# Somatic Generation of Hybrid Antibody H Chain Genes in Transgenic Mice via Interchromosomal Gene Conversion

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## Summary

We have constructed lines of mice with transgenes containing an antibody heavy (H) chain variable region ($V_HDJ_H$) gene and various amounts of natural immunoglobulin (Ig) and plasmid flanking DNA. In these lines, recombination of the transgene and the endogenous *Igh* locus takes place in B cells, leading to the expression of functional H chains partially encoded by the transgenic $V_HDJ_H$ gene. Here, we demonstrate that the transgenic $V_HDJ_H$ gene, and various amounts of flanking sequence are recombined with *Igh* locus DNA via interchromosomal gene conversion. The structures of the resulting "hybrid" transgene-*Igh* H chain loci are consistent with the 3′ end of the conversion occurring in regions of sequence identity, and the 5′ end taking place between regions of little or no homology. This mode of antibody transgene recombination with the *Igh* locus is fundamentally different from the previously reported "trans H chain class switching" that results in reciprocal translocations. In contrast, this recombination resembles events previously observed in mammalian tissue culture cells between adjacent homologous chromosomal sequences, or transfected DNA and a homologous chromosomal target. Our data indicate that this recombination takes place at a low frequency, and that the frequency is influenced by both the length and extent of homology between the transgene and the *Igh* locus, but is not greatly affected by transgene copy number. This recombination pathway provides a novel approach for the subtle alteration of the clonal composition of the mouse B cell compartment in vivo using $V_H$ genes with defined structures and functions.

Antigen receptor diversity in vertebrates results from the site-specific action of somatic DNA recombination and mutation systems. The V(D)J recombinase system catalyzes intrachromosomal joining of Ab and TCR V gene segments flanked by heptamer-nonamer signal sequence elements (1). Ab H chain class switching occurs because of recombination between highly repetitive "switch" DNA regions that flank Ab C region genes (2). Diversification of Ab $V_H$ and $V_L$ genes takes place in chickens and rabbits via gene conversion from V gene segment or V pseudogene segment donors (3, 4). Somatic hypermutation results in the accumulation of point mutations in the Ab $V_H$ and $V_L$ genes and immediate flanking sequences of many vertebrates (5).

Ab and TCR transgenes containing the necessary recognition elements have been shown to be substrates for many of these recombination and mutation systems. In B and T cells, V(D)J gene segment joining occurs within transgenic DNA bearing heptamer-nonamer recombination signal sequences (6–8). "Trans-isotype class switching" has been observed in B cells between an Ab H chain transgene containing $\mu$ switch DNA and the endogenous H chain locus (9). V gene somatic hypermutation can act on Ab transgenes containing sufficient amounts of natural 5′ and 3′ flanking DNA (10–12).

We have recently reported our observation of somatic recombination of Ab $V_HDJ_H$ transgenes and the endogenous *Igh* locus in B cells in vivo (13). Here, we show that this recombination is not directly mediated by the H chain isotype switch or V(D)J recombinase systems, but by a mechanism dependent on considerable sequence homology. Moreover, this recombination can occur between the *Igh* locus and transgene loci present at a variety of different chromosomal sites, resulting in nonreciprocal exchange of information between these loci.

## Materials and Methods

*Mice and Cell Lines.* Five of the transgenic lines of mice used in this study have been described previously (13, 14), one has not. All of the lines contain transgenic arrays resulting from the integration of pBluescript KS$^-$ constructs containing the anti-arsonate (Ars)[1] Ab 36-65 $V_HDJ_H$ gene and various amounts of natural 5′ and

[1] *Abbreviations used in this paper:* Ars, arsonate; FISH, fluorescence in situ hybridization.


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3′ flanking DNA linearized at a unique NaeI site in the vector. The PRH2 line of mice, not previously described, contains such a transgene with 2.5 kb of natural Ig DNA flanking the 5′ side of the V gene, and 0.6 kb of natural 3′ flanking DNA. In most of the lines, the transgenic array appears to consist of direct repeats of the input construct, however in the X1 line, the array contains both direct and inverted repeats. The lines have been maintained by backcrosses with A/J mice, resulting in mice heterozygous for the transgene, or by brother-sister mating of mice homozygous for the transgene, derived after three to four generations of backcrosses to A/J. Transgene-Igh linkage analysis was done by crossing these established lines to C57BL/6 mice to establish an $F_1$ generation, and then crossing the transgene bearing $F_1$ animals again to C57BL/6 to establish the N2 generation. Tail DNA digested with DraI was analyzed by Southern blotting using the J14B probe (see below) to distinguish N2 mice that were Igh heterozygous A/B6 from those that were homozygous B6. All inbred strains of mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME).

The transgenic hybridoma cell lines used have been described previously (13, 14). All of these hybridomas were derived by fusion of splenocytes from Ars-KLH immunized, transgene heterozygous mice with the Sp2/0 fusion partner, as described (15).

*Nucleic Acid Probes.* The probes used in this analysis, as well as their brief description and sources are as follows: pBluescript KS$^-$, Stratagene (La Jolla, CA); pVhX, pBluescript KS$^-$ containing a 2.2-kb insert from 150 bp 5′ of the 36-65 $V_HDJ_H$ gene to 1.6 kb 3′ of this gene; pVhR1, pBluescript KS$^-$ containing a 5.6-kb insert from 2.5 kb 5′ of the 36-35 $V_HDJ_H$ gene to 2.3 kb 3′ of this gene; enhancer, encompassing a 0.8-kb region 3′ of $J_H4$ that contains the intronic H chain enhancer; J14B, encompassing a 0.7-kb region 3′ of the Igh intronic enhancer and 5′ of $\mu$ switch DNA, obtained from Dr. Shermain Tilley (Public Health Research Institute, New York); $C_\alpha$, two λ phage clone inserts encompassing 12 kb of the $C_\alpha$ cluster of exons, obtained from Dr. Phil Tucker (University of Texas Southwestern Medical Center, Dallas, TX); $C_\epsilon$, a 1.5-kb fragment containing the $C_\epsilon$ gene, obtained from Dr. Wes Dunnick (University of Michigan, Ann Arbor, MI); $J_H1$, subcloned from the $pJ_o$ plasmid, obtained from Dr. Ken Marcu (State University of New York, Stony Brook, NY), and encompassing a region from 0.4 kb 5′ to 0.1 kb 3′ of the germline $J_H1$ gene segment; $J_H2$, also subcloned from $pJ_o$, containing a region from 0.16 kb 5′ to 0.2 kb 3′ of the germline $J_H2$ gene segment.

*Fluorescence In Situ Hybridization to Metaphase Chromosomes.* Fluorescence in situ hybridization (FISH) to mouse metaphase chromosome spreads obtained from splenocytes and hybridomas was done following previous protocols (16–18). Cells in metaphase were obtained from hybridoma cultures by treating cells growing in log phase with 0.01 $\mu$g/ml ethidium bromide for 30 min followed by treatment with 0.01 $\mu$g/ml colcimid (GIBCO BRL, Gaithersburg, MD) for 1–2 h. Splenocyte blasts were obtained by incubating spleen cells at $2 \times 10^6$ cells/ml in 50 $\mu$g/ml bacterial LPS, 10 $\mu$g/ml dextran sulfate, and 4 U/ml recombinant mouse IL-4 (Biosource, Camarillo, CA). 48 h after initiation of the culture, the blasts were treated with ethidium bromide and colcimid as described above, and harvested. Metaphase spreads were prepared by standard procedures (17) and stored for 2 wk at −20°C before use.

Probes were labeled via nick translation with either biotin-dUTP (BioNick kit; GIBCO BRL) or by random priming using digoxigenin-dUTP (Genius kit; Boehringer Mannheim Corp., Indianapolis, IN). The biotin labeled probes were elaborated with FITC-avidin DCS (Vector Labs., Inc., Burlingame, CA) and two consecutive sets of stainings with biotinylated goat anti-avidin Ab (Vector Labs., Inc.) followed by FITC-avidin DCS. The digoxigenin-labeled probes were detected with mouse anti-digoxigenin Ab (Boehringer Mannheim Corp.) followed by horse anti-mouse IgG-Texas red (Vector Labs., Inc.). Spreads were counterstained with either propidium iodide for FITC analysis or with 4,6-diamidino-2-phenylindole (Sigma Chemical Co., St. Louis, MO) for two-color FITC plus Texas red analysis, and observed through a triple-band pass filter. Spreads were photographed using Kodak Ektachrome 400 film.

The probes used in the FISH analyses were: pBluescript KS$^-$ (transgene specific), pVhR1, which is a pBluescript construct containing the transgene $V_HDJ_H$ gene and 2.5 and 2.3 kb of 5′ and 3′ flanking DNA, respectively (transgene and *Igh* locus specific), and λ phage insert DNAs, representing 12 kb of the $C_\alpha$ gene and flanking DNA (*Igh* locus specific). The pVhR1 and $C_\alpha$ probes were prehybridized with mouse "cot-1" DNA (GIBCO BRL) by denaturing at 70°C for 5 min and annealing at 37°C for 3 h before application to slides containing chromosomes.

*Electrophoresis and Southern Blotting.* Southern blot analysis was performed as described previously (15) using Zeta-bind nylon membranes (Cuno, Inc., Meriden, CT) and $^{32}$P-labeled probes generated either via nick translation or random priming using T7 DNA polymerase (United States Biochemical Corp., Cleveland, OH). Most probes were purified restriction fragments. Pulsed field gel electrophoresis was conducted using a DR-2 CHEF apparatus (Bio Rad Labs., Hercules, CA). Gels were 0.8% agarose in 0.5× TBE. Phage λ concatamers (New England Biolabs Inc., Beverly, MA) and chromosomes from *Saccharomyces cerevisiae* (Bio Rad Labs.) were used as molecular weight markers. Agarose plugs containing high molecular weight spleen and hybridoma DNAs were made using standard procedures, and digested with restriction enzymes using buffers and protocols provided by the manufacturer.

The chromosomal location of the X7 transgenic array was mapped using A × *Mus spretus* backcross panel blots obtained from Dr. Linda Siracusa (Jefferson Cancer Institute). The probe used in this analysis was derived from a region flanking the 5′ side of the hybrid locus in hybridoma X7-4G7, which was shown by Southern blotting to also be present immediately 5′ of the germline X7 transgene.

*Phage and Plasmid Cloning.* Regions of already described λ phage clones (13, 14) were subcloned into pBluescript KS$^-$ using standard procedures. A λ phage clone containing the hybrid locus of hybridoma X7-3C5 was isolated from a BglII digest of hybridoma DNA as described previously (13) using λ DASH II (Stratagene). Regions of the X7-3C5 λ clone were subcloned into pBluescript KS$^-$.

*Serology.* Amounts of anti-Ars and idiotope 107 expressing Abs in immune sera were determined by ELISA as previously described (15). Mice used in serological analyses were immunized with 100 $\mu$g of Ars-KLH in CFA intraperitoneally and bled 21 d later.

*Polymerase Chain Reaction.* PCR to detect hybrid loci in spleen DNA was conducted as described (13, 14). Splenic DNAs were assayed for their ability to support PCR amplification of a 500-bp region of the transgenic $V_H$ gene before further use. DNA was prepared from the spleens of naive transgenic mice, or from mice that had received an initial immunization with 100 $\mu$g of Ars-KLH in CFA intraperitoneally, followed 7 d later by three sequential i.p. injections of 50 $\mu$g of Ars-KLH in PBS, spaced at 3-d intervals.

*Nucleotide Sequencing.* Nucleotide sequencing was conducted as previously described (13, 14) using either the Sequenase II kit (United States Biochemical Corp.) or the fmole™ sequencing kit

(Promega Corp., Madison, WI). Sequence homology searches were conducted using the GCG software ("Fasta" program) and the combined GENBANK and EMBL sequence databases.

## Results

The results of previous analyses (13, 14) of the germline structure of the transgenic arrays in the X7, X1, PR15, PR19, and XR14 lines of mice, as well as the structure of expressed transgene-*Igh* hybrid H chain loci are summarized in Fig. 1. All of the arrays contain the $V_HDJ_H$ gene, associated leader exon and promoter from the anti-*p*-azophenylarsonate (Ars) hybridoma 36-65, and various amounts of 5′ and 3′ Ig flanking sequence, in the pBluescript $KS^-$ plasmid. All but the X7 line contain multicopy transgenic arrays. The structure of the hybrid loci was largely determined by conventional Southern blot analyses of DNA derived from hybridomas elicited by Ars-KLH immunization that were found to express transgenic $V_HDJ_H$ genes. However, several hybrid loci derived from the X7 line were molecularly cloned from hybridoma DNA and subjected to detailed restriction mapping and sequencing analyses (see below). Collectively, these studies have shown that formation of hybrid loci does not result in deletion or insertion of DNA in the $J_H$-enhancer region of these loci, and that different H chain isotypic classes can be expressed by hybrid loci (13, 14).

The transgenes contain two V(D)J recombination signal sequences with 23-bp spacers immediately 5′ of the unrearranged $J_H3$ and $J_H4$ segments. V(D)J joining of one of these signal sequences to one with a 12 base spacer in the *Igh* locus would result in a large net insertion or deletion of DNA in the hybrid locus $J_H$-enhancer region, which has never been observed. Joining of the transgenic $J_H3$ or $J_H4$ segment to its counterpart in the *Igh* locus would be expected to result in deletion of nucleotides and perhaps N region addition at the joint. Such small deletions or insertions near the $J_H$ segments in hybrid loci have not been seen. V gene "replacement" (19, 20) of $V_H$ genes in the endogenous *Igh* locus by the transgenic $V_H$ should result in hybrid $V_H$ genes containing a variety of different D and $J_H$ segments and junctional sequences. Many D and $J_H$ segments, as well as $V_H$-D and D-$J_H$ junctional amino acids, are compatible with specificity for Ars in the context of the 36-65 $V_H$ gene segment (21, 22). However, only transgenic DFL16.1 and $J_H2$ gene segments and junctional sequences are observed in the hybrid loci expressed by Ars-elicted hybridomas (13, 14). The transgenes lack switch DNA and therefore should not be substrates for the switch recombinase system. In addition, switch recombination resulting in hybrid locus formation should result in a deletion of the 3′ end of the Ig DNA included in the transgene. In all of the hybrid loci we have characterized, this has never been observed. In toto, these data suggest that neither the V(D)J recombinase system nor the isotype class switch recombinase system are directly responsible for formation of hybrid H chain loci. In contrast, the data point to a mechanism based on homologous recombination within the $J_H$-enhancer region.

*Analysis of the Frequency of Recombination of the $V_H$ Transgenes with Different Igh Alleles Supports a Homologous Recombination Mechanism.* To gain insight into the influence of length and extent of homology between the transgenes and the *Igh* locus on the frequency of functional hybrid locus formation, the X1, PR15, PR19, XR14, and PRH2 lines of mice (that have been maintained by backcrosses to A/J mice) were crossed to C57BL/6 (B6) mice. The PRH2 line, not previously described, has a transgene containing the 36-65 $V_HDJ_H$ gene and 2.5 and 0.6 kb of 5′ and 3′ natural Ig flanking sequence, respectively, in the pBluescript $KS^-$ vector. The resulting transgene-positive $F_1$ mice were crossed to B6 to obtain the N2 generation, in which A/J and B6 alleles would independently assort. The transgene Ig DNA is derived from an A/J mouse and contains allelic differences relative to B6 Igh DNA. Within the $J_H$ region, A/J and C57BL/10 (closely related to B6) Igh alleles are 99% homologous, containing mainly single nucleotide differences interspersed at 20–200-bp intervals (23). Restriction fragment length polymorphisms in a re-

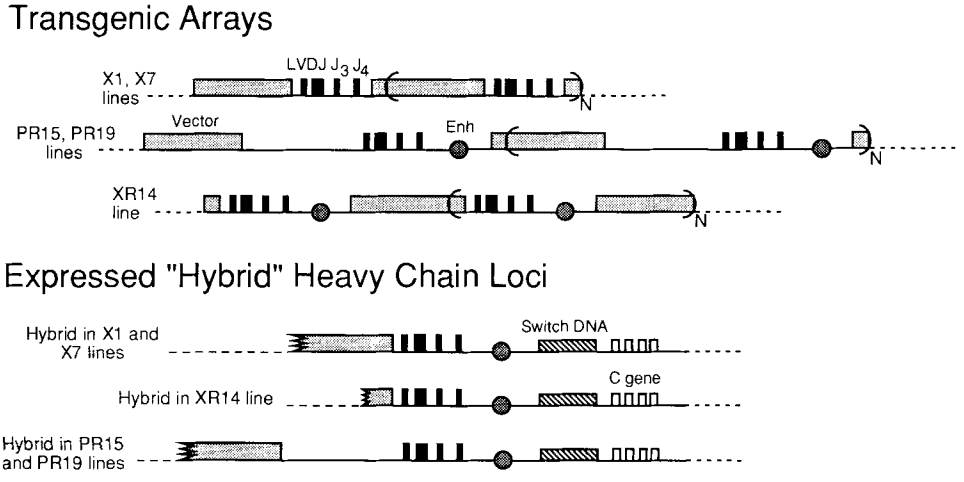


**Figure 1.** Schematic diagram of the structure of transgenic arrays and transgene-*Igh* hybrid H chain loci. Specific regions of DNA are indicated as follows: pBluescript vector (*gray boxes*); functional $V_H$ gene (*LVDJ*, including the leader exon [L], $VDJ_H2$ exon, and promoter), and unrearranged $J_H3$ and $J_H4$ segments (*black boxes*); Igh intronic enhancer (*filled circles*); switch DNA (*striped boxes*); constant region gene exons (*open boxes*); Igh DNA (*thin lines*); uncharacterized or incompletely characterized DNA (*dashed lines*). In the hybrid locus diagrams, the 5′ ends of vector sequences are shown as jagged ends because of the possibility that deletions of vector sequences at these ends took place (see text).

gion 3′ of the Igh intron enhancer were used to distinguish N2 mice heterozygous at the Igh locus from those homozygous for the B6 Igh allele.

The monoclonal antiidiotypic Ab 107 is specific for Abs partially encoded by the 36-65 (transgene) $V_HDJ_H$ gene because of the rare combination of $V_H$-D and D-$J_H$ junctional amino acid residues encoded by this $V_H$ gene (13, 24). Expression of the 107 idiotope in nonimmune normal A/J and transgenic sera is undetectable. After primary Ars immunization only ~5% of normal A/J mice express moderate levels of 107, whereas many Ars-immunized transgenic mice express moderate to high levels of 107. In addition, all of the hybrid locus-encoded mAbs we have characterized are $107^+$. Therefore, frequency of expression of the 107 idiotope by transgenic mice in $Ars^-$ immune primary sera is directly related to the frequency of generation and expression of hybrid H chain loci in these mice.

Fig. 2 shows the number of mice of the four Igh/transgene genotypes obtained in the N2 generations derived from each of the five transgenic lines (the denominator of each fractional value). Below each table in the figure is a schematic diagram of the transgenic array, in which transgene copy number and region of homologous overlap with the endogenous $J_H$-enhancer region (*striped bar*) are indicated. For all but the XR14 line (see legend to Fig. 2), these data demonstrate independent genetic assortment of the germline transgenic arrays and the A/J *Igh* locus. Also shown in each table in Fig. 2 are the number of mice of each genotype that expressed serum $107^+$ Abs after primary Ars immunization (the numerator of each fraction). As expected, all B6 Igh transgene-negative mice did not express $107^+$ Abs (the B6 *Igh* locus lacks the $V_H$ gene segment necessary to produce such Abs [25]). Heterozygous A/B6-Igh transgene-negative mice expressed the 107 idiotope at a very low frequency. All categories of transgene-positive mice expressed $107^+$ Abs, but at different frequencies. These frequencies were clearly lower in the groups of mice lacking an A/J *Igh* locus, indicating that formation of functional hybrid loci takes place less efficiently with the B6 *Igh* locus. For example, among X1 transgene-bearing N2 mice, the frequency of 107 expression was near unity among A/B6 Igh heterozygotes, but only one in seven of the X1 line B6 Igh homozygotes expressed 107. In addition, low frequencies of 107 expression were observed in A/B6-Igh transgene–positive mice of the PRH2 line, whose transgene contains only 0.6 kb of homologous overlap with the endogenous $J_H$-enhancer region. N2 mice of the PR15 line, containing the same transgenic construct as the PR19 line which expresses 107 at a high frequency, showed a low frequency of 107 expression. This could be due to a transgene integration site effect. However, the small number of PR15 N2 mice analyzed makes this conclusion tentative.

In general, it appears as though a larger region of homologous overlap can compensate for the decrease in frequency of hybrid locus formation due to absence of an isogenic Igh allele (e.g., compare the X1 and PR19 data). The frequency of 107 expression among the N2 mice does not seem to be greatly influenced by transgene copy number, nor does it appear to be affected by the amount of DNA flanking the 5′ side of the transgene $V_HDJ_H$. Whereas DNA 5′ of the transgene $V_HDJ_H$ is homologous to endogenous Igh DNA in the $V_H$ gene segment locus, recombination between these two

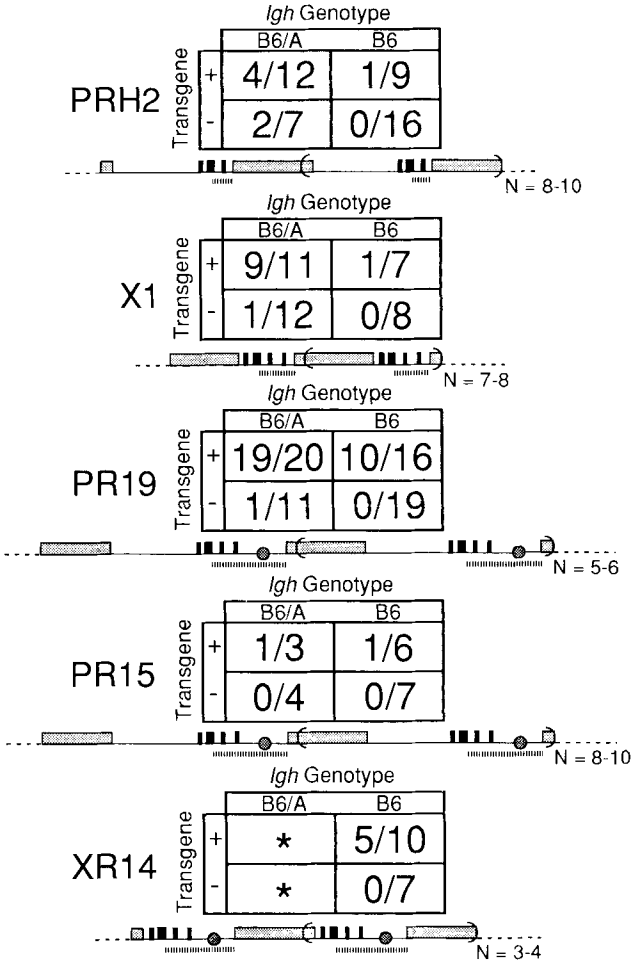


**Figure 2.** Analysis of genetic assortment of transgene and *Igh* loci and frequency of hybrid H chain locus formation and expression among mice of different genotypes. The figure is organized into five sections, each depicting the following data derived from a different transgenic line: a schematic diagram of the transgenic array, illustrated as in Fig. 1, showing approximate copy number (*N*) and regions of homology with the endogenous $J_H$-enhancer locus (*striped bar*); a table showing the number of mice of different transgene and Igh genotypes obtained from crossing transgenic × C57BL/6 $F_1$ transgene-positive mice to C57BL/6 mice with the value shown in the denominator of each fraction; and the number of each of these genetic classes of mice that produced $107^+$ Abs after primary immunization with Ars-KLH with the value shown in the numerator of each fraction. Ars-immune sera were also assayed for the presence of anti-Ars Abs, and the few mice that produced sera containing low levels of these Abs were excluded from the entire analysis. In the experiment using the XR14 line of mice, the $F_1$ transgene-positive mouse used to produce the N2 generation contained only the C57BL/6 *Igh* locus, and thus gave rise to no *Igh* heterozygotes (*). This was because in the XR14 line an equal frequency of B6 and A/J *Igh* loci are present. The embryos used for microinjection were A/J × C57BL/6 $F_1$, and sufficient backcrossing to A/J to insure that the loss of the C57BL/6 allele has not been done in the XR14 line. Subsequent mating of an XR14 × C57BL/6 transgene-positive $F_1$ mouse that was heterozygous at the *Igh* locus to a C57BL/6 mouse demonstrated independent assortment of the A/J *Igh* locus and the XR14 transgene (data not shown).

regions would not be expected to generate a functional H chain locus and, therefore, would not have been detected among our panel of Ars-elicited hybridomas. In toto, these observations are consistent with previous reports on extra, intra, and interchromosomal homologous recombination. These reports have documented the importance of both length of homologous overlap and degree of homology between strands, but lack of influence of concentration of homologous strands, on the frequency of these recombination events (26–31).

*Models for the Formation of Hybrid H Chain Loci.* Three classes of models based on homologous recombination that account for the generation of hybrid H chain loci are illustrated in Fig. 3. Gerstein et al. (9) have shown that recombination of complete Ab H chain transgenes present on chromosome 5 and the endogenous *Igh* locus on chromosome 12 via trans-class switching can result in reciprocal translocations, and that a single site for recombination is sufficient to account for their data. Since the transgenes in our lines of mice contain from 0.6 to 2.3 kb of homology with the *Igh* locus in the $J_H$-enhancer region, a single site homologous crossover could take place in this region leading to the generation of a hybrid locus and a reciprocal product. If the germline transgenic array was not syntenic with the *Igh* locus, a reciprocal translocation would result (Fig. 3 model *A*).

In Fig. 3 model *B* proposes a nonreciprocal exchange between the transgenic array and the *Igh* locus. This exchange might be nucleated in the $J_H$-enhancer region via homologous pairing, and resolved at one end in 5′ nonhomologous regions. This model is in keeping with the high frequency of gene conversion events observed between adjacent homologous chromosomal sequences, and transfected or microinjected DNA and a homologous chromosomal target in mammalian tissue culture cells (26–30). In both cases, one end of the conversion tract can be located in regions of little or no homology (32–35).

In Fig. 3 model *C* proposes that, because of the repetitive nature of the transgenic DNA, a "loop out" recombination event within an array could generate a circular DNA intermediate containing a unit repeat of the array. This intermediate could then integrate into the *Igh* locus via homology in the $J_H$-enhancer region, displacing endogenous Igh DNA to the 5′ side of the resulting hybrid locus. In the illustrations of all three models in Fig. 3, an X7-X1 type transgenic array and the endogenous germline *Igh* locus are shown for the sake of example. Endogenous *Igh* loci that had undergone either DJ or VDJ recombination could also serve as sites of hybrid locus formation since even if this recombination had been to the $J_H4$ gene segment, DNA with homology to the transgenic $J_H$ or $J_H$-enhancer region would remain.

*FISH Analyses of Transgene and Igh Loci before and after Hybrid Locus Formation Do Not Support a Single Site Homologous Crossover Model.* To characterize the structure and location of the transgenic arrays and *Igh* loci before and after formation of hybrid H chain loci, we conducted FISH to metaphase chromosome spreads obtained from mitogen-activated naive transgenic spleen cells and hybridomas expressing hybrid loci. Southern blot analysis of naive transgenic spleen DNA has

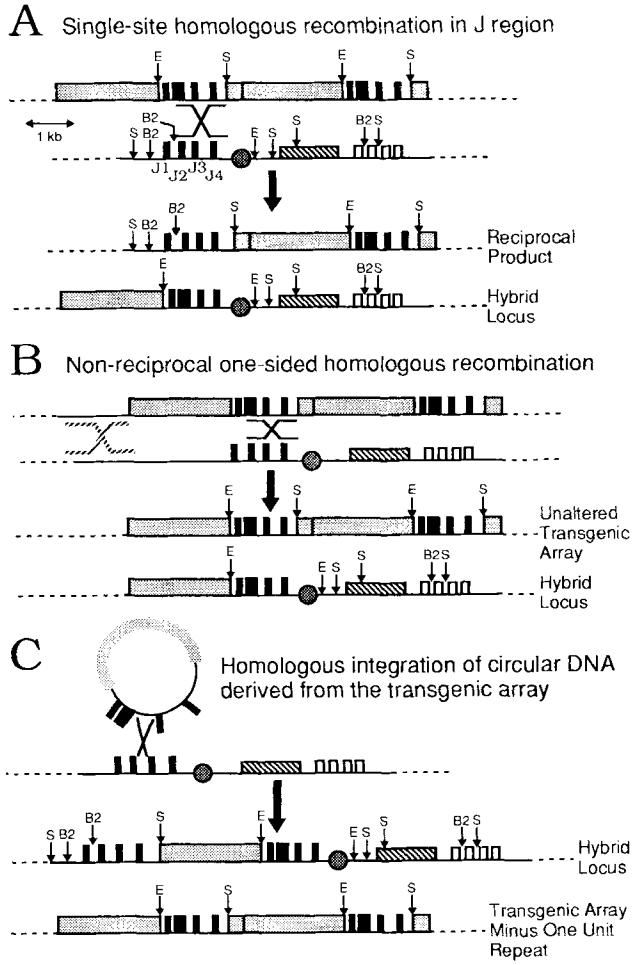


**Figure 3.** Homologous recombination models to account for hybrid H chain locus formation. Specific regions of DNA are illustrated as in Fig. 1. In all models, the homologous recombination site is indicated by solid crossed lines. In model *B*, two sites of recombination are illustrated; the homologous recombination site, and the nonhomologous recombination site, (*striped lines*). Germline transgenic arrays of the X7-X1 type are shown for the sake of example. The germline *Igh* locus is shown in each model, however recombination might also take place with *Igh* loci in DJ or VDJ configurations (see text). The location of cleavage sites of restriction enzymes used for Southern blot analysis of transgenic arrays and hybrid loci (see text) are indicated: (*B2*) BglII; (*E*) EcoRI; (*S*) SacI.

shown that hybrid loci are not detectable (Manser, T., unpublished results, and see Fig. 5). In addition, comparison of the results of Southern blot analysis of transgenic DNA obtained from naive spleen, tail, kidney, or liver has never revealed any differences. Thus, the location of transgene and *Igh* loci in total naive spleen cells is representative of their germline location.

Fig. 4 shows representative examples of the results obtained for spleen cells from four different transgenic lines (germline) and three corresponding hybridomas. All of the hybridomas were derived from mice heterozygous for the transgene. All but the XR14 spleen cell sample were obtained from mice homozygous for the transgene. In some analyses, a probe was used that detects both the transgenic array and the *Igh* locus (pVhR1). In others, probes specific for the transgenic array

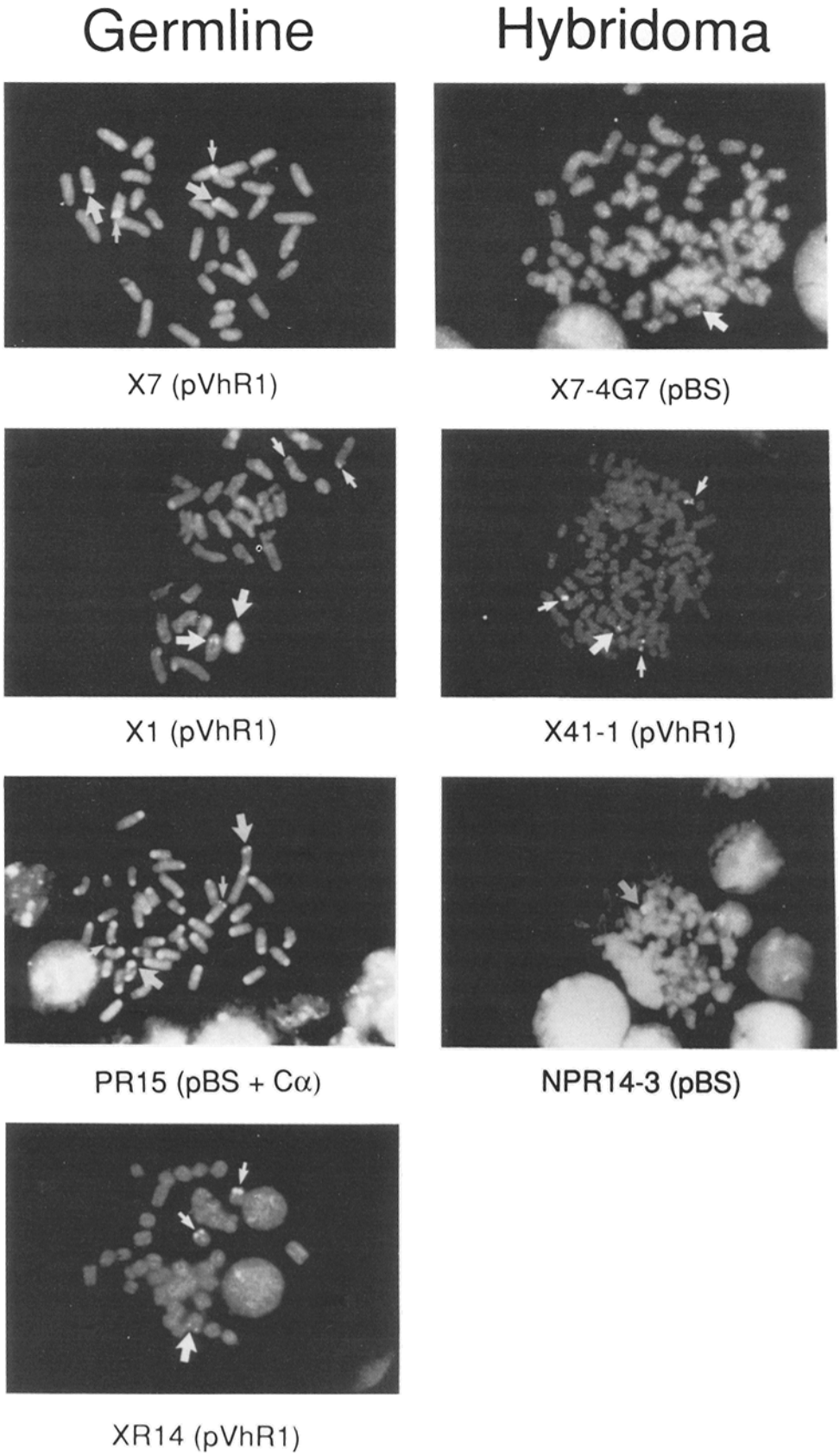


**Figure 4.** FISH analysis of transgene and *Igh* loci in metaphase chromosomes from transgenic mice. Each panel shows a representative metaphase spread obtained from mitogenically activated spleen cells (*germline*) or a hybridoma. The transgenic line from which the spleen was derived, or the name of the hybridoma, is indicated beneath each panel. Photographs of spleen and hybridoma metaphases derived from the same transgenic line are adjacent to one another, and were taken at either ×1,000 or ×1,200. (*Large arrow*) Location of each pair of transgene locus fluorescent signals; (*small arrow*) location of an *Igh* locus pair of signals. The probe(s) used for analysis in each case is shown in parenthesis beneath each panel: pBluescript (*pBS*), specific for the transgene; (*pVhR1*) which hybridizes to both the transgene and the *Igh* locus; and ($C_\alpha$) specific for the *Igh* locus. All of the analyses were done with biotin-labeled probes and elaborated with streptavidin-FITC except the PR15 spread, which was hybridized with pBS labeled with digoxigenin and $C_\alpha$ labeled with biotin, and developed with antidigoxigenin-Texas red and streptavidin-FITC. In some of the spleen cell analyses using pVhR1, the two pair of *Igh* loci and the single transgene pair were distinguished on the basis of signal intensity.

(pBluescript KS$^-$ pBS), or the *Igh* locus ($C_\alpha$ gene) were used singly, or in two-color combination (see legend to Fig. 4 and Materials and Methods for details). Consistent with the results of the B6 backcross experiment (Fig. 2), it can be seen that none of the transgenic arrays (Fig. 4, *large arrows*) are syntenic with the *Igh* loci on chromosome 12 (Fig. 4, *small arrows*). The data obtained from these experiments pertaining to transgene location, as well as the results of a mapping study of the X7 transgene using a strain A × *Mus spretus* backcross panel, are summarized in Table 1.

Comparison of the germline and hybridoma FISH results reveal no apparent differences in chromosomal location or signal intensity of either the transgenic arrays or the *Igh* loci. This comparison is particularly informative in the case of the X1 line, in which the germline transgenic array is centromeric. Since the *Igh* locus is telomeric, a reciprocal translocation as proposed in Fig. 3, model *A* would give rise to an abnormally large and an abnormally small chromosome. One or both of these abnormal chromosomes should hybridize with endogenous Igh and transgene-specific probes, depending on the location of the recombination in *Igh* and transgene loci and the Igh probe used. The degree of hybridization of the probes would also depend on these factors. In all spreads of the X41-1 hybridoma analyzed, chromosomes with these novel characteristics were never observed. Southern blot analysis of X41-1 DNA indicate that this hybridoma contains three *Igh* loci, which include one derived from the Sp2/0 fusion partner, and one that contains the hybrid locus (see below). Less detailed FISH analysis of another X1 line derived hybrid locus–expressing hybridoma, obtained from a different mouse than was X41-1, also showed no evidence of changes in the germline location or signal intensity of either the transgenic array of the *Igh* locus. In hybridomas derived from lines in which the transgene is telomeric or subtelomeric, a reciprocal translocation might not give rise to chromosomes of detectably different size, but could result in an additional chromosome hybridizing with a transgene-specific probe. However, only one chromosome was labeled with transgene-specific probes in all of the hybridomas we examined, and no Igh-containing chromosome of novel size was seen.

All of the hybridomas subjected to FISH analysis contain hybrid H chain loci as determined by sequencing of their H chain mRNA, Southern blotting, and PCR analysis of their DNA, and in the case of X7-4G7, molecular cloning. Nevertheless, we failed to unambiguously observe these loci via FISH. This could have resulted from the small amount of plasmid vector and transgene Ig DNA usually present in such loci (see below). Alterations of the structure of transgene and *Igh* loci due to insertion/deletion events of less than hundreds of kilobases would not be detectable with FISH analysis. Thus, if one of the *Igh* loci is converted to a hybrid locus by incorporation of small amounts of transgene-derived DNA, as Southern blot data suggest (see below), one-color FISH analysis using the $C_\alpha$ or pVhR1 probes would not reveal a difference between this locus and the other Igh allele(s) in the hybridoma. Detection of such a locus via two-color analysis using transgene and Igh probes should give two adjacent fluorescent signals of different colors, or a single signal of a novel color, assuming that the excitation and/or emission of one fluorochrome does not influence the other when they are separated by only a short distance. However, in our hands the use of the digoxigenin-Texas red system for labeling the probes led to approximately fivefold lower sensitivity than the biotin-FITC system. In addition, whereas the 12-kb $C_\alpha$ FITC-labeled probe reproducibly detected the single-copy $C_\alpha$ gene in the *Igh* locus, the transgene-specific 2.9 kb pBluescript FITC-labeled probe only reproducibly detected the transgenic arrays containing five or more transgene copies. These technical limitations precluded the detection of adjacent, single-copy transgene, and *Igh* loci.

*Conventional and Pulsed Field Gel Electrophoresis–Southern Blot Analyses Support a Nonreciprocal Homologous Recombination Model.* To further pursue the mechanism of hybrid H chain locus formation, conventional and pulsed field gel electrophoresis–Southern blot analyses were performed on hybridoma and naive spleen or kidney (germline) DNA. An extensive analysis of hybridomas X7-3C5, X7-3D12, X7-4G7 and X7-5D3, which express hybrid H chain loci and were derived from a single transgene–heterozygous mouse of the X7 line, was conducted. In this analysis, a variety of restriction enzymes and vector, $V_H$, $J_H$, and enhancer region–specific probes were used. Fig. 5 *A* shows a representative result from this analysis using SacI (which cuts once in the input transgene construct, see Fig. 3). Sequential hybridization was per-

**Table 1.** *Chromosomal Location of Transgenic Arrays*

| Transgenic line | Syntenic with *Igh* locus on 12? | Chromosomal location | Technique(s) used |
|---|---|---|---|
| X7 | No | Telomeric, Chromosome 8 | FISH, *Mus spretus* backcross |
| X1 | No | Centromeric | FISH, B6 backcross |
| PR15 | No | Telomeric | FISH, B6 backcross |
| PR19 | Not linked | Internal | FISH, B6 backcross |
| XR14 | No | Telomeric | FISH, B6 backcross |
| PRH2 | Not linked | ? | B6 backcross |

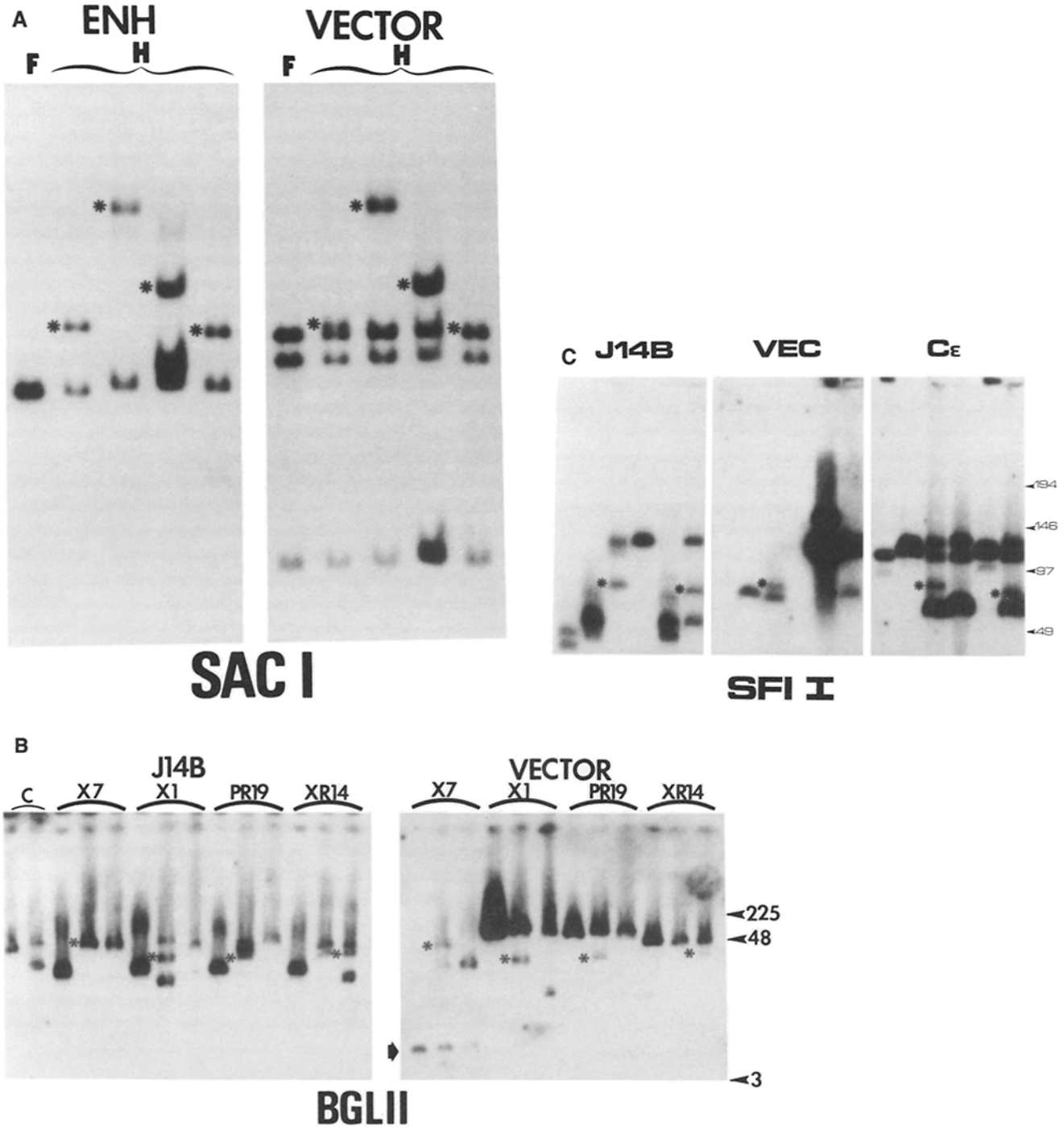


**Figure 5.** Conventional and pulsed-field Southern blot analysis of the products of hybrid H chain locus formation. (*A*) Analysis of X7-derived hybrid locus expressing hybridomas using SacI, and enhancer (*ENH*) and pBluescript (*VECTOR*) probes. In both the ENH and VECTOR panels, the lanes are: *F*, X7 founder liver DNA; *H*, DNAs from hybridomas, from left to right, X7-3C5, X7-3D12, X7-4G7, and X7-5D3. (*B*) Pulsed field gel analysis of spleen (germline) and hybrid locus expressing hybridoma DNA from four different lines of transgenic mice using BglII, and J14B and pBluescript (*VECTOR*) probes. Five groups of two to three lanes each are labeled. These contain the following DNAs, from left to right: *C* group, Sp2/0 (the fusion partner) and 36-65 (a nontransgenic anti-Ars hybridoma); *X7* group, H7-18 mouse spleen, hybridomas X7-4G7 and X7-5D3 (X7 line); *X1* group, S1-1 mouse spleen, hybridomas X41-1 and X42-6 (X1 line); *PR19* group, S19-4 mouse spleen, hybridomas PR18-1 and PR18-2 (PR19 line); *XR14* group, SNXR16 mouse spleen, hybridomas XR6-2 and XR6-3 (XR14 line). In the VECTOR panel the C group of digests is not included. The position of molecular weight markers (in kb) are indicated. An arrowhead indicates the band derived from the complete transgene in the X7 line. The additional band revealed by the vector probe in the X7-4G7 and X7-5D3 lanes is derived from a defective transgene copy, present in some X7 mice and not in others (see text). (*C*) Pulsed field gel analysis of DNA from X7- and X1-derived hybridomas X7-4G7 and X41-1 digested with SfiI; and J14B, pBluescript (*VEC*) and $C_\epsilon$ probes. Samples are, from left to right: A/J spleen; H7-18 spleen (X7 line), X7-4G7; Sp2/0; S1-1 spleen (X1 line), X41-1. Positions of molecular weight markers (in kb) are shown. The upper band in the S1-1 spleen lane of the VEC panel is a partial digestion product of the X1 transgenic array that was not reproducibly observed in SfiI digests. In *A–C*, bands expected from a hybrid locus are indicated (*). Most of the spleen DNAs were isolated from transgene homozygous mice. This may have resulted in more intense transgene germline bands than obtained from the transgene heterozygous hybridomas.

formed with the enhancer probe (*ENH*), specific for the endogenous *Igh* locus in this case, and a transgene-specific pBluescript probe (*VECTOR*). Comparison of the results obtained with the two probes reveals that the three transgene germline bands (Fig. 5 *A*, lanes *F*) are present in all the hybridoma DNA digests (Fig. 5 *A*, lanes *H*), as well as a single novel band in each digest, expected from the hybrid locus, which hybridizes with both probes at single-copy intensity (*, Fig. 5 *A*).

Another band of novel size (excluding the band derived from the Sp2/0 fusion partner) was often observed in hybridoma DNA digests using the enhancer and $J_H$ probes but not the vector probe (e.g., the third hybridoma lane, X7-4G7, Fig. 5 *A*). Two novel bands are routinely observed in conventional hybridoma DNA using $J_H$ or enhancer probes: one derived from the expressed, and the other from the nonproductively rearranged Igh allele of the B cell progenitor to the hybridoma. In our analysis of the X7 hybridomas, and many other hybridomas obtained from different transgenic lines, we have never observed more than one such band in any hybridoma DNA, indicating that one of the two *Igh* loci derived from the progenitor B cell was used to form the hybrid locus, and the other was not involved in hybrid locus formation, but in most cases had undergone V(D)J rearrangement. Only a small fraction of these bands also hybridized with a probe specific for the $J_H1$ segment and its 5′ flanking region, as expected of an *Igh* locus that had undergone V(D)J recombination, since the $J_H1$ region would be deleted if V(D)J recombination was to $J_H2$, $J_H3$, or $J_H4$ segments (data not shown).

As shown in Fig. 3, a reciprocal product of a single site homologous recombination in the $J_H$-enhancer region between one of the transgenes and germline *Igh* locus would contain adjacent $J_H1$ and transgene vector sequences. The vector DNA could be derived from different regions of pBluescript, depending on the orientation of the Ig DNA in the original transgene construct (see Fig. 1). Unlike the $J_H1$ DNA, this vector DNA should be present in a reciprocal product regardless of whether transgene recombination was with a germline, DJ, or VDJ *Igh* locus. Use of either the entire pBluescript vector or subregions of this vector as probes in Southern analysis failed to detect bands in any X7 hybridoma DNA digest other than those derived from the germline transgenic array and those expected from a hybrid locus. In addition, a Southern blot analysis of over 30 hybrid locus-expressing hybridomas derived from all of the transgenic lines was conducted using EcoRI, which would generate one novel band containing vector sequences from any reciprocal product of single-site recombination and a novel band containing both vector and $J_H1$ sequences if recombination was with the germline *Igh* locus (see Fig. 3). Only six of these hybridoma DNAs gave rise to any bands that hybridized with the $J_H1$ probe, and in two cases these bands comigrated with the band generated from the germline *Igh* locus (one of these cases was X7-3C5; see Fig. 5 *A*, lane *2* in *ENH* panel). None of the bands detected with the $J_H1$ probe also hybridized with a vector probe, nor were novel bands other than those expected from a hybrid locus detected with the vector probes (data not shown).

Fig. 5 *B* shows the results obtained via pulsed field gel electrophoresis–Southern blot analysis of a variety of DNAs of hybrid locus–expressing hybridomas obtained from transgene-heterozygous mice using BglII, which does not cut in any of the input transgene constructs (e.g., see Fig. 3). In this analysis, endogenous Igh J14B (specific for a region 3′ of the intronic enhancer and 5′ of $\mu$ switch DNA) and vector probes were used. In both panels, the lanes containing DNAs from each indicated transgenic line are arranged in groups of three: the germline DNA in the first lane, and two hybridoma DNAs in the second and third lanes. Single germline transgene bands are detected in each group of DNAs with the vector probe. These bands are identical in mobility within each transgene group and are of intensities expected of restriction fragments containing the entire transgenic array in each line. In addition, in many of the hybridoma DNAs, these germline transgene bands are accompanied by a novel band (*), expected from a hybrid locus, that hybridizes with both the vector and the J14B probes. In all cases, this novel band is of an intensity expected of a single-copy gene. The J14B probe also detects additional bands in the hybridoma lanes. Some of these bands are derived from the fusion partner (Fig. 5 *B*, first lane of the *C* group). Others appear to be derived from the *Igh* locus not involved in hybrid locus formation (see above). Additional bands that might be derived from a reciprocal product are not seen.

Fig. 5 *C* shows the result of a pulsed field electrophoresis–Southern blot analysis of hybrid locus expressing hybridomas X7-4G7 and X41-1, obtained from transgene-heterozygous mice of the X7 and X1 lines, respectively, using SfiI, which also does not cut in any of the input transgene constructs and cuts less frequently in genomic DNA than does BglII. In this analysis, two endogenous Igh-specific probes were used, J14B and a restriction fragment containing the $C_\epsilon$ gene. In each panel of Fig. 5 *C* the lane order is: A/J germline, X7 germline, X7-4G7 hybridoma, Sp2/0 (the fusion partner), X1 germline, and X41-1 hybridoma. As in the BglII analysis, identical germline transgene bands are detected in both naive spleen and hybridoma DNAs with the vector probe (*VEC*). In the hybridoma DNA digests, bands expected of a hybrid locus that hybridize with both vector and J14B probes (Fig. 5 *C*, *) are also observed. These hybrid locus bands are also detected with the $C_\epsilon$ probe. The $C_\epsilon$ gene is located ~55 kb 3′ of the C$\gamma$1 gene and ~30 kb 3′ of the C$\gamma$2b gene, the C genes expressed by the X7-4G7 and X41-1 hybrid loci respectively, indicating that substantial portions of the *Igh* C region locus are present in these hybrid H chain loci. The vector probe reveals no bands that might have been derived from a reciprocal product. Stripping and reprobing of this blot with the $J_H1$ probe resulted in germline bands in the germline DNA lanes, and no hybridization in the hybridoma lanes. Reprobing with a vector probe containing the subregion of pBluescript predicted to be present in a reciprocal product (see Fig. 3) again revealed only germline transgene bands (data not shown).

*Comparison of the Structure of Cloned Hybrid H Chain Loci with the Donor Transgenic Array Does Not Provide Support for an Integrative Recombination Model (Model C).* We have previously reported a partial analysis of two λ phage clones containing the hybrid loci of hybridomas X7-4G7 and X7-3D12, both derived from an Ars-immunized mouse of the X7 line (13, 14). These analyses showed that the region from the 3′ end of vector sequences to the Igh enhancer in these loci contains scattered nucleotide changes due to somatic hypermutation, but lacks insertions or deletions expected from V(D)J or isotype class switch recombination events (13, 14). Homologous recombination models A–C make different predictions regarding the structure of the region flanking the 5′ side of the vector sequences of these loci. Model A predicts that these sequences will be derived from the transgenic array and one flanking region; model B suggests that these regions need not be derived from the transgenic array; and model C predicts that these regions will be derived from the *Igh* locus, 5′ of C region DNA. To test these predictions, we subcloned and sequenced the region 5′ of vector sequences in the X7-4G7 hybrid locus, and isolated another λ phage clone containing the hybrid locus of hybridoma X7-3C5, a hybridoma derived from the same mouse as were X7-4G7 and X7-3D12. In addition, a detailed restriction mapping-Southern blot analysis of the X7 transgenic array in liver DNA obtained from the X7 line founder mouse was also carried out.

The results are summarized in Fig. 6. The X7 transgenic array contains only one complete copy of the input construct. The X7 founder also contained a defective copy of the input pVhX construct (data not shown) which appears to lack most of the Igh DNA present in this construct (i.e., hybridizes with vector but not $V_H$ or $J_H$ probes). This defective copy was observed to segregate independently from the complete transgene among offspring of the X7 founder, and so apparently integrated on a different chromosome than the complete transgene. The fact that this defective copy does not contain $V_H$ sequences excludes it as a donor for a hybrid locus.

The region 5′ of vector sequences in the X7-4G7 hybrid locus can be subdivided into two regions: a 3′ subregion that contains long tracts of di and tetra nucleotide repeats (Fig. 6, *checkered box*), and a 5′ subregion that is composed of unique sequence (Fig. 6, *crosshatched box*). The sequence of the 5′ subregion is not homologous to any other sequence in GenBank and, thus, is not derived from any characterized region of the *Igh* locus as predicted by model C. Southern blotting and restriction mapping of the X7-3D12 hybrid locus in hybridoma and phage clone DNA using the X7-4G7 5′ and 3′ subregions as probes indicated that the 5′ regions of the X7-3D12 and X7-4G7 hybrid loci are identical.

Southern blot analysis of the complete germline X7 transgene using the X7-4G7 5′ subregion probe (Fig. 6, *crosshatched box*) and vector probes showed no differences in its immediate 5′ flanking region as compared with the 5′ flanking regions of the X7-4G7 and X7-3D12 hybrid loci. This indicates that these hybrid locus 5′ regions were derived from the 5′ flanking region of the transgene, present on chromosome 8. However, Southern blot analysis of the X7-3C5 hybrid locus 5′ flanking region indicated no similarity to the 5′ flanking region of the transgene. Subcloning and sequencing analyses of the X7-3C5 hybrid locus 5′ region demonstrated the presence of the 3′ end of a LINE-1 element (Fig. 6, *brick-filled box*). Therefore, the immediate 5′ flanking region of the X7-3C5 clone is clearly not derived from the $J_H$ locus. The 5′ vector sequences in the X7-3C5 hybrid locus have also sustained a 5′ deletion of ~1 kb relative to the complete X7 transgene. An analogous 5′ deletion of vector sequences was observed in another less well characterized phage clone containing a hybrid locus isolated from another hybridoma, indicating that such deletions may often accompany hybrid locus formation.

In toto, the analysis of the structure of the germline X7 transgene and the 5′ regions of the X7-4G7, X7-3D12, and X7-3C5 hybrid H chain loci provide no support for the idea

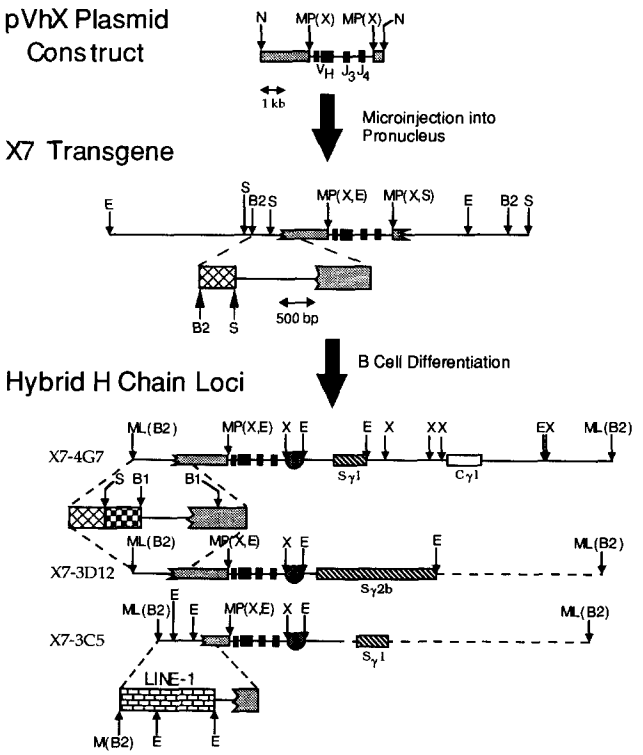


**Figure 6.** Molecular maps of the input construct, transgene and three hybrid H chain loci of the X7 transgenic line. Specific subregions of DNA are indicated as described in the legend to Fig. 1. The names of the hybridomas from which the λ phage clones containing the hybrid loci were derived are shown adjacent to the appropriate molecular map. Restriction sites are indicated as follows: (*N*) NaeI; (*X*) XbaI; (*E*) EcoRI; (*S*) SacI; (*B2*) BglII; (*B1*) BglI; (*MP*) multiple cloning site from pBluescript; (*ML*) multiple cloning site from λ DASH II. The ends of the hybrid locus maps are defined by BglII (*B2*) sites which represent the natural location of these sites in genomic DNA and which were used for cloning into *ML*. The 5′ regions of the X7 transgene and the phage clones are illustrated in expanded maps showing more structural detail. In these regions, boxes with different fill patterns indicate regions of DNA described in the text. Dotted lines indicate regions of the phage clones that were not completely restriction mapped. Only a subset of all the restriction sites mapped in the input construct, transgene, and cloned hybrid loci are shown.

that hybrid locus formation takes place via homologous integration of a circular transgene intermediate (model C). Indeed, it is difficult to imagine how such an intermediate could be generated from the single complete transgene in the X7 line. The structure of the X7-4G7 and X7-3D12 hybrid loci could be accounted for by both models A and B, but model A cannot easily account for the structure of the X7-3C5 hybrid locus. Because of the highly repetitive nature of LINE-1 elements, we could not determine whether such an element flanked either side of the complete X7 transgene. However, comparison of the order of restriction sites in these flanking regions with that of the 3C5 LINE-1 element revealed no similarities.

*PCR Analysis of Hybrid Loci in Transgenic Spleen DNA.* Cells expressing hybrid H chain loci might continually arise during pre-B differentiation, and the resulting mature B cells clonally selected to participate in the anti-Ars response. Such B cells could also be generated from clones already participating in the immune response. In support of the former idea, a few of the hybrid locus–expressing hybridomas we have characterized appear to contain an Igh allele that has not undergone V(D)J joining (see above). To our knowledge, antigen-induced hybridomas containing such Igh alleles have never been obtained from normal mice. The hybrid loci in these hybridomas were probably created during the pro-B stage of development. The resulting expression of a functionally H chain could then have allelically excluded V(D)J rearrangement at the other endogenous *Igh* locus (36).

To gain further insight regarding the frequency of B cells expressing hybrid loci before and after Ars immunization, hybrid locus–specific PCR analysis was performed on individual spleen DNAs obtained from naive and Ars-immunized mice of the X1 line. Primer pairs that hybridize in pBluescript vector (transgene specific) and the intronic enhancer region (endogenous *Igh* locus specific) were used (13). PCR reactions were subjected to Southern blot analysis using a probe specific for the $J_H2$ gene segment. Fig. 7 shows that despite their ability to support PCR amplification of the transgenic $V_HDJ_H$ gene (see Materials and Methods), none of the naive DNAs gave rise to an easily detectable 2.2-kb band expected from a hybrid locus. However, long exposures of the blot (data not shown) did reveal weak bands of the expected size for a hybrid locus in two of the naive DNA reactions. In contrast, all but one of the DNAs obtained from Ars-immunized transgenic mice gave rise to strong bands of the expected size and sequence content (see legend to Fig. 7).

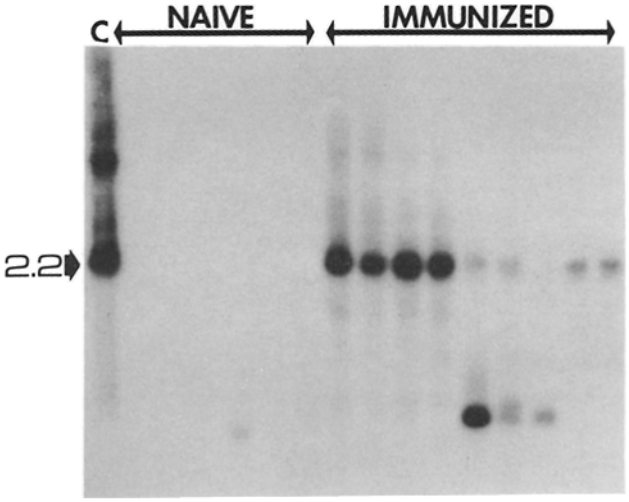


**Figure 7.** PCR analysis for the presence of hybrid H chain loci in spleens from transgenic mice of the X1 line. PCR was conducted as described (13) on DNA prepared from the spleens of individual mice that had either not been immunized (*NAIVE*), or that had been hyperimmunized with Ars-KLH (*IMMUNIZED*) as described in Materials and Methods, using primer combinations that result in specific amplification of X1 hybrid loci (13, 14). The reactions were analyzed by Southern blotting using a probe specific for the $J_H2$ gene segment and immediate flanking sequence. A 2-h exposure of the blot is shown. (Lane *C*) Contains a small fraction of a PCR reaction obtained from DNA of the hybrid locus expressing hybridoma X41-1. (*Arrow*) Location of the 2.2-kb band previously shown via cloning and sequencing to be derived from the hybrid locus (13, 14). The lower molecular weight bands present in several of the immunized samples appear to be artifactual PCR products as they do not hybridize with other probes specific for subregions of hybrid loci (data not shown). The upstream primers used for amplification hybridize to regions at the 3′ end of the pBluescript sequences present in the 5′ region of hybrid loci. Thus, even large 5′ deletions of this vector DNA (see text) should not affect the ability of a hybrid locus to be amplified.

## Discussion

The combined results of the FISH and Southern blot analyses provide compelling evidence for the absence of a hybrid locus reciprocal product in all transgenic hybridomas. In addition, these results show that no discernible changes in the transgenic arrays result from the formation of hybrid H chain loci. Taken together, these observations strongly suggest that models A and C, in their simple forms, cannot account for hybrid locus formation. Whereas random chromosome loss might have resulted in the absence of the reciprocal product predicted in model A in a subset of our hybridomas, this could not explain the universal absence we observe. Since all of the hybridomas we have analyzed were derived from transgene heterozygous mice, model A predicts that the transgenomes in these hybridomas cannot always be in germline configuration, yet this is what is observed. Double reciprocal exchange models are also not supported by the data for these same reasons. Whether hybrid locus formation takes place in G1 or G2 stages of the cell cycle does not influence these conclusions, since, at a minimum, in B cells selected for the expression of a hybrid locus a reciprocal product would cosegregate 50% of the time.

Model C predicts direct linkage of hybrid loci and their reciprocal products, resulting in obligate cosegregation of these loci. Random chromosome loss could therefore not account for the absence of the reciprocal products predicted by model C. Generation of the circular intermediate proposed in model C would be predicted to alter the size of the transgenic array, which is not what is observed. In addition, the absence of endogenous Igh DNA immediately 5′ of the cloned X7 hybrid loci suggests that if such a circular intermediate is involved in the formation of these loci, it cannot have the simple structure suggested in model C.

Whereas model B thus best accounts for formation of hy-

brid loci, direct proof of this model may be impossible because of an inability to unambiguously identify all the products of the recombination. Nevertheless, it seems inescapable that hybrid H chain loci can be generated in a manner that leaves the germline transgenic array unaltered, i.e., via a nonreciprocal (gene conversion) event. Moreover, the results of the Southern blot analyses and the structure of the cloned X7 hybrid loci strongly suggest that hybrid H chain loci contain only one copy of the transgene $V_HDJ_H$ and its 5′ flanking sequence, and that only one endogenous Igh allele is converted to a hybrid locus. The other Igh allele sometimes remains in a germline configuration, but most often is in a configuration accounted for by conventional V(D)J recombination.

Nonreciprocal hybrid locus formation could involve direct interaction of transgene and *Igh* loci, or might take place via a DNA or RNA intermediate. The transgenic arrays in all of the lines of mice appear to be transcriptionally active (14), and errors in DNA replication of the high copy number arrays might give rise to single or double stranded extrachromosomal copies of the transgene. The presence of a LINE-1 element 5′ of the X7-3C5 hybrid locus is intriguing in this regard, given that LINE-1 elements are retrotransposons (37). However, since homologous recombination can operate on loci present at different chromosomal locations (26), direct interchromosomal recombination represents the simplest mechanism given the available data.

Nonreciprocal intra and extrachromosomal homologous recombination in mammalian tissue culture cells are both well documented, but their mechanisms remain enigmatic (26, 32, 34, 35). Our data indicate that the frequency of interchromosomal formation of hybrid H chain loci is influenced by the length and degree of homology between the transgene and *Igh* locus, as previously found for intrachromosomal homologous recombination (26, 28). This implies that the rate-limiting step in hybrid locus formation is pairing of homologous strands. Resolution of the recombination at the 3′ (homologous) end of the gene conversion tract must take place with high fidelity, since despite the fact that most of the region between the $J_H2$ gene segment and the Igh enhancer is dispensable for H chain expression, no deletions or insertions are observed in this region in hybrid loci.

The regions immediately 5′ of vector sequences in the X7 hybrid loci we have characterized bear no homology to any region of the *Igh* locus. Other investigations (33, 34) have shown that mammalian gene conversion nucleated in regions of homology can proceed into regions of nonhomology. The X7-3C5 hybrid locus 5′ region appears to have been derived from neither the *Igh* locus, nor the immediate 5′ flanking DNA of the X7 transgene, as were the flanking regions of the X7-4G7 and X7-3D12 hybrid loci. In addition, the X7-3C5 hybrid locus contains 5′ vector sequences that have sustained a deletion relative to the X7 transgene, and this locus is flanked by a LINE-1 element. Although it is not known whether the deletion and LINE-1 acquisition took place during or subsequent to hybrid locus formation, these data indicate that nonhomologous recombination events can take place 5′ of hybrid loci, and are probably responsible for the resolution of the gene conversion event at its 5′ end. Determining whether repetitive retrotransposon elements such as LINE-1 are generally involved in these 5′ resolution events will require the molecular analysis of several other hybrid H chain loci.

Whereas our data demonstrate that V(D)J recombination and isotype class switch recombination cannot alone account for hybrid locus formation, components of these recombination systems may be indirectly involved. The V(D)J recombinase system has been shown to catalyze double strand breaks at heptamer-nonamer sites (38). All of our transgenes contain two heptamer-nonamer elements, and the *Igh* V segment locus contains many copies of these elements. The frequency of homologous recombination between *Igh* and transgene loci might be significantly increased because of the introduction of double strand breaks by the V(D)J recombinase at these sites in transgene DNA, endogenous Igh DNA, or both.

The transgenic arrays all lack any of the highly repetitive DNA involved in isotype class switch recombination. However, the *Igh* locus may be more recombinagenic than an average chromosomal locus because of the presence of extensive tracts of this switch DNA. In addition, the transcriptional activation of switch regions dramatically increases the frequency of class switch recombination within them (39, 40), and might increase the frequency of homologous recombination involving the *Igh* locus as well.

Gene conversion is responsible for the primary diversification of the V region repertoire in chickens (3), and contributes to V region diversity in rabbits (4). In contrast, primary diversification of the mouse V gene repertoire does not involve gene conversion (41, 42). In addition, during the course of T-dependent immune responses in mice, the diversification of V genes expressed by responding B cell clones appears to be largely due to untemplated nucleotide substitution (i.e., somatic hypermutation) (43, 44). Nevertheless, it is possible that the somatic hypermutation mechanism in mice is initiated by heteroduplex formation between an expressed V gene and an unexpressed V gene segment or V pseudogene, as a gene conversion event would be. Gene conversion initiated at such a heteroduplex by an error-prone DNA recombination system could result in mainly de novo nucleotide substitutions, with an occasional templated substitution. Indeed, regions of clustered base changes for which potential V segment donor sequences exist have been observed in somatically mutated mouse V genes (45, 46). Our data demonstrate that somatic gene conversion can indeed take place between Ig sequences in mouse B cells in vivo, consistent with previous observations made in B cell hybridomas in culture (47). However, while $V_H$ genes present in hybrid loci do contain somatic mutations, these mutations appear to be introduced after, not concurrently with the gene conversion events that generate these loci (14). Determining whether components of the machinery responsible for gene conversion that results in hybrid H chain locus formation are also utilized during somatic hypermutation requires further investigation.

Regarding the precursor frequency of B cells expressing hybrid loci, several observations are relevant. The presence

of hybrid loci in bulk naive spleen DNA could not be unequivocally demonstrated via PCR. We have also not observed a significant difference in the frequency of expression of 36-65 $V_H$ (transgene) encoded idiotypes among panels of hybridomas derived from polyclonally activated transgenic and normal B cells (Manser, T., unpublished observations). The identification of several Ars-induced hybrid locus–expressing hybridomas containing $J_H$ loci in germline configuration supports the idea that hybrid locus formation can take place during the antigen-independent stages of B cell development. Since, after Ars immunization, strong antigenic selection results in clonal expansion of B cells expressing hybrid loci, we cannot directly determine whether hybrid locus formation takes place at a higher frequency during antigen-dependent or -independent stages of B cell differentiation. However, since the number of hybridomas expressing anti-Ars Abs isolated from transgenic and nontransgenic mice is similar (Manser, T., unpublished observations), the overall frequency of functional hybrid locus formation must be low.

Finally, the recombination mechanism we have described provides a new approach for the introduction of novel $V_H$ genes and gene constructs into a natural context within the *IgH* locus in vivo. This approach has a potential advantage over embryonic stem cell–based gene targeting technology (27) for this purpose: hybrid H chain locus formation takes place somatically at a low frequency, resulting in only subtle alteration of the expressed Ab repertoire. Thus, the impact of the expression of a defined $V_H$ gene on a "background" of a normal V region repertoire and B cell compartment can be examined. This approach should prove particularly useful for studies concerning the influence of Ab V region structure and specificity on B cell clonal selection and differentiation.

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