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A Pivotal Role for DNase I-Sensitive Regions 3b and/or 4 in the Induction of Somatic Hypermutation of IgH Genes

Akiko Terauchi,* Katsuhiko Hayashi,† Daisuke Kitamura,† Yuko Kozono,* Noboru Motoyama,2* and Takachika Azuma3*

Chimeric mice were prepared from embryonic stem cells transfected with IgH genes as transgenes and RAG-2-deficient blastocysts for the purpose of identifying the cis-acting elements responsible for the induction of somatic hypermutation. Among the three transgene constructs used, the VH promoter, the rearranged VH-D-JH, an intron enhancer/matrix attachment region, and human CpG were common to all, but the 3′-untranslated region in each construct was different. After immunization of mice with a T cell-dependent Ag, the distribution and frequency of hypermutation in transgenes were analyzed. The transgene lacking the 3′ untranslated region showed a marginal degree of hypermutation. Addition of the 3′ enhancer resulted in a slight increase in the number of mutations. However, the transgene containing DNase I-sensitive regions 3b and 4 in addition to the 3′ enhancer showed more than a 10-fold increase in hypermutation, reaching levels comparable to those observed in endogenous VH186.2 genes of C57BL/6 mice. The Journal of Immunology, 2001, 167: 811–820.

Diversity in the Ig V region of mice and humans is generated by the combinatorial joining of germline V, D, and J gene segments, the deletion and addition of nucleotides at the junction of these segments during joining, and somatic hypermutation of resulting V(D)J genes (1). Somatic hypermutation was shown to be related to the affinity maturation of Abs and frequently has been observed after stimulation of T cell-dependent (TD) Ags (2–10). The inductions and frequencies of hypermutation in transgenes were analyzed. The transgene lacking the 3′ untranslated region showed a marginal degree of hypermutation. Addition of the 3′ enhancer resulted in a slight increase in the number of mutations. However, the transgene containing DNase I-sensitive regions 3b and 4 in addition to the 3′ enhancer showed more than a 10-fold increase in hypermutation, reaching levels comparable to those observed in endogenous VH186.2 genes of C57BL/6 mice.

To identify the component(s) important in raising the frequency of hypermutation in the IgH gene, we used a RAG-2-deficient (RAG-2−/−) blastocyst complementation system developed by Chen et al. (31). A series of transgene constructs that differed only in the 3′ region flanking CpG were transfected into embryonic stem (ES) cells that were microinjected into RAG-2−/− blastocysts. The chimeric mice obtained with this system were immunized with a TD Ag. Analyses of the frequency of somatic hypermutation in the VH-D-JH region of transgenes revealed a pivotal role for the DNase I-sensitive (HS) regions 3b and/or HS4 (32–34) in the induction of high-frequency somatic hypermutation.

Materials and Methods

Mice

RAG-2−/− mice (35) with a BALB/c background were obtained from Dr. Y. Shinkai (Kyoto University, Kyoto, Japan) and maintained at this institute. Females 4–12 wk old were used as blastocyst donors. C57BL/6 and BALB/c mice were obtained from the Tokyo Animal Center (Tokyo, Japan).

Construction of IgH transgenes

IgH transgenes carrying human CpG with different 3′-flanking regions were constructed. A fragment containing the V′H17.2.25 promoter (0.55 kb) with KpnI and Apol sites and another containing Eμ/MAR (1 kb) with XhoI and SalI sites were cloned by PCR from the construct used previously to create CAT-D-JH mice (21). The rearranged VH-D-JH gene (2.0 kb) containing Apol and XhoI sites was also cloned by PCR with 5′-CAGGACTAGTGATCATTGAGCACTGGCGCTCTAAC-3′; HS3-anti Spol, 5′-AGGACTAGTGATCATTGAGCCTCGGCTTCACCTAC-3′; HS4-anti Spol, 5′-CTAGCCTGAGGCACAGACTCTGGACACTGGATATTGGGG-3′ in which the enzyme sites are underlined. These fragments were inserted at the 3′ end of 3′E.

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3 Abbreviations used in this paper: TD, T cell dependent; CAT, chloramphenicol acetyltransferase; Eμ, 1-C intron enhancer; MAR, matrix attachment region; 3′E, 3′ enhancer; ES, embryonic stem; NP, (4-hydroxy-3-nitrophenyl)acetyl; POD, peroxidase; CGG, chicken γ-globulin; GC, germinal center; HS, DNase I-sensitive.

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**Transfection of IgH genes into J558L cells**

Linearized IgH gene constructs (30 μg) with NotI were electroporated into J558L cells with the pSV2-gpt (1.5 μg) vector, and transfected cells were cultured in the presence of a mixture of hypoxanthine, xanthine, and mycophenolic acid (37). The drug-resistant IgH gene transfectants were subjected to limiting dilution, and clones were selected on the basis of Ab production. For the quantitative analysis of Ab production, several clones of transfectants derived from each transgene construct were cultured at 2 × 10^5 cells/ml for 12 h in a total volume of 200 μl IMDM containing 10% FCS in flat-bottom 96-well trays. The supernatants of various dilutions with PBS containing 1 mg/ml BSA were analyzed by ELISA with polyvinyl plates coated with NP34-BSA. Peroxidase (POD)-labeled anti-mouse λ-chain Abs (Southern Biotech, Birmingham, AL) or anti-human μ-chain Abs (Zymed, San Francisco, CA) were used for detection of bound Abs.

**Generation of chimeric mice**

The linearized IgH transgene constructs (30 μg) as well as the EcoRI fragment of pGKneo (1.5 kb, 1 μg) were electroporated into 1 × 10^7 E14 cells (38) with a Gene Pulser (Bio-Rad, Richmond, CA). The transfected cells then were selected with G418 (150 μg/ml). Colonies were screened by Southern blotting with the human Cμ gene as a probe and by PCR using the primers, 5'-GACTCAGGAGGACTCTAGTT-3' and 5'-GCTGTC CCTAGTCTTTCATG-3', which hybridized to DNA sequences located in the VμJH12.25 promoter and Jμ2, respectively. PCR amplification was performed for 30 cycles of 95°C for 30 s, 65°C for 30 s, and 72°C for 1 min. Copy numbers of integrated pvehc3'E were estimated by Southern blotting. The copy number of the other transgenes, pvehcΔ3'E and pvehc3'EHS3b/4, were estimated by PCR analysis with DNA from pvehc3'-transfected ES cells as a standard.

ES cell colonies containing the IgH transgene were injected into blastocysts from RAG-2^-/-/ mice (35) and transplanted into uteri of ICR foster mothers. The complementation of the immune system by B and T cells that originated from ES cells in chimeric mice was examined by flow cytometry after staining cells with PE-anti-CD3 Abs or biotin-anti-B220 streptavidin-FITC. The amount of IgM in the sera of chimeric mice was measured by ELISA with plates coated with rat anti-mouse IgM mAb. POD-labeled goat anti-mouse μ-chain Abs were used for detection of bound Abs. For analysis of Abs bearing human Cμ plates were coated with goat anti-human IgM Ab (Southern Biotech), and POD-labeled anti-human IgM mAb (Zymed) was used for detecting bound Abs. Culture supernatants of J558L transfectants, a human Waldenstrom IgM, and anti-NP IgM mAb, B4-3, were used as control Abs.

**Ags, immunization, and Ab production**

NP34-chicken γ-globulin (NP34-CGG) and NP-BSA with a different NP valence were prepared as described previously (39). Mice were administered NP34-CGG (100 μg/mouse) in CFA and were boosted with the same amount in IFA. Three days after the final administration of NP34-CGG in PBS, antisera were collected from the immunized mice. Mice then were sacrificed, and tissue samples were obtained. For measuring anti-NP Ab production and affinity maturation of these Abs, polyvinyl plates coated with NP34-BSA or NP16-BSA were used. POD-conjugated goat anti-mouse IgG was used for detection of bound Abs. Of the anti-NP mAbs, F8 was used as a control for immature Ab and C6 for a maturated mAb control (9, 10).

**Flow cytometry**

Single-cell suspensions from spleens of NP34-CGG-immunized chimeric mice were depleted of erythrocytes by treatment with 0.83% NH4Cl. In some experiments, T cells also were depleted by treatment with anti-Thy 1 Abs (T24/40 and HO13.4), followed by treatment with rabbit complement. Cells were stained with PE-conjugated anti-CD45R/B220, or FITC-conjugated anti-mouse IgM. The peritoneal cells were stained with biotin-conjugated anti-CD5/FITC-conjugated streptavidin and PE-conjugated anti-CD45R (B220). The CD45R (B220)^-IgM^-, CD45R (B220)^-IgM^+, or CD5^-CD45R (B220)^+ cells were fractionated by flow cytometry on a FACSVantage. For control experiments, CD4^- and/or CD8^- cells were obtained from thymus after staining and sorting.

**RT-PCR and nucleotide sequence analysis**

Total RNA was prepared from cytometrically fractionated spleen or peritoneal cells using TRIzol (Life Technologies, Rockville, MD) according to the manufacturer’s instructions. cDNA was prepared from total RNA using oligo(dT) as a primer and Superscript II reverse transcriptase (Life Technologies) according to the manufacturer's instructions. cDNA was prepared from total RNA using oligo(dT) as a primer and Superscript II reverse transcriptase (Life Technologies). Each cDNA sample was PCR-amplified with the Vμ186.2 primer, 5'-GCGTGTC CCTAGTCTTTCATG-3', and the human Cμ primer, 5'-GCGTGTC CCTAGTCTTTCATG-3' for transgenes and the Vμ186.2 primer and the mouse Cμ primer, 5'-GGGCGGAATTCGAGTACGCGACGTCGAC-3' for endogenous mouse Ig genes, respectively. The reaction protocol consisted of 30 cycles of 95°C for 1 min, 62°C for 1 min, and 72°C for 1 min. PCR products of transgenes and endogenous IgH

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**FIGURE 1.** A, IgH mutant transgene constructs (not to scale) used to generate chimeric mice. B, Locations and directions of primers used to amplify the cDNA are shown by arrows.
Results

Structural features of transgenes

The transgene constructs used in this work contain the V region, which is encoded by mouse V<sub>H</sub>186.2, the dominant V<sub>H</sub> involved in the response to NP hapten (2). The rearranged mouse V<sub>H</sub>186.2-DF1L6.1-J<sub>H</sub> gene from A6, an anti-NP hybridoma (9, 10), was linked to human C<sub>µ</sub>, thereby allowing for easy discrimination between transgene-encoded and endogenous mouse H chains. In addition, the transmembrane exon was removed from C<sub>µ</sub> to prevent expression on the cell surface, which might cause skewed B cell development. Transgenes contained only C<sub>µ</sub> but not those encoding other H chain isotypes.

All transgene constructs contained, as cis-acting elements, the V<sub>H</sub> promoter and E<sub>µ</sub>/MAR from the mouse J-C<sub>µ</sub>-MAR intron, both of which had been used for CAT transgene construction (21). A V<sub>H</sub> promoter sequence (550 bp) from V<sub>H</sub>7.2.25 (22), which was shorter than that of the CAT transgene (2 kb), was used in the present experiment. Therefore, differences in the structure of the transgenes were restricted to the 3′-flanking region (Fig. 1). One of the constructs, pvehc<sub>Δ3E</sub>, lacked the entire untranslated 3′ region and was driven by the V<sub>H</sub> promoter and E<sub>µ</sub>/MAR. Another construct, pvehc<sub>3E</sub>, contained 3′E (29) in addition to the V<sub>H</sub> promoter and E<sub>µ</sub>/MAR. The BamHI fragment (4 kb) containing 3′E was used for the gene construction. The addition of HS3b and HS4 to pvehc<sub>3E</sub> gave rise to a construct referred to as pvehc<sub>3E</sub>HS3b/4. HS3b (1.2 kb) and HS4 (1.4 kb) were obtained from genomic DNA of a B cell lymphoma, MPC11 (32), after amplifying by PCR. 3′E, HS3b, and HS4 were linked in tandem without any intervening Ig intron sequences. Endogenous Ig<sub>H</sub> genes contained an additional cis-acting element, referred to as HS3a (40), with identical nucleotide sequences to those of HS3b, but this element was in an inverted form (34). Our transgene constructs did not contain this cis-acting element.

Expression of transgene constructs in J558L cells

Transgene constructs were designed to encode chimeric H chains possessing the V<sub>H</sub> region from a mouse anti-NP mAb and the C region from human Ig, which were expected to assemble with mouse λ<sub>1</sub>-chains and give rise to anti-NP Abs. To estimate their transcriptional activity of these constructs, they were transfected into a mouse myeloma cell line, J558L, and anti-NP Ab production was measured by ELISA (Fig. 2). All constructs were actively translated in J558L cells and produced anti-NP Abs by pairing with endogenous λ<sub>1</sub>-chains. Among these constructs, pvehc<sub>Δ3E</sub> showed rather weaker production compared with the others. The addition of 3′E (pvehc<sub>3E</sub>) resulted in a significant increase in Ab production. However, further addition of HS3b and HS4 seemed to have no effect. Although little information was available concerning the dependence of transcription on the copy number of each transfected DNA, we assumed that the amount of Ab production reflected the transcriptional activity of the constructs, which did not differ significantly between pvehc<sub>3E</sub> and pvehc<sub>3E</sub>HS3b/4.

Generation of chimeric mice and immunization

To eliminate a possible effect from the positioning of the integrated transgenes as well as random mouse-to-mouse variation, we used at least two independently transfected ES clones for microinjection into the RAG-2<sup>−/−</sup> blastocysts to generate chimeric mice. We also used at least two chimeric mice per transgene construct for analyses of somatic hypermutation. The transfected ES cell lines and chimeric mice used in this experiment are shown in Table I.

To examine whether the immune system was reconstituted with lymphocytes derived from ES cells, the amounts of IgM in pre-immune sera of chimeric mice A4-3, 2-1, and 2-3 were measured by ELISA. Similar amounts of mouse IgM were detected in all sera of these mice, irrespective of transgene constructs, although there was less IgM than in normal BALB/c mice (Fig. 3A). However, we were unable to detect Abs bearing human C<sub>µ</sub> encoded by transgenes in preimmune or immune sera of mice immunized with NP<sub>34</sub>-CGG (Fig. 3B). As will be presented elsewhere (A. Terauchi and T. Azuma, manuscript in preparation), hybridomas producing anti-NP mAbs (γ1λ1) were prepared from the pvehc<sub>3E</sub>HS3/4 mice immunized with NP<sub>34</sub>-CGG. None of these secreted Abs bearing human C<sub>µ</sub>, although some of them synthesized intracellular H chains having human C<sub>µ</sub>. Therefore, it was suggested that
the transgenes were transcribed and translated in B cells of the chimeric mice. However, the H chain products secreted were under detectable levels. Next, the Ab response of chimeric mice to NP34-CGG, was examined by ELISA. Although titers varied, all mice produced anti-NP IgG Abs, indicating that their immune systems were responsible for the production of anti-NP Abs (Fig. 3C). We also examined the ratio of binding of these Abs to NP16-BSA relative to binding to NP9-BSA as a measure of affinity maturation. As shown in Fig. 3D, for a control mAb, F8, which showed no somatic hypermutation and has an association constant (Ka) to NP-Cap of 2 × 10^5 M^-1, the ratio was 0.29. In contrast, C6, a well-maturated mAb with a Ka of 2 × 10^7 M^-1 (10), had the ratio of around 1. Because all antisera from the chimeric mice showed ratios similar to that of C6, affinity maturation of anti-NP Abs proceeded to a similar extent in all chimeric mice after immunization with NP34-CGG.

Somatic hypermutation of transgenes in spleen or peritoneal B cells

Although immune systems were reconstituted in the chimeric mice, it was rather difficult to obtain a sufficient number of PNA^high^IgG^+^ B cells, known to be germinal center (GC) cells (41), from a single chimeric mouse. Therefore, we sorted IgM^+^B220^+^ cells among which we expected to select isotype-
FIGURE 5. Mutation in the transgenes of pvehcΔ3E (A) and pvehc3'E (B) in IgM spleen B cells from chimeric mice immunized with NP34-CGG. DNA sequences of cDNA clones from two individual chimeric mice are shown. The top line shows the unmutated nucleotide sequences of V_{HV}186.2-DFL16.1-J_{H}2 genes of the transgene, which are numbered according to Kabat et al. (61). Nucleotide sequences identical to those of the transgene construct are indicated by dashes. Location of CDRs are shown by shadowed letters. Although only data from a chimeric mouse are shown in this figure, essentially similar results were obtained with the other mice.
switched memory B cells by flow cytometry with a FACSVantage (BD Biosciences, San Jose, CA) after immunization with NP34-CGG. Somatic hypermutation in transgenes was examined by cloning and sequencing of cDNA prepared by RT-PCR with primers hybridizing to either \(V_H\)186.2 or human \(\gamma\mu\). Specific amplification of transgenes by use of this combination of primers was confirmed by the facts that PCR products only were observed in spleen cells from chimeric mice and not in those from C57BL/6 mice immunized with NP34-CGG (Fig. 4), and that all sequences had identical junctional diversity in CDR3, which is characteristic of the A6 DNA used for constructing transgenes (Figs. 5 and 6). Mouse-to-mouse variation in the RT-PCR products of endogenous Ig genes (Fig. 4) was explained in terms of cross hybridization of the \(V_H\)186.2 primer to \(V_H\)186.2-related \(V_H\) genes, because the \(V_H\)186.2 gene was absent in germline genes of 129/O1a mice (Igh\(^a\)) from which the ES cells were derived.

Although the frequency was low, we found a few nucleotide changes resulting from somatic hypermutation in \(V_H\)186.2-DFL16.1-\(J_H\)2 of pveh\(^D\)3\(E\) (Fig. 5A). The frequency of mutation was estimated to be 0.17% (Table II), which was in agreement with that observed in CAT-transgenic mice and confirmed the previous conclusion that the \(V_H\) promoter and/or \(\gamma\mu/MAR\) were responsible for the induction of somatic hypermutation (21). In contrast, we were unable to detect any nucleotide changes in transgenes from CD4\(^+\)/CD8\(^+\) thymus cells, suggesting that the somatic hypermutation machinery did not operate in these cells and that nucleotide changes attributable to PCR error or to mutation during generation of chimeric mice were negligible under the experimental conditions used (data not shown).

FIGURE 6. Mutation in the transgene of pveh\(^C\)EH3b/4 in IgM\(^+\) spleen B cells from two chimeric mice immunized with NP34-CGG. DNA sequences of cDNA clones from two chimeric mice are shown. The top line shows the unmutated nucleotide sequences of \(V_H\)186.2-DFL16.1-\(J_H\)2 genes of the transgene. Nucleotide sequences identical to those of the transgene construct are indicated by dashes. Location of CDRs are shown by shadowed letters. Although only data from a chimeric mouse are shown in this figure, essentially similar results were obtained with the other mouse.
Table II. Mutations in V region sequences of spleen IgM B cells of chimeric mice

<table>
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<tr>
<th>Transgene</th>
<th>No. of ES Clones</th>
<th>No. of Sequences Analyzed</th>
<th>No. of Mutations Observed</th>
<th>% of Mutation in CDR&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% of Mutation Frequency&lt;sup&gt;b&lt;/sup&gt;</th>
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<sup>a</sup> Percent of mutated sequences in CDR = total no. of point mutations in CDR/total no. of point mutations.

<sup>b</sup> Mutation frequency = total no. of point mutations/total no. of base pairs.

<sup>c</sup> Two chimeric mice were analyzed.

The transgene, pvehc<sub>3</sub>E, showed an essentially similar distribution of hypermutation (Fig. 5B), although the frequency increased slightly compared with that of pvehc<sub>Δ3</sub>E (Table II). However, it was still lower than that observed in endogenous V<sub>H</sub> genes, suggesting that 3<sub>E</sub> did not contribute significantly to induction of somatic hypermutation.

The addition of HS3b/4 to pvehc<sub>3</sub>E resulted in a dramatic increase in the frequency of somatic hypermutation in V<sub>H</sub>186.2-DFL16.1-J<sub>H</sub>2 genes from the transgene construct pvehc<sub>3</sub>EHS3b/4. All PCR clones sequenced contained nucleotide changes despite the fact that they were selected randomly. These changes were found with high frequency in the area around CDR2 and CDR3 (Fig. 6 and Table II).

The distribution and frequency of somatic hypermutation among the transgene constructs in spleen B cells are compared in Fig. 7. The data obtained from two chimeric mice corresponding to each transgene construct are shown in this figure and are in good agreement with each other. A high frequency of hypermutation was apparent in pvehc<sub>3</sub>EHS3b/4. However, mutation was clearly absent in peritoneal B1 cells from the same chimeric mouse (Fig. 7B).

Table III shows the nature of substitutions identified in the transgenes. A comparison of IgH transgenes reveals that transitions and transversions occur at approximately equal frequency. We also analyzed the occurrence of somatic hypermutation in the V<sub>H</sub>186.2 transgene at the location of the sequence referred to as RGYW motif (A/G, G, C/T, A/T) (42, 43). The germline V<sub>H</sub>186.2 gene...
(294 bp) contains the RGYW/WRCY motif (93 bp), which corresponds to 32% of the total bp. The frequency of somatic hypermutation in this motif was found to be 34%, which would not be significantly higher than that of the other \(V_{H}\) regions.

**Discussion**

In the present experiments, we used RAG-2\(^{-/-}\) blastocyst complementation (31) and prepared chimeric mice carrying IgH transgenes with different \(cis\)-acting elements. This method has several advantages over the conventional transgene technology, especially its superiority in terms of the low copy number of transgenes transfected into ES cells. In fact, the copy number of transgenes in the ES cell transfectants was estimated to be three copies at most by Southern blotting and PCR (Table I). To eliminate the possibility of a positional effect from the placement of integrated transgenes and mouse-to-mouse variation, we used at least two chimeric mice per transgene construct with ES cell transfectants prepared by independent electroporation. Complementation of the RAG-2\(^{-/-}\) mouse immune system with that of ES cells was confirmed by the appearance of lymphocytes in peripheral blood and production of Ab to NP\(_{34}\)-CGG. The cooperation of T and B cells in the immune response to NP\(_{34}\)-CGG was expected to proceed normally, because affinity maturation of endogenous anti-NP Abs occurred without any significant difference between mice carrying pvehc3'E and those carrying pvehc3'EHS3b/4 (Fig. 3C).

To elucidate the mechanisms responsible for somatic hypermutation, it is essential to identify the \(cis\)-acting elements responsible for its induction (44). Experiments with transgenic mice carrying \(\kappa\)-chain or \(H\) chain transgenes revealed that \(E_{\mu}/MAR\) is critical for induction of hypermutation (14, 19). IgH gene exons are not required for the induction, as we previously showed with transgenic mice carrying the CAT gene driven by the \(V_{H}\) promoter and \(E_{\mu}/MAR\). However, the rather low frequency of hypermutation in CAT-transgenic mice suggested that other components are important in raising the frequency of hypermutation (21). Johnston et al. (45) created transgenic mice carrying, in addition to \(E_{\mu}\), a 727-bp fragment of 3'E from Wistar rat genomic DNA. However, the transgene did not induce a high frequency of hypermutation.

We prepared chimeric mice carrying IgH transgenes with different \(cis\)-acting elements and examined somatic hypermutation after immunization with NP\(_{34}\)-CGG. We used at least two chimeric mice per transgene and obtained essentially similar results, suggesting that mouse-to-mouse variation or effects of positioning of integration sites of transgenes were negligible in our case. In a prototype transgene, pvehc3Δ3'E, we found only a marginal number of hypermutations and confirmed previous findings that the \(cis\)-acting elements, \(V_{H}\) promoter and \(E_{\mu}/MAR\), were not sufficiently for induction of a high frequency of hypermutation. Addition of a 3'E fragment (4 kb) to pvehc3Δ3'E resulted in an \(~30\%\) increase in the hypermutation frequency (Table II). This may be related to an enhanced transcription activity of pvehc3'E compared with that of pvehcΔ3'E in B cells as observed with J558L cells (Fig. 2). However, the degree of hypermutation was still low compared with that of endogenous \(V_{H}\) genes (46, 47). Finally, we introduced HS3b and HS4 to the 3'E end of C genes were thought to be important for increasing the hypermutation frequency. With transgenic mice carrying mutant \(\kappa\)-chain transgenes, Betz et al. (14) showed that 3'E\(_{\kappa}\) helped to enhance the frequency of hypermutation. However, recent experiments with mice lacking 3'E\(_{\kappa}\) showed that it was not involved in the induction of hypermutation of \(\kappa\)-chain genes (50). The discrepancy between the results of these two experiments may have arisen from a difference in the size of 3'E used. Betz et al. (14) used a 1.2-kb fragment to construct transgenes, and Gorman et al. (51) deleted 808 bp from 3'E\(_{\kappa}\). Therefore, the element responsible for a high frequency of hypermutation may exist outside of 3'E\(_{\kappa}\) (808 bp) but instead in the 1.2-kb fragment made up of 3'\kappa-chain transgenes. Our present findings suggested that HS3b/4 but not 3'E\(_{\kappa}\) (HS1, 2) contributed to the significant enhancement of the hypermutation frequency in H chain transgenes, although we have no direct evidence of whether HS3b/4 alone without 3'E\(_{\kappa}\) was capable of inducing a high frequency of somatic hypermutation. The validity of our prediction currently is being examined by preparing chimeric mice carrying either HS3b or HS4.

3'H5 consisting of HS3a, HS1.2, HS3b, and HS4, acts as a locus control region (32, 52–54). 3'E corresponds to HS1.2 and was shown to be an active transcriptional enhancer in B-lineage cells (47, 55–57). In fact, pvehc3'E displayed higher transcriptional activity than pvehcΔ3'E in J558L cells, suggesting that 3'E augmented the \(E_{\mu}/MAR\) effect. However, it is unlikely that HS3b/4 also acted as enhancer elements in J558L cells because a further increase in activity was not observed with the addition of HS3b/4 to pvehc3'E. This transcriptional activity of mutant transgene constructs in plasma cell lines may not be suitable for measuring the capability of \(cis\)-acting elements to induce hypermutation because hypermutation occurs only at certain stages of B cell development, such as the point at which centroblasts appear in the GC (58–60). Information on factors that interact with HS3b/4 at GC stage of B lineage cells currently is being examined by preparing chimeric mice carrying either HS3b or HS4.
promoter/Eκ and HS regions. In endogenous Ig μ-chain genes, the distance was ~200 kb, whereas it was less than 20 kb in the case of pvehc3' EHS3b/4 because 3′E and HS3b/4 directly flanked 3′ of Cκ, similar to the situation in class-switched endogenous Ig genes (32). It was expected that such a short distance between the Vμ promoter/Eμ and HS regions enabled pvehc3' EHS3b/4 to induce a high frequency of somatic hypermutation.

Because our transgenes lacked membrane exons, they were unable to be expressed on the B cell surface and to be subjected to Ag selection. Therefore, results obtained from the transgenic mice revealed the distribution and frequency of somatic hypermutation without Ag selection. Hypermutation in pvehc3' EHS3b/4 was frequent around CDR2 and 3 but was less so in CDR1. This is in contrast to the distribution in endogenous Vμ, in which somatic hypermutation accumulated around CDR1 and CDR2 (7, 61, 62). A particularly high frequency of mutation found at position 33 corresponding to the codon TGG has been shown to result from Ag selection (7) because a nucleotide change from TGG to TTG gives rise to the replacement of Trp with Leu, accompanied by a 10-fold increase in affinity. Therefore, the fact that no mutation occurred in pvehc3' EHS3b/4 at position 33 clearly was explained in terms of the absence of Ag selection. A high frequency of mutation around CDR3 also was evident in the transgene. Because CDR3 is at the junction of Vμ, D, and Jμ segments, complex relationships among nucleotide sequences in this site, resulting in the addition or deletion of nucleotides, makes the assignment of somatic hypermutation difficult, and little information on its frequency in CDR3 was gained from the analysis of endogenous Ig genes. The results obtained with the transgene clearly showed that CDR3 is the hypermutation target. As for the nature of the nucleotide substitutions, transitions and transversions occur with approximately equal frequency (Table III). In addition, we were unable to detect a preferential occurrence of somatic hypermutation in the RGYW motif of the Vμ, Jκ2,2 transgene (data not shown). We have no clear explanation for the discrepancy between our finding and those of other investigators (42, 43). We are currently examining the relationship between the positions of double-strand breaks and somatic hypermutation with pvehc3' EHS3b/4-transgenic lines.

We were unable to detect Abs harboring transgene-encoded μ-chains in chimeric mice despite the fact that the transgene-encoded anti-NP Abs were secreted by the transfected J558L cells. Because transgene-encoded μ-chains were found in the cytoplasm of some hybridomas, it was anticipated that they were synthesized but not secreted by Ab-forming cells (AFCs) in chimeric mice (A. Terauchi and T. Azuma, manuscript in preparation). It is likely that the transgene products consisting of mouse Vμ, D, and Jμ segments, complex relationships among nucleotide sequences in this site, resulting in the addition or deletion of nucleotides, makes the assignment of somatic hypermutation difficult, and little information on its frequency in CDR3 was gained from the analysis of endogenous Ig genes. The results obtained with the transgene clearly showed that CDR3 is the hypermutation target. As for the nature of the nucleotide substitutions, transitions and transversions occur with approximately equal frequency (Table III). In addition, we were unable to detect a preferential occurrence of somatic hypermutation in the RGYW motif of the Vμ, Jκ2,2 transgene (data not shown). We have no clear explanation for the discrepancy between our finding and those of other investigators (42, 43). We are currently examining the relationship between the positions of double-strand breaks and somatic hypermutation with pvehc3' EHS3b/4-transgenic lines.

As a result of this study, we are now able to define the minimal elemental unit necessary for the somatic hypermutation of IgH chain genes. The germine transmission of the transgene, pvehc3' EHS3b/4, made it possible to establish a transgenic mouse line carrying this gene. This model will be useful for gaining further insight into the mechanism of somatic hypermutation.

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References


