1/K in liver DNA. DNA in each lane is as follows: (a) CBA/N liver; (b) SL2-1; (c) SL2-1-52; (d) BALB/c liver; (f) AT11-2-24-6-99.

fragment different in size from that in liver (shown in Figure 8A and 8B). The above result and the lack of Bgl I restriction fragments with sizes other than germline when hybridized with probe h indicate that two γ_{2b}-producing cell lines have undergone class-switch upstream of $S_{\gamma 2b}$. In addition, in the case of SL series, parent nonproducer, SL2-1, showed the same pattern as γ_{2b}-producing subclone, SL2-1-52 (Figure 8A), which indicated that the switch recombination site remained unaffected before and after γ_{2b} production. Furthermore, Bst EII cut on the 3′ side of J_3 and within the C_H2 domain of the C_μ gene in embryonic DNA (Figure 9) (Köhler et al., 1982). As Bst EII sites between J_3 and J_4 were not eliminated in either SL2-1-52 or AT11-2-24-6-99, but the C_H2 Bst EII sites were eliminated in both clones because of C_μ deletion (data not shown), it was suspected that in Bst EII digests of two γ_{2b} producer DNAs, the new fragments hybridizing to probe g might also hybridize with the J_H probe (probe b). Filters of Bst EII digests from DNAs of two γ_{2b} producers were washed and rehybridized to the J_H probe (Figure 8C). Each newly produced fragment, an 11 kb fragment in SL2-1-52 and a 10 kb fragment in AT11-2-24-6-99 also hybridized with the

Figure 6. Comparison of the Context of C_μ–J_H Region in DNA from SL2-1 Series (A) and AT11-2- Series (B)

DNAs were digested with Eco RI, Kpn I, or Xba I and hybridized with the J_H probe or the C_μ probe. DNA in each lane is as follows: (a) CBA/N liver; (b) SL2-1; (c) SL2-1-52; (d) BALB/c liver; (e) AT11-2-24-6; (f) AT11-2-24-6-99.


A.
JH
Cμ
EcoRI
Xba I
Kpn I
Kpn I
a b c
a b c
a b c
a b c
7.4–
6.6–
3.1–
2.6–
11.5–
5.8–
3.8–
3.2–
0.9–
25–
14–
12–
25–
14–
B.
JH
Cμ
Eco RI
Kpn I
Kpn I
EcoRI
d e f
d e f
d e f
d e f
6.6–
4.5–
14–
12.5–
6.7–
18–
14–
12.5–
12.5–
11–



Percentage of γ_{2b} producing cells at DNA preparation

	Hybridoma	% γ_{2b} positive cells
1	2C4-G9	100
2	2C5-C11	100
3	2A5-F8	100
4	4B2-B6	30
5	5C1-H3	20
6	4C6-B8	0
7	5A2-H11	0

Figure 7. Comparison of the Context of the J_H Gene in Several Hybrid Clones by the Fusion of SL2-1-52 and P_3U_1

DNAs were digested with Eco RI and hybridized with the J_H probe. Table shows the percentage of γ_{2b}-producing cells in each hybrid clone at DNA preparation. Numerals (1–7) shown in lanes and table correspond with each other.

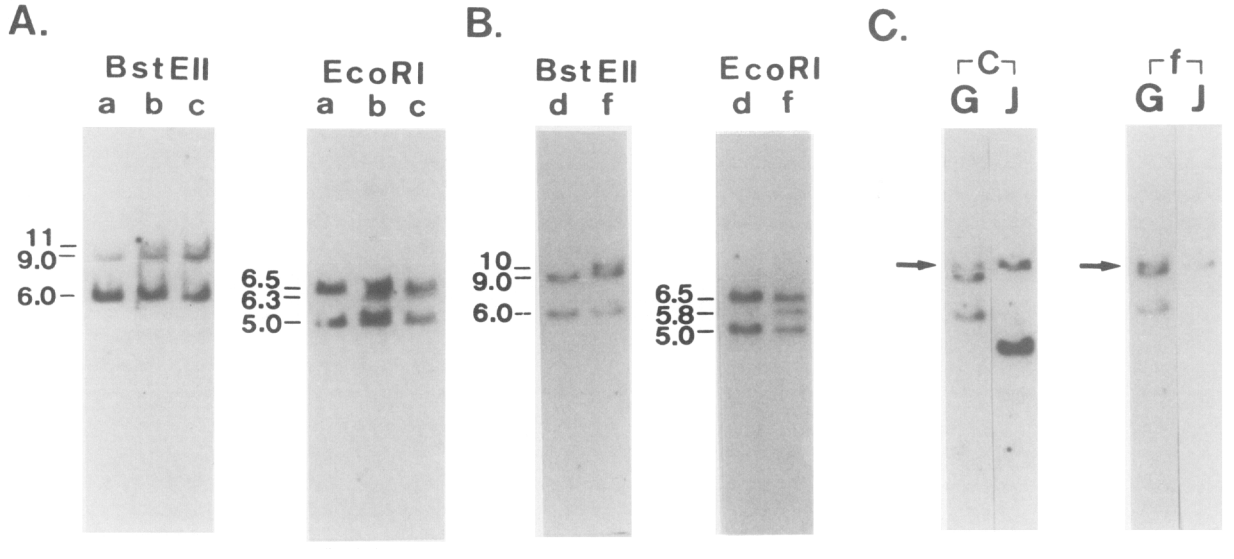


Figure 8. Hybridization of DNA from SL2-1 Series (A) and AT11-2-24-6-99 (B) with the 3.0 kb Bam HI Fragment (probe g in Figure 2) of Ch-M-Igγ_{2b}-69

DNA in each lane is as follows: (a) CBA/N liver; (b) SL2-1; (c) SL2-1-52; (d) BALB/c liver; (f) AT11-2-24-6-99. Filters of Bst EII-digested DNAs from SL2-1-52 (c) and AT11-2-24-6-99 (f) were washed and rehybridized with the J_H probe (probe b) (C). Lanes G and J are the Southern blots hybridized with probe g and the J_H probe respectively. The common fragments are indicated by arrows.

J_H probe. Taken together, these data strongly suggest that two γ_{2b}-producing cell lines rearranged the $C_{\gamma 2b}$ gene immediately adjacent to the J_H gene and deleted all the intervening C_H genes (Figure 9).

Discussion

This study presented several new findings with respect to the isotype switching in immature B precursor cell lines, i.e., one, isotype switcing can occur in pre-B cells; two, isotype switching may be operated by a C_H gene deletion mechanism even in pre-B cells; three, isotype switch rearrangements in pre-B cell lines can take place outside of the S regions; and four, isotype commitment may be able to occur in advance of the functional variable region gene formation.

Isotype Switching Can Operate at the Stage of Pre-B Cell

The possibility that isotype switching can operate at the pre-B cell stage has been proposed for a few years. Some patients with pre-B cell leukemia had leukemic cells that

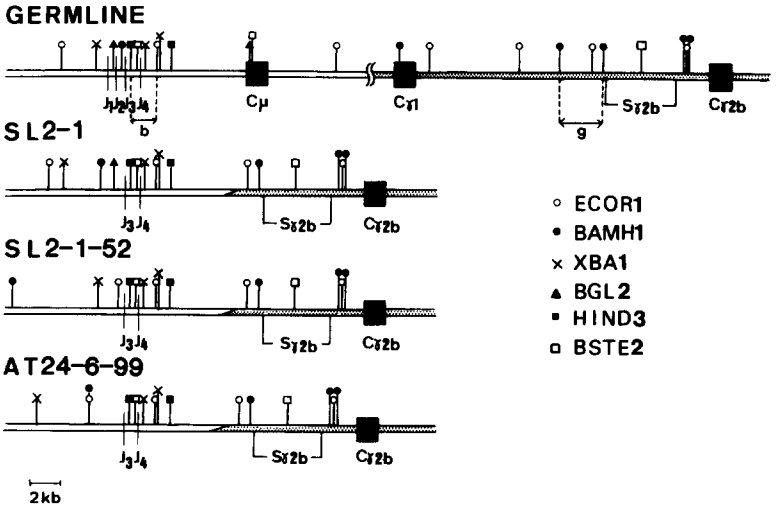


Figure 9. Restriction Map Comparisons in SL2-1, SL2-1-52, and AT11-2-24-6

These restriction endonuclease cleavage sites were identified by Southern blot analysis. Restriction sites in germline DNA were extracted from the published data although a Bst EII site in 5′ flanking sequences of the $C_{\gamma 2b}$ gene was deduced from this study.

expressed cytoplasmic γ or α chains, although in a very small proportion (Vogler et al., 1978). Vogler et al. (1981) reported the expression of a non-μ heavy chain isotype in some leukemic cells with features of pre-B cells. However, it was uncertain whether leukemic cells with cytoplasmic γ or α chains represented the pre-B cell stage, since no homogeneous cell population was available for the analysis at a molecular level. Alt et al. (1982) demonstrated that an A-MuLV-transformed cell line, 18–81, had undergone the class switch from μ to γ_{2b}. According to their study at the molecular level, the cell line that switched from μ to γ_{2b} employed the same V_H region for the both heavy chains and retained two copies of the C_μ genes. This suggests the possibility that the class switch from μ to γ_{2b} may occur without the deletion of the intervening C_H genes and instead by some sort of RNA processing mechanism. However, as these authors point out, it is not clear whether the class switch observed in the 18–81 cells indeed reflects such events in natural pre-B cells because the 18–81 cell line can give rise to subclones having the rearranged κ chain genes and the γ_{2b}-producing subclone already has undergone κ gene rearrangement on both chromosomes. In contrast to 18–81 cells, SL2-1-52 cells employed in the present study carry the κ genes in the embryonic configuration and therefore may be judged to be a closer representative of pre-B cells (Kearney, 1980). The result also indicates that the class switch can occur without antigen stimulation or T cell help.

C_H Gene Deletion Mechanism Can Operate even in the Pre-B Cell Stage

Several recent studies have shown the presence of two different mechanisms of isotype switching, C_H gene deletion (Honjo and Kataoka, 1978; Cory and Adams, 1980; Rabbitts et al., 1980; Yaoita and Honjo, 1980) and RNA splicing mechanisms (Alt et al., 1982; Yaoita et al., 1982). A deletion model has been elucidated from studies done in myelomas or hybridomas secreting various isotypes that represented the terminally differentiated stage of B lymphocytes. On the other hand, an RNA splicing mechanism has been recently proposed for dual expression of IgM and IgD in $\mu^+\delta^+$ lymphomas and in $\mu^+\delta^+$ hybridomas from the studies based on molecular genetic analyses (Moore et al., 1981; Maki et al., 1981). In addition, as described above, Alt et al. (1982) presented an example of class switch from μ to γ_{2b} production without deletion of the C_μ gene in an A-MuLV-transformed immature B cell line, 18–81. Furthermore, by studying the organization of C_H genes in sorted $\mu^+\epsilon^+$ B lymphocytes, Yaoita et al. (1982) reported that $\mu^+\epsilon^+$ lymphocytes retained the C_μ, C_δ, C_γ, and C_ϵ genes, suggesting that the simultaneous expression of the C_μ and C_ϵ genes could be mediated by an RNA splicing mechanism. These observations implied that heavy chain genes other than the C_μ gene might be expressed without deletion of the C_μ gene, as a result of an RNA splicing mechanism at the stage of pre-B cells or B cells. In the present study, however, we showed in cloned cells that intracytoplasmic γ_{2b} production was accomplished by deletion of all the intervening C_H genes, indicating that C_H gene deletion events could occur even in the pre-B cell stage. Isotype switch by a C_H gene deletion mechanism in pre-B cells indicated that isotype commitment of B cells could occur early in pre-B cells.

Non-Ig-producers which Have Two Rearranged J_H Alleles Are Not Abortive but Intermediate

In the present study, γ_{2b}-producing cells, SL2-1-52, were derived spontaneously from non-Ig-producing cells, SL2-1, which had already undergone rearrangements including J_H segments in both heavy chain alleles. The result of DNA analysis of γ_{2b} producers and nonproducers indicated that γ_{2b} production was accompanied by an additional alteration within the J_H gene cluster on one chromosome. Thus it appears that two rearranged J_H segments observed in nonproducers are not abortive but intermediate and further rearrangements involving J_H segments are required for heavy chain expression. In addition, further rearrangement of J_H segments occurred on the chromosome that had

already deleted the C_μ gene in this particular cell line. Southern blot analysis of hybridomas obtained by fusion of SL2-1-52, γ_{2b} producer, with a myeloma cell line, P_3U_1, revealed that the expressed γ_{2b} gene was assigned on the chromosome that had undergone the further rearrangement of the J_H gene. To examine the nature of the secondary J_H-associated rearrangement, organization of the J_H segment was compared with nonproducer (SL2-1) and γ_{2b} producer (SL2-1-52) using Southern blot analysis. In the nonproducer, rearrangement seems to have occurred with J_3 on the C_μ-deleted chromosome and the γ_{2b} producer also seems to use J_3 on the same chromosome. This result suggests the possibility that the secondary J_H-associated rearrangement may be $V_H \rightarrow DJ_H$ joining. We experienced the spontaneous differentiation of a A-MuLV-transformed cell line (AT11-2) from the μ^- to μ^+ stage while growing in culture. The differentiation from the μ^- to μ^+ stage was accompanied by further rearrangements of J_H segments (Sugiyama et al., 1983). From the sequencing analysis it is shown that further rearrangements were $V_H \rightarrow DJ_H$ joinings (Yaoita and Honjo, personal communication). The result showed that isotype switching, deletion of the intervening C_H genes, could precede the formation of functional V regions in SL2-1 series and explained the reason why SL2-1 gave rise to γ_{2b} producer, SL2-1-52, without the stage of μ production. This indicates that prior expression of μ chain is not obligatory for the expression of other isotypes.

Rearrangement of Heavy Chain Genes Is Confined to One Allele

A deletion model proposed by Honjo and Kataoka (1978) suggested that isotype switching took place on one of the homologous chromosomes with the nonexpressed chromosome remaining unchanged. However, several observations (Cory and Adams, 1980; Rabbitts et al., 1980) showed that rearrangement of the C_H genes was not confined to one chromosome in myelomas and hybridomas. In the present study, although SL2-1 had rearrangement of two J_H loci, its γ_{2b}-producing subclones revealed further rearrangement of a J_H segment on one chromosome. Furthermore, C_H gene deletion was confined to one chromosome in both SL2-1 and AT11-2 series. Thus it might be possible that C_H gene deletion and functional V-D-J joining take place in one chromosome and later, during proliferation, the deletion on nonexpressed chromosome may take place.

Class Switch Rearrangements Can Take Place Outside of the Switch Regions

It has been shown that heavy chain class switch takes place at the switch region which is located 5′ to each constant region of the heavy chain genes from the analysis of nucleotide sequences surrounding the recombination sites of rearranged heavy chain genes (Sakano et al., 1980; Nikaido et al., 1981; Nikaido et al., 1982). A model is presented in which short sequences common to all S regions mediate C_H switch by homologous recombination. However, in this study, two γ_{2b}-producing pre-B cell lines did not use the γ_{2b} switch region for the switch to γ_{2b}. In hybridoma cells, class switch without employing the switch region is also demonstrated (Sablitzku et al., 1982). Several γ_{2b}-producing hybrid clones by fusion of SL2-1-52 and P_3U_1 did not show any change in $S_{\gamma 2b}$ as compared with SL2-1-52 in spite of a large amount of γ_{2b} production equivalent to that produced in the anti-DNP hybridomas (unpublished data). These results indicate that class switch recombination can occur at the sites other than the switch region, although it is not known whether this can operate in normal B cells.

Experimental Procedures

Cells

A-MuLV-transformed cell lines were established by in vivo infection of newborn CBA/N mice or BALB/c mice as described previously by Sugiyama et al., (1982). These cell lines were examined for heavy chain synthesis by immunofluorescence. Intracytoplasmic γ_{2b}-positive cells were detected in two cell lines, SL2-1 (non-Ig-producer) derived from a CBA/N mouse, and AT11-2-24-6 (μ-producer) derived from a BALB/c mouse. These two cell lines were subcloned and 100% γ_{2b}-positive subclones, SL2-1-52 and AT11-2-24-6-99, were obtained respectively.

Antibodies and Antisera

FITC-conjugated goat anti-mouse IgM serum (Cappel Laboratories), rabbit anti-mouse IgG_1 serum (Miles Laboratories), rabbit anti-mouse IgG_{2a} serum (Litton Bionetics), rabbit anti-mouse IgG_{2b} serum (Miles Laboratories), rabbit anti-mouse IgG_3 serum (Litton Bionetics), rabbit anti-mouse IgA serum (Miles Laboratories), rabbit anti-mouse κ serum (Miles Laboratories), and rabbit anti-mouse γ serum (Miles Laboratories) were purchased. Anti-γ_{2b} serum was made mono-specific by immunoadsorption with appropriate myeloma Ig conjugated to Sepharose 4B beads. Monoclonal anti-δ was purified from the culture fluid of hybridoma (Oi et al., 1978) and was biotinized as described (Heitzmann and Richards, 1974). Rabbit anti-mouse ϵ chain antiserum was prepared by immunization of rabbit with mouse monoclonal IgE purified from the culture fluid of anti-DNP IgE-producing hybridomas (Böttcher et al., 1980).

Immunofluorescence

To detect cytoplasmic μ chains, cells were fixed with acid–alcohol, stained with FITC-conjugated goat anti-mouse IgM serum for 30 min at 37°C, washed with phosphate buffered saline (PBS), and examined by fluorescent microscope (Zeiss Standard 18). To examine cytoplasmic γ_3, γ_1, γ_{2b}, γ_{2a}, ϵ, α, κ, and λ chains, fixed cells were reacted with each anti-serum for 30 min at 37°C. After washing with PBS, cells were stained with FITC-conjugated sheep anti-rabbit IgG (Cappel Laboratories) for 30 min at 37°C and examined by fluorescent microscope. To detect cytoplasmic δ, fixed cells were stained with biotinized mouse anti-δ antibody for 30 min at 37°C, and after washing with PBS they were stained again with FITC-conjugated avidin (Vector Laboratories) for 30 min at 37°C. To examine surface IgM, cells were incubated with FITC-conjugated goat anti-mouse IgM serum for 30 min at 4°C, washed, and examined by fluorescent microscope. Surface IgD was examined by indirect immunofluorescent staining. Cells were incubated with biotinized anti-mouse δ antibody for 30 min at 4°C, washed, and stained with a FITC-conjugated avidin for 30 min at 4°C. In examination of surface γ_3, γ_1, γ_{2b}, γ_{2a}, ϵ, α, κ, and λ chains, cells were treated first with each unlabeled antiserum for 30 min at 4°C and washed. The procedure was then repeated with a FITC-conjugated anti-rabbit IgG, and examined by fluorescent microscope.

Biosynthetic Labeling and Immunoprecipitation

Biosynthetic labeling and immunoprecipitation were carried out as described elsewhere (Witte and Baltimore, 1978; Kikutani et al., 1981). Five to ten million cells were incubated in 2 ml methionine-free RPMI1640

containing 10% dialysed fetal calf serum and 100 μCi ^{35}S-methionine for 3 hr in CO_2 incubator. After incubation, cells were washed three times with cold minimum essential medium, lysed in 0.8 ml of 10 mM Tris-HCl (pH 7.5)/1 mM $MgCl_2$/0.5% triton X-100/1 mM PMSF and centrifuged at 2000 g at 4°C for 15 min. The cell lysate was adjusted to 0.01 M NaH_2PO_4 (pH 7.5)/01. M NaCl/0.1% SDS/0.5% Na-deoxycholate/1% triton X-100/1 mM PMSF (precipitation buffer) and ultracentrifuged at 100,000 G at 4°C for 30 min. The supernatant was precleared by incubation with 5 μl normal rabbit serum and 50 μl of goat anti-rabbit IgG-coupled sepharose. The precleared cell lysate was reacted with anti-IgG_{2b} and anti-IgM serum overnight and followed by incubation with 20 μl of goat anti-rabbit IgG-coupled Sepharose for 3 hr at 4°C. Immunoprecipitate was washed three times with precipitation buffer and twice with 0.5 M NaCl/5 mM EDTA/50 mM Tris-HCl (pH 7.5)/ 0.5% NP-40/1 mM PMSF. Labeled immunoglobulin was eluted by boiling for 2 min in 2.3% SDS/10% glycerol/5% 2-mercaptoethanol/0.0625 M Tris-HCl (pH 6.8) and subjected to 9% SDS polyacrylamide slab gel.

Somatic Cell Hybridization

SL2-1-52 ($BudR^R$) was a mutant of SL2-1-52 cells that could proliferate in the presence of 30 μg/ml of 5-BudR, but could not proliferate in a selective HAT medium. P_3U_1 (P3/X63-Ag8U_1) was a mutant of X63 cells (P3/X63-Ag8) (Köhler and Milstein, 1975) and did not secrete either γ_1- or κ-chain. Cell hybridization was performed according to the modified method as described by Yoshida et al. (1982). Briefly, 2×10^7 SL2-1-52 ($BudR^R$) cells were hybridized with 1×10^7 P_3U_1 cells in 0.2 ml of 50% PEG-4,000 (Sigma Chem. Comp.) for 4 min at 37°C. Hybrid cells were selected in HAT medium and cloned by limiting dilution.

Preparation of High Molecular Weight Cellular DNA

DNA was prepared by modification of the method described previously by Yaoita and Honjo (1980). Mouse livers and A-MuLV-transformed cells were homogenized in 50 mM Tris·HCl (pH 8.0)/10 mM EDTA/0.15 M NaCl with a Potter-Elvehejm homogenizer at 0°C. NaCl was added to a final concentration of 0.5 M to a whole homogenate, followed by SDS to 0.3%. The homogenate was extracted with water-saturated phenol/chloroform/isoamyl alcohol (25:24:1 vol/vol). The water phase was separated by centrifugation, dialysed against 0.1 × SSC (15 mM NaCl, 15 mM Na-citrate) overnight, digested with RNAase (100 μg/ml) for 1 hr at 37°C and then extracted with phenol. After dialysis, the water phase was used as DNA sample.

Southern Blot Analysis

DNA digests with restriction enzymes were electrophoresed in 0.5% agarose gels, transferred to nitrocellulose filters (Southern, 1975), hybridized with nick-translated probes and autoradiographed. Hybridization was performed at 65°C in 1 M NaCl/50 mM Tris·HCl (pH 7.4)/10 mM EDTA/0.2% bovine serum albumin/0.2% Ficoll/0.2% polyvinylpyrrolidone/0.1% SDS/ 100 μg/ml sonicated denatured E. coli DNA. After hybridization, filters were washed in 0.1 × SSC plus 0.1% SDS (four times) for 40 min each at 65°C.

Immunoglobulin Gene Probes

A J_H region probe was the 1.5 Kb Hind III/Eco RI fragment containing the J_{H4}. A μ gene probe was the 1.2 Kb Hind III fragment that contained the C_H3 and C_H4 domains of the μ-chain gene clone. A δ gene probe was the 1.5 Kb Bam HI/Eco RI fragment of δ-89, located around 6 kb 3′ to the C_δ gene. A γ_3 gene probe was the 2.8 kb Kpn I fragment of the γ_3-chain gene. A γ_1 gene probe was the 6.6 kb Eco RI fragment containing the γ_1-chain gene (IgH2). A γ_{2b} gene probe was the 4.0 kb Xba I/Hha I fragment of the γ_{2b}-chain gene (IgH22). The 3.0 kb Bam HI fragment of Ch·M·Igγ_{2b}-69 was used to examine recombination site between the γ_1 and γ_{2b} genes. Details about these probes are described by Shimizu et al. (1981, 1982). A C_κ probe was the 3.0 kb Hind II–Bam HI fragment containing C_κ which was subcloned from a clone containing both J_κ and C_κ gene. This clone was isolated from Charon 28 library containing partial Sau 3A digest of newborn mouse DNA. All DNA probes were kindly provided by Dr. T. Honjo, Osaka University.

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