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Somatic mutation in constant regions of mouse λ1 light chains

(immunoglobulin)

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ABSTRACT **To study the distribution of somatic mutation, we determined nucleotide sequences of rearranged λ1-chain genomic DNA from four hybridomas obtained from C57BL/6 mice that had been immunized with (4-hydroxy-3-nitrophenyl)acetyl-conjugated chicken gamma globulin. In total, 114 nucleotide substitutions were observed, with neither insertion nor deletion. Sixty-one mutations occurred in the variable–joining region genes ($V_{\lambda 1}$–$J_{\lambda 1}$) and 49 in joining–constant ($J_{\lambda 1}$–$C_{\lambda 1}$) introns. Although frequency decreased with distance from the $V_{\lambda 1}$–$J_{\lambda 1}$ coding region, somatic mutations occurred in the entire $J_{\lambda 1}$–$C_{\lambda 1}$ intron and even in the $C_{\lambda 1}$ region. We found four nucleotide substitutions in $C_{\lambda 1}$ genes, all of which were replacement mutations. Therefore, the mechanism responsible for somatic mutation is operative into the $C_{\lambda 1}$ exons. Nucleotide sequences of rearranged but inactive λ2-chain genes from two hybridomas were also examined and compared with those of λ1-chain genes. The clustering of replacement mutations in complementarity-determining regions in the inactive λ2-chain genes similar to the active λ1-chain genes suggested a mechanism that induces somatic mutation preferentially in this region even in the absence of antigenic selection.**

Sequence diversity in the variable (V) domains of immunoglobulins is generated by random recombination of *V*, diversity (*D*), and joining (*J*) gene segments of heavy (H) chains or *V* and *J* segments of light (L) chains (1). Insertion of the *N* region and flexibility in the recombination sites also result in the variation in the V domain sequences (2). Somatic mutation introduces additional variation to V_H and V_L domains (1, 3–6). It has been shown that somatic mutation is triggered by stimulation with thymus-dependent antigens (3, 4, 6, 7) and is found in rearranged immunoglobulin genes both active and inactive (8–12). The somatic mutation mechanism is as yet unclear, although several models have been proposed (13–15). To evaluate possible mechanisms, information on the distribution of somatic mutations is undoubtedly required (5, 16–19). Both murine and human immunoglobulin gene sequences show a lack of somatic mutation in the constant (*C*) region exons of H and κ chains (5, 17, 20, 21). However, Cleary *et al.* (22) reported the occurrence of somatic mutation in the human C_λ exon. Because of the complex genetics and gene structure of human immunoglobulins, it is rather difficult to distinguish between nucleotide variations arising from somatic mutation and those from other mechanisms.

Murine λ chains are particularly useful for examining the distribution of somatic mutations because of their simple gene structures (23–25). The λ-chain genes possess only two V_L genes ($V_{\lambda 1}$ and $V_{\lambda 2}$), which recombine with $J_{\lambda 1}$, $J_{\lambda 2}$, or $J_{\lambda 3}$. Selective recombination of $V_{\lambda 1}$ to $J_{\lambda 1}$–$C_{\lambda 1}$ or $J_{\lambda 3}$–$C_{\lambda 3}$ has been commonly observed (26, 27). Therefore, a variation in sequence as a result of somatic mutation could be easily distinguished from that generated by other mechanisms. Furthermore, the *J–C* introns of λ-chain genes differ from those of H- and κ-chain genes in that the former is shorter and lacks an intron enhancer. Since elements in the *J–C* intron such as the enhancer and matrix association region (MAR) may play important roles in somatic mutation mechanism, the distribution of somatic mutations in λ1-chain genes would be expected to differ from that of H- and κ-chain genes. Therefore, findings obtained from the analysis of λ-chain gene structure should provide information about the functional role of the *J–C* intron in the somatic mutation mechanism of immunoglobulin genes.

MATERIALS AND METHODS

Hybridomas. Hybridomas producing anti-(4-hydroxy-3-nitrophenyl)-acetyl (NP) monoclonal antibodies (mAbs) were prepared at 5 wk (5E2, γ2b-λ1), 12 wk (C6-8-2, γ1-λ1), 42 wk (E3-19, γ2a-λ1; E11-14, γ2b-λ1) after a primary immunization of C57BL/6 mice (8-wk-old at immunization) with NP-conjugated chicken gamma globulin in complete Freund's adjuvant, followed by restimulation with the same antigen in phosphate-buffered saline 3 days before fusing with SP2/0-Ag14 cells (28).

Polymerase Chain Reaction (PCR), Cloning, and Sequencing. DNAs were prepared from liver and hybridoma cells. Genomic DNA (1 μg) was amplified in a Perkin–Elmer/Cetus Thermal Cycler using the Gene-Amp kit. The oligonucleotide primers used for the cloning of λ-chain genes are shown in Fig. 1*A*. For amplification of λ1-chain genes, primers 1 and 6 at concentrations of 0.5–1 μM were used. The thermal protocol included 30 cycles of denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min, and extension at 72°C for 2 min. The amplified DNA was digested with *Eco*RI and subjected to 1% agarose gel electrophoresis. The DNA was purified with silica beads and ligated with pBluescript SKII(+). The ligation mixture was transfected into *Escherichia coli* XL1-Blue. DNA from putative recombinants was analyzed by restriction enzyme digestion to confirm that a fragment of the appropriate size had been cloned.

For cloning of rearranged and unexpressed λ2-chain genes, DNAs were amplified by using primers 1 and 2. Although both λ1- and λ2-chain genes were amplified, these products were separated from each other by agarose gel electrophoresis. To obtain germ-line nucleotide sequences corresponding to the $J_{\lambda 1}$–$C_{\lambda 1}$ intron, DNA from liver was amplified by using primers 6 and 16.

Cloned DNA was sequenced by the dideoxy method with primers as shown in Fig. 1*B*. To minimize cloning artifacts, PCR and cloning were performed twice independently. At least two clones from each PCR were subjected to sequencing, and only the consensus nucleotide sequences produced in independent experiments are shown in this paper.

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Abbreviations: CDR, complementarity-determining region; H, heavy; L, light; MAR, matrix association region; mAb, monoclonal antibody; NP, (4-hydroxy-3-nitrophenyl)-acetyl; V, variable; C, constant; D, diversity; J, joining.

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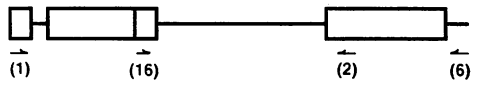
A



Primer(1); CTC/GAA/TTC/ATG/GCC/TGG/ATT/TCA/CTT/AT
Primer(2); CTC/GAA/TTC/TTC/AGA/GGA/AGG/TGG/AAA/CA
Primer(6); CTC/GAA/TTC/TCA/GCC/TCT/GTG/GGT/AAA/ATG/AAG
Primer(16); CAT/GAA/TTC/TGG/GTG/TTC/GGT/GGA/GGA/ACC

B

SK; CTA/GGT/GAT/CAA/GAT/CT
KS; CGA/GGT/CGA/CGG/TAT/CG
Primer(2); CTC/GAA/TTC/TTC/AGA/GGA/AGG/TGG/AAA/CA
Primer(3); ACC/TGG/TGA/AAC/AGT/CAC/ACT/CAC
Primer(4); CTC/GAA/TTC/CCA/TCA/CAG/GGG/CAC/AGA/CTG/AGG
Primer(5); ACT/TCA/CAC/TAG/GTA/AAG/AAT/TTC
Primer(7); GAT/GTT/GAA/CTT/GGA/GAG/CAC
Primer(9); CCA/ATA/CAG/ACA/CCT/GAC/CCT
Primer(17); CAC/ACC/TGG/GTA/GAA/ATC/AGT

FIG. 1. Oligonucleotide primers used for PCR and cloning (*A*) and for sequencing (*B*). The positions of the primers for sequencing were: primer(2), 1662 ← 1681; primer(3) 189 → 206; primer(4), 377 → 400; primer(5), 575 → 599; primer(7), 851 → 871; primer(9), 1419 ← 1439; primer(17), 1731 ← 1751, respectively. The arrows indicate the direction of sequencing. Position numbers are shown in Fig. 2.

RESULTS

Somatic Mutations in Active λ1-Chain Genes. Nucleotide sequences of rearranged λ1-chain genes are shown in Fig. 2, which includes the published germ-line sequences for $V_{\lambda 1}$ and $J_{\lambda 1}$ (29–31). The sequences of the $J_{\lambda 1}$–$C_{\lambda 1}$ intron and $C_{\lambda 1}$ exon were determined by using liver DNA from a C57BL/6 mouse and were in complete agreement with those published by Bothwell *et al.* (31). One hundred and fourteen nucleotide substitutions were observed in a total of 7760 bases with no insertions or deletions. More than half (53.5%) of the mutations were observed in $V_{\lambda 1}$–$J_{\lambda 1}$ genes, including the leader–$V_{\lambda 1}$ introns. Although nucleotide substitutions were scattered over the coding region, they occurred primarily in complementarity-determining regions (CDRs) 1 and 2 (CDR1 and CDR2). At some positions in the CDRs, somatic mutations were observed in more than two λ1 chains. Three of the four λ1-chain genes showed a change from cytosine to thymidine at position 254, which was accompanied by amino acid replacement of alanine with valine. Such a high mutation frequency was not observed in CDR3 or in the $J_{\lambda 1}$ regions.

The $J_{\lambda 1}$–$C_{\lambda 1}$ intron consists of 1153 nucleotides, in which no elements analogous to MAR were detected (32–34). Somatic mutations occurred throughout the region, although a higher frequency was observed 5′ to the $J_{\lambda 1}$–$C_{\lambda 1}$ intron adjacent to $J_{\lambda 1}$ genes, accompanied by a decrease in frequency with distance.

Somatic mutations occurred less frequently 3′ to the $J_{\lambda 1}$–$C_{\lambda 1}$ intron and $C_{\lambda 1}$ exons. We found four substitutions in the $C_{\lambda 1}$ exons of two λ1-chain genes (positions 1641, 1715, 1800, and 1898), with accompanying replacement of amino acids (Lys → Arg, Thr → Ser, Gln → Pro, Thr → Ser, respectively). As described in *Materials and Methods*, these were not due to cloning artifacts. Independent experiments confirmed these mutations.

Fig. 3 shows the distribution of somatic mutation in λ1-chain genes. The percent mutation per 50 nucleotides is plotted against position number. High numbers of mutations are seen in $V_{\lambda 1}$–$J_{\lambda 1}$ coding regions in addition to the adjacent 3′ flanking region. The mutation frequency decreased with distance from the $V_{\lambda 1}$–$J_{\lambda 1}$ genes and became constant around 1.1 kilobases (kb) downstream from the leader region. Although mutations were detected less frequently further downstream, they could still be found in the $C_{\lambda 1}$ genes. The percent mutations of $V_{\lambda 1}$–$J_{\lambda 1}$ including the leader–$V_{\lambda 1}$ intron, $J_{\lambda 1}$–$C_{\lambda 1}$ intron, and $C_{\lambda 1}$ were 3.32%, 1.06%, and 0.31%, respectively.

Somatic Mutation of Rearranged Unexpressed $V_{\lambda 2}$–$J_{\lambda 2}$ Genes. Rearrangement of λ2-chain genes occurred in two of four hybridomas. The nucleotide sequences of rearranged $V_{\lambda 2}$–$J_{\lambda 2}$ were compared with their germ-line counterparts (Fig. 4). The germ-line sequence of C57BL/6 mice was assumed to be identical to that of BALB/c mice (29). The $V_{\lambda 2}$–$J_{\lambda 2}$ genes were not expressed because of the presence of an extra base, cytosine, at the joining site. Somatic mutations were observed in these unexpressed genes. We found 25 and 15 mutations in E3-19 and E11-14 hybridoma $V_{\lambda 2}$–$J_{\lambda 2}$ genes, respectively. These numbers were similar to those of their $V_{\lambda 1}$–$J_{\lambda 1}$ counterparts (22 and 13 for E3-19 and E11-14 mAbs, respectively).

Distribution of Somatic Mutations in $V_{\lambda 1}$–$J_{\lambda 1}$ and $V_{\lambda 2}$–$J_{\lambda 2}$ Genes. Since λ2 chains were not expressed in these anti-NP hybridomas, they never underwent antigenic selection as immunoglobulin receptors. Therefore, analysis of sequence data would provide information concerning the effect of antigenic selection on somatic mutation. The number of mutations per 10 nucleotides was plotted against the position numbers of $V_{\lambda 1}$–$J_{\lambda 1}$ and $V_{\lambda 2}$–$J_{\lambda 2}$. A high mutation frequency was evident in CDR1 and CDR2 in $V_{\lambda 1}$–$J_{\lambda 1}$ (Fig. 5*A*). A similar tendency for mutation frequency was observed in CDR1 and CDR2 of $V_{\lambda 2}$–$J_{\lambda 2}$, although this was not as remarkable as in $V_{\lambda 1}$–$J_{\lambda 1}$ (Fig. 5*B*). Table 1 compares the ratio of replacement mutations between CDR and the framework region. A higher ratio of replacement mutations was observed in the CDRs of both λ1- and λ2-chain genes.

DISCUSSION

The nucleotide sequences of rearranged genomic λ1-chain genes derived from anti-NP hybridomas prepared at a late stage of immunization with NP-conjugated chicken gamma globulin showed a number of nucleotide substitutions with no insertions or deletions. The total number of mutations found in λ1-chain genes was 114, which is sufficient to allow statistical analysis of the sequence data. We cloned λ1- and λ2-chain genes with the aid of PCR. Ennis *et al.* (35) estimated the error frequency of PCR to be 1/1421 and recommended that three or four clones be sequenced to obtain a relevant consensus sequence. In our experiments the error frequency was found to be 25 in the 25,468 nucleotides sequenced (error frequency is 1/1108). Therefore, sequencing four clones derived from independent PCR seemed to be sufficient to eliminate the possibility of PCR error.

We found four somatic mutations in two $C_{\lambda 1}$ genes accompanied by amino acid replacements. Previously no somatic mutation was observed in $C_{\lambda 1}$ of S43, an anti-NP hybridoma, although three occurred in $V_{\lambda 1}$–$J_{\lambda 1}$ (31). Since the frequency of somatic mutations in $C_{\lambda 1}$ (0.31%) was less than 1/10th of that in $V_{\lambda 1}$–$J_{\lambda 1}$ (3.32%), it was difficult to detect nucleotide changes in the $C_{\lambda 1}$ gene when the total number of somatic mutations was low as in the S43 λ1-chain. Although the frequency of mutation in $C_{\lambda 1}$ was low, it was higher than the basal mutation frequency (11, 36–38). Therefore, the somatic mutator mechanism was definitely active in the $C_{\lambda 1}$ exon.

No somatic mutation was reported in C_H and C_κ exons, although a high frequency in V_H–D–J_H or V_κ–J_κ was observed as seen in $V_{\lambda 1}$–$J_{\lambda 1}$. Therefore, the absence of somatic mutations in C_H and C_κ genes cannot be explained in terms of a low total number of somatic mutations as in the S43 λ1 chain but might be related to a difference in gene structure. λ-Chain genes differed from H- and κ-chain genes in the length of *J*–*C* introns and the absence of an intron enhancer. The $J_{\lambda 1}$–$C_{\lambda 1}$ intron was about 1.1–1.2 kb, which is shorter than that of H-

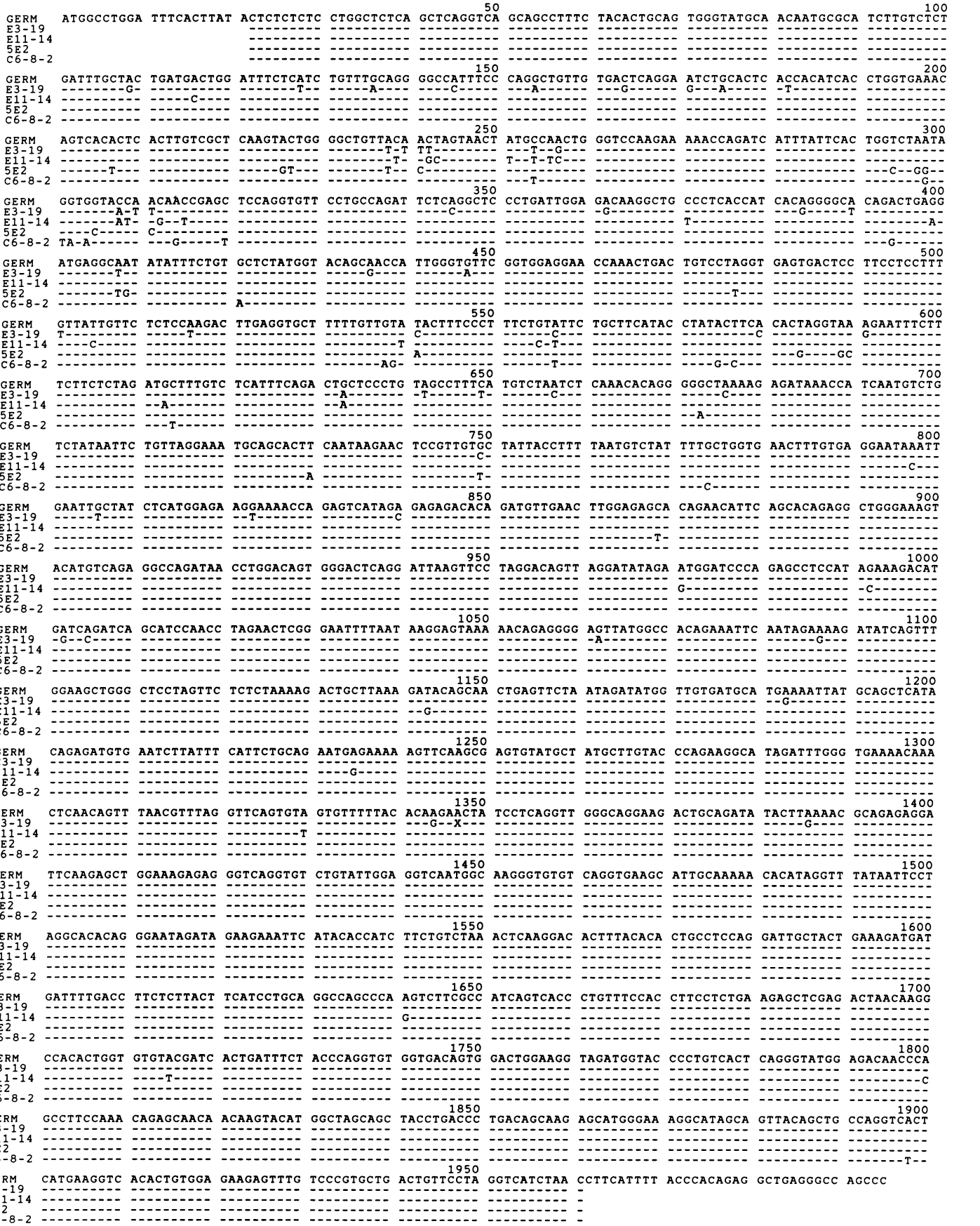


FIG. 2. Nucleotide sequences of mutated λ1-chain genes. The sequences of somatically mutated λ1-chain genes expressed in anti-NP mAbs are aligned with the germ-line sequence of the λ1-chain gene (29). Nucleotides identical to the germ-line λ1-chain gene are indicated by dashes. X indicates uncertainty.

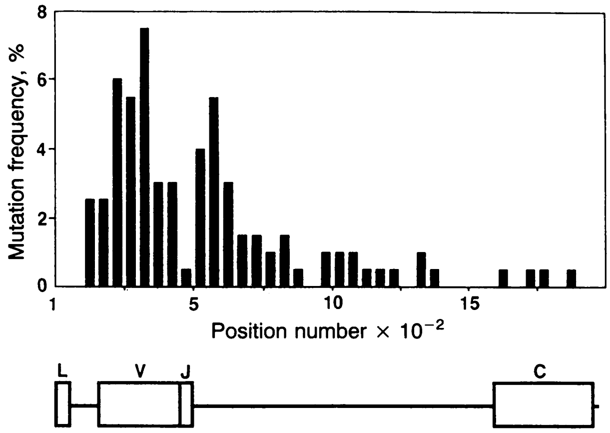


FIG. 3. (*Upper*) Distribution of somatic mutations in λ1-chain genes. Mutation frequency (%) is the number of mutations per 50 bases sequenced, which is plotted against the position number from the start codon ATG. (*Lower*) Diagram showing the leader, $V_{\lambda1}$–$J_{\lambda1}$, and $C_{\lambda1}$ gene.

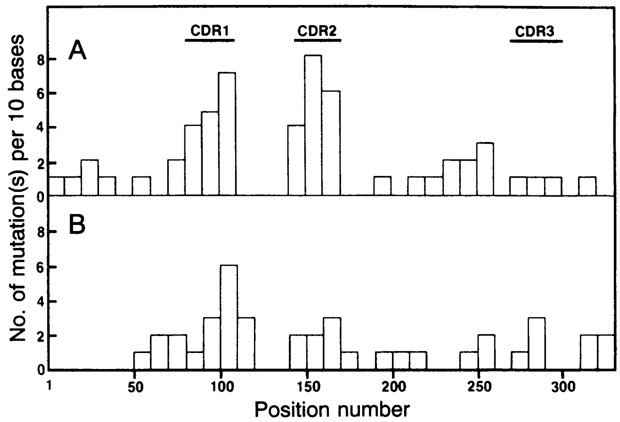


FIG. 5. Frequency of somatic mutations in expressed $V_{\lambda1}$–$J_{\lambda1}$ and nonexpressed $V_{\lambda2}$–$J_{\lambda2}$ genes. The number of mutations per 10 nucleotide increments is plotted against the position number.

or κ-chain genes (6.5 kb for H-chain genes and 3 kb for κ-chain genes). In addition, we could not find sequences corresponding to MAR that exist in H- and κ-chain genes. Two models may explain the absence of somatic mutation in *C* exons of H- and κ-chain genes: (*i*) separation of the *C* exon from the *V–J* exon protects it from targeting by the somatic mutator mechanism, and (*ii*) an enhancer or MAR provides a signal to terminate somatic mutation. It is difficult to determine which model accounts for the absence of somatic mutations in the C_κ and C_H exon at present. It is likely, however, that the length of the *J–C* intron is related to the lack of somatic mutations in C_κ and C_H exons because several mutations were observed in the enhancer and MAR regions of H-chain genes (18). Occurrence of somatic mutations in the $C_{\lambda1}$ exon could be explained in terms of a short intron insufficient in length for preventing mutation beyond the intron. The results also suggest that no particular signal sequences are present in *J–C* introns to inhibit somatic mutation in *C* exons.

Somatic mutations in $C_{\lambda1}$ and $V_{\lambda1}$ domains must be subject to selection based on function. Interaction with the H chain is one of the important functions of the $C_{\lambda1}$ domain. In fact, low expression of λ1-chains in SJL mice is thought to be due to amino acid substitution at position 155 (Gly-155 → Val), which results in an inability to interact with γ chains (39). Since mutated λ1 chains assembled into functionally active anti-NP mAbs, somatic mutations found in the present experiments do not have any serious effects on immunoglobulin assembly. On the other hand, mutations in the V_λ domains are expected to be subject to antigenic selection. In fact the affinity to NP haptens of C6-8-2 and E3-19 was 500–1000 times higher than that of mAbs prepared 1 wk after immunization (40). Such selection of B cells bearing high affinity immunoglobulin receptors is believed to bring about an apparent predominance of somatic mutation in CDR regions in addition to an increase in the ratio of replacement mutation relative to silent mutation. At first we thought that frequent somatic mutations in CDR1 and CDR2 as well as a high ratio of replacement mutation in CDRs resulted from antigenic selection during immunization (25, 41). However, a similar pattern of mutation was observed in the nucleotide sequences of unexpressed λ2-chain genes. No significant differences were observed in the numbers of somatic mutations and in the ratio of replacement mutations between active $V_{\lambda1}$–$J_{\lambda1}$ and inactive $V_{\lambda2}$–$J_{\lambda2}$. Therefore, the high mutation rates as well as

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          -19                                 -10                                      1
GERM LINE ATG GCC TGG ACT TCA CTT ATA CTC TCT CTC CTG GCT CTC TGC TCA GGA GCC AGT TCC CAG GCT GTT GTG ACT CAG
E3-19                                 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
E11-14                                --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                       10                                      20                                      30
GERM LINE GAA TCT GCA CTC ACC ACA TCA CCT GGT GGA ACA GTC ATA CTC ACT TGT CGC TCA AGT ACT GGG GCT GTT ACA ACT
E3-19     --- --- --- --- --- --- --- --- --- --- --- --- -G- --- --c --- --- --- -C- -A- --- --- --- T-- ---
E11-14    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --c --- --- --- --- --- --- --- --- --- ---

          CDR1                                 40                                      50             CDR2
GERM LINE AGT AAC TAT GCC AAC TGG GTT CAA GAA AAA CCA GAT CAT TTA TTC ACT GGT CTA ATA GGT GGT ACC AGC AAC CGA
E3-19     -C- --G -T- -T- --t --- --c --- --- --- --- --- --- --- --- --- --- --t T-- --- -A- T-- -A- --- ---
E11-14    --- --G -C- -T- --t --- --c --- -G- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                           60                                      70                                      80
GERM LINE GCT CCA GGT GTT CCT GTC AGA TTC TCA GGC TCC CTG ATT GGA GAC AAG GCT GCC CTC ACC ATC ACA GGG GCA CAG
E3-19     A-- --- --- --- --- --- --- --- --- --t --t --- --- --- --- --- --c --- --- --- --- --- --- --- ---
E11-14    -G- -T- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- T--

                                               90             CDR3        V <--|--> J 100
GERM LINE ACT GAG GAT GAT GCA ATG TAT TTC TGT GCT CTA TGG TAC AGC ACC CAT TTT     GTT TTC GGC GGT GGA ACC AAG GTC
E3-19     --- --- -G- --- --- --- --- --- --- --- --- --- -TT --- --- --- --- C   --- --- --- --- --- --- --- ---
E11-14    --- --- --- --- -A- --- --- --- --- --- --- --A --- -A- --- --- --- C   --- --- --- --- --- --- --- ---

GERM LINE ACT GTC CTA GGT
E3-19     G-- --- --- C--
E11-14    T-- --- --c ---
```

FIG. 4. Nucleotide sequences of the coding region of unexpressed λ2-chain genes from hybridomas E3-19 and E11-14 were compared with the published sequence for the coding region of the BALB/c germ-line sequence (30). Nucleotides identical to the germ-line sequence are indicated by dashes. Capital and small letters show replacement and silent mutations, respectively.

Table 1. Frequency of replacement mutations in CDRs and framework regions (FWRs) of $V_{\lambda 1}$–$J_{\lambda 1}$ and $V_{\lambda 2}$–$J_{\lambda 2}$

	$V_{\lambda 1}$–$J_{\lambda 1}$			$V_{\lambda 2}$–$J_{\lambda 2}$		
Hybridoma	Total mutations, no.	CDR, %	FWR, %	Total mutations, no.	CDR, %	FWR, %
E3-19	22	82	55	25	93	40
E11-14	13	73	50	15	86	63
5E2	13	88	20	—	—	—
C6-8-2	9	100	100	—	—	—

the high ratio of replacement mutations in CDRs are not necessarily due to antigenic selection but may be related to the mechanism of somatic mutation as proposed by Manser (15).

Nucleotide substitutions common to E11-14 and E3-19 mAbs were observed at several positions in the sequences of either λ1- or λ2-chain genes. These shared somatic mutations in addition to the similarity in the manner of nonproductive rearrangement of $V_{\lambda 2}$–$J_{\lambda 2}$ genes suggested a sibling lineage for these hybridomas—i.e., they may have arisen from the same parent clone, since these hybridomas were obtained from the same mouse. Recently Tao and Bothwell demonstrated that oligoclonal nature of the primary anti-NP response in C57BL/6 mice (42).

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