VH GENE FAMILY UTILIZATION IS REGULATED BY A LOCUS OUTSIDE OF THE VH REGION

By Gillian E. Wu* and Christopher J. Paige*†

From the Basel Institute for Immunology, CH-4005 Basel, Switzerland; the *Department of Immunology University of Toronto, Toronto, Ontario, M5S 1A8; and †the Ontario Cancer Institute, Toronto, Ontario M4X 1K9, Canada

The Ig heavy chain is composed of four regions, Vh, Dh, Jh, and CH. Each region is encoded by discrete gene segments which, in the germline state, are widely separated on the chromosome (1). During differentiation along the B cell pathway, three of these gene segments, namely, V, D, and J, become juxtaposed, forming a complete heavy chain gene with the ensuing deletion of the intervening DNA sequences. In the mouse, there are >100 germline-encoded heavy chain VH gene segments categorized into at least nine families based upon sequence homology to representative DNA probes (2–4). Whereas the genetic mechanism involved in the joining process has received much attention, little is known concerning the basis for the selection of a particular VH gene segment (5–7). Models based upon transformed cell lines and/or total RNA suggest that the process is developmentally ordered and related to the position of the V gene segment relative to the DJ-C complex (5). Thus some families are underrepresented in B cell hybridomas from fetal or neonatal mice as well as in Abelson-transformed pre-B cell lines, while other families, notably the Jh most proximal gene family, Vh7183, are overrepresented (5). In this communication we provide evidence that a genetic locus (or loci) lying outside the VH region plays a regulatory role in VH selection. This conclusion is based upon the analysis of VH family usage in primary B cell colonies derived from congenic mice carrying the Igha or Ighb haplotype.

Materials and Methods

Colony-forming B cells were grown as previously described (8–10) from the spleen cells of adult mice obtained from the breeding facilities of the Basel Institute for Immunology. The RNA blots were hybridized with Cμ and eight VH family–specific DNA fragments as previously described (8), and the resulting filters were autoradiographed for 5–14 d. The VH family–specific probes were isolated from appropriately digested plasmids using standard procedures (9). The plasmids were kindly provided by F. Alt, G. Yancopoulos, E. Dzierzak, R. Riblet, P. Brodeur, R. Perlmutt, J. Adams, and G. Lennox.

Interpretation of RNA dot blot analyses can be hampered by nonspecific background hybridizations. These difficulties were overcome in the RNA colony blot assay by rigorously testing our probes and procedures. We found the following details important: frag-
ment size ranging from 200 to ~1,000 bp was optimal; the specificity of each fragment used was confirmed each time it was reisolated using cell lines of known V\(_H\) regions. *Escherichia coli* DNA (100 \(\mu\)g/ml) and poly A (100 \(\mu\)g/ml) were included in the prehybridization and hybridization solutions; long prehybridization (overnight), hybridization (36 h), and washing procedures (3–4 h) were used.

Control experiments probing the same colony with more than one V\(_H\) family probe showed hybridization to only one probe. Furthermore, we failed to detect hybridization to probes encompassing sequences not found in mature Ig mRNA (e.g., 3' V\(_H\)J558, C\(_\mu\) intervening sequence probes). These controls demonstrated that transcriptions that may occur during B cell development, such as those due to germline V gene transcription, aberrant or sterile C\(_\mu\) transcription, and V\(_H\) gene replacement, are not detectable in our assay. We emphasize that at the time of assay, B cell colonies contain mature Ig-secreting cells. Transcriptions that could have been more abundant at earlier times in colony development may well be undetectable at the time of assay.

**Results and Discussion**

We have used the RNA colony blot assay to characterize the V\(_H\) family usage of B cell colonies derived from surface Ig\(^+\) (sIg\(^+\)) spleen cells (CFU-B) and sIg\(^-\) fetal liver cells (CFU-pre-B) with eight V\(_H\) family-specific DNA probes (9). We found that V\(_H\) family usage was independent of whether the cell that initiated colony formation was a CFU-B or CFU-pre-B. However, we did find that the V\(_H\) family usage was highly strain dependent. Discrete patterns of V\(_H\) family usage are particularly prominent when C57BL/6 and BALB/c mice are compared and can be most easily illustrated by analysis of two V\(_H\) gene families, namely V\(_H\)J558, the largest family, and V\(_H\)7183, the most J\(_\mu\) proximal family in BALB/c mice. C57BL/6 mice used V\(_H\)J558 most frequently (mean in six experiments monitoring >2,500 individual colonies: 45%) and V\(_H\)7183 far less frequently (mean: 10%). In contrast, BALB/c mice used the V\(_H\)7183 family most frequently (mean in nine experiments monitoring >700 individual colonies: 22%) and used V\(_H\)J558 less often (mean: 13%). To simplify this comparison we express these data as a single parameter, i.e., the V\(_H\)J558/V\(_H\)7183 ratio. In these experiments this ratio was 4.7:1 in C57BL/6 mice and 0.6:1 in BALB/c mice.

As a first approach to identify the basis for this genetic difference, we analyzed V\(_H\) usage in B cell colonies derived from (C57BL/6 \(\times\) BALB/c)\(F_1\) mice and (BALB/c \(\times\) C57BL/6)\(F_1\) mice. Based upon analysis of 2,343 colonies from the \(F_1\) mice we found that the percent utilization detected was intermediate between the parental patterns in every V\(_H\) family for which parental differences had been noted (9). This point is best illustrated by comparing \(F_1\) and parental mice with respect to the V\(_H\)J558/V\(_H\)7183 ratio (Fig. 1). This result rules out the possibility that the V\(_H\) usage is determined by a dominant diffusible gene product encoded by either C57BL/6 or BALB/c genes. Further, since similar V\(_H\)J558/V\(_H\)7183 values were found in the reciprocal \(F_1\) hybrids, the influence of maternally inherited factors must be negligible in the determination of the V\(_H\) family.

An intermediate response in the \(F_1\) hybrids could be explained if the Igh locus itself could determine V\(_H\) family usage. Then colonies expressing the BALB/c Igh allele would show the V\(_H\) preference of BALB/c, while those expressing the C57BL/6 allele would show the C57BL/6 preference. This possibility was tested by analyzing mice congenic at the Igh locus. We used two congenic strains for
this analysis: B.C-Iga (C57Ig*), which has the Igh locus of BALB/c on a C57BL/6 background; and CB20, which has the Igh locus of C57BL/6 on a BALB/c background (11). The VH genotype of the congenic mice was verified by Southern blot analysis of kidney DNA (Fig. 2).

The frequency of VH family usage was determined for the congenic and parental strains and the VHJ558/VHJ183 ratios are shown in Fig. 3. The ratios fall into two groups: a BALB/c type group (BALB/c, CB20) and a C57BL/6 type group (C57BL/6, C57Igh*). The BALB/c group has low VHJ558/VHJ183

FIGURE 1. Plot of the VHJ558/VHJ183 ratios in individual parent and F1 hybrid experiments. Each circle is the ratio determined from one complete VH family usage experiment in which triplicate blots were assessed for the hybridization to μ or the VH family probes. The mean ratio (± SEM) of each group resulting from the analysis of >500 individual colonies in every case is 5.03 ± 1.73 for C57BL/6, 0.68 ± 0.14 for BALB/c, and 2.41 ± 0.30 for F1 hybrids. Application of the Mann-Whitney U-test to these data reveals that each of these groups differ significantly from each other (p > 95%). BALB/c (●), C57BL/6 (○), F1, with a BALB/c mother and a C57BL/6 father (□). F1, with a C57BL/6 mother and a BALB/c father (◇). (-) Arithmetic mean.

FIGURE 2. Southern blot analysis of parental and congenic DNA. Approximately 5 μg of genomic DNA was digested with Eco RI fractionated on a 0.8% agarose gel and transferred to GeneScreenPlus filters. Triplicate blots were assayed for hybridization to 32P-labeled VHJ52, VHJ606, and VHJ183 gene family probes (9). After hybridization and washing the blots were exposed for 7 d with intensifying screens. The VHJ52 probe consists of an ~300 bp Eco RI–Bam HI fragment from pVHJ52Hha.1 and contains the VH coding region and 20 JH nucleotides. The VHJ183 probe consists of an ~1,000-bp Eco RI–Bam HI fragment from pVHJ52APC-15 containing the VH coding region and some 5' flanking sequences. The probe VHJ606 contains an ~600-bp Eco RI–Bam HI fragment from pBV14J606 containing the VH coding region and 5' flanking sequences. Sizes in kilobases of the marker DNA λ cut with Hind III, are noted on the right of the autoradiographs.
FIGURE 3. Plot of the $V_H^{J558}/V_H^{7183}$ ratios in C57BL/6 and BALB/c parents, and CB20 are C57Igα congenic mice. Each circle is the ratio determined from one complete $V_H$ family usage experiment in which triplicate blots were assessed for hybridization to $\mu$ or the $V_H$ family probes. The mean ratio (±SEM) of each group, resulting from the analysis of >500 individual colonies in every case is 3.86 ± 0.27 for C57BL/6, 0.94 ± 0.22 for BALB/c, 1.35 ± 0.12 for CB20 and 3.22 ± 0.23 for C57Igα. Application of the Mann-Whitney U-test to these data revealed that the following groups differ significantly (p > 95%) from each other: C57BL/6 vs. BALB/c; C57BL/6 vs. CB20; C57Igα vs. BALB/c; C57Igα vs. CB20. In contrast, the following groups were found not to differ significantly: BALB/c vs. CB20 and C57BL/6 vs. C57Igα.

ratios (1.1:1, 1.4:1) and correlates with the BALB/c background; the C57BL/6 group has high $V_H^{J558}/V_H^{7183}$ ratios (3.9:1, 3.3:1) and correlates with the C57BL/6 background.

These data demonstrate that polymorphisms of the $V_H$ locus are not the principle determinants of $V_H$ family usage. Thus, differences in the putative recombination sequences, the overall organization of the $V_H$ locus, linear map distance from combinatorial elements or in the quantity and quality of $V_H$ genes themselves, do not explain strain differences in $V_H$ family usage. Rather, one (or more) locus outside of the Igh locus regulates the relative frequency at which $V_H$ gene families are used.

In our experiments, 3–5% of BALB/c spleen cells (~5–10% of sIg− B cells) form colonies, whereas 10–15% of C57BL/6 spleen cells (~20–30% of sIg+ B cells) form colonies. To determine whether the cloning efficiency was related directly to $V_H$ gene family usage, we studied recombinant inbred mice derived from BALB/c × C57BL/6 parents. We found the cloning efficiency and $V_H$ gene family usage varied independently. In four experiments analyzing >1,000 colonies, BALB/c and C57BL/6 controls had values for cloning efficiency similar to those reported previously (3 and 10% of input spleen cells, respectively) as well as typical J558/7183 ratios (1.2 and 4.3, respectively). In contrast, recombinant inbred strains CXBH and CXBI exhibited C57BL/6 type J558/7183 ratios (4.6 and 4.3, respectively) with low-to-intermediate cloning efficiencies (5 and 6%, respectively). These results demonstrate that the loci that determine cloning efficiency are unlinked to the loci that determine $V_H$ gene family usage.

Shultze and Kelsoe (11) have also examined $V_H$ usage in primary B cell colonies derived from spleen. In their system splenic cells are grown on filter paper discs in the presence of LPS and thymocytes. In this system they have determined the usage of three $V_H$ families, namely X24, Q52, and J558, in both C57BL/6 and BALB/c strains. Although they note a slightly higher usage in BALB/c of the Q52 family this difference is not significant in their system and they conclude that strain-specific differences are not found. The discrepancy between these data and our own may well be due to technical differences in the assays. The filter paper disc method requires colony sizes to exceed 100 cells
for a signal to be detected, whereas the RNA colony blot assay detects colonies that have achieved the 30-cell stage. Perhaps because of this difference 1% of the input spleen cells (from C57BL/6) are detectable in the filter paper disc method, whereas 10–15% of the input spleen cells are detectable in the RNA colony blot method. Further, although both methods rely on LPS as the principal proliferative stimulus, thymocytes are present in the filter paper disc assay and not in the RNA colony blot assay, whereas agar mitogen is present in the latter and not in the former. These differences make direct comparisons difficult. It should also be noted that the polymorphism detected in our assay may well be due to a trait that has already diverged within either the BALB/c or C57BL/6 mouse strain.

The experiments reported in this paper demonstrate a stable and reproducible phenotypic difference, detected as the probability of $V_H$ usage, which can be traced through parental, $F_1$, congenic, and recombinant inbred strains. The discovery of an unexpected genetic region outside the Ig loci that influences the probability of $V_H$ usage leads to the prediction that important regulatory mechanisms exist and may be mapped based upon the assay described herein. These might involve genes encoding the recombination enzymes or genes that regulate their expression, differences in factors affecting DNA topology, or even post-translational differences that would result in intracellular selection.

Summary

We have used the RNA colony blot method to examine $V_H$ usage in colonies derived from primary splenic B cells. We found that there are strain-specific differences in the pattern of $V_H$ usage. Using parental $F_1$, congenic, and recombinant inbred strains we demonstrate that the genetic element that causes the observed phenotype is: (a) stably expressed; (b) not due to maternal influence; (c) not due to dominate diffusible factors; (d) not linked to cloning efficiency; and (e) outside the Ig locus.

We thank B. Grossenbacher and H. Dick for technical assistance; C. Plattner for preparing this manuscript; Christopher Coleclough and Michael H. Julius for helpful suggestions and comments.

Received for publication 21 December 1987 and in revised form 2 February 1988.

References

1. Tonegawa, S. 1983. Somatic generation of antibody diversity. Nature (Lond.). 302:575.
2. Brodeur, P., and R. Riblet. 1984. The immunoglobulin heavy chain variable region (Igh-V) locus in the mouse 1. One hundred Igh-V genes comprise seven families of homologous genes. Eur. J. Immunol. 14:922.
3. Livant, D., C. Blatt, and L. Hood. 1986. One heavy chain variable region gene segment subfamily in the Balb/c mouse contains 500–1000 or more members. Cell. 47:461.
4. Rathburn, G. A., J. D. Capra, and P. Tucker. 1987. Organization of the murine immunoglobulin $V_H$ complex in the inbred strains. EMBO (Eur. Mol. Biol. Organ.) J. 6:2931.
5. Yancopoulos, G., S. Desiderio, M. Paskind, J. Kearney, D. Baltimore, and F. Alt. 1984. Preferential utilization of the most JH-proximal VH gene segments in pre-B cell lines. Nature (Lond.). 311:727.

6. Yancopoulos, G., and F. Alt. 1985. Developmentally controlled and tissue-specific expression of unrearranged VH gene segments. Cell. 40:271.

7. Hesse, J. E., M. R. Lieber, M. Gellert, and K. Mizuuchi. 1987. Extrachromosomal DNA substrated in preB cells undergo inversion or deletion at immunoglobulin V-(D)-J joining signals. Cell. 49:775.

8. Paige, C., G. Wu, and C. L. Castiglia. 1986. Detection of RNA transcripts in normal lymphoid and myeloid colonies. J. Immunol. Methods. 93:37.

9. Wu, G., and C. Paige. 1986. VH gene family utilization in colonies derived from B and pre-B cells detected by the RNA colony blot assay. EMBO (Eur. Mol. Biol. Organ.) J. 5:3475.

10. Paige, C., H. Skarvall, H. Sauter, and S. Magasiny. 1985. Development of B-cell progenitors in semisolid agar cultures. In Immunological Methods. Vol. 3. I. Lefkovits and B. Pernis, editors. Academic Press, New York. 221.

11. Schultze, D. H., and G. Kelsoe. 1987. Genotype analysis of B cell colonies by in situ hybridization. J. Exp. Med. 166:163.