Altering the Antibody Repertoire via Transgene Homologous Recombination: Evidence for Global and Clone-autonomous Regulation of Antigen-driven B Cell Differentiation

By Kalpit A. Vora and Tim Manser

From the Department of Microbiology and Immunology and The Jefferson Cancer Institute, Thomas Jefferson Medical College, Philadelphia, Pennsylvania 19107

Summary

Antibody V_H transgenes containing small amounts of natural 5′ and 3′ flanking DNA undergo nonreciprocal homologous recombination with the endogenous *Igh* locus in B cells. The resulting "hybrid" heavy chain loci are generated at a low frequency but are fully functional, undergoing somatic hypermutation and isotype class switching. We have used this recombination pathway to introduce a somatically mutated variable (V) region with an unusually high affinity for the hapten *p*-azophenylarsonate (Ars) into the preimmune antibody repertoire. The affinity of this V region for Ars is 100-fold higher than any unmutated anti-Ars antibody previously characterized. Expression of the transgene-encoded V region did not affect many aspects of antigen-driven B cell differentiation, including somatic hypermutation, in either Ars-specific transgene- or endogenous V gene–expressing clones. Thus, the regulation of these processes appears to operate in a "global" fashion, in that the mechanisms involved are imperceptive of the relative affinities for antigen of the antibodies expressed by B cell clones participating in the immune response. In contrast, the selection of V region mutants leading to affinity maturation and memory cell formation was found to be strongly influenced by the transgenic V region, but only in clones expressing this V region. Hybridomas derived from transgene- and endogenous V region–expressing memory cells were isolated at similar frequencies from individual transgenic mice. The V regions expressed by hybridomas in both of these groups had 2- to 30-fold greater affinity for Ars than their unmutated precursors, despite the fact that the transgene-encoded precursors had 100-fold higher affinity than their endogenous counterparts. These results show that the criterion for entry into the memory compartment is established not by the affinity of a B cell's V region relative to all other V regions expressed during the response, but by the affinity of this V region relative to its unmutated precursor. Thus, the development of B cell memory is regulated in a "clone-autonomous" fashion.

The nature of the B cell response to T cell–dependent antigens is extremely dynamic: extensive changes in both the clonal composition of the responding B cell population and the structure and function of the antibodies expressed by this population occur with time (1–3). Isotype class switching alters the structure of antibody-constant regions, and somatic hypermutation of V region genes alters the structure and function of antibody V domains. A stringent process of antigen selection, in concert with the hypermutation process, accounts for much of the phenomenon of antibody affinity maturation (4–8).

The hypermutation and affinity-based selection processes appear to take place largely in germinal centers (GC)[1] (9–11). GCs form within the B cell follicles of secondary lymphoid organs after immunization and have long been recognized as sites of H chain class switching and memory B cell (B_m) development (12, 13). Models have been proposed suggesting that V gene hypermutation is induced and sustained in the rapidly proliferating population of GC B cells termed centroblasts (14–17). These cells are then thought to exit the cell cycle and migrate to a region of the GC rich in follicular dendritic cells (FDC). FDC can retain antigen–antibody complexes on their surfaces for extended periods (18, 19). The B cells, now termed centrocytes, are "tested" for the ability to bind with increased affinity to antigen present in these immune complexes. Cells that "fail" the test die via apoptosis; cells that "pass" either reenter the mutation–selection cycle or become memory cells. Most B_m cells express antigen receptors with increased affinity for antigen due to this mutation–selection process (20–24).

[1] *Abbreviations used in this paper:* AFC, antibody-forming cell; Ars, *p*-azophenylarsonate; B_m, memory B cell; C, constant; FDC, follicular dendritic cell; GC, germinal center; R, replacement; S, silent.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/95/01/0271/11 $2.00
Volume 181 January 1995 271-281

A direct relationship between relative sIg affinity for antigen and degree of B lymphocyte proliferation and differentiation is so fundamental to clonal selection–based models for the maturation of the B cell response that this concept is often taken for granted. The importance of a requisite affinity for the recruitment of clones at the onset of the response, as well as their ongoing participation in the mutation–selection process, is well established (for example, see references 25 and 26). Yet, the molecular mechanisms that translate affinity differences among B cells into proliferative or survival differences at these stages of differentiation remain vague. Moreover, whether relative affinity differences directly influence clonal decisions regarding (*a*) entry into antibody-forming cell (AFC) versus B_m pathways in the primary response, (*b*) entry into and exit from GCs, (*c*) activity of the hypermutation process, and (*d*) establishment of the B_m cell compartment remain subjects of speculation at present.

We have described a somatic nonreciprocal homologous recombination pathway leading to the incorporation of one copy of an antibody V_H transgene, initially present in a variety of chromosomal locations and copy numbers, into the endogenous *Igh* locus in B cells (27, 28; Fig. 1). The resulting transgene-IgH "hybrid" loci encode fully functional H chains whose V_H regions undergo somatic hypermutation and constant (C) regions undergo class switching upon immunization with an antigen for which the transgenic V_H confers specificity (29). Allelic exclusion appears to be operative in B cells expressing hybrid loci, as hybridomas derived from such cells express only one H chain isotype and, in some cases, lack endogenous V_H gene segment rearrangements (28; Vora, K. A., and T. Manser, unpublished results). Due to the low frequency of these "transgene homologous targeting" events, only a small fraction of all B cells present in naive transgenic mice express transgenic V_H regions. B cells in which this recombination has not taken place do not express the transgene and, consequently, rearrange and express only endogenous V_H gene segments. This recombination pathway provides for the subtle alteration of the antibody repertoire by use of novel V_H genes and gene constructs, thus allowing the fate of the B cell clones that express these transgenes to be studied in the context of a naturally diverse immune repertoire. This approach differs fundamentally from conventional Ig transgene approaches and embryonic stem cell–based Ig gene replacement strategies that result in major alterations of the antibody repertoire.

Materials and Methods

Transgenic Mice. Transgenic mice were produced as described previously (27). Fertilized eggs from matings of C57BL/6xC3H F_1 mice were injected with a linearized (at a unique NaeI site in vector sequences) pBluescript KS(−) (Stratagene Inc., La Jolla, CA) plasmid construct containing the 36-71 V_H gene and 150 bp and 1.6 kbp of natural 5′ and 3′ flanking DNA, respectively (Fig. 1). The plasmid lacked any "switch" or constant (C) region DNA.

Immunization, Serology, and Generation of Hybridomas. Mice were given a primary i.p. injection of 100 μg *p*-azophenylarsonate (Ars)-KLH in CFA. For serological analyses, mice were bled on days 7, 14, and 24, boosted on day 30 with 100 μg Ars-KLH in PBS, bled on day 40, and the levels of various antibodies were determined by ELISAs. Hybridomas were generated from spleen cells as described (30). Spleens were taken either 4, 7, or 12 d after primary immunization, 3 d after an i.p. injection of 100 μg Ars-KLH in PBS, administered 1 mo after primary immunization; or 3 d after three sequential i.p. injections of 50 μg of Ars-KLH in PBS, spaced at 3-d intervals, beginning 1 wk after primary immunization (hyperimmunized). Total spleen cells were fused to Sp2/0. Hybridoma supernatants were screened for mAbs reactive with Ars-BSA, and monoclonal anti-Id E4 by ELISA. Hybridomas in wells showing E4 reactivity were cloned by limiting dilution. The isotypes of the mAbs were determined by ELISA by use of an isotyping kit (Hyclone Laboratories Inc., Logan, UT).

DNA Isolation and Southern Blot Analysis. DNA was isolated from hybridomas, tails, and spleens as described earlier (31). Sequential Southern blotting analysis was done as described (30) with an XbaI-EcoRI fragment containing the IgH enhancer, then pBluescript KS(−).

RT-PCR and Genomic DNA Amplifications. Total RNA was prepared from hybridomas as described by Chomczynski and Sacchi (32). 1–5 μg of total RNA was reverse transcribed by use of a 3′ primer complimentary to a region in the C_H1 exon of the appropriate C region gene. Subsequent PCR amplifications were carried out with a 5′ primer homologous to the leader exon of the V_HId^{CR} gene segment. The light chain mRNA was reverse transcribed by use of a 3′ C_κ primer and subsequently PCR amplified by use of a 5′ V_κ primer specific for a region of the leader exon of the canonical $V_\kappa10$ gene segment.

DNA flanking the 3′ side of V_H genes in "hybrid" IgH loci was PCR amplified by use of hybridoma DNA and a nested primer approach. The 5′ primer pair hybridized upstream of the CDR2 region of the canonical V_HId^{CR} gene segment. The 3′ primer pair hybridized in the 3′ intronic enhancer region of the endogenous *Igh* locus.

Nucleotide Sequencing. Nucleotide sequencing was done by use of the "fmol" PCR sequencing kit (Promega Biotech Corp., Madison, WI). PCR products were gel purified (Seaplaque; FMC Bioproducts, Rockland, ME) and sequenced by use of ^{32}P end-labeled internal primers according to the manufacturer's instruction.

Antibody Purification and Affinity Measurements. mAbs were purified from hybridoma supernatants by affinity chromatography on Ars-bovine gamma globulin Sepharose 4B as described previously (33). Their affinities for Ars-tyrosine were determined by fluorescence quenching, also as described previously (34).

PCR Analysis of "Hybrid" Loci and Estimation of Precursor Frequencies. PCR detection of transgene V_H-*Igh* "hybrid" loci in transgenic spleen DNA was accomplished by use of a nested primer approach, with primers described previously (29) that were specific for transgenic vector sequences and the enhancer region of the endogenous *Igh* locus, and the protocol of Liu et al. (35). Target copy number DNA standards were created by doing serial dilutions of DNA from a hybridoma containing a "hybrid" locus into DNA obtained from a mouse macrophage cell line. The amount of ethidium bromide fluorescence of the hybrid locus containing 2.2-kb bands obtained from the standard dilutions was compared with that obtained from identical amounts of transgenic spleen DNA, thus allowing estimation of the frequency of spleen cells containing hybrid loci. This frequency was estimated to be $1/10^4$ to $1/10^5$ for five of seven HAM29 transgenic mice. The fact that DNA from some transgenic mice did not give rise to a band predicted from a hybrid locus indicates that in these mice such loci were not present in the spleen at the time of killing.

The V_HId^{CR} gene segment is expressed at a frequency of

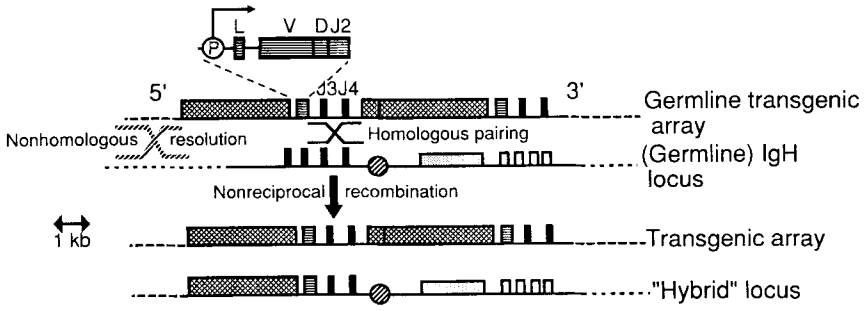

Figure 1. Schematic diagram of the recombination pathway leading to the formation of transgene–IgH hybrid loci. Two sites of recombination are illustrated: the homologous recombination site (*solid lines*) and the nonhomologous recombination site (*striped lines*). Regions of DNA are indicated by boxes filled in different patterns. The germline *Igh* locus is shown; however, rearranged endogenous VDJ and DJ loci may also be substrates of hybrid locus formation. B cells in transgenic mice express either a hybrid or a conventional H chain locus and also contain the original transgenic array due to the nonreciprocal nature of this recombination pathway. ▩, pBluescript vector; ▤, VDJ gene and leader intron/exon; ⊘, IgH enhancer/MAR; ■, unjoined J segments in J_H region; □□□□, constant region exons; ▨, switch DNA.

~1/200 among B cell hybridomas isolated from the polyclonally activated splenic B cells of A/J mice. Sequencing of the V_H genes expressed by such hybridomas reveals a rather random utilization of D and J_H gene segments (36). Therefore, we estimate that the frequency of expression of canonical V_H genes among naive B cells in A/J mice is ~$1/10^4$ (1/200 V_Hs × 1/15 Ds × 1/4 J_Hs). The influence of N region addition has been ignored in this calculation, since canonical V genes contain few N region nucleotides at both V_H-D and D-J_H junctions, and many amino acid combinations encoded at these junctions are compatible with specificity for Ars (37).

Results

During the anti-Ars response in A/J mice, antibodies encoded by a single combination of V_H (V_HId^{CR}), D (DFL16.1), J_H (J_H2), V_κ (V_κIdCR-V_κ10), and J_κ ($J_\kappa1$) gene segments become predominant (21). We term this combination, and the antibodies it encodes, "canonical." The prototype unmutated canonical mAb is expressed by hybridoma 36-65. Canonical V regions undergo hypermutation and antigen affinity–based selection during the anti-Ars response, resulting in an anti-Ars B_m compartment in which mutated canonical V regions with 5- to 20-fold increased affinity relative to their unmutated counterparts predominate (8, 21).

The somatically mutated canonical mAb produced by hybridoma 36-71 (38) has a K_a for Ars-tyrosine of 3–5 × 10^7 M^{-1} (34, 39), the highest affinity anti-Ars mAb from A/J mice yet characterized. The affinity of 36-71 is at least 100-fold higher than mAbs encoded by the unmutated combination of canonical V segments, such as 36-65 (K_a 2–4 × 10^5 M^{-1}). Three amino acid substitutions in the V_H region of 36-65 reproduce much of this 100-fold higher affinity: two substitutions in CDR 2 (T58 → I; K59 → T) and a third at the D-J junction in CDR3 (Y100 → K). Mutations present in the 36-71 V_κ do not play a major role in the affinity increase, as the 36-71 H chain can combine with the 36-65 L chain, resulting in little change in affinity compared with 36-71 (39). An x-ray crystal structure of 36-71 Fab is available and has been refined to 1.85 Å (40). B cells expressing a V region encoded by both a hybrid H chain locus containing a 36-71 V_H transgene and an endogenous canonical V_κ gene would bear a structurally characterized, somatically mutated, high affinity antigen receptor representative of an ultimate product of the mutation–selection pathway leading to the differentiation of B_m cells.

Transgenic mice were generated by use of a plasmid construct containing the 36-71 V_H gene and 150 bp and 1.6 kbp of natural 5′ and 3′ flanking DNA (derived from a genomic clone of the 36-65 V_H gene), respectively, including the V_H promoter and the J_H3-J_H4 region, but lacking the intronic enhancer and switch and C region DNA (Fig. 1). Founder lines were backcrossed at least twice to A/J mice to obtain the mice used here. Three lines were used in our studies containing 8–10 (HAM29), 5–6 (HAM61), and 2–3 (HAM57) copies of the transgene, although the HAM29 line was used most extensively.

We could detect the transgene–IgH hybrid loci resulting from homologous recombination of the HAM29 transgenic array with the endogenous *Igh* locus by PCR analysis of spleen DNA. Standards indicated that spleen cells containing such loci were present at a frequency of $1/10^4$ to $1/10^5$. Transgene-encoded antibody could not be detected in preimmune sera of any of the lines of mice. Four fusions of lipopolysaccharide plus dextran sulfate–stimulated splenic B cells from HAM29 transgenic mice yielded ~5,000 hybridomas, none of which secreted transgene-encoded Ars-binding antibody (data not shown). These results suggest that V_H 36-71–transgenic mice have a very low frequency of cells in their "naive" B cell compartment that have generated and express transgene–IgH hybrid loci. A low precursor frequency has also been estimated for canonical V_H regions encoded by endogenous V gene segments (36; see Materials and Methods for calculations).

Models for Participation of 36-71 V_H Transgene-expressing B Cells in the Anti-Ars Response. Models for the participation in an immune response to Ars of a B cell initially expressing a V region encoded by the 36-71 V_H transgene and a canonical, unmutated V_κ gene are shown schematically in Fig. 2. Since it expresses a "memory type" antigen receptor, this cell might be directly recruited into the B_m compartment, effectively bypassing the GC/mutation–selection pathway (Fig. 2 *A*). A cell expressing this receptor might efficiently capture soluble antigen, resulting in predominant induction and focusing of T cell help early in the response before GC formation. This might lead to extensive clonal expansion fol-

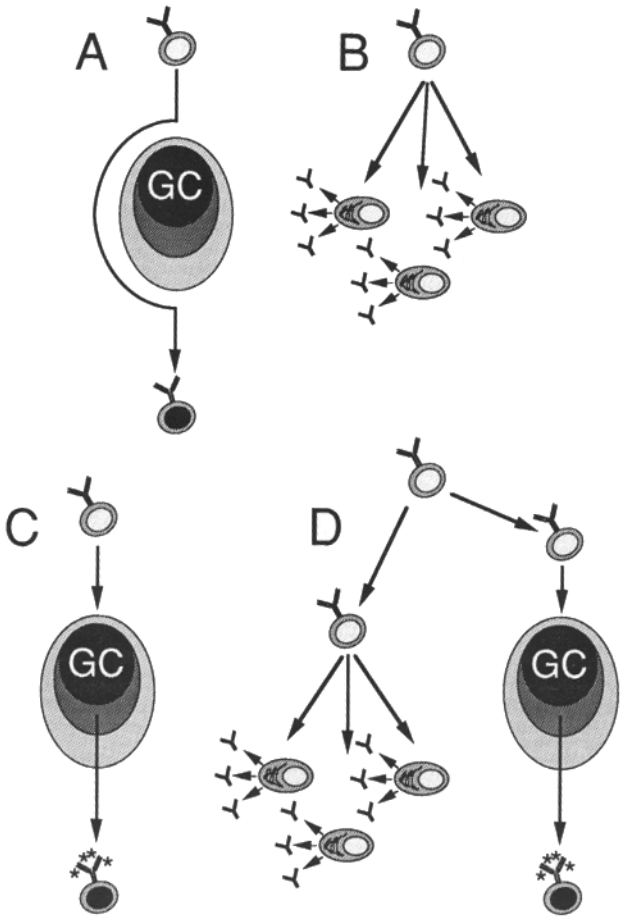

Figure 2. Models for the antigen-driven differentiation of a B cell expressing a 36-71–transgenic V_H gene and an endogenous canonical V_κ gene. *GC*, germinal centers; *, V region somatic mutations. "Naive" B cells are shown containing light gray nuclei. Memory B cells are shown containing black nuclei. AFCs are illustrated as ellipsoid cells. (*A*) Direct recruitment to memory, bypassing or only transiently passing through germinal centers. (*B*) Proliferation and direct differentiation into AFCs. (*C*) Exclusive and precocious entry into germinal centers. (*D*) Participation in both GC/B_m and AFC pathways.

lowed by rapid terminal differentiation to AFC phenotype (Fig. 2 *B*). B cell clones expressing this receptor might seed GCs quickly, leading to the precocious somatic mutation and affinity maturation of their V regions (Fig. 2 *C*). Finally, the effect of the high affinity "memory type" antigen receptor might be simply quantitative, allowing dominant participation of B cells expressing it in both the primary AFC and GC/B_m pathways (Fig. 2 *D*).

Expression of Serum Anti-Ars Antibodies in V_H 36-71 Transgenic Mice. 12 HAM29 transgenic mice and 8 transgene-negative littermates were immunized with Ars-KLH and bled 7, 14, and 24 d later, and then boosted with Ars-KLH and bled 10 d later. Sera were analyzed for levels of anti-Ars and anti-KLH antibodies and idiotopes recognized by the mAbs E4 and AD8. E4 recognizes all unmutated and mutated forms of canonical V regions that have been tested (30, 41, 42; Vora, K. A., and T. Manser, unpublished observations). The combination of a 36-71 H chain and an unmutated canonical L chain is $E4^+$ but does not express detectable levels of the AD8 idiotope (42). All antibodies encoded by the V_HId^{CR} gene segment in unmutated form are $AD8^+$ (36).

Transgenic mice and their nontransgenic littermates showed similar kinetics of expression of anti-Ars antibodies and the two idiotopes (Table 1). The anticarrier (KLH) response was indistinguishable in the two groups (data not shown). Significant levels of anti-Ars or idiotype-bearing antibodies were not detected on day 7 in either group. However, levels of anti-Ars and $E4^+$ antibodies in the transgenic mice were higher than in the littermates at days 14 and 24 of the primary and in the secondary response. Over 50% of the binding of the anti-Ars antibodies present in pooled day-24 sera from the transgenic animals was inhibited by low concentrations of free Ars-tyrosine (10–100 μM), indicating that a major subfraction of this antibody had high affinity for Ars. This high affinity subfraction was absent from pooled day-24 sera obtained from the nontransgenic littermates (data not shown).

Since transgene-encoded antibodies are $AD8^-$, expression of the AD8 idiotope provides a direct measure of the endogenous (particularly the primary) anti-Ars response. The level

Table 1. *Serum Antibody Levels in V_H 36-71–transgenic Mice and Nontransgenic Littermates*

		Primary day 14			Primary day 24			Secondary day 10		
		ARS	AD8	E4	ARS	AD8	E4	ARS	AD8	E4
			μg/ml			*μg/ml*			*μg/ml*	
TG^+	Range	36–197	13–138	0–30	30–2,400	14–851	15–1,500	666–10,000	35–1,100	198–4,900
	Average	89	55	9	489	215	465	3,364	433	1,273
TG^-	Range	20–107	10–100	0–25	49–219	35–276	7–1,160	800–2,900	49–2,400	99–3,300
	Average	55	43	6	108	126	238	1,534	972	1,081

12 HAM29 transgenic (TG^+) and 8 nontransgenic littermates (TG^-) were immunized with Ars-KLH and bled at various times as described in Materials and Methods. Serum levels of Ars-BSA binding antibody (*ARS*) and AD8 and E4 idiotopes were determined by ELISA and are expressed as micrograms per milliliter equivalents of the mAb 36-65. The range and average of these levels obtained for each group of mice at each time point are shown.

of expression of this idiotope did not vary more than approximately twofold at any time between transgenic and nontransgenic animals, demonstrating that participation of B cells expressing endogenous $V_H Id^{CR}$–encoded antibodies in the AFC response was not greatly altered due to the participation of transgenic V_H–expressing clones. Moreover, these data suggest that the majority of the additional, high affinity serum anti-Ars antibody in the transgenic mice is derived from 36-71 V_H–IgH hybrid locus–expressing B cells.

Hybridoma Analysis of the Participation of V_H 36-71–expressing B Cell Clones in the Primary and Secondary Anti-Ars Responses. B cell hybridomas were generated from HAM29 transgenic mice at days 4, 7, and 12 during the primary anti-Ars response and were screened for the secretion of anti-Ars antibody and the E4 idiotope. Use of E4 allowed equally efficient identification of wells containing canonical antibodies encoded by endogenous V_H segments or the transgenic V_H. Of 12 fusions performed, 10 yielded Ars^+ hybridomas (2–10 positive wells each), and of these, 5 yielded $E4^+$ hybridomas. Among hybridomas obtained from the latter fusions, ~20% of the Ars^+ primary hybridomas were also $E4^+$. Representative $E4^+$ hybridomas were subcloned and their expressed V_H and V_κ genes were sequenced. All seven of the V_H regions analyzed (one from day 4, two and three from day 7, and one from day 12) are transgene encoded and are expressed as either IgG3 (one hybridoma) or IgG1 (six hybridomas) H chains. These V_H regions lack somatic mutations in addition to those encoded in the 36-71 transgene. Southern blot analysis of genomic DNA isolated from the $E4^+$ hybridomas confirmed the presence of transgene–IgH hybrid loci. All of these hybridomas also express unmutated, canonical V_κ genes identical to those expressed by conventional A/J mice (data not shown). These results contrast with those we obtained from mice containing a 36-65 V_H transgene capable of forming hybrid loci, in which both $E4^+$ hybridomas expressing canonical endogenous or transgenic V genes were isolated at day 7 of the primary anti-Ars response at a low frequency. In these experiments, 36-65 V_H transgene–expressing hybridomas were isolated at a frequency of ~5% of all Ars^+ hybridomas (Manser, T., unpublished observations).

To study the participation of transgene-expressing B cells in the anti-Ars B_m pathway, 10 36-71 V_H–transgenic mice (1 mouse each of the HAM61 and HAM57 lines and 8

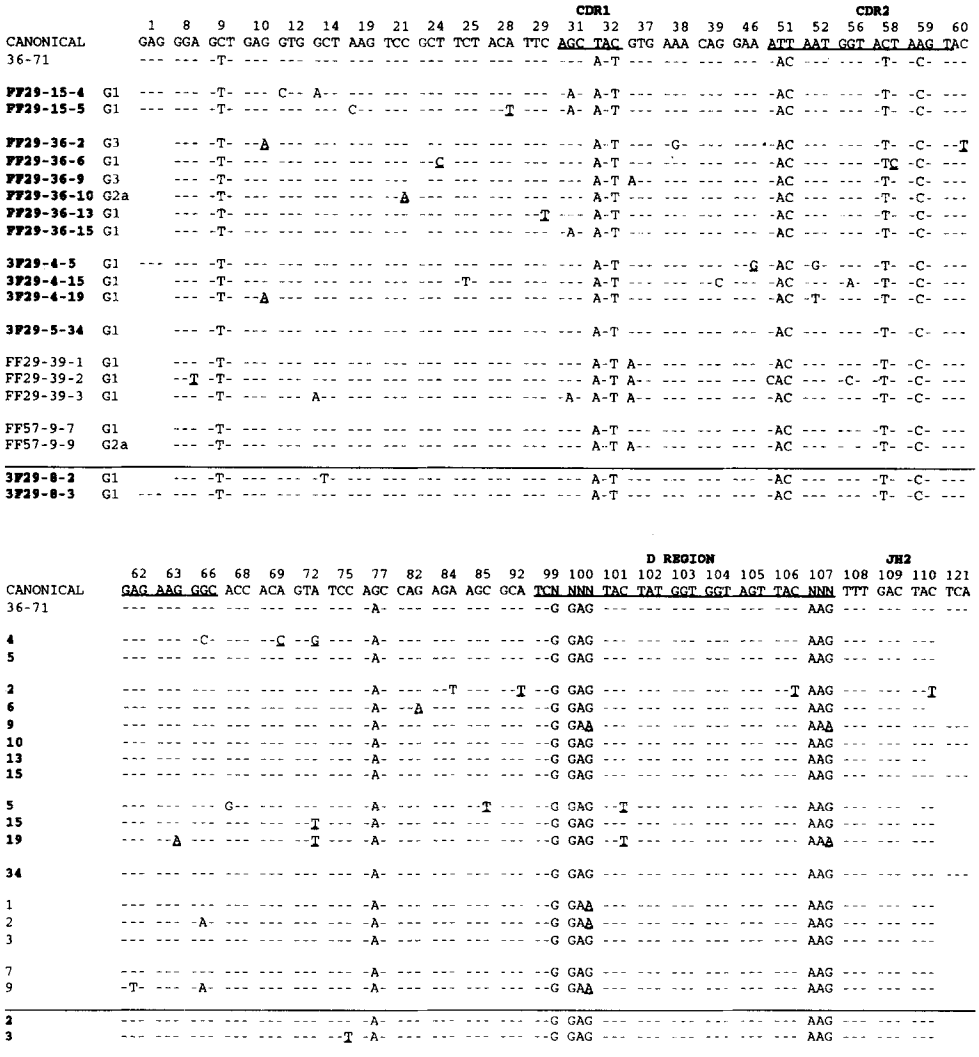

Figure 3. Nucleotide sequences of the 36-71 V_H transgenes expressed by hybridomas isolated from secondary and hyperimmune anti-Ars responses. The sequences are compared with the prototype sequence of unmutated canonical V_H genes (*CANONICAL*) and the 36-71 V_H transgene (*36-71*). Amino acid codons are numbered sequentially, beginning with the mature amino terminus. Sequence identity to the canonical sequence is indicated by a dash; differences are shown explicitly. Translationally silent mutations are underlined. Only the codons in which mutations were observed are shown, with the exception of codons 1 (the mature amino terminus), 121, and those encoding the D region. The position of CDR codons (*underlined*) and regions encoded by D and J_H2 gene segments are also indicated. Sequences derived from individual mice are shown in groups. The name of each hybridoma, as well as the isotype of the antibody it expresses, are listed at the beginning of each sequence. Sequences derived from mice from which endogenous, canonical V_H-expressing hybridomas were also isolated are indicated by names in bold lettering. The two sequences obtained from the single hyperimmunized mouse are separated by a solid line. The prototype sequence data are available from EMBL/GenBank/DDBJ under accession numbers M20274 and M31909.

HAM29 mice) were immunized with Ars-KLH, rested for 1 mo, and then boosted with Ars-KLH and spleen cells used for hybridoma formation. One HAM29 mouse was hyperimmunized with Ars-KLH (see Materials and Methods) before fusion. Hybridoma screening and V_H and V_κ sequencing were carried out as described above. Nine of the fusions gave rise to $E4^+$ hybridomas, and roughly equal proportions of these hybridomas were subsequently found to express transgenic or endogenous V_H regions. To rule out the possibility that some transgene-encoded antibodies were not being detected in the screens due to loss of the E4 idiotope, the expressed V_Hs of representative Ars^+E4^- hybridomas from two of the HAM29 fusions that yielded $E4^+$ transgene-encoded mAbs were subjected to partial sequencing analysis. Of 17 such hybridomas, none were found to express transgene-encoded V_H genes.

Representative transgene-expressing hybridomas chosen from all of the secondary fusions for detailed V gene sequencing express IgG3 (2 hybridomas), IgG2a (2 hybridomas), and IgG1 (15 hybridomas) H chains (Fig. 3). The other $E4^+$ hybridomas express canonical V regions entirely encoded by endogenous V gene segments, in the form of IgG3 (2 hybridomas) and IgG1 (16 hybridomas) H chains (Fig. 4). In six of the mice, both hybridomas expressing transgene or endogenous V_H gene–encoded V regions were isolated (e.g., the 3F29-4 group). All the $E4^+$ hybridomas express canonical V_κ genes, identical except for somatic mutations to those expressed by conventional A/J mice (data not shown). Results from related studies on 36-65 V_H–transgenic mice showed that secondary anti-Ars $E4^+$ hybridomas isolated from these mice express hybrid loci at a lower frequency (10–20%) than was found for the 36-71 V_H–transgenic mice (Vora, K. A., and T. Manser, manuscript in preparation).

Analysis of Somatic Mutations in 36-71 V_H Transgenes and Endogenous V_H Genes Expressed by Secondary Hybridomas. Analysis of both 36-71 transgenic V_H–expressing and endogenous V_H–expressing secondary $E4^+$ hybridomas revealed extensive somatic mutation of their functional V_H and V_κ

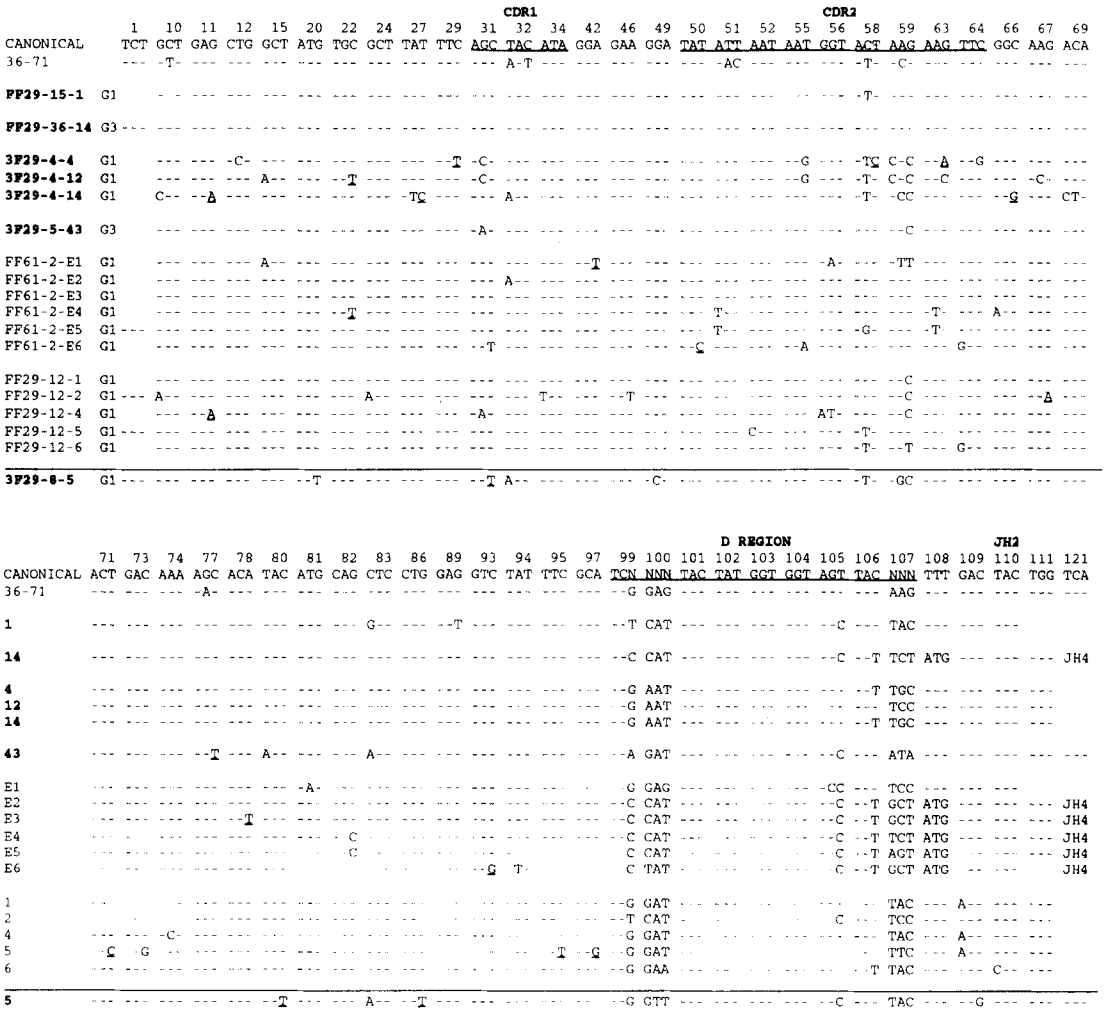

Figure 4. Nucleotide sequences of the endogenous canonical V_H genes expressed by hybridomas isolated from the secondary anti-Ars response of 36-71 V_H–transgenic mice. Sequences are illustrated as described in the legend to Fig. 3. The names of hybridomas isolated from mice from which transgene V_H–expressing hybridomas were also isolated are shown in bold lettering. The prototype sequence data are available from EMBL/GenBank/DDBJ under accession numbers M20274 and M31909.

genes, a hallmark of the GC/B_m pathway (Figs. 3 and 4, and data not shown). However, the mutation frequency in the transgenic V_H genes is twofold lower than in the endogenous canonical V_H genes. Moreover, the majority of mutations in the transgenic V_H sequences are translationally silent (S) (Figs. 3 and 4, *underlined mutations*), while the major fraction of mutations in the endogenous V_H genes encode amino acid replacements (R) (Fig. 4). The frequency, type, and distribution of mutations in these endogenous V_H genes are similar to what have been observed previously in the canonical V_H genes expressed by secondary anti-Ars hybridomas isolated from conventional A/J mice (43).

An analysis of the distribution and type of somatic mutations observed in the V_H genes expressed by the secondary hybridomas isolated from the transgenic mice is summarized in Table 2. The frequency of S mutations should not be affected by antigen selection, so positive selection for R mutations will result in a large R/S ratio, while negative selection of such mutations will result in a small R/S ratio (44). Mutations in the endogenous canonical V_H genes have an overall R/S ratio that is more than three times greater than the mutations in the transgenes. Moreover, the R/S ratio of mutations in CDRs of endogenous V_H genes is ~10-fold higher than in the CDRs of the transgenes. However, the overall frequencies of S mutations in the two types of V_H gene are comparable. These data suggest that while the extent of mutational alteration of transgenic and endogenous V_H genes was similar during the generation of the B_m compartment, most R mutations in the transgenes were selected against. Concurrently, positive selection of R mutations in CDRs took place in endogenous V_H genes.

Table 2. *Analysis of Somatic Mutations in Canonical V_H Genes Expressed by Secondary Hybridomas*

V_H gene	Mutation frequency	R/S ratio
	%	
Transgene encoded	0.79 (S = 0.40)	0.89
CDRs	1.17	1.20
FWs	0.64	0.81
J_H2-J_H3 3′ flank	1.70	
Endogenous	1.48 (S = 0.35)	3.1
CDRs	2.39	10.25
FWs	1.07	1.65

The percent frequency of mutation (total mutations/total nucleotides sequenced × 100) and the R/S ratio (number of mutations causing amino acid replacements/number that do not) were calculated for the transgenic and endogenous V_H genes whose sequences are presented in Figs. 3 and 4, as well as for the CDR (1, 2, and 3) and FW subregions of these genes. The frequency of silent (S=) mutations in these V_H genes is also shown, as is the frequency of mutations in the 500 bp of DNA flanking the 3′ side of four of the transgenic V_H genes (J_H2-J_H3 3′ flank).

This conclusion is substantiated by the results of an analysis of the mutation frequency in the 500 bp of DNA flanking the 3′ side of transgenic V_Hs present in the hybrid loci of secondary hybridomas. This region has been shown to be as "mutable" as V_H coding sequence (29, 45) but does not encode protein that can be subjected to selective forces. The frequency of mutation in this region is 1.7%, approximately twice the frequency observed in the adjacent coding sequence but analogous to the 1.48% frequency of mutation observed in coding sequence of endogenous V_H genes (Table 2). In addition, the frequencies and R/S ratios of mutations in endogenous canonical V_κ genes expressed by the transgenic and endogenous V_H–expressing hybridomas are very similar (0.77% and 2.0; 1.0% and 1.69, respectively).

Affinity Measurements of V_H 36-71 Transgene-encoded and Endogenous V_H Gene-encoded Canonical Antibodies. The intrinsic affinities of mAb produced by representative secondary hybridomas isolated from the transgenic mice for Ars-tyrosine, which represents the major arsonate B cell epitope on Ars-KLH, were measured by fluorescence quenching. Monoclonal antibody FF29-34-1, expressed by a hybridoma isolated on day 4 of the primary response, whose V region is encoded by the unmutated 36-71 transgenic V_H and an endogenous unmutated canonical V_κ gene, was used to determine the "germline" affinity of B cell antigen receptors of this type.

Table 3. *Affinities of V_H 36-71 Transgene and Endogenous V_H Gene-encoded Canonical Antibodies*

Monoclonal antibody	Response	Transgene encoded?	Ka (M^{-1})
FF29-34-1	Primary	+	2.9×10^7
FF29-15-1	Secondary	–	4.8×10^6
FF29-15-4	Secondary	+	3.3×10^7
FF29-36-14	Secondary	–	4.8×10^5
FF29-36-2	Secondary	+	9.4×10^7
FF29-36-9	Secondary	+	4.0×10^7
FF29-36-15	Secondary	+	6.7×10^7
3F29-4-4	Secondary	–	1.1×10^6
3F29-4-12	Secondary	–	4.6×10^6
3F29-4-14	Secondary	–	1.1×10^7
3F29-4-5	Secondary	+	8.8×10^7
3F29-4-15	Secondary	+	5.6×10^7
3F29-5-43	Secondary	–	9.4×10^6

The affinity of each purified mAb for Ars-tyrosine was determined by fluoresence quenching and is shown as a molar association constant (K_a). The anti-Ars response from which the hybridoma producing each mAb was isolated is indicated (*Response*) as is whether the mAb is encoded by the 36-71 transgene (*Transgene encoded?*). mAbs derived from individual mice and the data pertaining to these mAbs are shown in groups as indicated.

The affinity of 2.9 × 10^{-7} M^{-1} obtained (Table 3) is consistent with previous affinity measurements of the parent 36-71 mAb (34, 39) and with the finding that mutations in the 36-71 V_κ do not contribute greatly to the high affinity of this mAb (39).

The K_a's for Ars-tyrosine of unmutated canonical V regions have been previously shown to range from 5 × 10^4 to 2 × 10^6, depending on the combination of amino acids present at the V_H-D and D-J_H junctions (39, 41). Most of the mAbs encoded by endogenous V segments have affinities at the high end of or exceeding this range, and all of these antibodies have K_a's equal to or greater than the median of this range. Unexpectedly, all of the transgene-encoded antibodies have affinities equal to or higher than their unmutated counterparts, as represented by the FF29-34-1 mAb. Moreover, both endogenous and transgene V_H–encoded antibodies expressed by hybridomas isolated from the same mice (grouped in Table 3) display this property of increased affinity relative to their unmutated precursors.

Discussion

At the outset of these experiments, we anticipated that expression of the unusually high affinity antibody encoded by the 36-71 V_H transgene could influence the outcome of the anti-Ars response via both (*a*) *trans* effects on B cell clones expressing endogenous antibody genes mediated by direct or indirect interaction of these clones with transgene-expressing clones or by secreted transgene-encoded antibody; and (*b*) *cis* effects of the transgenic antibody on the transgene-expressing clones themselves. The kinetics of serum antibody production, extent and type of isotype class switching, and intrinsic degree of V gene somatic hypermutation were indistinguishable in transgene- and endogenous V gene–expressing anti-Ars clones (Tables 1 and 2 and Figs. 3 and 4). Moreover, none of these parameters varied appreciably from those measured in nontransgenic A/J mice. This demonstrates a lack of either *cis* or *trans* effects of transgene expression on the respective underlying processes, indicating that relative affinity of sIg for antigen does not play a role in the regulation of these processes. This regulation appears to act in a "global" fashion with respect to all responding B cell clones. Previous observations support the notion that regulation of class switching occurs in such a global fashion (46, 47).

The finding that somatic hypermutation takes place to the same extent, and apparently with similar timing in transgene- and endogenous V_H–expressing clones, was unexpected. We reasoned that after the initiation of the GC reaction, clones expressing transgene-encoded canonical V regions might either rapidly exit GCs, since the affinity of their sIg relative to endogenous affinities would be extremely high, or be capable of increased iterations of the mutation–selection cycle relative to endogenous clones due to an enhanced intra-GC proliferative or survival potential afforded by their high affinity sIg. Given these scenarios, if the GC microenvironment is requisite for hypermutation, then transgenic V regions would display either reduced or increased mutation frequency compared with their endogenous counterparts. Neither was the case, indicating that the affinity of a B cell clone's sIg does not directly affect the hypermutation process in this clone. It has been suggested that hypermutation takes place predominantly within the GC centroblast population, before antigen selection of centrocyte derivatives of this population (48). The spatial and temporal uncoupling of hypermutation and selection predicted by this model, with a corollary predicting limited reentry of centrocytes into the centroblast pool, could explain our observations.

The model shown in Fig. 2 *D* best accounts for the behavior in the anti-Ars response of clones initially expressing unmutated forms of the 36-71 V_H gene and a canonical V_κ gene. The high affinity of these clones' sIg appears to lead to predominant participation in the AFC response. This is probably due to a high relative efficiency of antigen capture, leading to focusing of CD4 T cell help. Nevertheless, this predominant participation does not significantly alter the AFC response mounted by endogenous clones. The high affinity of the sIg of transgene-expressing clones also ensures participation in the GC/B_m pathway, but again does not greatly alter participation of endogenous clones in this pathway. Thus, the degree of participation in both the AFC and GC/B_m pathways appears to be regulated only in *cis* by the transgene. Our data do not rule out an effect of differences in precursor frequency between B cells expressing transgene-encoded and endogenous canonical V regions, although both frequencies are clearly low (see Materials and Methods). Experiments using several lines of mice containing a 36-65 V_H transgene that forms hybrid loci have shown that transgene-expressing clones give rise to lower frequencies of primary and secondary hybridomas than are observed in 36-71 V_H–transgenic mice. This argues that predominant participation of 36-71 transgene–expressing clones in the anti-Ars response is largely due to the very high affinity for Ars endowed by expression of this transgene.

The models shown in Fig. 2, *A* and *B*, can be ruled out, as major fractions of primary anti-Ars serum antibody have high affinity for Ars and are $E4^+$, i.e., they appear to be transgene encoded, and secondary transgene-expressing hybridomas display extensive somatic mutation of their V_H and V_κ genes. The model shown in Fig. 2 *C*, exclusive and precocious participation of transgene-expressing clones in the GC/B_m pathway, is also not supported by the serological analyses. Moreover, none of the transgene-expressing primary hybridomas were found to express mutated V genes, demonstrating that the somatic hypermutation process was not accelerated in transgene-expressing clones. Modified forms of this model proposing multiple stages of GC development could be considered, however. Indeed, it has been suggested that many B cells exit the GC to form AFC before the initiation of hypermutation (49).

Perhaps the most striking conclusion to be made from our analyses is that the affinity requisite for formation of B_m cells by a clone is not determined by the "average" affinity of the antibodies produced by all responding clones. Rather, this requisite affinity seems to be defined by the affinity of the antibodies expressed by only this clone before the hypermu-

tation period. This was made evident by two observations indicating *cis* effects of the transgene: (*a*) all mAbs produced by transgene-expressing secondary hybridomas tested had K_a's larger than their "starting" affinities (2.9×10^7), while all mAbs produced by endogenous V region–expressing hybridomas tested had K_a's substantially lower than this value and in the range expected for antibodies expressed by secondary hybridomas isolated from Ars-immunized conventional A/J mice (21) (Table 3); (*b*) secondary hybridomas expressing transgenic V_H or endogenous canonical V_H genes were isolated at similar frequencies. Moreover, many of the endogenous V region–expressing hybridomas were isolated from mice that also yielded transgene-expressing hybridomas.

It is well documented that GCs are formed by only a small number of B cells (47, 50). The oligoclonality of newly formed GCs appears to be further reduced before the onset of hypermutation (47), perhaps due to an affinity "cutoff" imposed by the loss of sIgD (12, 51, 52). The resulting pauciclonality of GCs during the mutation selection period would make it extremely unlikely that two clones specific for the same epitope were resident in the same GC. Physical isolation of multiple clones expressing sIg specific for identical epitopes but of different affinities would allow "clone-autonomous" regulation of the postmutation affinity-based B_m selection process.

Current data, however, do not provide a satisfactory explanation for the mechanism of such a clone-autonomous selection process. If deposition of circulating Ag:Ab complexes on the surface of FDCs is a prerequisite for GC formation (53), then the antibody present in these complexes should be of polyclonal origin. In the 36-71 V_H–transgenic mice, "testing" of the affinity of sIg expressed by GC B cells relative to this polyclonal Ig should have resulted in a normalization of the sIg affinity of transgene and endogenous V_H–expressing clones, i.e., endogenous affinities would increase and transgene affinities would decrease. The latter was not observed. One possible explanation is that upon entry into the GC, before hypermutation, B cell clones secrete antibody that binds, perhaps monovalently, to a limited number of available epitope sites on FDC-bound Ag:Ab complexes. After hypermutation, competition for binding to this limited number of sites between the secreted antibody and mutant antigen receptors could allow selection of sIg mutants with increased affinity. Alternatively, we have previously proposed a model for affinity maturation based on sequential expression and testing of different mutant forms of a V region by single B cells (54). Due to DNA replication of expressed V genes in the absence of cell division, during which the replication of only one strand was assumed to be error prone, this model allowed for reversion to the expression of an unmutated V gene by individual cells. Successive cycles of expression of mutated and unmutated V regions by a single B cell might allow this cell to detect the acquisition of a mutant V region with increased affinity relative to its unmutated precursor, based on increased relative signaling through the antigen receptor complex. Detailed analysis of the interaction of B cells and FDCs during the GC reaction, and determination of whether affinity-based selection takes place exclusively as a consequence of this interaction, will be required to gain insight into the mechanism of clone-autonomous affinity maturation and B_m generation.

Regarding the mechanism of hypermutation, the finding that mutation took place to similar extents in transgenic and endogenous V_H genes reinforces previous observations that extensively mutated V genes remain good substrates for hypermutation (55). It has been suggested that gene conversion between functional V genes and unrearranged V gene segments of V pseudogenes over short stretches of DNA may play a role in the hypermutation process (56). While it is now widely accepted that most of the mutations in mouse V genes and their flanking sequences arise de novo, the possibility that a subset of somatic mutations are templated has received some support (57, 58).

The B cell precursors to the secondary hybridomas expressing endogenous canonical V_H gene contained a large "reservoir" of potential donors for "microconversion" events in the form of the multicopy 36-71 V_H–transgenic array. In addition, hybrid loci are formed by interchromosomal "macro" gene conversion events between such transgenic arrays and the *Igh* locus (28). Furthermore, three of the codons present in the 36-71 V_H encode amino acids that individually and additively confer increased affinity for Ars when introduced into an unmutated canonical V region (39), and so could be selected by antigen. Two of these codons arose in the 36-71 V_H via somatic mutation (58 ACT to ATT, 59 AAG to ACG), and one is present at the D-J_H junction (107 AAG). Fig. 4 shows that none of the 18 endogenous canonical V_H sequences contains AAG at codon 107. Several of these sequences contain the ATT change at codon 58, and one contains the ACG change (ACC in this case) at codon 59. However, both of these changes are recurrently observed among canonical V regions isolated from conventional A/J mice (43). Of particular significance, none of the sequences contain both of these changes, despite that fact that a "micro" gene conversion event involving only two adjacent codons could simultaneously introduce them. Of the other base changes present in the 36-71 V_H transgene, only one is found in the endogenous V_H sequences: a TAC to AAC change at codon 32. However, this change is also recurrently observed in the canonical V_H genes of conventional A/J mice (43), and the codon present at position 32 in the 36-71 V_H is an AAT. The third position C-to-T change is silent, yet none of the endogenous sequences that contain the first position T-to-A change also contain this silent change. In toto, these observations suggest that micro gene conversion events between the 36-71 V_H transgenic array and expressed endogenous canonical V_H genes did not take place.

Finally, the differences in mutation frequencies and R/S ratios observed between the endogenous and transgene-encoded V_H genes (Table 2) suggest that there are limited "mutational solutions" to acquiring increased affinity among canonical anti-Ars V regions, and the number of such solutions decreases with increasing affinity. Indeed, smaller affinity increases relative to precursors were observed among transgene-

encoded secondary antibodies than among endogenous secondary antibodies (Table 3), implying that transgene-expressing clones may have been approaching a "global affinity optimum" (59, 60). Nevertheless, affinity maturation did take place within 36-71 V_H transgene–expressing clones in a short period of time, providing further testimony to the exquisite stringency of the affinity-based selection process (8, 26, 61). Indeed, our data suggest that what limits affinity maturation in normal mice is not antigen selection, but the generation of V region mutants. This is surprising given the exceedingly high rate at which hypermutation takes place (62).

We thank Alexander Krasev and Paul Henriksen for technical support, Judith Morgan for construction of transgenic founders, Bice Perussia for the use of her spectrofluorimeter, Jackie Sharon for the V_H 36–71 construct, and all members of the Manser laboratory for their many indirect contributions to this work. We also thank Larry Wysocki, Ann Marshak-Rothstein, and Alan Perelson for helpful discussions.

This work was supported by grants AI-23739 from the National Institutes of Health and 3763 from the Council for Tobacco Research.

Address correspondence to Tim Manser, Jefferson Cancer Institute, BLSB 708, 233 S. 10th Street, Philadelphia, PA 19107.

Received for publication 11 August 1994 and in revised form 13 September 1994.

References

1. Rajewsky, K., I. Forster, and A. Cumano. 1987. Evolutionary and somatic selection of the antibody repertoire in the mouse. *Science (Wash. DC).* 238:1088–1094.
2. Berek, C., and C. Milstein. 1988. The dynamic nature of the antibody repertoire. *Immunol. Rev.* 105:5–26.
3. Manser, T., L.J. Wysocki, T. Gridley, R.I. Near, and M.L. Gefter. 1985. The molecular evolution of the immune response. *Immunol. Today.* 6:94-101.
4. Eisen, H.N. 1966. The immune response to a single antigenic determinant. *Harvey Lect.* 60:1–33.
5. Siskind, G.W., and B. Benaceraff. 1969. Cell selection by antigen in the immune response. *Adv. Immunol.* 10:1–50.
6. Sablitzky, F., G. Wildner, and K. Rajewsky. 1985. Somatic mutation and clonal expansion of B-cells in an antigen-driven immune response. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:345–350.
7. Berek, C., G.M. Griffiths, and C. Milstein. 1985. Molecular events during maturation of the immune response to oxazolone. *Nature (Lond.).* 316:412–418.
8. Sharon, J., M.L. Gefter, L.J. Wysocki, and M.N. Margolies. 1989. Recurrent somatic mutations in mouse antibodies to p-azophenylarsonate increase affinity for hapten. *J. Immunol.* 142:596–601.
9. Jacob, J., G. Kelsoe, K. Rajewsky, and U. Weiss. 1991. Intraclonal generation of antibody mutants in germinal centres. *Nature (Lond.).* 354:389–392.
10. Berek, C., A. Berger, and M. Apel. 1991. Maturation of the immune response in germinal centers. *Cell.* 67:1121–1129.
11. Küppers, R., M. Zhao, M.L. Hansmann, and K. Rajewsky. 1993. Tracing B-cell development in human germinal centers by molecular analysis of single cells picked from histological sections. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:4955–4967.
12. Butcher, E.C., R.V. Rouse, R.L. Coffman, C.N. Nottenberg, R.R. Hardy, and I.L. Weismann. 1982. Surface phenotype of Peyers patch germinal centers in B-cell differentiation. *J. Immunol.* 129:2698–2707.
13. Coico, R.F., B.S. Bhogal, and G.J. Thorbecke. 1983. Relationship of germinal centers in lymphoid tissue to immunological memory. VI. Transfer of B-cell memory with lymph node cells fractionated according to their receptors for peanut agglutinin. *J. Immunol.* 131:2254–2257.
14. Berek, C., and C. Milstein. 1987. Mutation drift and repertoire shift in the maturation of the immune response. *Immunol. Rev.* 96:23–41.
15. Kocks, C., and K. Rajewsky. 1989. Stable expression and somatic hypermutation of antibody V-regions in B-cell development pathways. *Annu. Rev. Immunol.* 7:537–559.
16. MacLennan, I.C.M., Y.J. Liu, S. Oldfield, J. Zhang, and P.J.L. Lane. 1990. The evolution of B-cell clones. *Curr. Top. Microbiol. Immunol.* 159:37–63.
17. Nossal, G.J.V. 1992. The molecular and cellular basis of affinity maturation in the antibody response. *Cell.* 68:1–2.
18. Nossal, G.J.V., G.L. Ada, and C.M. Austin. 1964. Antigens in immunity. IV. Cellular localization of ^{125}I and ^{131}I-labelled flagella in lymph nodes. *Aust. J. Exp. Biol.* 42:311–330.
19. Tew, J.G., R.P. Phipps, and T.E. Mandel. 1980. The maintenance and the regulation of humoral immune response: persisting antigen and the role of follicular antigen-binding dendritic cells as accessory cells. *Immunol. Rev.* 53:175–201.
20. Griffiths, G.M., C. Berek, M. Kaartinen, and C. Milstein. 1984. Somatic mutation and the maturation of the immune response to 2-phenyloxazolone. *Nature (Lond.).* 312:271–275.
21. Manser, T., L.J. Wysocki, M.N. Margolies, and M.L. Gefter. 1987. Evolution of antibody variable region structure during the immune response. *Immunol. Rev.* 96:141–162.
22. Weiss, U., and K. Rajewsky. 1990. The repertoire of somatic antibody mutants accumulating in the memory compartment after primary immunization is restricted through affinity maturation and mirrors that expressed in the secondary response. *J. Exp. Med.* 172:1681–1689.
23. McHeyzer-Williams, M.G., G.J. Nossal, and P.A. Lalor. 1991. Molecular characterization of single memory B-cells. *Nature (Lond.).* 350:502–505.
24. Clarke, S., R. Rickert, M.K. Wloch, L. Staudt, W. Gerhard, and M. Weigert. 1991. The BALB/C secondary response to the Sb site of influenza virus hemagglutinin: non-random silent mutation and unequal number of V_H and V_κ mutations. *J. Immunol.* 145:2286–2296.
25. Klinman, N.R. 1972. The mechanism of antigenic stimulation of primary and secondary clonal precursor cells. *J. Exp. Med.* 136:241–260.
26. Fish, S., M. Fleming, J. Sharon, and T. Manser. 1991. Different epitope structures select distinct mutant forms of an antibody

variable region for expression during the immune response. *J. Exp. Med.* 173:665–672.
27. Giusti, A.M., R. Coffee, and T. Manser. 1992. Somatic recombination of heavy chain variable region transgenes with the endogenous immunoglobulin heavy chain locus in mice. *Proc. Natl. Acad. Sci. USA.* 89:10321–10325.
28. Giusti, A.M., and T. Manser. 1994. Somatic generation of hybrid antibody H-chain genes in transgenic mice via interchromosomal gene conversion. *J. Exp. Med.* 179:235–248.
29. Giusti, A.M., and T. Manser. 1993. Hypermutation is observed only in antibody H-chain V-region transgenes that have recombined with endogenous immunoglobulin H DNA: implications for the location of *cis*-acting elements required for somatic mutation. *J. Exp. Med.* 177:797–809.
30. Manser, T. 1989. Evolution of antibody structure during the immune response: the differentiative potential of a single B lymphocyte. *J. Exp. Med.* 170:1211–1230.
31. Alt, F.W., and D. Baltimore. 1982. Joining of immunoglobulin heavy chain gene segments: implications from a chromosome with evidence for three D-J_H fusions. *Proc. Natl. Acad. Sci. USA.* 79:4118–4120.
32. Chomczynski P., and N. Sacchi. 1987. Single step method for RNA isolation by acid guanidium thiocyanate-phenol chloroform extraction. *Anal. Biochem.* 12:156–159.
33. Sharon, J. 1990. Structural characterization of idiotypes by using antibody variants generated by site directed mutagenesis. *J. Immunol.* 144:4863–4869.
34. Rothstein, T.L., and M.L. Gefter. 1983. Affinity analysis of idiotype positive and idiotype negative Ars binding hybridoma proteins and Ars immune sera. *Mol. Immunol.* 20:161–168.
35. Liu, A.H., G. Creadon, and L.J. Wysocki. 1992. Sequencing heavy and light-chain variable genes of single B-hybridoma cells by total enzymatic amplification. *Proc. Natl. Acad. Sci. USA.* 89:7610–7614.
36. Manser, T., S.Y. Huang, and M.L. Gefter. 1984. Influence of clonal selection on the expression of immunoglobulin variable region genes. *Science (Wash. DC).* 226:1283–1288.
37. Manser, T. 1990. Limits on heavy chain junctional diversity contribute to the recurrence of an antibody variable region. *Mol. Immunol.* 27:503–511.
38. Marshak-Rothstein, A., M. Siekevitz, M.N. Margolies, M. Mudgett-Hunter, and M.L. Gefter. 1980. Hybridoma proteins expressing the predominant idiotype of the anti-p-azophenylarsonate response of A/J mice. *Proc. Natl. Acad. Sci. USA.* 77: 1120–1124.
39. Sharon, J. 1990. Structural correlates of high antibody affinity: three engineered amino acid substitutions can increase the affinity of an anti-p-azophenylarsonate antibody 200-fold. *Proc. Natl. Acad. Sci. USA.* 87:4814–4817.
40. Strong, R.K., R. Campbell, R.D. Rose, G.A. Petsko, J. Sharon, and M.N. Margolies. 1991. Three-dimensional structure of murine anti-p-azophenylarsonate Fab 36-71 1. X-ray crystallography, site directed mutagenesis, and modeling of the complex with hapten. *Biochemistry.* 30:3739–3748.
41. Parhami-Seren, B., L. Wysocki, and M.M. Margolies. 1989. The amino acid residues at the V_H-D-J_H junctions affect the affinity of anti-p-azophenylarsonate antibodies. *J. Immunol.* 143:4090–4097.
42. Parhami-Seren, B., J. Sharon, and M. Margolies. 1990. Structural characterization of H-chain associated idiotypes of anti-p-azophenylarsonate monoclonal antibodies. *J. Immunol.* 144: 4426–4433.
43. Manser, T. 1991. Regulation, timing and mechanism of antibody V-gene somatic hypermutation: lessons from the arsonate system. *In* Somatic Hypermutation in V-Regions. E.O. Steele, editor. CRC Press, Boca Raton, FL. 42–54.
44. Shlomchik, M.J., A.H. Aucoin, D.S. Pisetsky, and M.G. Weigert. 1987. Structure and function of anti DNA autoantibodies derived from a single autoimmune mouse. *Proc. Natl. Acad. Sci. USA.* 84:9150–9154.
45. Lebecque, S.G., and P.J. Gearhart. 1990. Boundaries of somatic mutation in rearranged immunoglobulin genes: 5′ boundary is near the promoter, and 3′ boundary is ~1 kb from V(D)J gene. *J. Exp. Med.* 172:1717–1727.
46. McHeyzer-Williams, M.G., M.J. McLean, P.A. Lalor, and G.J.V. Nossal. 1993. Antigen-driven B cell differentiation in vivo. *J. Exp. Med.* 178:295–307.
47. Jacob, J., J. Pryzylepa, C. Miller, and G. Kelsoe. 1993. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. III. The kinetics of V region mutation and selection in germinal center B cells. *J. Exp. Med.* 178:1293–1307.
48. Liu, Y.J., J. Zhang, P.J.L. Lane, Y.T. Chan, and I.C.M. MacLennan. 1991. Sites of specific B-cell activation in primary and secondary responses to T-cell dependent and independent antigen. *Eur. J. Immunol.* 21:2951–2962.
49. Tew, J.G., R.M. Dilosa, G.F. Burton, M.H. Kosco, L.I. Kupp, A. Masuda, and A.K. Szakal. 1992. Germinal centers and antibody production in bone marrow. *Immunol. Rev.* 126:99–112.
50. Kroese, F.G.M., A.S. WuBenna, H.G. Seijen, and P. Nieuwenhuis. 1987. Germinal centers develop oligoclonally. *Eur. J. Immunol.* 17:1069–1072.
51. Havran, W.L., D.L. DiGiusto, and J.C. Cambier. 1984. mIgM: mIgD ratios on B-cells: mean mIgD expression exceeds mIgM by 10-fold in most splenic B-cells. *J. Immunol.* 132:1712–1716.
52. George, J., and L. Claflin. 1992. Selection of B-cell clones and memory B-cells. *Semin. Immunol.* 4:11–17.
53. Nossal, G.J.V. 1994. Differentiation of the secondary B-lymphocyte repertoire. The germinal center reaction. *Immunol. Rev.* 137:172–183.
54. Manser, T. 1990. The efficiency of antibody maturation: can the rate of B-cell division be limiting? *Immunol. Today.* 11: 305–308.
55. O'Brien, R.L., R.L. Brinster, and U. Storb. 1987. Somatic hypermutation of an immunoglobulin transgene in κ-transgenic mice. *Nature (Lond.).* 326:405–409.
56. Maizels, N. 1989. Might gene conversion be the mechanism of somatic hypermutation of mammalian immunoglobulin genes? *Trends Genet.* 5:4–8.
57. Allen, D., A. Cumano, R. Dildrop, C. Kocks, K. Rajewsky, N. Rajewsky, J. Roes, F. Sablitzky, and M. Siekevitz. 1987. Timing, genetic requirements and functional consequences of somatic hypermutation during B-cell development. *Immunol. Rev.* 96:5–22.
58. David, V., N.L. Folk, and N. Maizels. 1992. Germline variable regions that match hypermutated sequences in genes encoding murine antihapten antibodies. *Genetics.* 132:799–811.
59. Kauffman, S.A., and S. Levin. 1987. Towards a general theory of adaptive walks on rugged landscapes. *J. Theor. Biol.* 128:11–45.
60. Macken, C.A., and A.S. Perelson. 1989. Protein evolution on rugged landscapes. *Proc. Natl. Acad. Sci. USA.* 86:6191–6195.
61. Kocks, C., and K. Rajewsky. 1988. Stepwise intraclonal maturation of antibody affinity through somatic hypermutation. *Proc. Natl. Acad. Sci. USA.* 85:8206–8210.
62. McKean, D., K. Huppi, M. Bell, L. Staudt, W. Gerhard, and M. Weigert. 1984. Generation of antibody diversity in the immune response of BALB/c mice to influenza virus hemagglutinin. *Proc. Natl. Acad. Sci. USA.* 81:3180–3184.