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Immunology

Somatic recombination of heavy chain variable region transgenes with the endogenous immunoglobulin heavy chain locus in mice

(transgenic mice/antibody genes)

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ABSTRACT **Transgenic lines of mice were derived by using plasmid constructs containing DNA encoding an antibody heavy chain variable–diversity–joining region (V_H–D–J_H) and various amounts of 5′ and 3′ flanking DNA but lacking any repetitive isotype switch (S) or constant (C) region DNA. Unexpectedly, many of the antibody V_H regions expressed by B-cell hybridomas generated from immunized transgenic mice were found to be of transgenic origin. Further analyses showed that somatic events had generated hybrid genomic loci in the mice containing the transgenic V_H–*D*–J_H gene and plasmid sequences 5′ of endogenous heavy chain C region genes. Thus, V_H–*D*–J_H transgenes lacking S and C region DNA can recombine with endogenous *Igh* DNA, leading to the expression of transgene-encoded antibody.**

Antibody variable (V) region diversity in mice is generated by the combinatorial joining of germ-line *V* gene segments that are members of heterogeneous multigene families; the deletion and *de novo* addition of nucleotides at the junctions of these *V* gene segments during joining; and hypermutation of the resulting *V* genes (1). During the immune response of strain A/J mice to the hapten *p*-azophenylarsonate (Ars), antibody V regions encoded by a single combination of *V* gene segments (termed "canonical") become predominant and hypermutated (2). As part of an investigation of the cis-acting elements that direct the hypermutation process to antibody *V* genes, we generated transgenic mice using plasmid vectors containing a canonical heavy chain *V* gene (V_H).

MATERIALS AND METHODS

Transgenic Mice. Transgenic mice were produced by using published protocols (3). Fertilized eggs were from matings of C57BL/6 × A/J F_1 female mice and A/J male mice.

Immunization and Generation of Hybridomas. Mice were injected i.p. with 100 μg of Ars-conjugated keyhole limpet hemocyanin (Ars-KLH) in complete Freund's adjuvant. One week later three i.p. injections of 100 μg of Ars-KLH in phosphate-buffered saline were given at 3-day intervals. Three days after the final injection, spleen cells were used in fusions to Sp2/0 cells as described (4). Idiotype and isotype assays on supernatants were done as described (4).

DNA Isolation and Southern Blot Analysis. DNA was isolated from hybridomas, purified λ phage, tails, or spleens as described (5). Sequential Southern blotting analyses were done (4) with the following probes: pBluescript KS(−) (Stratagene); VhUp [an *Xba* I–*Pst* I fragment specific for sequences upstream of the A/J anti-Ars hybridoma 36-65 (6) V_H coding region ending within the leader exon]; Vh133 [an *Ava* II–*Rsa* I fragment encompassing codons 15–59 in the hybridoma 36-65 V_H gene (7)]; an *Xba* I–*Eco*RI fragment containing the *Igh* enhancer; J14B, a *Hin*dIII fragment 5′ of the enhancer and 3′ of the μ heavy chain switch (*S*) region (8); pγ1/EH10.0 specific for γ1 heavy chain *S*-region DNA ($S_{\gamma 1}$) (9); pγ1/A5, specific for the γ1 chain constant (C) coding region ($C_{\gamma 1}$) (10); and pγ2b/E6.6, specific for $S_{\gamma 2b}$ DNA (9).

Serology. Normal A/J and transgenic mice were immunized i.p. with 150 μg of Ars-KLH in complete Freund's adjuvant. Twenty-one to 23 days later serum was taken. Levels of Ars-binding antibodies and idiotopes were assayed essentially as described (11).

cDNA and Genomic PCR Amplifications. Reverse transcription–PCR was done as described (12) with a Thermocycler model 60 (Coy Laboratory Products, Ann Arbor, MI). V_H cDNA was amplified by using a 3′ primer for the appropriate C_H1 exon and a 5′ primer homologous to the leader exon of the 36-65 V_H gene. A 2.2-kilobase (kb) region within transgenic *V–D–J–Igh* hybrid loci was amplified with a nested primer strategy by using primers specific for pBluescript sequences and for a site 3′ of the *Xba* I site in the *Igh* enhancer region (see Fig. 3). All products were cloned in pBluescript KS(−).

Nucleotide Sequencing. Sequencing of immunoglobulin RNA was performed as described (13). pBluescript insert DNA was sequenced by using the Sequenase Version II kit (United States Biochemical) as described by the manufacturer.

Phage Cloning of *V–D–J–Igh* Hybrid Loci. The hybrid loci from the hybridomas X7-4G7 and X7-3D12 were cloned by enrichment for *Bgl* II restriction fragments, which hybridized to pBluescript, 36-65 V_H, and J14B, by size fractionation on sucrose gradients. The appropriate fractions were ligated to *Bam*HI-digested phage λ Dash II arms (Stratagene) and packaged by using extracts from Promega, all according to the manufacturers' instructions. The resulting λ libraries were screened primarily by using the *Igh* enhancer probe and secondarily by using the Vh133 probe. Phage positive for hybridization with both probes were purified by standard procedures.

RESULTS

Construction and Characterization of V_H Transgenic Mice. Linearized plasmid constructs containing the unmutated canonical V_H–*D*–J_H gene from the A/J anti-Ars hybridoma 36-65 (6) and various amounts of 5′ and 3′ flanking sequence but lacking any highly repetitive isotype *S* or *C* region DNA were used for the construction of transgenic mice (Fig. 1). The constructs contain the promoter elements required for transcription in B cells (14). Founders were backcrossed to

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Abbreviations: V, variable; D, diversity; J, joining; V_H and J_H, V and J region heavy-chain genes; C, constant; Ars, *p*-azophenylarsonate; Ars-KLH, Ars-conjugated keyhole limpet hemocyanin; S, switch.

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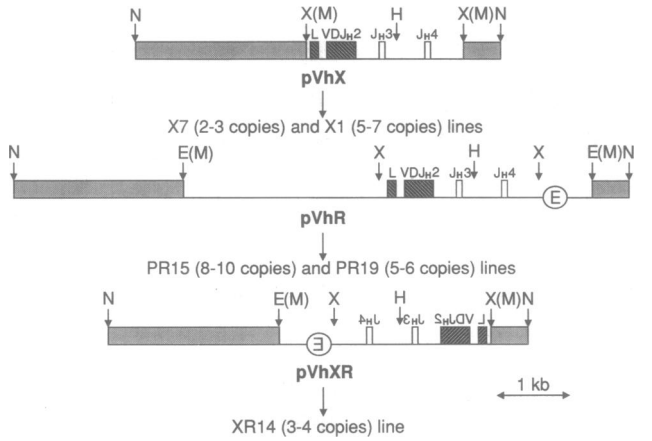

FIG. 1. pBluescript KS(−) constructs with various amounts of 5′ and 3′ immunoglobulin DNA flanking the hybridoma 36-65 V_H region were used for the generation of transgenic mice. Vector sequences are denoted as grey bars; immunoglobulin coding sequences, as striped boxes; and the enhancer, as a circled "E." E, *Eco*RI; H, *Hin*dIII; N, *Nae* I; X, *Xba* I; M, multiple cloning site.

A/J mice. All transgenomes have passed from parent(s) to offspring at frequencies expected for single chromosomal insertion sites. Transgene copy numbers vary from 2 to 10 among the lines, and all transgenic loci appear to be composed of tandem repeats of the input construct.

Hybridoma Isolation and Expressed V_H Sequencing. Since the transgenomes lack *C* region exons, we expected that they would not "allelically exclude" rearrangement of endogenous V_H gene segments. Thus, some transgenic B cells were expected to contain a functional endogenous heavy chain gene with a canonical V_H gene as well as a nonfunctional,

	D							
	TCN	NNN	TAC	TAT	GGT	GGT	AGC	TAC	NNN
36-65	--G	GTC	---	---	---	---	--T	---	TAC
X7-3D12*	--G	GTC	---	---	---	---	--T	---	TAC
X7-3C5	--G	GTC	---	---	---	---	--T	---	TAC
X7-4G7	--G	GTC	---	---	---	---	--T	---	TAC
X7-5D3	--G	GTC	---	---	---	---	--T	---	TAC
X41-1	--G	GTC	---	---	---	---	--T	---	TAC
X41-2	--G	GTC	---	---	---	---	--T	---	TAC
X42-1	--G	GTC	---	---	---	---	--T	---	TAC
X42-6	--G	GTC	---	---	---	---	--T	---	TAC
X42-8	--G	GTC	---	---	---	---	--T	---	TAC
PR14-3	--G	GTC	---	---	---	---	--T	---	TAC
PR14-2*	--G	GTC	---	---	---	---	--T	---	TAC
PR18-1*	--G	GTC	---	---	---	---	--T	---	TAC
PR18-2	--G	GTC	---	---	---	---	--T	---	TAC
XR6-1	--G	GTC	---	---	---	---	--T	---	TAC
XR6-2	--G	GTC	---	---	---	---	--T	---	TAC
XR6-3	--G	GTC	---	---	---	---	--T	---	TAC
XR26-2	--G	GTC	---	---	--A	---	--T	---	TAC
NX20-1	--C	CAT	---	---	---	---	---	---	GAC
NX20-4	--C	GAG	---	---	---	---	--T	--T	AAC
PR11-4	--T	CTT	---	---	---	---	---	---	GCC
PR11-6	--G	TAT	---	---	---	---	--T	--T	GCA
XR26-3	--G	GTC	---	---	---	---	--T	--T	TTT

FIG. 2. Sequences of the V_H–D–J_H junctional region in the canonical V_H genes expressed by transgenic mice. Hybridoma names denote mouse origin: X7 and NX20 hybridomas, from two X7 line mice; X41 and X42, from two X1 line mice; PR11 and PR14, from two PR15 line mice; PR18, from one PR19 line mouse; and XR6 and XR26, from two XR14 line mice. Asterisks indicate hybridomas from which heavy chain RNA was directly sequenced. Sequences are compared with the consensus sequence of canonical V_H genes and the anti-Ars hybridoma 36-65 V_H gene in this region. The sequences begin with the first junctional codon 3′ of V_H gene segment coding sequence and end with the junctional codon just 5′ of J_H2 coding sequence. Variable nucleotides are indicated by "N."

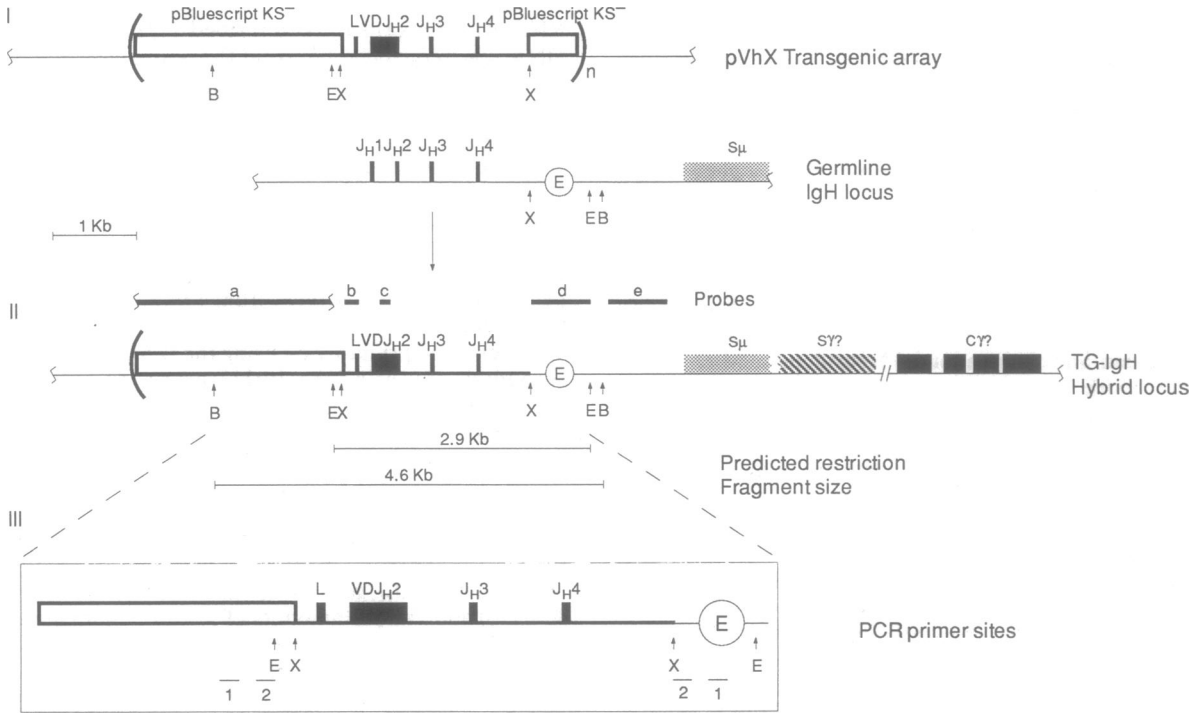

FIG. 3. Generation of a transgenic *V–D–J–Igh* hybrid locus. Line I is a schematic drawing of the transgenic array in X1 or X7 transgenic lines and the endogenous *Igh* locus. A single repeat unit of the transgenic array is shown. The germ-line *Igh* locus is illustrated for the sake of example. Vector sequences are denoted as open bars; immunoglobulin coding sequences, as filled boxes; the enhancer region, as a circled E; transgenic sequences, as a boldface line; endogenous DNA, as a thin line; and *S* regions, as stippled and striped boxes. Line II shows the resulting hybrid locus and predicted novel 2.9-kb *Eco*RI and 4.6-kb *Bgl* I restriction fragments. Probes used for the detection of such a hybrid locus are designated as horizontal bars labeled with the following letters: a, pBluescript KS(−); b, VhUp; c, Vh133; d, *Xba* I–*Eco*RI fragment in the enhancer region; e, J14B. Line III shows the "nested" primers used to amplify the hybrid locus. B, *Bgl* I; E, *Eco*RI; X, *Xba* I.

canonical transgenic V_H gene. To generate hybridomas from such B cells, first-generation transgenic offspring were immunized with Ars-KLH, and hybridomas were constructed and screened for expression of canonical V regions using an anti-idiotypic antibody (E4) that is specific for such V regions (11, 15). All hybridomas were found to produce IgG. The sequences in the V_H region of hybridoma heavy chain RNA were determined by direct RNA sequencing with a *C* region primer or by sequencing of reverse transcription–PCR clones obtained with *C* region and V_H leader primers. As expected, the genes are somatically mutated canonical V_H genes (data not shown). Unexpectedly, however, few of these V_H genes contain the diverse junctional nucleotides characteristic of conventional canonical V_H genes (16); most contain junctional nucleotides identical to those of the 36-65 transgenic V_H (Fig. 2). We have previously shown that among 30 independently isolated hybridomas that express canonical *V* regions derived from Ars-KLH immunized normal A/J mice, this junctional nucleotide combination is observed only once (16).

Explanations for the High Frequency of Transgenic Junctional Sequences Among Functional Antibody V_H Genes. Since the plasmid constructs used to generate our transgenic mice do not encode complete heavy chains, these results indicated that the transgenes were influencing the nature of junctional residues expressed among endogenous canonical anti-Ars heavy chains. Previous explanations for such transgene effects differ regarding whether information transfer between DNAs (e.g., recombination), RNAs (e.g., trans-splicing), or proteins (e.g., idiotype selection) is involved (17–19).

Fig. 3 illustrates that a hybrid chromosomal locus generated via recombination of homologous sequences within the X1 or X7 transgenes and the endogenous *Igh* locus would give rise to novel *Eco*RI and *Bgl* I restriction fragments of predictable size (2.9 and 4.6 kb) and sequence content. Fig. 4*a* shows that an *Eco*RI fragment of 2.9 kb, which hybridized with an enhancer and V_H, but not a vector probe could be detected in DNA from Ars-induced hybridomas derived from the X7 line. Fig. 4*b* shows that a *Bgl* I fragment of 4.6 kb that hybridized with all three probes could be detected in the DNA of hybridomas derived from the X7 and X1 lines (lanes 1 and 2). In addition, bands of the sizes expected for transgene–*Igh* hybrid loci were observed in the DNA of Ars-induced hybridomas constructed from our other transgenic lines of mice (indicated with asterisks).

DNA derived from hybridomas expressing V_H genes with junctional sequences that differ from the transgene gave rise

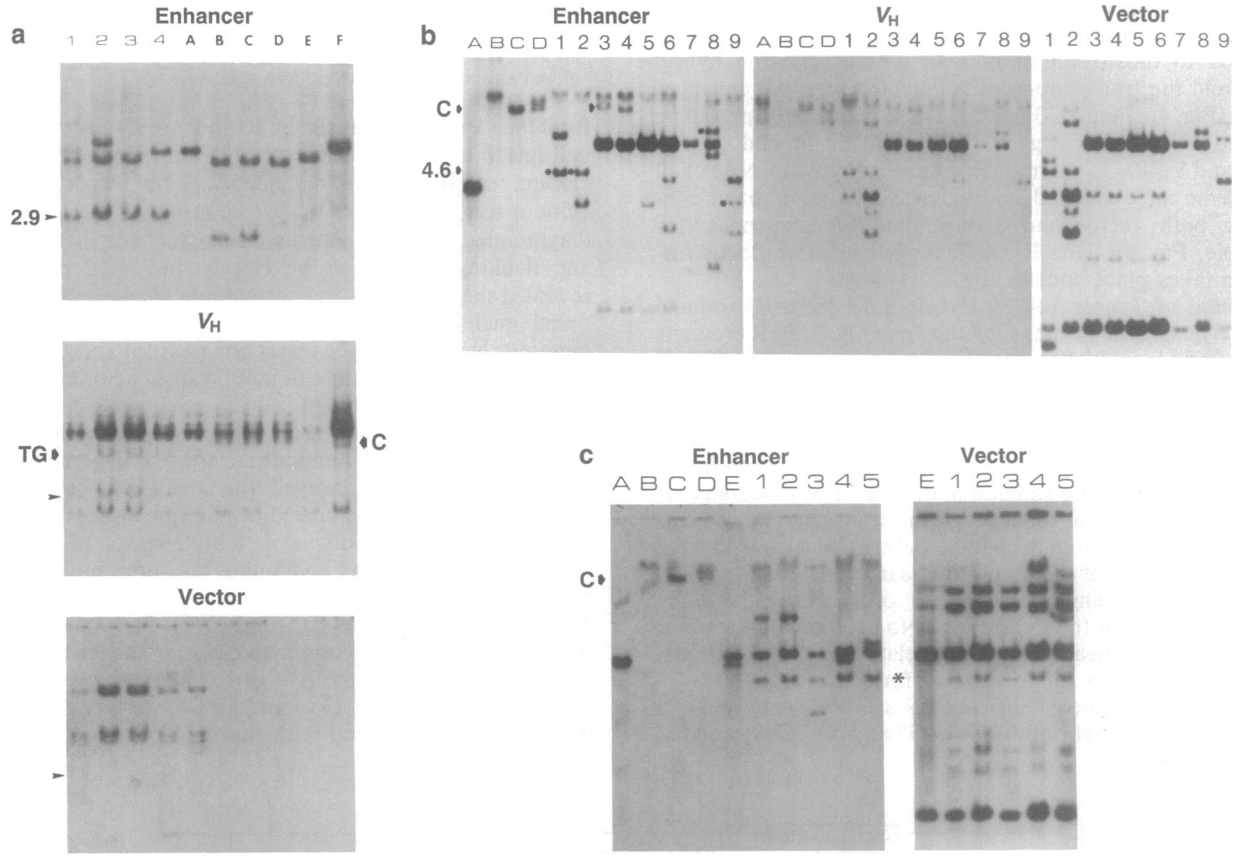

FIG. 4. Southern blot analyses of transgene and V_H region gene rearrangements in transgenic mice and hybridomas. *Eco*RI (*Left*) and *Bgl* I (*Right*) blots were analyzed sequentially with the following probes: enhancer (probe d in Fig. 3), VhUp (probe b in Fig. 3, used for *b*), Vh133 (probe c in Fig. 3, used for *a*) and vector (probe a in Fig. 3). "C" indicates the endogenous canonical V_H locus and bands expected from the hybrid locus are indicated with asterisks. (*a*) *Eco*RI blot. Controls (lanes A–F): X7 founder tail (lane A), HIP 19 (lane B), HIP-16 (lane C) and 36-65 (lane D) (A/J anti-Ars hybridomas expressing the canonical *V* region), Sp2/0 (lane E), A/J kidney (lane F); hybridomas from a single X7 offspring (lanes 1–4): X7-5D3 (lane 1), X7-4G7 (lane 2), X7-3D12 (lane 3), X7-3C5 (lane 4). The arrow indicates the 2.9-kb *Eco*RI fragment from the hybrid locus; TG designates the band generated from the transgenic array. (*b*) *Bgl* I blot. Controls (lanes A–D): A/J kidney (lane A), Sp2/0 (lane B), 36-65 (lane C), HIP-16 (lane D); transgenic hybridomas (lanes 1–9): X7-4G7 (X7 line) (lane 1), X41-2 (X1 line) (lane 2), PR11-4 (lane 3), and PR11-6 (lane 4) (same mouse PR15 line), PR14-2 (PR15 line) (lane 5), PR18-3 (lane 6), PR18-1 (lane 7) and PR18-2 (lane 8) (same mouse PR19 line), XR6-3 (XR14 line) (lane 9). Detection of a hybrid fragment in the PR transgenic lines is difficult because the fragment is only several hundred base pairs larger than that generated from the transgenic array. The unexpectedly large fragment in lane 8 could be due to a change in the *Bgl* I site downstream of the enhancer region because of isotype switching. (*c*) *Bgl* I-digested DNA from anti-Ars hybridomas generated from a single XR14 offspring. Controls (lanes A–E): A/J kidney (lane A), Sp2/0 (lane B), 36-65 (lane C), HIP-16 (lane D), NXR6 tail (lane E); hybridomas (lanes 1–5): XR6-1 (lane 1), XR6-2 (lane 2), XR6-3 (lane 3), XR6-4 (lane 4), XR6-5 (lane 5).

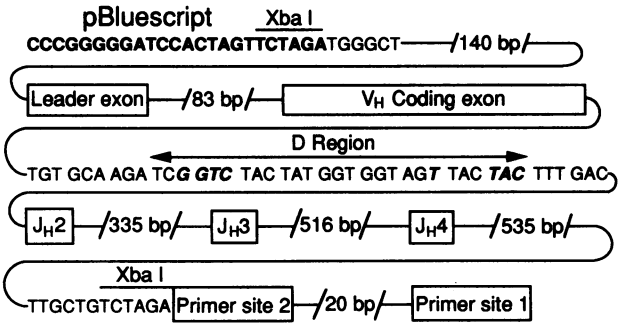

FIG. 5. Schematic diagram of hybrid loci cloned from hybridomas X7-5D3 and X7-3C5 via PCR. Sequences derived from pBluescript of transgenic origin (bold lettering), the 5′ end of immunoglobulin sequences, the *D* region of V_H 36-65 (junctional nucleotides shown in boldface italics), the 5′ end of J_H2, and the 5′ end of the *Igh* enhancer region are shown explicitly. Exons are shown as open boxes, as are the downstream J_H segments and the two 3′ primer sites in the enhancer region used for amplification.

to a band consistent with the structure of a conventional canonical V_H locus (indicated with a "C"). In Fig. 4*b* two such bands are indicated in lanes 3 and 4 by an arrow. All hybridomas that contain hybrid loci did not give rise to such a band. Also, in many of the hybridoma DNAs, a band in addition to those consistent with a transgene–*Igh* hybrid locus and the transgenome was detected with the enhancer probe (Fig. 4*c*, lanes 1–3 and 5). These bands appeared to be derived from either a germ-line *Igh* allele or endogenous *D*–J_H and V_H–*D*–J_H rearrangements. Finally, tail DNAs of all transgenic mice showed no evidence of a novel band containing both vector and endogenous *Igh* sequences (for example, Fig. 4*c*, lane E), indicating that hybrid locus formation takes place somatically.

Cloning of Transgene–*Igh* Hybrid Loci from Hybridoma DNA. By using the primer sites shown in Fig. 3, PCR products from hybrid loci (2.2 kb) were obtained from DNA of X7-5D3 and X7-3C5, two hybridomas derived from a single mouse of the X7 line, and were cloned and sequenced. Results are shown schematically in Fig. 5. The sequences in the V_H coding region of these clones agree with those obtained from heavy chain RNA, revealing somatic mutations. The junctional sequences are identical to those of the transgene V_H. Vector sequences derived from the hybrid loci are shown in bold type, and the structures of the 3′ ends of the inserts are indicative of the primer sites being immediately 3′ of the *Xba* I site in the enhancer region of the hybrid loci. No deletions, inversions, insertions, or duplications of DNA relative to the input transgene or endogenous *Igh* locus were found.

To eliminate the possibility of PCR artifact, the hybrid loci present in the X7 hybridomas X7-4G7 and X7-3D12, isolated from the same mouse as the PCR clones and expressing IgG1 and IgG2b antibodies, respectively, were cloned by λ phage cloning techniques. Fig. 6 summarizes the structures of the clone inserts, structures that are consistent with the model diagrammed in Fig. 3. Nucleotide sequences of the *V*–*D*–*J* and flanking regions of the clones (indicated by "Seq") revealed that the *V*–*D*–*J* genes contain transgene-type junctional nucleotides and that these regions are somatically mutated. No deletions or insertions (>1 bp) and no duplications or inversions relative to input transgene or endogenous *Igh* DNA were observed.

Table 1. Levels of the idiotopes recognized by the monoclonal anti-idiotypic antibodies AI and 107 in primary Ars-KLH immune sera from transgenic and normal mice

Normal A/J mice				Transgenic mice			
Mouse	Ars+	107+	AI+	Mouse*	Ars+	107+	AI+
1	100	<5	10	2NX59	675	681	1000
2	200	100	117	2NX62	725	300	785
3	455	<5	<5	2NX66	425	<5	<5
4	210	<5	18	2NX69	830	38	145
5	118	<5	<5	2NXR25	625	265	833
6	2220	1	159	2NXR26	1030	94	611
7	500	5	318	2NXR27	1040	625	1720
8	641	8	6	NPR47	135	<5	<5
9	975	8	8	NPR48	295	295	172
10	1300	<5	13	2NPR33	90	14	23
11	990	60	40	2NPR35	320	93	110
12	565	260	87	2NPR36	375	382	275
13	200	<5	<5	2NPR39	225	180	212
14	925	9	7				
Avg	671	37	56		522	228	453

*The mice used were from the X7 (2NX59 and 2NX62), X1 (2NX66 and 2NX69), XR14 (2NXR25, 2NXR26, and 2NXR27), PR15 (NPR47 and NPR48), and PR19 (2NPR33, 2NPR35, 2NPR36, and 2NPR39) transgenic lines. Names beginning with "2" indicate second generation offspring. All others are first generation offspring. The values shown are μg equivalents of the 36-65 monoclonal antibody per ml. Avg, average.

Transgene–*Igh* Hybrid Loci Encode Predominant Serum Antibodies. Ars-KLH immune sera from transgenic and normal A/J mice were assayed for levels of the idiotopes identified by the monoclonal anti-idiotypic antibodies AI and 107. These antibodies displayed a high degree of specificity for canonical *V* regions containing the rare combination of heavy chain junctional amino acid residues present in the 36-65 (transgene) V_H (refs. 20 and 21; B. Parhami-Seren and M. Margolies, personal communication). The frequency and level of expression of the AI and 107 idiotopes were greatly increased among Ars-KLH immune sera derived from transgenic mice as compared with normal A/J mice, while the

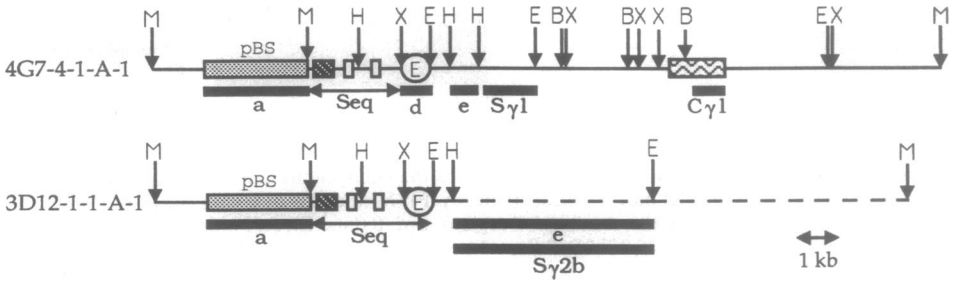

FIG. 6. Molecular maps of the transgenic *V*–*D*–*J*–*Igh* hybrid loci of the X7-4G7 and X7-3D12 hybridomas cloned in λ phage. The regions that hybridize with pBluescript (a) are designated as stippled bars. V_H–*D*–J_H2, unrearranged J_H, and *C* coding regions are shown as black striped, open, and wave-filled boxes, respectively. Probes used for the analysis are designated by horizontal bars labeled "a–e" as in Fig. 3, underlying the regions to which they hybridize. In addition, probes specific for the $S_{\gamma 1}$ and $S_{\gamma 2b}$ switch DNA regions and the $C_{\gamma 1}$ gene were used. The enhancer region is indicated by a circled E. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; M, multiple cloning site. A region in the 3D12 clone that was not completely mapped is shown as a dashed line.

average levels of total anti-Ars antibodies in sera of these two groups of mice were similar (Table 1). In some transgenic mice, transgene-encoded *V* regions appeared to compose a majority of the anti-Ars antibody, an observation that is not unexpected since in normal A/J mice canonical *V* regions dominate the anti-Ars response (2).

DISCUSSION

Several clues to the nature of the mechanism responsible for generation of V_H–*D*–J_H transgene-IgH hybrid loci are provided by our data. Since the input transgenes lacked the highly repetitive *S* region DNA in which isotype switch breakpoints are usually found, the factors responsible for isotype switching, implicated in the trans-switching of a transgenic IgM-encoding V_H gene into the endogenous *C* region locus by Selsing and coworkers (18), may not be involved. The transgenes lack a 5′ "heptamer–nonamer" element, required for conventional *V*–(*D*)–*J* joining (22). Since the V_H–*D* and *D*–J_H junctional sequences in hybrid loci are always identical to those of the transgene, V_H replacement nucleated at heptamer-like elements within V_H gene segments (23, 24) is ruled out.

The result of transgene–Igh recombination is a locus indistinguishable 3′ of V_H from functional endogenous *Igh* loci. Indeed, apparently normal isotype switching has taken place 3′ of all of the hybrid loci we have characterized. Since vector sequences are always found 5′ of the V_H, classical gene conversion cannot account for hybrid locus formation. Collectively, these data suggest that a homologous recombination event nucleated 3′ of V_H (e.g., in the J_H locus) took place during hybrid locus formation and that one-sided homologous recombination best accounts for formation of these loci. This type of recombination has been observed between transfected DNA and homologous endogenous loci in culture cells (25), being apparently resolved by illegitimate recombination outside of the region of mutual homology. In this regard, we have not observed a band in Southern analyses that could correspond to the reciprocal product of the transgene–Igh hybrid, a product that should be generated by a conventional single-site homologous cross-over event. Since hybrid locus formation takes place in five independent lines of mice and transgenic DNA has always been observed to integrate at random genomic sites (26), formation of some hybrid loci probably occurs via interchromosomal events. Indeed, we have obtained evidence with fluorescence *in situ* hybridization indicating that the germ-line transgenome and *Igh* loci in the PR15 and X1 lines of transgenic mice are on different chromosomes.

Several other observations are relevant to the question of whether recombination of transgenes and homologous endogenous loci occurs in other lines of transgenic mice. Idiotopes encoded by canonical V_H–*D*–J_H genes cannot be detected in transgenic nonimmune sera, and the frequency of expression of antibodies encoded by the canonical V_H gene segment in hybridoma populations derived from polyclonally activated normal or transgenic B cells does not differ (T.M., unpublished observations). Fluorescence-activated cell-sorting analysis of transgenic spleen cells reveals normal lymphocyte numbers, subset distribution, morphology, and cell-surface phenotype. We have never observed a band representative of a hybrid locus in Southern analyses of DNA derived directly from the spleens of naive or Ars-KLH immunized mice; PCR amplification is required to reveal the presence of such a locus. Finally, while all of our transgenic lines of mice have shown evidence of hybrid locus formation, not all individual members of these lines give rise to hybridomas that express hybrid loci. Thus, the frequency of formation of hybrid loci in the B-cell population of transgenic mice is low. Antigen selective forces are probably responsible, in large part, for the high frequency of hybridomas we have observed to express hybrid loci. Somatic recombination of transgenic and endogenous loci may, therefore, be a general phenomenon, but the frequency of these events may preclude their detection. Nevertheless, immunoglobulin transgenes can no longer be assumed to influence the immune system only in trans via the heavy or light chains they may encode.

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