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Molecular cloning of the primary IgH repertoire: a quantitative analysis of V_H gene usage in adult mice

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The generation of the primary antibody repertoire requires the somatic rearrangement of germline gene segments. It is not known, however, whether all functional V and J gene segments have an equal probability of contributing to this initial set of antibody specificities. To address this issue, we have examined the relative utilization of V_H and J_H gene segments of the mouse. We have constructed V_H cDNA phage libraries from $C\mu$ transcripts obtained from polyclonally activated spleen cells of the BALB/c and C57BL/6 strains. We show that probes specific for either one, two or three functional V_H gene segments hybridize to cDNAs at frequencies directly proportional to the number of functional germline V_H genes detected by each probe. In contrast, the representation of 10 V_H gene families within each library indicates that certain families are under-represented relative to their estimated germline gene number. These families must either have extraordinary proportions of nonfunctional genes or are influenced by as yet unidentified regulatory mechanisms or constraints on rearrangement.

Key words: immunoglobulin genes/Ig repertoire/V_H gene families/V_H gene utilization

Introduction

Studies of the mechanisms of antibody diversification have elucidated the contribution of germline encoded gene segments in the development of the antibody repertoire (reviewed in Alt *et al.*, 1987). The production of functional immunoglobulins requires the rearrangement of V_H, D and J_H segments. In the mouse there are four J_H, 12 known D and >100 V_H gene segments that can potentially contribute to the antibody combining sites available prior to selection by exogenous antigens. The developing repertoire is dependent on the relative contribution of these various germline elements to generate this primary repertoire.

The classification of V_H gene segments into at least 11 V_H families on the basis of sequence homology (Brodeur and Riblet, 1984; Winter *et al.*, 1985; Kofler, 1988; Reininger *et al.*, 1988) has provided a useful parameter by which the content of the *Igh-V* locus can be assessed and compared with the expressed repertoire. For example, analyses of B lineage cells from fetal and neonatal mice show biased utilization of the V_H families most proximal to the D and J_H subregions (Yancopoulos *et al.*, 1984, 1988; Perlmutter *et al.*, 1985a; Jeong and Teale, 1988). In contrast, the repertoire of adult splenic B cells has been reported to reflect more closely the estimated size of each V_H family (Dildrop *et al.*, 1985; Wu and Paige, 1986; Schulze and Kelsoe, 1987; Jeong *et al.*, 1988; Yancopoulos *et al.*, 1988). This suggests a shift during ontogeny from a biased to a probabilistic utilization of V gene segments. However, the relative expression of V_H families varies among inbred strains (Wu and Paige, 1986; Jeong *et al.*, 1988; Yancopoulos *et al.*, 1988), and the number of functional V_H genes within most families is unknown. Therefore, the question of whether all functional V_H genes have an equal probability of being expressed remains.

We describe here a molecular cloning strategy to study the expressed V_H repertoire. We report the detailed characterization of two V_H cDNA libraries and the relative utilization of 10 V_H gene families. We demonstrate that the functional members of two families (V_HX24 and V_HS107) are expressed at frequencies consistent with equivalent expression of individual functional V_H genes. These findings are discussed in the context of the strain associated patterns of V_H gene family usage, the shift in V_H gene usage during early development and estimates of the germline content of the *Igh-V* locus of the mouse.

Results

V_H gene cDNA libraries: construction and characterization

To study the V_H repertoire of adult mice, we have sampled the IgM heavy chain transcripts of polyclonally activated splenic B cells using a direct cDNA cloning approach. Briefly, cDNAs were synthesized using polyadenylated mRNAs obtained from adult spleen cells after 3 days in culture with LPS. First strand synthesis reactions were specifically primed with a $C\mu$ region specific oligonucleotide and the resultant cDNAs were size selected from polyacrylamide gels. The cDNAs were ligated into the phage vector λgt10, packaged *in vitro* and the phage plated on the bacterial host C600 *Hfl*. An amount of 5 μg of RNA yielded 2.2×10^5 and 9×10^4 recombinant phage for the BALB/c and C57BL/6 libraries, respectively. Approximately one-half of each library was used for amplification to obtain high-titer stocks of recombinant phage.

The recombinant phage of the two libraries were analyzed by plaque lift hybridization using a $C\mu$ probe (p3741, Marcu *et al.*, 1980) and oligonucleotide probes specific for each of the four J_H segments. As summarized in Table I, approximately one-third of the recombinant phage in the BALB/c library and one-fourth in the C57BL/6 library hybridized with the J_H probes (J_H^+). The proportion of $C\mu$-hybridizing phage ($C\mu^+$) was not altered during the amplification of the libraries.

To characterize more fully the $C\mu^+$ cDNAs within the libraries, 98 $C\mu^+$ BALB/c and 100 $C\mu^+$ C57BL/6 derived phages were isolated from the non-amplified libraries for

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detailed analyses. The phage were spotted onto bacterial lawns by pipetting $1-2$ μl of each phage isolate within a 10×10 grid and incubating at 37°C until large plaques (~5 mm) had formed. The plaques were lifted onto 132 mm nitrocellulose filters and hybridized with radiolabeled probes for 10 V_H gene families, the four J_H segments and $C\mu$ (Figures 1 and 2).

Analysis of the BALB/c $C\mu^+$ panel (Figure 1) showed that most (94/98) of the $C\mu^+$ phages are J_H^+ and that only a small fraction (5/94) are positive for two different J_H segments. Furthermore, most of the J_H^+ phages (93/94) contain V_H sequences as determined by hybridization. Only five phages (E2, G3, G8, H2, J1) are positive with two V_H probes, four of which are among the five phage containing two different J_H segments. Therefore, each of the isolates which hybridize to more than one probe either contains two independent cDNA inserts or includes two distinct phage populations.

Analysis of the C57BL/6 $C\mu^+$ panel (Figure 2) gave similar results—most phages are J_H^+ (98/100), a small fraction (1/100) contain sequence for two J_H segments (E9) and the vast majority of J_H^+ phages have V_H sequences homologous to one of the 10 V_H gene families (93/98). None of the C57BL/6 $C\mu^+$ panel has scored positive for more than a single V_H family. The results of screening the $C\mu^+$ panels of both BALB/c (Figure 1) and C57BL/6 (Figure 2) are summarized in Table II. These data demonstrate that most, and perhaps all, of the J_H^+ recombinant phages contain V_H sequences and that most phages contain a single V_H cDNA insert. Most importantly, these internally controlled analyses clearly show that non-cross hybridizing sets of V_H gene segments are identified under the conditions described.

Table I. Composition of V_H cDNA libraries

Library	Strain	No. of recombinant phages	$\%J_H^{+a}$	Total J_H^+ phages
c45	BALB/cByJ	2.2×10^5	33.9	7.5×10^4
b48	C57BL/6J	9.0×10^4	26.3	2.4×10^4

[a] J_H^+phages hybridizing with one of four J_H segment specific oligonucleotide probes.

Accuracy of screening V_H cDNA libraries

The quantitative assessment of V_H gene representation within the V_H cDNA libraries is one of the principal advantages to this approach as thousands of recombinant phages from the amplified libraries can be sampled for screening with a variety of probes. To verify directly the accuracy of V_H family scoring on large scale screening, lifts of plates containing $1000-2000$ J_H^+ phages each were screened by hybridization with a probe specific for the

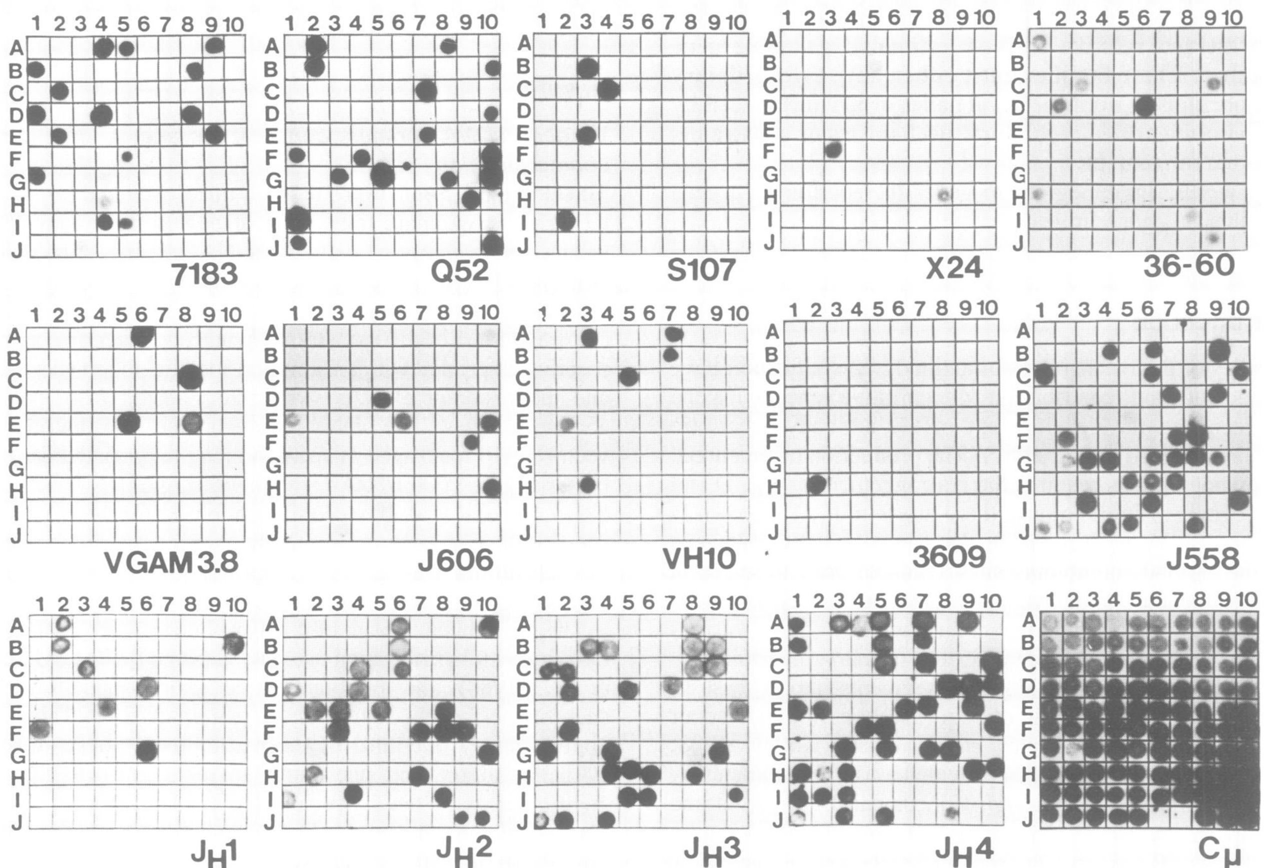

Fig. 1. Analysis of $C\mu^+$ BALB/c cDNA. $C\mu^+$ phage were isolated from the BALB/c V_H cDNA library (c45) and individual isolates plated on bacterial lawns within 10×10 grids on 150 mm plates. The resulting plaques (~5 mm diameter) were screened by plaque lift hybridization using probes for V_H gene families, J_H segments and $C\mu$. Phage grown in J6 and J7 of the grid are $C\mu^-$ controls.

V_HS107 family. A panel of 96 recombinant phages, counted as positive for V_HS107 cDNAs, was selected and plated within 10 × 10 grids as described above. Lifts of the resulting plaques were hybridized with the V_HS107 probe or probes of the four families most closely related by nucleotide sequence to V_HS107 (V_H7183, V_HJ606, V_HX24, V_H10).

The results (Figure 3) show that 93 of the 96 selected phages contain cDNAs of V_HS107 genes. Of these 93 phages, two also hybridize with the V_H7183 probe and one also hybridizes with the V_H10 probe suggesting that these three phages contain two V_H cDNA inserts. Three phages do not hybridize with the V_HS107 probe; of these, one hybridizes with the V_H7183 probe whereas the other two

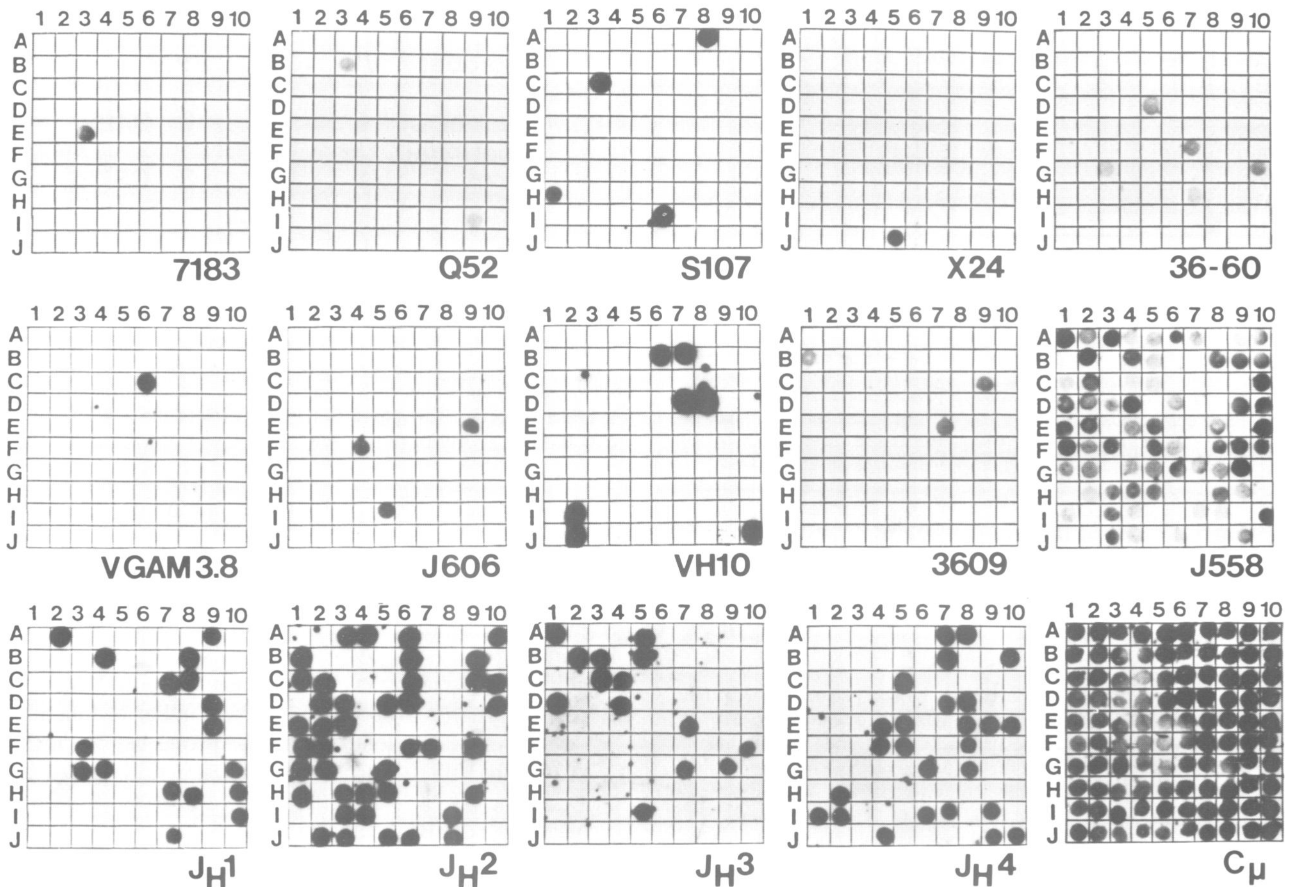

Fig. 2. Analysis of $C\mu^+$ C57BL/6 cDNA library. $C\mu^+$ phages were isolated from the C57BL/6 V_H cDNA library (b48) and hybridized with V_H, J_H and $C\mu$ probes as described in Figure 1.

Table II. Summary of V_H and J_H utilization for $C\mu^+$ phage panels[a]

Strain	V_H gene family									
	7183	Q52	S107	X24	36-60	VGAM	J606	V_H10	3609	J558
BALB/c	17	18	4	2	8	4	8	7	1	29
C57BL/6	1	2	4	1	5	1	3	7	3	66

Strain	J_H segment				summary totals				
	J_H1	J_H2	J_H3	J_H4	$C\mu^+$	J_H^+	V_H^+	$C\mu^+J_H^-$	$J_H^+V_H?$
BALB/c	8	24	28	39	98	99[b]	98[c]	4	2
C57BL/6	17	41	14	26	100	98[d]	93	3	5

[a]Data taken from Figures 1 and 2.
[b]Five BALB/c $C\mu^+$ phages (E2, G8, H2, I1, J1) hybridize with two J_H probes.
[c]Five BALB/c $C\mu^+$ phages (E2, G3, G8, H2, J1) hybridize with two different V_H family probes.
[d]One C57BL/6 $C\mu^+$ phage isolate (E9) hybridizes with two J_H probes.

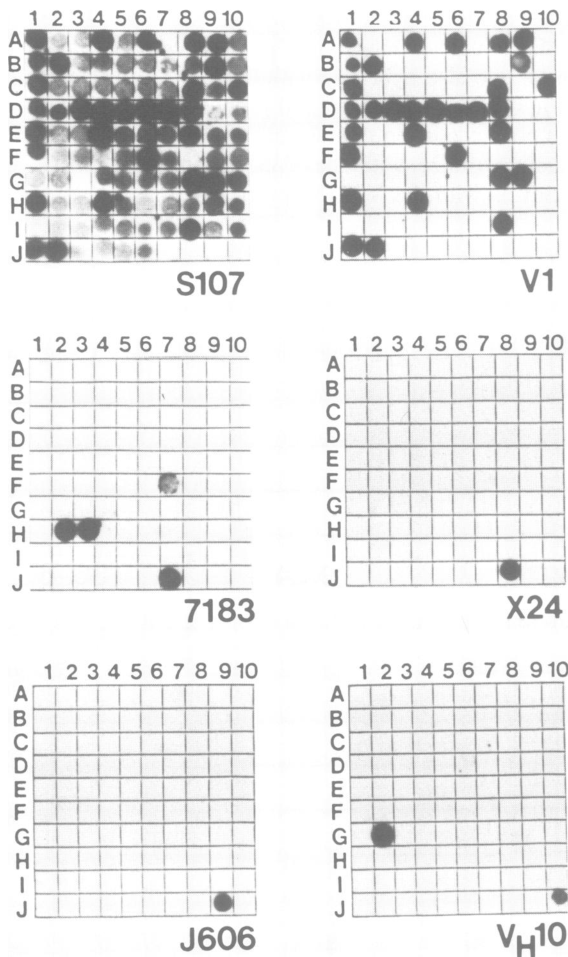

Fig. 3. Analysis of V_HS107^+ phages isolated and plaque purified from the BALB/c V_H cDNA library. Phages hybridizing with the V_HS107 family probe were isolated and plated in 10 × 10 grids on 150 mm plates. Lifts were screened with the probe for the V_HS107 family, the V1 oligonucleotide probe and probes of V_H gene families most closely related to V_HS107. Phage grown in J7–J10 of the grid represent control phage for each non-V_HS107 family: (J7) V_H7183; (J8) V_HX24; (J9) V_HJ606 and (J10)V_H10.

may represent false (artifactual) positives. We conclude that, using the conditions of hybridization described, closely related V_H families can be accurately distinguished in screening thousands of recombinant phages.

Evidence that functional members of two V_H gene families are utilized equivalently

We sought to assess the utilization of a single functional V_H segment relative to that of its V_H gene family. Accordingly, an oligonucleotide probe specific for the V1 gene, one of the three functional members of the V_HS107 family (Crews *et al.*, 1981), was prepared. As shown in Figure 3, one-third (31/93) of the V_HS107^+ phage hybridized with the V1 oligonucleotide, demonstrating the specificity of this probe and suggesting the equivalent representation of V_HS107 genes in the BALB/c V_H cDNA library. Screening of this library showed the frequency of the J_H^+ cDNAs hybridizing with the V1 probe to be 1.1% (76/6883), approximately one-third the frequency of J_H^+ cDNAs scoring positive with the V_HS107 probe (2.9%, 117/3975).

Since the V_HX24 family consists of two genes, both of which are known to be expressed by BALB/c plasmacytomas

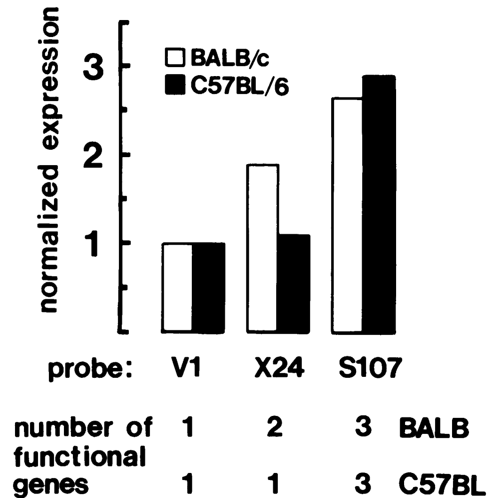

Fig. 4. Expression of one, two or three functional V_H gene segments. The BALB/c and C57BL/6 V_H cDNA libraries were screened with an oligonucleotide probe which hybridizes to a single V_H gene segment (V1). The representation of the V1 sequence is compared to the frequencies of V_HX24 and V_HS107 family sequences within the libraries (data from Figure 6). The data are presented normalized to V1 representation within each library (V1 = 1). The frequency of V1 hybridizing recombinant phage is 1.1 and 0.7% of the J_H^+ phages for BALB/c and C57BL/6, respectively.

(Hartman and Rudikoff, 1984), we had the opportunity to compare the frequency of cDNAs identified by probes recognizing one (V1 oligo), two (V_HX24 probe) or three (V_HS107 probe) well characterized functional V_H gene segments of the BALB/c strain. The frequency of cDNAs hybridizing with the V_HX24 probe is 2.1% (104/4915), thereby demonstrating that the frequencies of cDNAs in the BALB/c library is proportional to the number of functional V_H gene segments identified by the probe used—the V1, V_HX24 and V_HS107 probes hybridizing to 1.1, 2.1 and 2.9% of the BALB/c J_H^+ phages, respectively (Figure 4).

As in the BALB/c strain, the C57BL/6 genome contains three functional V_HS107 family genes (Perlmutter *et al.*, 1985b), one of which hybridizes to the V1 oligonucleotide. About one-third the number of C57BL/6 cDNAs hybridize to the V1 oligonucleotide probe (0.66%, 25/3775) compared to the number hybridizing to the V_HS107 probe (1.9%, 194/10045). That the frequencies of cDNAs in the C57BL/6 library which hybridize to the V1 oligonucleotide and V_HX24 probes (0.74%, 67/9074) are nearly equivalent (Figure 4) suggested the possibility that only one of the two C57BL/6 V_HX24 genes is functional. Indeed, the recent cloning and sequencing of the two V_HX24 genes in this strain has revealed that one contains a stop codon and cannot be functionally expressed (A.Hartman, personal communication). Therefore, the frequency of cDNAs in both the BALB/c and C57BL/6 libraries hybridizing to the V1, V_HS107 and V_HX24 probes is proportional to the number of functional V_H gene segments detected by each probe.

Representation of V_H gene families in the V_H cDNA libraries

The representation of 10 V_H gene families within the BALB/c and C57BL/6 libraries was assessed by plaque lift hybridization of 150 mm plates containing 1000–3000 J_H^+ phages each. Multiple filters were hybridized with each V_H

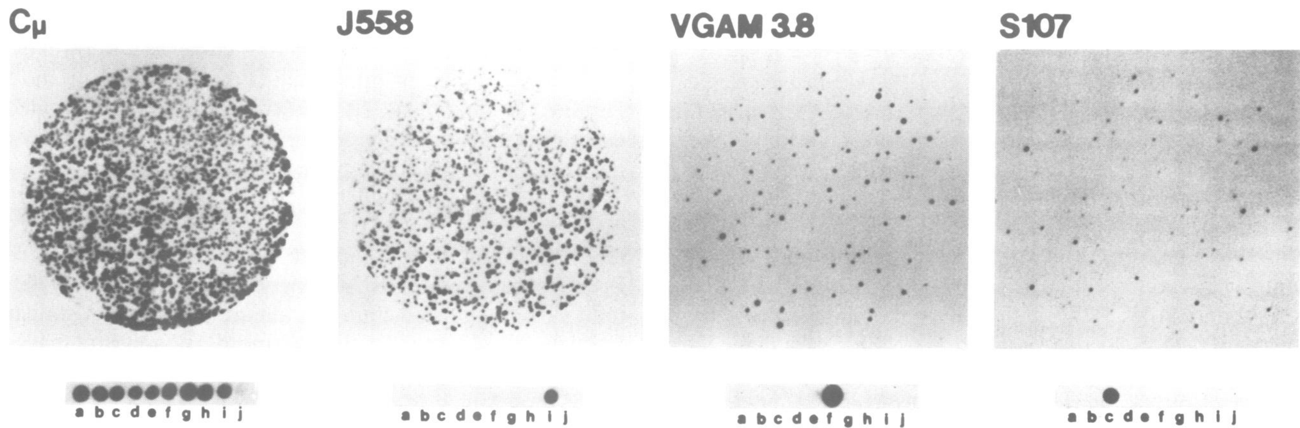

Fig. 5. Representative filters from V_H family screening of the C57BL/6 V_H cDNA library. Each filter was hybridized with the indicated probe. The total number of p.f.u. was determined for each plate and used to calculate the number of J_H^+ phages screened. Control filter strips were included in each hybridization: **(a)** V_H7813; **(b)** V_HQ52; **(c)** V_HS107; **(d)** V_HX24; **(e)** V_H36-60; **(f)** VGAM 3-8; **(g)** V_HJ606; **(h)** V_H3609; **(i)** V_HJ558; **(j)** $C\mu^-$ control.

family probe such that at least 3000 J_H^+ cDNAs were screened with each probe. Representative lifts are shown in Figure 5. Lifts of plaque purified phage representing the 10 V_H families were included in each hybridization as internal specificity controls.

Figure 6 summarizes the representation of 10 V_H gene families in both the BALB/c and C57BL/6 libraries. In general, the V_H families most frequently expressed are those families having the greatest complexity—that is, the greatest number of restriction fragments identified with a prototypic V_H probe (Brodeur and Riblet, 1984). However, there are notable exceptions to this pattern; for example, the small V_H10 family (Kofler, 1988) has a complexity similar to those of the V_HS107 and V_HX24 families (two or three restriction fragments) yet it is the second most highly represented family in the C57BL/6 library. In contrast, the V_H3609 probe, which detects one of the larger families based on Southern blot analysis (~ 15 *Eco*RI fragments in the BALB/c genome), hybridizes to only $\sim 2\%$ of the J_H^+ phages in both BALB/c and C57BL/6 libraries.

The percentages of J_H^+ cDNAs which hybridize to V_H family probes total 72% and 81% of the BALB/c and C57BL/6 libraries, respectively (Figure 6). It is possible that, with a given probe, we fail to detect cDNAs of some members of the larger and more diverse families. However, preliminary results indicate that these numbers reflect the existence of other V_H gene families not represented in the panel of 10 probes used in our study: e.g. V_H11 (Reininger *et al.*, 1988) and the V_H gene expressed by the CH27 lymphoma (Pennell *et al.*, 1988).

The representation of the V_H families shows strain associated patterns of utilization as previously reported by others (Wu and Paige, 1986; Jeong *et al.*, 1988; Yancopoulos *et al.*, 1988). Most striking is the difference in usage of the two most D-proximal V_H gene families, V_H7183 and V_HQ52, and the most D-distal V_H gene family, V_HJ558 (Rathbun *et al.*, 1987; Brodeur *et al.*, 1988). BALB/c mice more frequently utilize V_H7183 and V_HQ52 family members than do C57BL/6, whereas C57BL/6 mice express V_HJ558 family members more frequently than do BALB/c. These disparities in V_H gene family usage between BALB/c and C57BL/6 mice reported here,

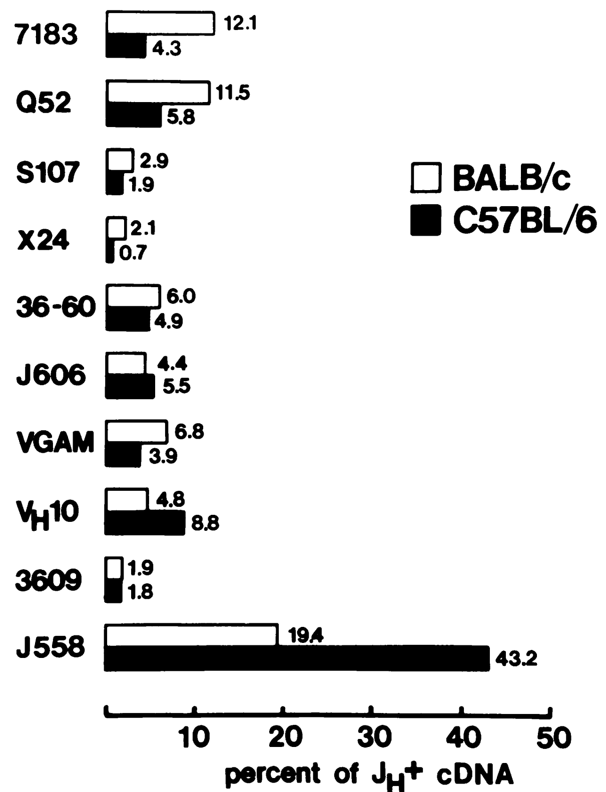

Fig. 6. Representation of V_H gene families within the BALB/c and C57BL/6 V_H cDNA libraries. The data are presented as the percentage of J_H^+ phages which hybridize with V_H probes of each family. A minimum of 3000 J_H^+ phages were screened with each probe on three or more individual 132 mm filters. The relative complexity (see text) of each V_H gene family in BALB/c and C57BL/10 (BALB/C57BL) is: V_H7183, 12/10; V_HQ52, 14/8; V_HS107, 4/4; V_HX24, 2/2; V_H36-60, 6/7; VGAM 3.8, 5/4; V_H10, 3/2; V_HJ606, 10/4; V_H3609, 15/9 (Brodeur and Riblet, 1984; Brodeur *et al.*, 1988). These estimates are based on *Eco*RI digested DNA except for V_HX24 (*Pst*I) and V_H36-60 (*Hin*dIII). As noted in the text, the V_HJ558 family has been estimated to have 60–500 members.

especially the greater use of V_HJ558 in C57BL/6, are generally consistent with previously reported differences despite the diverse experimental approaches.

Evidence that the V_H cDNA libraries reflect a non-antigen selected repertoire

It is possible that some proportion of LPS responsive splenic B cells have been influenced by exposure to antigen and, therefore, represent an antigen selected repertoire. We reasoned that, since the utility of an individual V region gene product in a specific immune response is usually dependent on its association with a particular J_H segment (Bothwell *et al.*, 1981; Crews *et al.*, 1981; Wysocki *et al.*, 1986), comparing the representation of J_H segments associated with a unique V_H gene with that of the complete library would reveal selective expansion of a particular VJ combination by antigen.

A panel of V1-hybridizing phage isolates was prepared from the BALB/c V_H cDNA library and screened by hybridization with the four J_H oligonucleotide probes. Comparison of the distribution frequencies of the J_H segments within the $V1^+$ panel with those of the entire BALB/c library showed no obvious selection for a particular J_H segment (data not shown). This finding, together with the proportional representation of functional V_H genes described above, supports the notion that our V_H cDNA libraries are representative of the non-antigen selected adult antibody repertoire.

Discussion

We consider the V_H cDNA libraries described in this report to be representative of the primary adult repertoires of BALB/c and C57BL/6 mice. LPS is a potent polyclonal stimulator of mouse B cells; approximately one-third of splenic B cells are activated *in vitro* by LPS (Anderson *et al.*, 1977) and it is, therefore, not highly selective. That LPS stimulates virgin B cells is revealed by the general lack of somatic mutation among V genes expressed in the IgM fraction of the LPS induced response (Manser, 1987). In addition, we have used a $C\mu$ specific primer in the synthesis of the V_H cDNA libraries and thereby excluded isotype-switched cells of the memory B cell pool. We are aware of potential problems intrinsic to assaying IgH transcripts to determine the utilization of individual V_H genes since both transcriptional activity and steady state mRNA levels are subject to distinct regulatory processes (Perry and Kelley, 1979; Yuan and Tucker, 1984; Kelley and Perry, 1986; Gerster *et al.*, 1986; Jäck and Wabl, 1988). However, the general agreement between our V_H family expression data and that obtained using *in situ* hybridization (Jeong and Teale, 1988) together with our finding that individual V_HX24 and V_HS107 family members appear to be expressed at comparable frequencies, indicate that the V_H cDNA libraries provide an accurate representation of V_H gene usage at the single cell level. Finally, our finding that J_H segments associated with a particular V_H gene (V1) show a similar pattern of J_H segment usage as the entire BALB/c library is consistent with an antigen independent sampling of the repertoire.

V_H gene family expression studies have generally been discussed in relation to the estimated size of each family (Dildrop *et al.*, 1985; Wu and Paige, 1986; Schulze and Kelsoe, 1987; Jeong *et al.*, 1988; Yancopoulos *et al.*, 1988). The relative size, or complexity of most families is an approximation based on the number of specifically hybridizing fragments resolved by Southern blot analyses (Brodeur and Riblet, 1984; Brodeur, 1987). In general, V_H family complexity and usage are positively correlated. However, since the number of functional V_H genes within most families is unknown, such correlations are of limited usefulness in determining the contribution of individual V_H genes to the expressed repertoire.

We have asked whether all functional V_H genes have equal probabilities of being expressed in the primary repertoire. Our results indicate that, at least for the five BALB/c and four C57BL/6 functional V_H genes analyzed, individual V_H genes within a strain are expressed at similar frequencies (Figure 4). Furthermore, it appears that a non-functional V_H gene (the C57BL/6 V_HX24 pseudogene) is not represented at a significant level. This is consistent with the observations that although transcriptional rates of productive and nonproductive Ig alleles are comparable (Kelley *et al.*, 1986), non-functional Ig transcripts appear to be considerably less stable and consequently have much lower steady state levels than functional Ig mRNAs (Baumann *et al.*, 1985; Mason *et al.*, 1988).

The implication of equivalent expression is obvious: the frequency of V_H gene family utilization may directly reflect the number of functional V_H gene segments in each family. If so, the V_H3609 and V_HJ558 families must contain an extraordinary proportion of pseudogenes. The representation of V_H3609 related sequences in the BALB/c cDNA library is only 1.9%, just 2-fold greater than the V1 gene, although the V_H3609 probe hybridizes to $\sim$15 *Eco*RI fragments in this strain (Brodeur *et al.*, 1988). Similarly, the V_HJ558 family has a complexity of $\sim$60 (Brodeur and Riblet, 1984) yet only $\sim$20% of the J_H^+ BALB/c cDNAs hybridize with a V_HJ558 probe (Figure 6). The V_HJ558 family is thought to encompass significantly more V_H genes than indicated by Southern blot analysis (Maizels and Bothwell, 1985; Schiff *et al.*, 1985; Blankenstein *et al.*, 1987) and may contain 500–1000 genes (Livant *et al.*, 1986). Therefore, the expression of the V_HJ558 and V_H3609 families appears to be restricted and may reflect either a limited number of functional V_H genes or a lower frequency of expression of individual V_H genes in these families.

The expression of V_H gene families may be influenced by parameters other than gene number. Chromosomal position, originally proposed as a major influence in the fetal and neonatal repertoires (Yancopoulos *et al.*, 1984; Perlmutter *et al.*, 1985a), might also influence the adult repertoire, as indicated by the under-representation of the D-distal V_H families V_H3609 and V_HJ558 in the V_H cDNA libraries and the unexpectedly high rearrangement frequencies of two J_H-proximal V_H genes (Lawler *et al.*, 1987). Even so, there is as yet no direct evidence that the position of a V gene *per se* has any bearing upon its frequency of rearrangement and no positional influence is evident over the limited physical distance defined by V_HS107 and V_HX24 (Brodeur *et al.*, 1988).

The different utilization of V_H gene families by BALB/c and C57BL/6 mice (Figure 6) is consistent with previous reports describing strain associated patterns of V_H expression (Wu and Paige, 1986; Jeong *et al.*, 1988; Yancopoulos *et al.*, 1988). However, differences between BALB/c and C57BL/6 V_H gene usage vary among published reports and, in one study (Schulze and Kelsoe, 1987), no significant differences were reported in comparing the expression of three V_H families in BALB/c and

C57BL/6 mice. Although the basis of these discrepancies is not known , it is possible that in some cases it is due to the particular cell population assayed. For example, the B cell colony assays of Wu and Paige (1986) and Schulze and Kelsoe (1987) may read-out discrete B cell subsets. The studies of Jeong *et al.* (1988) and Yancopoulos *et al.* (1988), and the work described here all assayed LPS-stimulated adult splenic B cells, suggesting that discrepancies among these reports are due to technical factors intrinsic to the detection of V_H gene families, such as the particular probe used and the hybridization conditions employed.

The relative map positions of the V_H gene families in *Igh*[a] and *Igh*[b] haplotypes are indistinguishable (Brodeur *et al.*, 1988) and it is unlikely, therefore, that the differential expression of V_H families in BALB/c and C57BL/6 mice is due to differences in V_H gene organization. Strain associated patterns of V_H family utilization may, however, be attributable to disparities in functional V_H gene content, as evidenced by the V_HX24 families of BALB/c and C57BL/6, or to the influence of non-*Igh* linked genes in the utilization of V_H families recently demonstrated by Wu and Paige (1988).

In summary, we have described a powerful approach to study the adult immunoglobulin repertoire using polyclonally activated splenic B cells and assaying IgM RNA transcripts by $C\mu$ oligonucleotide primed cDNA phage libraries. We have presented evidence that the V_HS107 and V_HX24 families are represented in the V_H cDNA libraries in direct proportion to the number of functional V_H genes in the genomes of BALB/c and C57BL/6 mice, and suggest that the most D-distal V_H families, V_H3609 and V_HJ558, are under-represented relative to their apparent germline content.

Materials and methods

Mice

BALB/cByJ and C57BL/6J mice were purchased from The Jackson Laboratories (Bar Harbor, ME) and maintained in the Tufts University School of Medicine animal facility.

Cell culture

Spleens were aseptically removed from 17-week BALB/cByJ or 10-week C57BL/6J female mice. Single cell suspensions of five pooled spleens were cultured in Iscove's Modified Dulbecco's Medium (Gibco, Grand Island, NY) supplemented to contain 10% fetal bovine serum (Hy-Clone Laboratories, Logan, UT), 5×10^{-5} M 2-mercaptoethanol, 200 U/ml penicillin, 200 μg/ml streptomycin (Irvine Scientific, Santa Ana, CA), 50 μg/ml gentamicin (Hazelton Research, Lenexa, KS) and 50 μg/ml lipopolysaccharide (*Escherichia coli* 0111:B4, Difco, Detroit, MI) at 10^6 cells/ml. The cultures were incubated at 37°C in humid air containing 5% CO_2 for 3 days.

RNA preparation

Total RNA was prepared according to the method of Auffrey and Rougeon (1980). Briefly, cultured splenocytes were washed twice with DPBS (Gibco), resuspended in 6 M urea, 3 M LiCl at $25-35 \times 10^6$ cells/ml and homogenized for 2 min on ice. The lysate was kept at 0°C overnight then pelleted by centrifugation and resuspended in 10 mM Tris, pH 7.6; 0.5% SDS. The RNA was extracted twice with phenol:chloroform (1:1) and ethanol precipitated. The RNA was resuspended in diethylpyrocarbonate (DEPC)-treated H_2O and stored at −80°C. Polyadenylated RNA was prepared by a single passage of total RNA over an oligo-(dT) cellulose column (Pharmacia-LKB), ethanol precipitated and stored in DEPC-treated H_2O at −80°C.

cDNA synthesis

V_H cDNAs were synthesized by modification of the method of Gubler and Hoffman (1983). First strand synthesis was carried out using 5 μg polyA$^+$ RNA and 50 ng CH_2 oligonucleotide primer in a 50 μl reaction containing 50 mM Tris−HCl, pH 8.3, at 42°C; 140 mM KCl; 10 mM $MgCl_2$; 10 mM dithiothreitol; 4 mM sodium pyrophosphate; 1 mM each dATP, dCTP, dGTP, dTTP; and 50 U AMV Reverse Transcriptase (Promega, Madison, WI) incubated at 42°C for 45 min and at 50°C for 15 min. The CH_2 primer is a $C\mu$-specific 19mer oligonucleotide primer of sequence 5′-GCGTGGTGCAGGGCCAGAG-3′.

For second strand synthesis the first strand reaction was adjusted to a volume of 300 μl containing 10 mM Tris−HCl, pH 8.0, at 16°C; 100 mM KCl; 4 mM $MgCl_2$; 4 mM dithiothreitol; 500 μM each dATP, dGTP, dTTP; 250 μM dCTP; 50 μCi [α-^{32}P]dCTP (800 Ci/mmol, New England Nuclear, Boston, MA); 75 U *E.coli* DNA polymerase I (Bethesda Research Laboratories, Gaithersberg, MD); 2.3 U *E.coli* RNase H (BRL) incubated at 16°C for 2 h. The reaction was heated at 65°C for 5 min to terminate synthesis. The reaction was adjusted to 1 mM each dATP, dCTP, dGTP, dTTP to which was added 5 U *E.coli* DNA polymerase, large fragment (BRL) and incubated for 15 min at room temperature. After extraction with phenol:chloroform (1:1) the cDNAs were ethanol precipitated.

The cDNAs were methylated at *Eco*RI sites with R1 methylase (Promega) and reprecipitated. *Eco*RI linkers (New England Biolabs, Beverly, MA) were ligated to the cDNAs with T4 DNA ligase (BRL). The cDNAs were size fractionated on 5% polyacrylamide gels, exposed to Kodak XAR-5 film, and the band appearing at 800−850 bp was eluted and ethanol precipitated. These size selected cDNAs were ligated into arms of the bacteriophage vector λgt10 (T4 DNA ligase, BRL) and packaged *in vitro* (Gigapack Gold, Stratagene, La Jolla, CA). Phages were stored in TNM 10/50/10 (10 mM Tris, pH 8, 50 mM NaCl, 10 mM $MgSO_4$) over $CHCl_3$ at 4°C and titered on *E.coli* indicator strain *C600 Hfl*. Approximately one-half of each library was amplified to obtain high titer stocks (Maniatis *et al.*, 1982).

Phage analysis

Phages were plated onto *C600 Hfl* indicator strain bacteria and lifted onto nitrocellulose filters (Schleicher and Schuell, Keene, NH) in a modification of the method of Benton and Davis (1977). Briefly, phages were adsorbed onto filters which were dried briefly at room temperature, placed onto absorbent paper (BlotBlock, Schleicher and Schuell) saturated with 0.5 N NaOH, 1.5 M NaCl for 2 min and then transferred to two consecutive 5-min baths of 1 M Tris, pH 7.0; 0.5 M NaCl. Filters were washed in 2 × SSC for >20 min, dried at room temperature and baked at 80°C for 2 h *in vacuo*.

Phage isolates were plated by spotting 1−2 μl of a 1:2 dilution of isolate stocks in TNM 10/50/10 onto freshly plated *C600 Hfl* and incubated at 37°C until 4−5 mm plaques were obtained. Lifts were prepared as described above.

Hybridization of filters with $C\mu$ and V_H family probes was carried out at 68°C in 3 × SSC, 10 × Denhardt's solution, 50 μg/ml sonicated salmon sperm DNA, 0.5% SDS, 5 mM EDTA for 16−20 h. Filters were washed twice in 2 × SSC, 0.2% SDS at 70°C for 15 min and once in 0.1 × SSC, 0.2% SDS at 72°C for 90 min.

Filters were hybridized with J_H and V1 oligonucleotide probes in 5 × SSC, 2 × Denhardt's solution, 100 μg/ml sonicated salmon sperm DNA, 0.5% SDS for 16−20 h at 50°C (V1), 56°C (J_H2, J_H3) or 60°C (J_H1, J_H4). Filters were washed three times for 30 min at room temperature in 2 × SSC, 0.2% SDS and twice for 15 min at hybridization temperatures in 1 × SSC, 0.2% SDS. Autoradiographs of the filters were obtained by exposure on Kodak XAR film with intensifying screens (Lightning Plus, Dupont, Wilmington, DE) at −80°C.

Probes

Restriction fragment probes representing nine V_H families and constant region ($C\mu$) were prepared as previously described (Brodeur *et al.*, 1988). The V_H10 probe was prepared as a 450 bp *Pst*I−*Dde*I fragment from the MRL-DNA4 clone (Kofler, 1988). Fragments were labeled to high specific activity with [α-^{32}P]dCTP (800 Ci/mmol, New England Nuclear) by random primer labeling (Feinberg and Vogelstein, 1983).

The oligonucleotides used were prepared by the Department of Molecular Biology and Microbiology, Tufts University School of Medicine. The V1 probe is a 21mer of sequence (5′-TGCAGCAATCCACTCCAGTCT-3′) corresponding to codons 44−50 of the BALB/c V1 gene as determined by Crews *et al.* (1981). The J_H probes were the kind gift of Drs Dominic Picarella and Naomi Rosenberg and have the following sequences: J_H1 (5′-GTGGTCCCTGCGCCCCAGACATCGAAGTACCA-3′); J_H2 (5′-TGAGGAGACTGTGAGAGTGGTGCCTTG-3′); J_H3 (5′-TGCAGA-GACAGTGACCAGAGTCCCTTG-3′); J_H4 (5′-GGTGACTGAGGT-TCCTTGACCCCAGTAGTCCATAGC-3′). Oligonucleotide probes were end-labeled to high specific activity with [γ-^{32}P]ATP (New England Nuclear) using T4 polynucleotide kinase (BRL).

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