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

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Summary

Mice with transgenes containing an antibody H chain V region (V_HDJ_H) gene were used in an analysis of the *cis*-acting elements required for hypermutation of immunoglobulin (Ig) V genes. These transgenes can somatically recombine with endogenous IgH DNA, leading to the formation of functional heavy (H) chains partially encoded by the transgenic V_HDJ_H. The transgenomes in the five different lines of mice analyzed contain as little as 150 bp, and as much as 2.8 kb of natural DNA flanking the 5′ side of the V_H and either 1.5 or 2.3 kb (including the intronic enhancer and 5′ matrix attachment region [MAR]) flanking the 3′ side of V_H. Hybridomas were constructed from immunized transgenic mice, and transgenes present in these hybridomas that had or had not recombined to form functional H chain loci were sequenced. The data obtained show that: (*a*) the recombined transgenes contain hypermutated V_H genes; and (*b*) among such transgenes, even those containing only 150 bp of natural V_H 5′ flanking sequence and several kilobases of 5′ plasmid vector sequence display a frequency, distribution, and type of mutation characteristic of conventional IgH loci. The data also indicate that transgenic V_HDJ_H genes that have not recombined with endogenous IgH DNA are not substrates for hypermutation, even if they are flanked by 2.8 kb of natural 5′ DNA, and 2.3 kb of natural 3′ DNA, including the J_H2-J_H4 region, a MAR, and the intronic enhancer. Collectively, the data suggest that sequences 5′ of the V_H promoter are dispensable, a V_H promoter and the intronic IgH enhancer region are not sufficient, and a region(s) within or 3′ of the IgH constant region locus is requisite, for hypermutation of Ig V_H transgenes.

Antibody V region diversity in mice and humans is generated by the combinatorial joining of germline V gene segments that are members of heterogeneous multigene families, the deletion and de novo addition of nucleotides at the junctions of these V segments during joining, and hypermutation of the resulting V genes (1). While characterization of the *cis*- and *trans*-acting factors involved in the rearrangement of V gene segments and the generation of junctional sequences has proceeded rapidly in recent years (2, 3), the mechanism of hypermutation continues to remain enigmatic.

Current evidence suggests that hypermutation is induced during an immune response (4–6), introduces mainly single-nucleotide replacements at a rate estimated to be 10^{-3} per base pair per cell division (7), acts efficiently only in and immediately around fully rearranged V genes (8–14), and is not causally linked to isotype switching (15, 16). Since V gene hypermutation plays a central role in the affinity maturation of antibodies (6, 17, 18), and is intimately associated with the development of B cell memory (17, 19–23), the complete elucidation of its mechanism, as well as how this mechanism is regulated, is of central importance to an understanding of the development of antibody specificity and humoral immunity.

While direct approaches to the characterization of the *trans*-acting factors involved in the hypermutation process continue to remain elusive, transgenic mouse technology has allowed investigations of necessary *cis*-acting elements. Two groups have demonstrated that the V genes in Ig transgenes encoding functional κ L chains are mutable (24, 25), demonstrating that the *cis*-acting elements requisite for mutation of V_κ genes must lie in reasonably close proximity (24, 26). Indeed, the recent work of Sharpe et al. (25) has implicated a requirement for the downstream κ enhancer in the mutation process.

During the course of the immune response of strain A/J mice to the hapten *p*-azophenylarsonate (Ars),[1] antibody V regions encoded by a single combination of V region gene

[1] *Abbreviations used in this paper:* Ars, *p*-azophenylarsonate; MAR, matrix attachment region.

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Volume 177 March 1993 797–809

segments become predominant (20). We term such V regions "canonical." The distribution and type of somatic mutations in the canonical V regions expressed by hybridomas isolated during the anti-Ars response are well characterized, as are the effects of recurrently observed mutations on the affinity of such V regions for Ars (18). We found that in mice bearing transgenomes comprised of a canonical V_H gene and various amounts of natural 5′ and 3′ flanking DNA, the transgenic V_H and associated 5′ flanking region could recombine with endogenous IgH DNA, leading to the expression of transgenic V_H-encoded antibody (27). Sequence analysis of the transgenes present in hybridomas derived via Ars immunization of these transgenic mice has allowed us to evaluate the influence of proximal 5′ and 3′ flanking regions, and whether the V_H gene is part of a functional H chain locus, on V_H hypermutation.

Materials and Methods

Transgenic Mice. The construction and characterization of the transgenic mice used in this study have been described previously (27). Briefly, plasmids containing an unmutated canonical V_HDJ_H gene cloned from the 36-65 anti-Ars hybridoma, and various amounts of natural 5′ and 3′ flanking sequence, were used to construct the mice. The following lines, containing the amount of V_H 5′ and 3′ flanking DNA and transgene copy numbers indicated, were used in this study: X1 and X7 lines, 150 bp of 5′ flank and 1.5 kb of 3′ flank, five to seven and two to three copies, respectively; PR15 and PR19 lines, 2.8 kb of 5′ flank and 2.3 kb of 3′ flank, 8–10 and 5–6 copies respectively; and XR14, 150 bp of 5′ flank and 2.3 kb of 3′ flank, three to four copies. The founder mice were derived from matings of $(A/J \times C57BL/6)F_1$ female mice to A/J male mice, and the mice used in this study were obtained by backcrossing these founders to A/J mice.

Immunization and Generation of Hybridomas. Transgenic mice were given an intraperitoneal injection of 100 μg Ars-KLH in CFA. 1 wk later, the mice received three intraperitoneal injections of 50 μg Ars-KLH in PBS at 3-d intervals. 3 d after the final injection, the mice were killed and their spleens used in fusions to Sp2/O as previously described (15). Hybridoma supernatants were screened for reactivity with an anticanonical V region mAb, E4, and the isotype of antibodies expressed by positive clones was determined by ELISA, all as described previously (15).

cDNA and Genomic PCR Amplifications. Total RNA was prepared from hybridoma cells by a guanidinium isothiocyanate–CsCl procedure described previously (28, 29). The V_H RNA was converted to cDNA using a 3′ primer complimentary to sequences within the first 60 nucleotides of the appropriate C_H1 exon and amplified using a 5′ primer homologous to sequences in the leader exon of the 36–65 variable region. 1–5 μg of total RNA was subjected to reverse transcriptase (RT)-PCR essentially as described (30) using a thermocycler (model 60; Coy Laboratory Products Inc., Ann Arbor, MI). Each of the primers contained restriction sites (XhoI in the case of constant region primers and SacI for V_H leader region primers [VhL]) to facilitate the subsequent cloning of the amplification products into the pBluescript KS(−) vector (Stratagene, La Jolla, CA).

Canonical V_H genes in genomic DNA were amplified with V_H leader and J_H2 primers (both containing restriction sites) for 35 cycles at 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min. The products were digested with the appropriate restriction enzymes and cloned into the polylinker of pBluescript KS(−).

Nucleotide Sequencing. Direct sequencing of Ig RNA was performed as described previously (4). pBluescript plasmid DNA was sequenced with ^{35}S-dATP using the Sequenase Version II kit (U.S. Biochemicals Corp., Cleveland, OH) with oligonucleotide primers as described by the manufacturer. Some of the sequences were also obtained using the "Taq DyeDeoxy" kit (Applied Biosystems, Inc., Foster City, CA), and the automated sequencer (Applied Biosystems, Inc.), or the "fmole" PCR sequencing kit (Promega Biotec, Madison, WI).

RNA Analysis. Northern blot analyses were performed with formaldehyde agarose gels using standard procedures. RNase protection assays were conducted as described (31). Antisense RNA was synthesized from a 486-bp StuI-MscI restriction fragment cloned behind the T3 promoter in pBluescript KS(−). This fragment was obtained from the genomic V_H clone of the 36–65 hybridoma and encompasses from V_H codon 41 to 186 bp 3′ of the end of J_H2. An RNA transcription kit (Stratagene, La Jolla, CA) and α-[^{32}P]UTP were used to synthesize the antisense probe from plasmid linearized with SacI. After hybridization and RNase digestion, protected fragments were analyzed on a 6% polyacrylamide/urea gel and visualized by autoradiography.

Results

Experimental Strategy. Clues to the location of the *cis*-acting elements requisite for V_H hypermutation are provided by previous findings that: (*a*) mutations are observed at high frequency only within a region encompassing the rearranged V_H gene and ~300 bp 5′ of the V coding region and 1 kb 3′ of the coding J segment, irrespective of which J segment is used (8, 11, 12); and (*b*) hypermutation appears mechanistically unrelated to H chain class switching since mutation can take place in a single IgH locus both before and after switching mediated by DNA deletion to a variety of different C region genes (15, 16). These results suggested that a rearranged V_H gene and only limited amounts of 5′ and 3′ flanking DNA might contain all the *cis*-acting elements required for full activity of the hypermutation process.

We have constructed mice bearing multicopy transgenomes containing an unmutated canonical V_HDJ_H gene, various amounts of natural 5′ and 3′ flanking sequence, and plasmid vector sequences. We previously demonstrated that in such mice a transgenic V_HDJ_H gene, along with 5′ flanking Ig and plasmid sequences, could become juxtaposed next to IgH C region genes in B cells, leading to the production of anti-Ars antibodies partially encoded by the transgenic V_H (27). Hybridomas expressing canonical V regions that are isolated from Ars-KLH–immunized transgenic mice often contain a "hybrid" transgene-IgH locus that encodes their functional H chains, and always contain other copies of the original transgene. The configuration of the transgenes present in our various transgenic hybridomas is diagrammed in Fig. 1.

The transgenic V_H gene and 5′ flanking DNA present in the X1, X7, and XR14 lines of mice somatically recombines with endogenous IgH DNA to give rise to an expressed H chain locus containing 150 bp of 5′ transgenic Ig DNA followed by plasmid DNA. In two X7 hybrid loci we have characterized in detail, the plasmid DNA is ~2.2 kb in length and is flanked on the 5′ side by at least 1.1 kb of DNA that bears

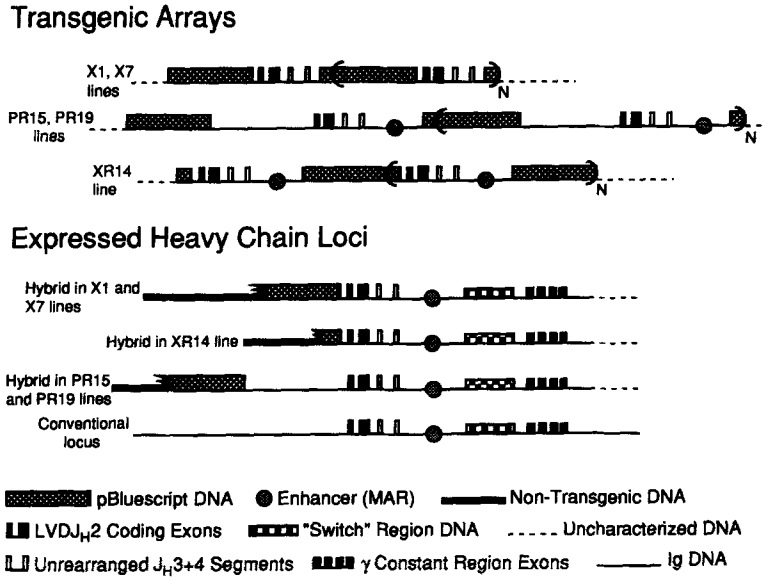


Figure 1. Schematic diagram of the structure of transgenomes, transgene-IgH hybrid loci, and conventional H chain loci. Two copies of each repeat unit of each type of transgenic array are shown; (*N*) the unit repeat. Each transgenic hybridoma has either a hybrid or conventional expressed H chain locus containing a canonical V_H gene, as well as additional copies of the original transgene that remain present as tandem repeats. The length of plasmid sequences present in most hybrid loci are as yet undefined (*jagged edge*), but always appear to be flanked by sequences not derived from transgenic DNA (*thick lines*). Our data indicate in all hybrid loci only one transgenic V_H copy has been juxtaposed next to endogenous IgH DNA (see text).

no homology to transgenic or Ig DNA. The transgenic V_H gene and 5′ flanking DNA present in the PR15 and PR19 lines somatically recombines with IgH DNA giving rise to an expressed locus with several kilobases of natural 5′ Ig DNA, followed by plasmid sequences. In both types of hybrid loci, regions 3′ of the transgenic V_H appear identical to those observed in conventional IgH loci that encode IgG H chains. That is, the unrearranged J_H3 and J_H4 segments are present, followed by the matrix attachment region (MAR)/enhancer region, followed by a region containing switch DNA, and then the Cγ exons (27; and A. M. Giusti and T. Manser, unpublished results). Canonical V_H-expressing hybridomas isolated from all of the lines of transgenic mice contain one of these types of hybrid loci or a conventional endogenous IgH locus, in addition to copies of the transgene that have not recombined with IgH DNA. Conventional and pulsed field gel electrophoresis combined with Southern blotting analysis strongly suggests that only one transgene V_H copy is associated with IgH DNA in the hybrid loci expressed in all of our hybridomas. Moreover, fluorescence in situ hybridization analysis of metaphase chromosomes from normal B cells and hybridomas obtained from the X1, XR14, PR15, and PR19 lines indicates that the majority of transgene copies are present in their germline chromosomal location in cell lines containing a hybrid locus. In all cases this germline location is not syntenic with the IgH locus (A. M. Giusti and T. Manser, manuscript in preparation).

Since many of the hybridomas contain a copy of a canonical V_HDJ_H transgene that has recombined with IgH DNA, and copies that have not, sequencing of such transgenes should provide insight into IgH elements not included in the transgenes that may be necessary for V_H hypermutation. Further, since the transgenes in different lines of mice contain varying amounts of natural 5′ and 3′ sequence flanking the V_HDJ_H, such an analysis would also allow an evaluation of the requirements for proximal V_H 5′ and 3′ flanking regions in the hypermutation process.

Sequencing of V_H Genes Expressed by Ars-induced Transgenic Hybridomas. The origin and isotypic class of antibodies expressed by the canonical V region–expressing transgenic hybridomas used in our sequencing analyses are shown in Table 1. The V_H regions of H chain RNA expressed by these hybridomas were sequenced, either directly using RT and an appropriate C region primer, or by PCR amplification of V_H region cDNA followed by cloning and sequencing of the PCR product. The data in Fig. 2 show that the V_H genes contain numerous somatic mutations and many contain transgene-

Table 1. *Origins and Characteristics of mAbs Generated from Ars-KLH-immunized Transgenic Mice*

Construct	Founder	Mouse	Hybridoma	Subclass
pVhX	X7 (female)	NX7 (female)	X7-5D3	G1,κ
			X7-4G7	G1,κ
			X7-3D12	G2b,κ
			X7-3C5	G1,κ
		NX20 (female)	X20-4	G1,κ
	X1 (male)	NX41 (female)	X41-1	G2b,κ
			X41-2	G2b,κ
pVhR	PR15 (male)	NPR11 (male)	PR11-4	G1,κ
		NPR14 (male)	PR14-3	G1,κ
	PR19 (female)	NPR18 (female)	PR18-1	G2b,κ
pVhXR	XR14 (male)	NXR6 (female)	XR6-1	G3,κ
		NXR26 (female)	XR26-3	G1,κ

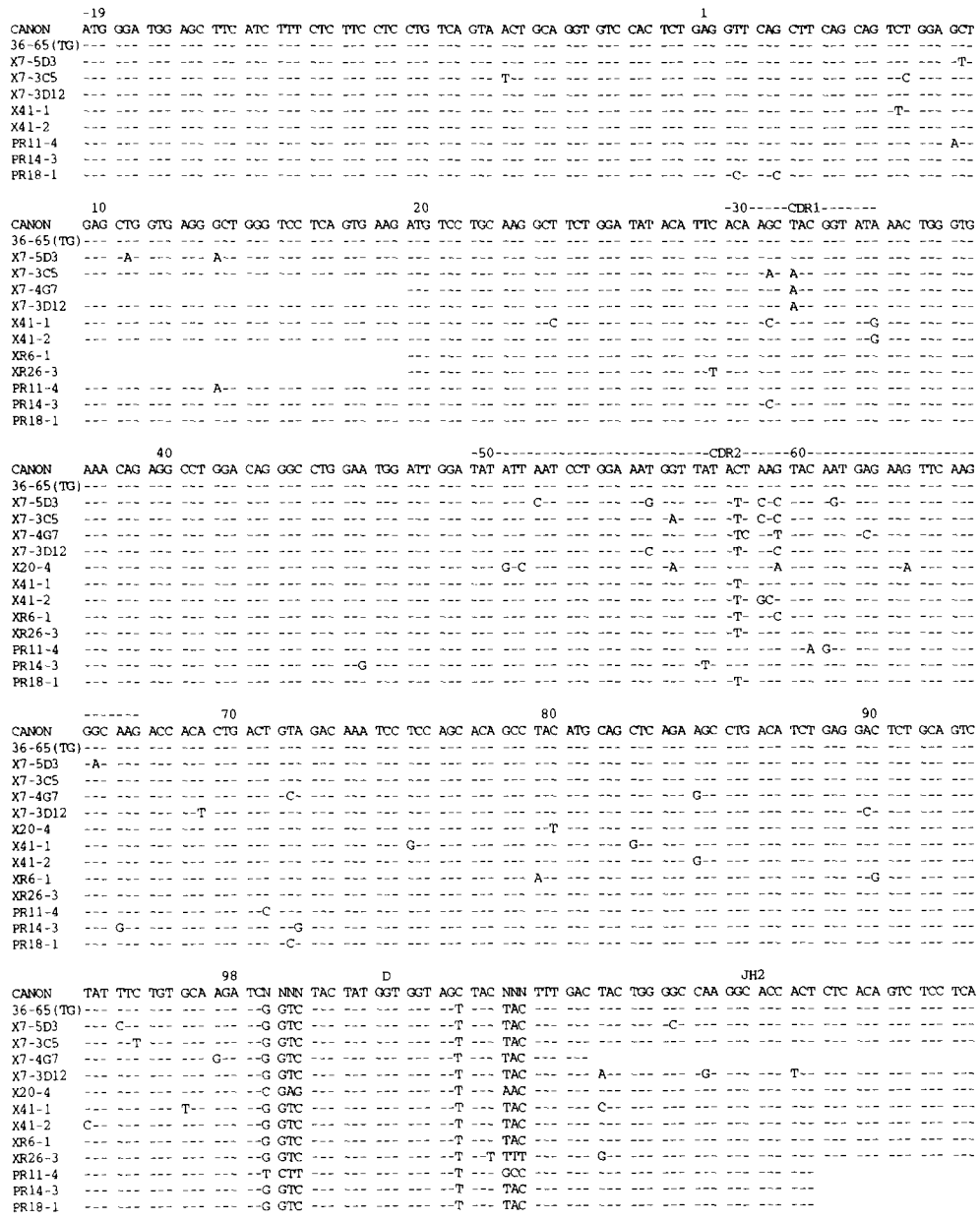


Figure 2. Sequences in the V_H coding region of V_H mRNA expressed by transgenic hybridomas. The sequences are compared with the consensus sequence of unmutated canonical V_H genes (*CANON*) and the unmutated sequence of the 36–65 transgene V_H gene [*36–65(TG)*]. Sequence identity is indicated by a dash, differences are shown explicitly. The position of the mature amino-terminal codon is indicated (*1*), as are the location of CDRs and regions encoded by D and J_H2 gene segments.

type junctional nucleotides. Previous Southern blotting analyses have shown that in the hybridomas expressing V_H genes with transgene-type junctional nucleotides, these V_H genes are present in transgene-IgH hybrid loci (Fig. 1). In contrast, such analyses have shown that the canonical V_H genes containing other types of junctional nucleotides reside in conventional, endogenous IgH loci (27, and A. M. Giusti and T. Manser, unpublished results). The distribution and type of somatic mutations in canonical V_H genes present in both conventional and hybrid loci indicate that antigen selection acted on the products of these genes. A large fraction of the mutations cause amino acid substitutions, mutations are present at a higher frequency in CDRs than in other regions of the genes, and mutations are recurrently observed that cause amino acid substitutions previously shown (18) to result in increases in affinity for Ars (e.g., ACT to ATT at codon 58). Overall, the frequency, distribution, and type of mutations observed in canonical V_H genes present either in conventional or hybrid loci are similar.

Transgene-IgH Hybrid Loci Containing Small Amounts of Natural 5′ Flanking DNA Are Hypermutated. We previously obtained PCR clones of the V_HDJ_H and immediate flanking regions present in the hybrid loci of the hybridomas X7-5D3 and X7-3C5, and λ phage clones of the hybrid loci in the hybridomas X7-3D12 and X7-4G7 (27). These four cell lines were isolated from a single Ars-KLH–immunized transgenic mouse of the X7 line. The nucleotide sequences of V_HDJ_H and flanking regions of these clones are shown in Fig. 3. The sequences within the V_H coding region of the clones agree with those obtained from RNA (Fig. 2), revealing many mu-

tations. Numerous mutations also exist in the DNA flanking the V_H coding regions in these hybrid loci. Some of these mutations observed in the 3C5 and 5D3 clones might have resulted from *Taq* polymerase errors. However, the frequency of *Taq* errors we observe in control experiments conducted under the conditions used to amplify the X7-5D3 and X7-3C5 hybrid loci is never >0.1% (E. Ingersoll and T. Manser, unpublished observations). Since the frequency of mutation in the clones is >1% (in both coding and much of the noncoding regions), the majority of the observed nucleotide changes are probably not due to *Taq* errors. Moreover, the distribution of mutations in flanking regions in the PCR clones is nonrandom (see below), and several of the mutations are shared with the phage clones.

In toto, the transgene-IgH hybrid loci reveal a frequency, distribution, and type of mutation that is characteristic of conventional Ig loci (8–14). The frequency of mutation in the 5′ transgene–derived plasmid sequences is 0%, in the region from the promoter to the 3′ end of the leader intron it is 0.4%, in the V_H coding region it is 2.0%, in the J_H2-J_H3 3′ flanking DNA it is 1.8%, in the J_H3-J_H4 region it is 0.7%, and in the DNA 3′ of J_H4 it is 0%. Such a "3′ skewing" of the distribution of mutation relative to the V coding region appears to be a trademark of the somatic mutation process (11, 13, 14). The mutations are mainly nucleotide replacements, nucleotide transitions are predominant, and several mutations in noncoding regions are shared among the clones, indicating a common mutational genealogy. Again, these attributes are all characteristic of somatically mutated natural Ig loci (32–34).

Transgenic V_H Genes Present in IgH Hybrid Loci Are Hypermutated, but Other Transgenic V_H Genes Are Not. All of our hybridomas contain an expressed canonical V_H gene associated with IgH C region DNA (either due to transgene rearrangement or conventional VDJ rearrangement), as well as other copies of the transgene present on different chromosomes than the IgH locus. We therefore could evaluate the action of hypermutation that had occurred in a single B cell clone on an expressed conventional or hybrid locus V_H gene and on transgenic V_H genes that are not part of functional H chain loci. PCR primer combinations that would allow amplification of all canonical V_HDJ_H genes were used in reactions containing hybridoma genomic DNA, and the resulting PCR products were cloned and sequenced. To control for possible *Taq* polymerase errors, each hybridoma DNA was subjected to two independent amplifications and clonings.

Sequences of canonical V_H clones derived from hybridomas X7-5D3 (X7 line) and X41-1(X1 line) are shown in Figs. 4 and 5 as compared with the 36–65 transgene [36–65 (TG)] and the sequences derived from H chain RNA (Fig. 2) expressed by these hybridomas (*M*). Most cloned V_H genes are members of one of two classes; those that contain only occasional, unique base changes at a frequency expected for *Taq* polymerase error, and those that agree nearly entirely with the corresponding somatically mutated H chain RNA sequence. The frequency of V_H clones that are members of the former class correlates with the estimated copy number of the transgenes in the hybridomas. These data suggest that only the canonical V_H gene that is part of the transgene-IgH hybrid locus has undergone hypermutation. This conclusion is strongest in the case of X7-5D3, since the transgenic copy number in the X7 line is two to three, and the 16 PCR clones we have analyzed makes it highly likely that all canonical V_H genes would have been sampled.

Since the X1 and X7 lines contain the least amount of natural 5′ (150 bp) and 3′ (1.5 kbp; no enhancer or MAR) flanking sequence of all of the lines we constructed, their transgenes could be lacking a *cis*-acting element(s) necessary for hypermutation that is present in the transgenes containing more extensive flanking DNA. To test this idea, the canonical V_H genes present in genomic DNA of hybridomas PR11-4 (PR15 line), PR14-3 (PR15 line), and PR18-1 (PR19 line) were PCR amplified, cloned, and sequenced as described above. The mice from which these hybridomas were derived all contain V_H transgenes with 2.8 kb of 5′ DNA, and 2.3 kb of 3′ DNA (including the intronic enhancer and 5′ MAR) in 5–10 copies. The PR11-4 hybridoma contains a conventional IgH locus that encodes a canonical H chain. Fig. 6 shows that no genomic PCR clone sequences were obtained that agree with the mutated RNA sequences. Since the transgene copy numbers in the PR lines of mice are high, and the PCR primers used will amplify any canonical V_H gene, this is not an unexpected result. However, only 1 of the 17 genomic sequences contained a mutation, a frequency easily accounted for by *Taq* polymerase error.

Transcription of the Transgenomes. Analysis of the transcriptional activity of the transgenomes is complicated by the fact that while functional V_H promoters and sometimes intronic enhancers are present, natural 3′ splice acceptor and transcription termination/polyadenylation sites are not. Thus, transcripts that may be initiated from the V_H promoter in unrearranged transgenes would be expected to be of heterogeneous length, and would lack a 3′ poly(A) tail. Such transcripts would most probably be extremely unstable.

Nevertheless, since the transcriptional activity of a V_H gene may influence its susceptibility to hypermutation, we performed Northern blot and RNase protection analysis on total RNA isolated from the spleens of X1, PR15, PR19, and XR14 lines of transgenic mice. Using a vector probe, diffuse signal in the low molecular weight range of the gels was obtained from these RNAs in Northern analysis (data not shown). In the RNase protection studies, RNA probes derived from the VDJ region and immediate 3′ flanking DNA of the transgene were used. Probe fragments were protected by some of the splenic RNAs of sizes (243 bp) indicative of the presence of spliced transcripts derived from the transgenomes (Fig. 7, see legend for details). Since transgene-IgH hybrid loci can not be detected in splenic DNA of unimmunized transgenic mice (27; and A. M. Giusti and T. Manser, unpublished results), these transcripts are most probably derived from unrearranged transgenes. The small amount of 243-bp fragment protected by transgenic spleen as compared with 36–65 (the transgene donor) RNA, taken together with the data obtained from Northern blots, suggests that transgenome-derived transcripts are very unstable.

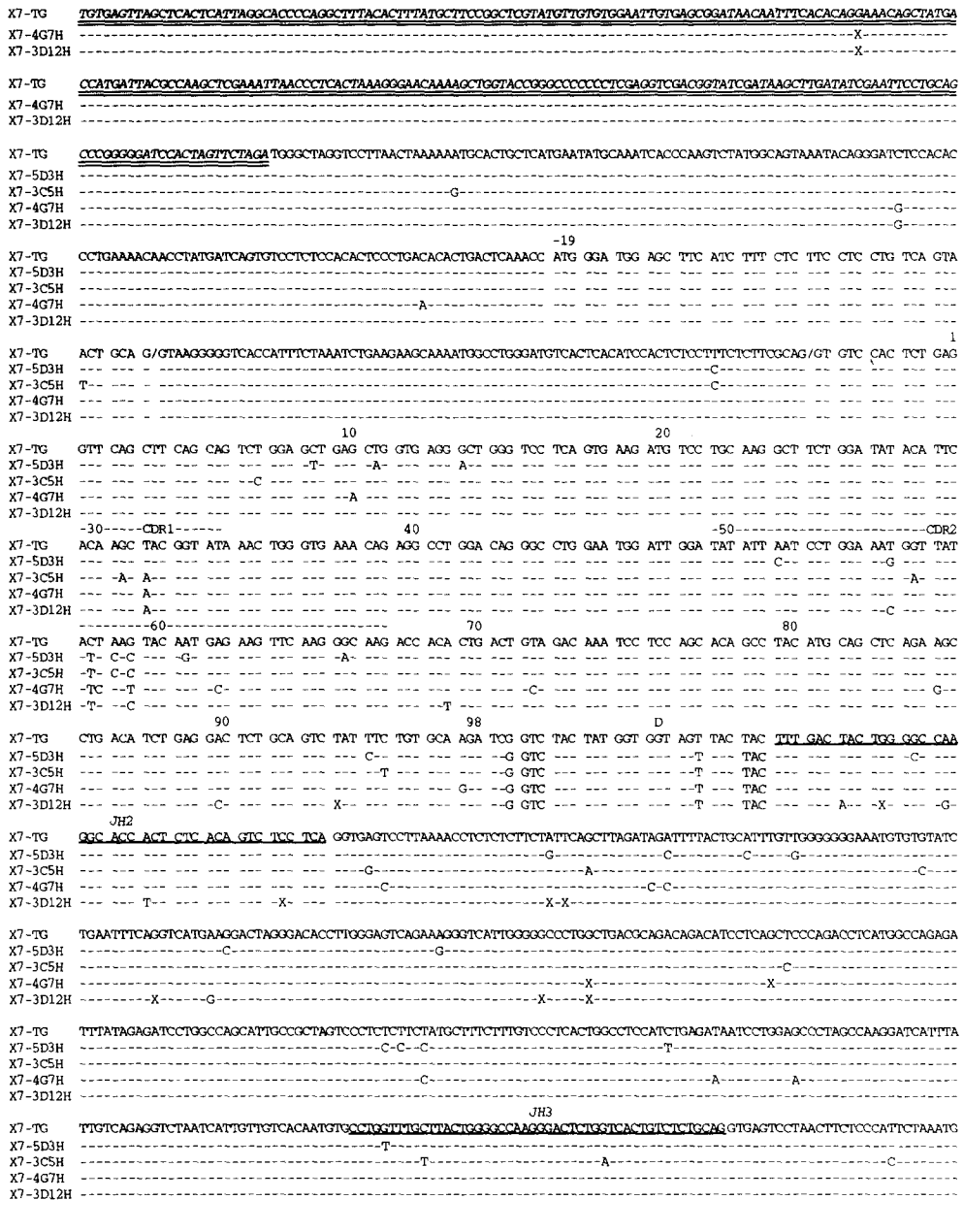
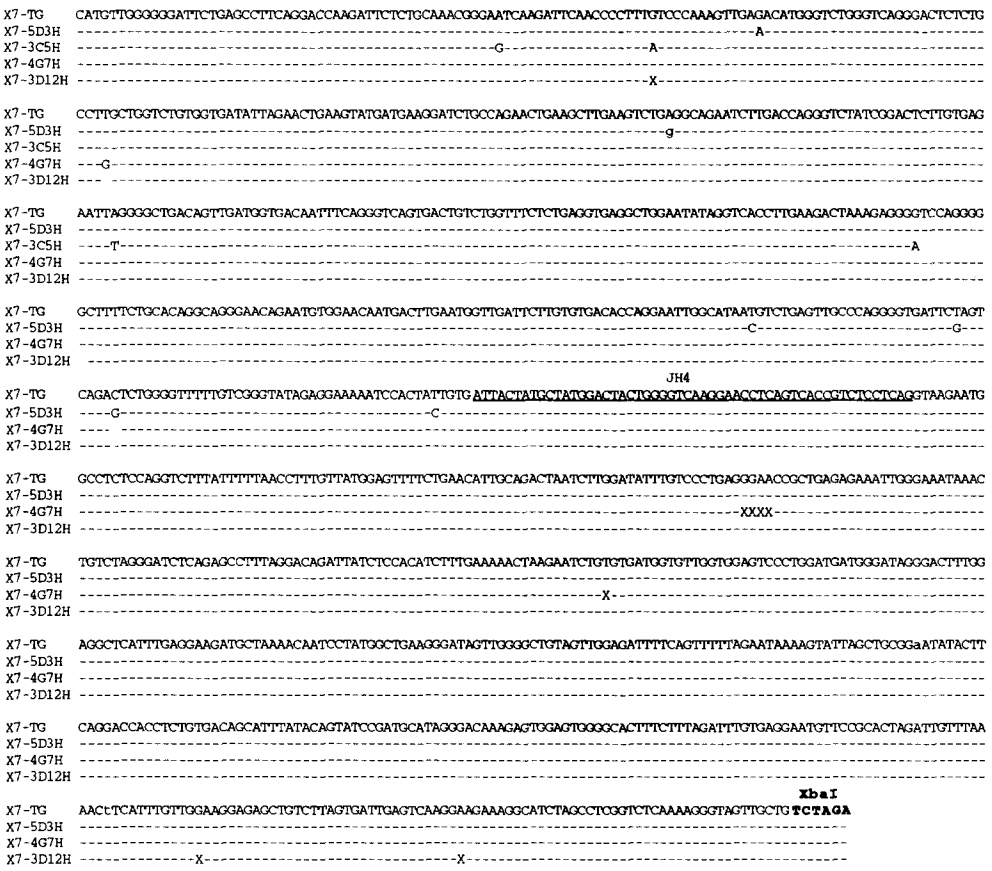


Figure 3. Sequences of the V_H genes and 5′ and 3′ flanking regions present in transgene-IgH hybrid loci cloned from Ars-KLH–induced transgenic hybridomas derived from the X7 line of mice. Sequences are shown as described in Fig. 2, in comparison with the sequence of the plasmid used to construct the X7 line (*X7-TG*). pBluescript vector sequence is double underlined, and the J_H gene segments are single underlined. The 3′ XbaI site used in cloning the 5D3 and 3C5 PCR products is shown in bold type. Sequence was obtained at least 1 kb 3′ of this site in the 4G7 and 3D12 phage clones, and, when compared with the sequence of 36–65 DNA in this region, revealed no evidence of somatic mutations. Ambiguous nucleotides are indicated (*X*).

Discussion

The B cell hypermutation process appears to act on canonical V_H transgene-IgH hybrid loci containing only small amounts of natural 5′ flanking DNA in a manner indistinguishable from conventional Ig loci. This conclusion is based on the premise that most of the mutations observed in such hybrid loci took place after recombination of the transgenes with endogenous IgH DNA. Collectively, our data strongly suggest that this was the case. The formation of transgene-IgH hybrid loci occurs at a very low frequency (such loci can not be detected in unimmunized mice, and only can be detected via PCR or hybridoma technology in Ars-immunized mice), and it is unlikely that four independent recombination events took place in the B cell population of mouse NX7, giving rise to the hybrid loci in hybridomas X7-3C5, X7-3D12, X7-4G7, and X7-5D3, and their many unique mutations. These hybrid loci contain shared somatic mutations in noncoding regions, suggesting derivation from a common recombined and mutated "precursor hybrid locus." Some of the V_H genes present in hybrid loci that we have sequenced do not contain mutations (data not shown), indicating that the mechanism that results in transgene-IgH recombination is not responsible for the high frequency of mutation we have observed. Finally, all of the transgenes that are not part of hybrid loci that we have analyzed contain base changes at a frequency not greater than that expected from PCR error.

The 150 bp of natural 5′ DNA flanking V_H coding se-



quences in X1 and X7 hybrid loci contain only the purine-rich block of nucleotides, the "heptamer," octamer, and TATA box (all known to be important for efficient promoter function; reference 35), and the RNA cap site. Analysis of λ phage clones containing X7 hybrid loci has shown that ~2.2 kb of pBluescript vector DNA is present immediately 5′ of the promoter region, and no other Ig locus or transgene DNA is present within 1.1 kb 5′ of this vector DNA (see Fig. 1). Thus, sequences immediately 5′ of the V_H promoter can be replaced by unrelated sequences without noticeable effect on the mutation process, indicating that *cis*-acting elements necessary for hypermutability do not exist 5′ of the promoter. Therefore, our data do not support models for the somatic mutation process that propose that 5′ *cis*-acting elements are important (36), but leave open the possibility that a functional V_H promoter is requisite for mutation.

It is somewhat more difficult to interpret the low frequency of mutation, entirely accountable by PCR errors, in the transgenes that have not recombined with endogenous IgH DNA. Explanations based on absence of the *trans*-acting factors necessary for hypermutation are unlikely since the V_H genes within a transgene-IgH hybrid or conventional IgH locus that were present in the same B cell clone are observed to be mutated. However, our current ignorance of the nature of the hypermutation process limits further conclusions. Whether mutation occurs "processively," such that several nucleotide changes are introduced nearly simultaneously in a single V gene, or in a stepwise "distributive" fashion over the course of many cell divisions, is a current subject of controversy (6, 7, 37–40). If the former case were true, the lack of a high frequency of mutation in all of our V_H transgenes that have not recombined with endogenous IgH DNA could not be easily interpreted as evidence that these transgenes lack the *cis*-acting elements necessary for hypermutability. In this case, the stringent action of antigenic selection, which could operate only on loci encoding functional H chains, would further complicate matters. If "processive" mutational events took place infrequently, the only such events that would be represented among antigen-induced hybridomas would be those that resulted in an antibody with increased affinity for antigen, i.e., those that took place in the functioning V_H gene. For such a scenario to account for the data, the number of mutations introduced per "processive" event would have to be large. For example, in the case of the X7-5D3 and X41-1 hybridomas (Figs. 4 and 5), 13 and 9 mutations, respectively, occurred in the hybrid locus V_Hs, while few or no mutations took place in the many other V_Hs present in unrearranged transgene copies.

In contrast, a number of previous observations support a "distributive" model for hypermutation. In hybridomas expressing hypermutated V genes, V_H and V_L genes are usually observed to be mutated to similar extents, even when only translationally silent mutations are considered (6, 15, 17, 32, 37). Moreover, productively and nonproductively rear-

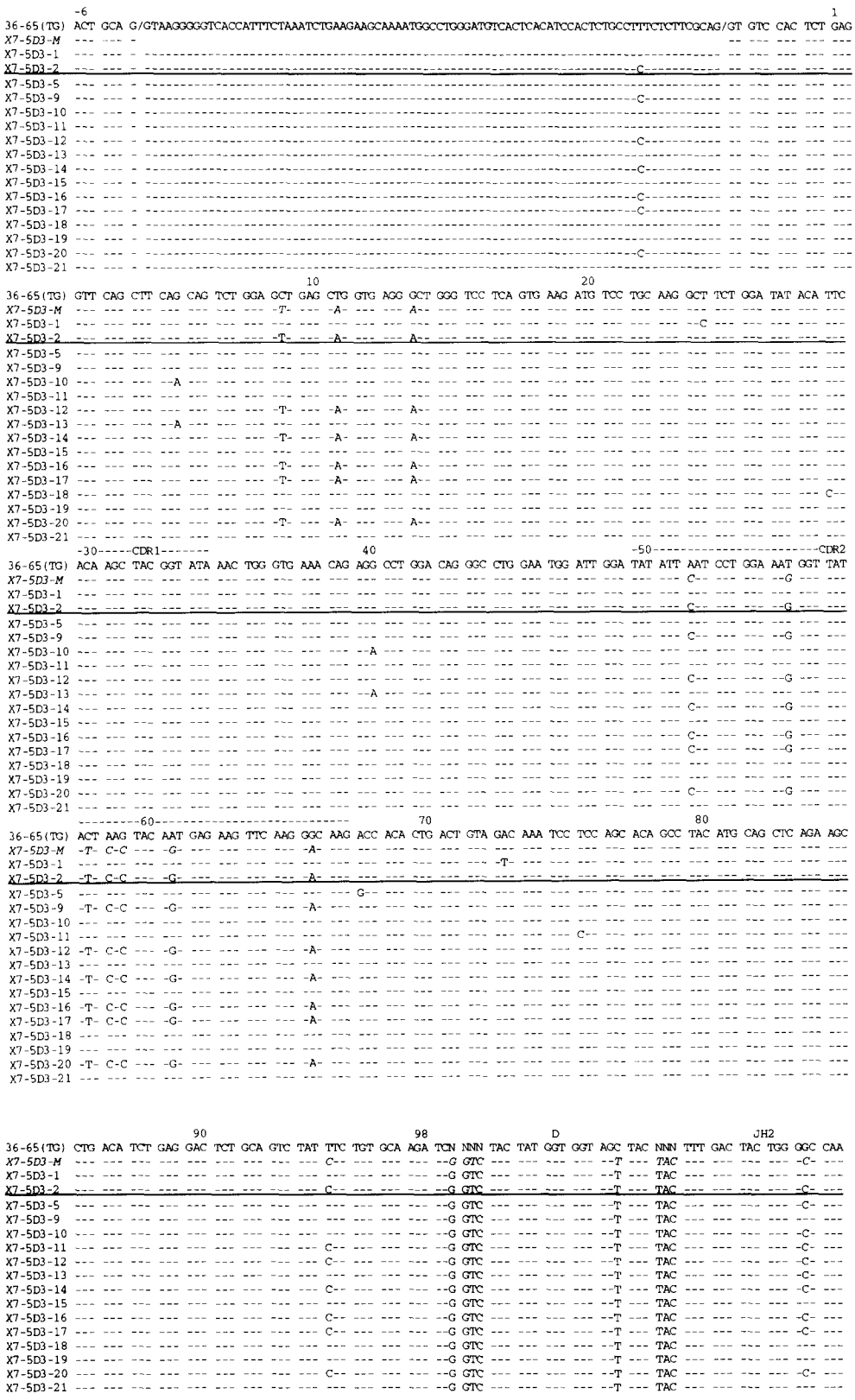


Figure 4. Sequences of the V_H PCR clone inserts obtained from the X7-5D3 hybridoma. Sequences are shown as in Figs. 2 and 3, as compared with the sequence of the 36–65 transgene and the sequence of the H chain mRNA expressed by X7-5D3 (*X7-5D3-M*). Sequence of clones derived from the two independent amplifications and clonings are separated by a solid line. The clone insert present in X7-5D3-9 appears to have resulted from artifactual exchange of DNA between unmutated and mutated V_H loci during PCR since this insert lacks the cluster of mutations centered at codon 11. The recurrent G to C change in the J_H2 region of the sequences may also represent a PCR artifact of this type, since this mutation does not always cosegregate among the clones with mutations present in the rest of the RNA sequence.

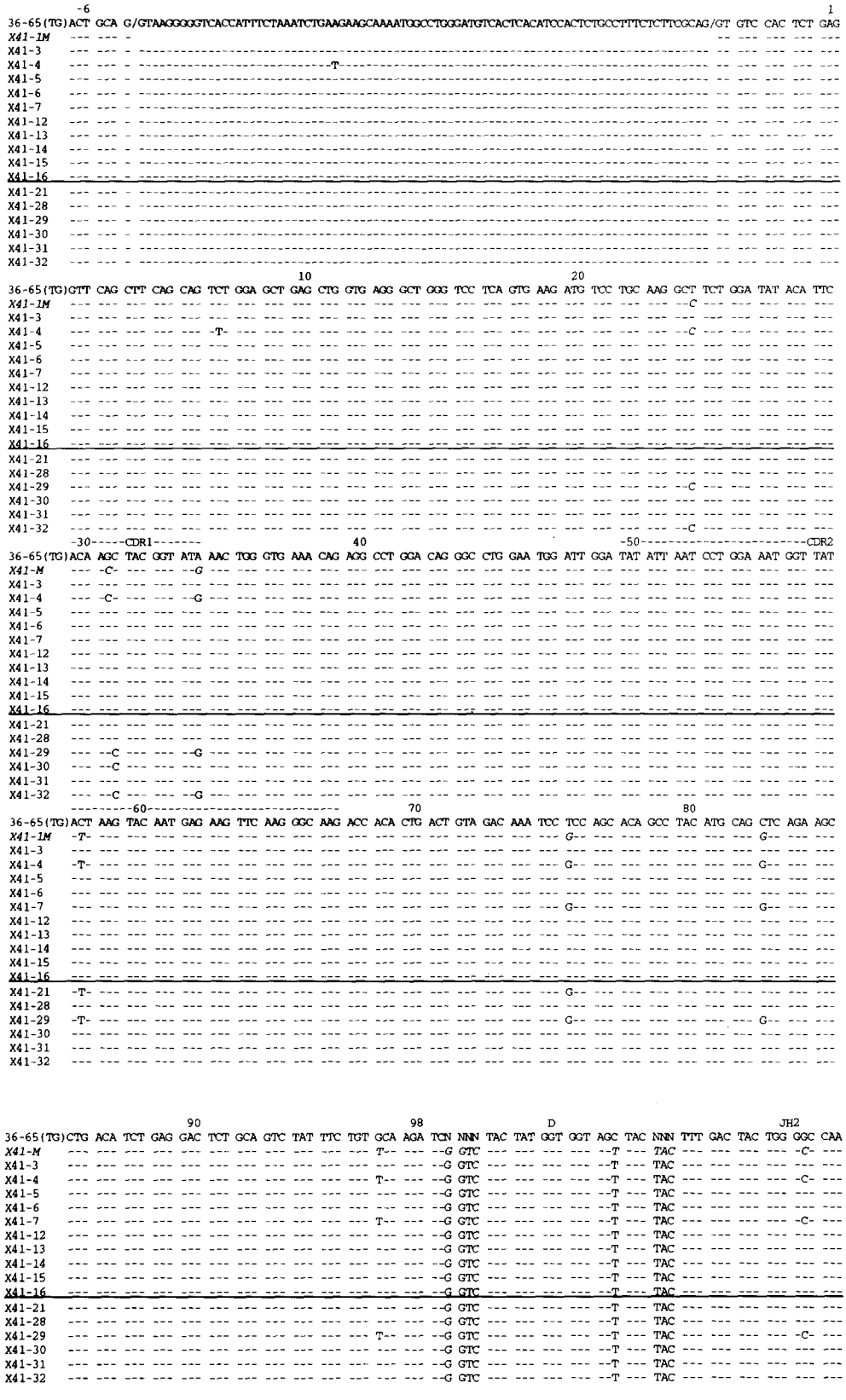


Figure 5. Sequences of the PCR clone inserts obtained from the transgenic hybridoma X41-1. Sequences are shown as described in Fig. 4. Clone inserts X41-4 and 29 share all of the mutations present in the H chain RNA. Clones X41-7 and 21 share mutations with the mRNA only in discrete V_H subregions and appear to have resulted from artifactual exchange of DNA between mutated and unmutated canonical V_H loci during PCR.

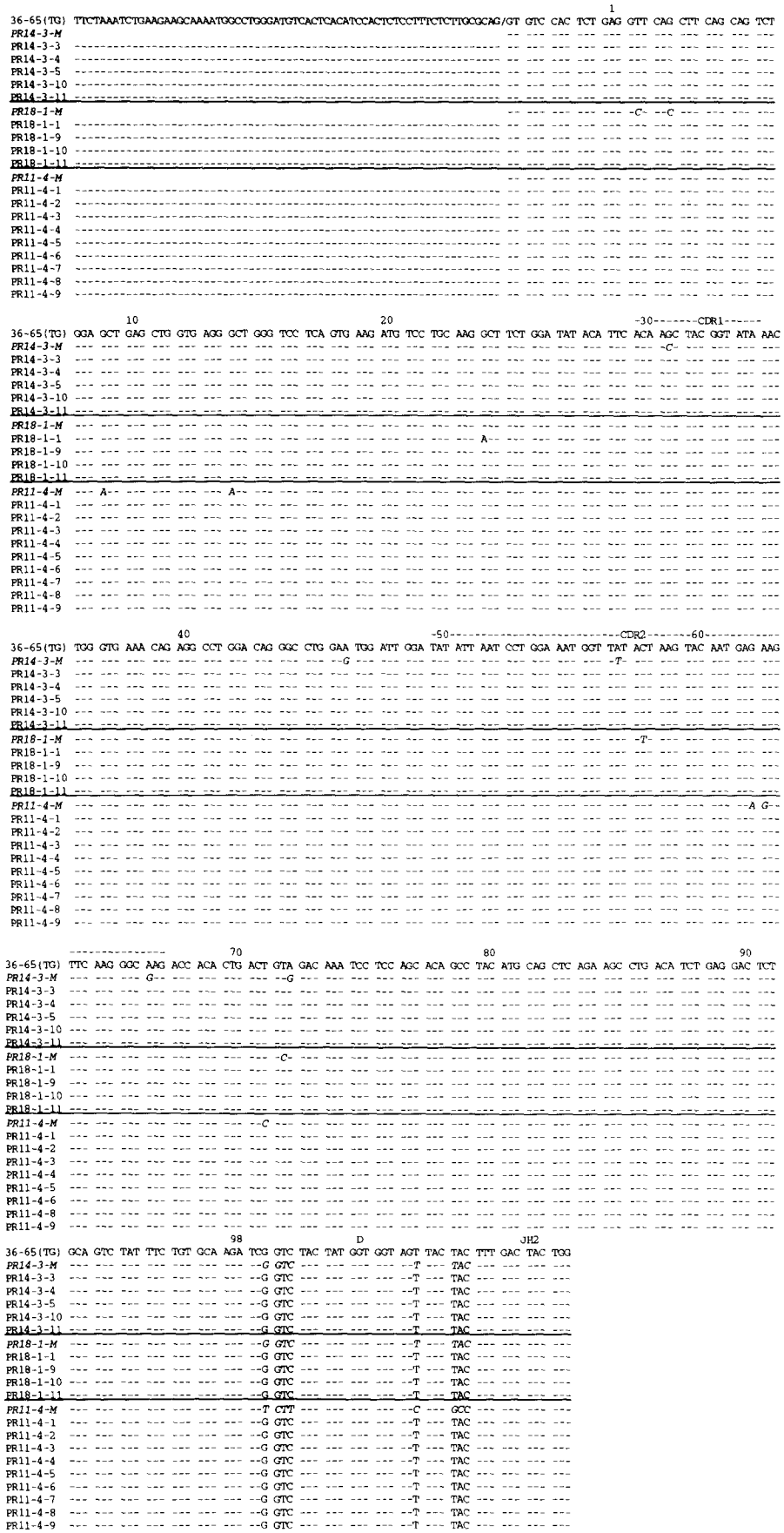


Figure 6. Sequences of the PCR clone inserts obtained from the PR14-3, PR18-1, and PR11-4 hybridomas. Sequences are shown as described in Fig. 4, except that sequences derived from the different hybridomas are separated by solid lines.

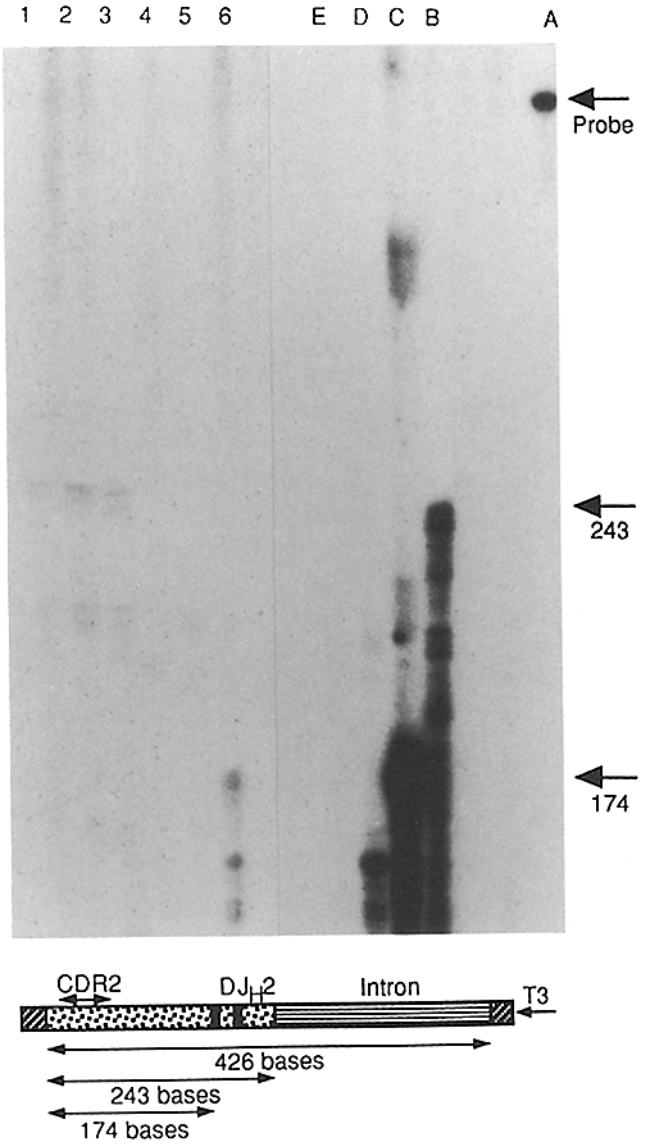


Figure 7. RNase protection analysis of total splenic RNA derived from transgenic mice. The analysis was performed as described in Materials and Methods. The schematic diagram indicates the sequences present in the probe: (*diagonal striped boxes*) pBluescript-derived sequences; (*horizontal striped box*) natural IgH sequence flanking the V_H region; (*stippled boxes*) V_H coding sequence. Within coding sequence the D, J_H2, and CDR2 regions are indicated. The V_H-D and D-J_H junctional residues are indicated by black bars. Some of the potential protected fragments of the probe are indicated: 426 bases would be protected by an unspliced transcript with junctional nucleotides complementary to the probe; 243 bases would be protected by a spliced transcript with junctional residues complementary to the probe; 174 bases would be protected by any transcript with V_H-D junctional nucleotides that were not complementary to the probe. The lanes correspond to 5 μg of each total RNA, hybridized with identical amounts of probe, obtained from the indicated sources. Controls: lane *A*, undigested probe; lane *B*, 36–65 hybridoma; lane *C*, NX20-1 hybridoma (V_H-D junctions of functional H chain RNA not complementary to probe); lane *D*, NX41-1 hybridoma (junctional nucleotides of functional H chain RNA complementary to probe); lane *E*, spleen from a naive A/J mouse. Experimentals: lane *1*, B19-17 naive spleen (PR19 line); lane *2*, B15-4 naive spleen (PR15 line); lane *3*, H15-35 naive spleen (PR15 line); lane *4*, S2-2 naive spleen (PRH2 line, contains a transgene with extensive natural sequence flanking the 5′ side of the 36–65 V_H gene but no IgH enhancer [A. M. Giusti and T. Manser, unpublished results); lane *5*, S1-24 naive spleen (X1 line); lane *6*, spleen from an A/J mouse 3 d after a secondary immunization with Ars-KLH. The RNA from hybridoma NX41-1 does not protect the expected 243-base fragment, apparently due to the many nucleotide differences from the probe in its expressed H chain RNA resulting from somatic mutation. Autoradiographic exposures of lanes *1–6* and *E–A* were for 7 and 1 d, respectively.

ranged V genes present within the same B cell have been shown to have similar frequencies of mutation (11, 12). Finally, the sequences of hypermutated V genes expressed by hybridomas derived from the same expanded B cell clone can often be related to one another via a multi-tiered mutational geneology, indicating that mutation was active in the clone over an extended period(s) of cell division (7, 17, 32, 37, 38). Given that hypermutation can take place in this fashion, i.e., multiple times at multiple genomic sites during the growth of a B cell clone, our data indicate that the combination of a V_H promoter (included in as much as 2.8 kb of natural 5′ flanking DNA) and the intronic IgH enhancer is not sufficient, and a region 3′ of this enhancer is requisite, for hypermutability of V_H transgenes.

The possibility that the unrearranged transgenes are not as "accessible" to the putative *trans*-acting factors required for hypermutation as are functional H chain loci can not be directly evaluated due to the current lack of information regarding the nature of these factors. However, a "suppressive" effect of particular transgene integration sites seems unlikely since we have examined transgenes present in four independently derived transgenic lines of mice. Inactivation of the transgenic loci for hypermutation due to sex-specific parental "imprinting" (41) also seems unlikely since, as shown in Table 1, the hybridomas we have examined were derived from first generation offspring obtained from both male and female founders (i.e., X7 and PR19 founders were females, and PR15 and X1 founders were males). Preliminary analyses of the extent of cytosine methylation in the transgenic arrays indicate that these arrays are not hypermethylated. Finally, our Northern blot and RNase protection (Fig. 7) analyses show that the unrearranged transgenes can be transcribed in spleen, indicating accessibility to the transcriptional machinery.

Data obtained by us (15) and others (16) have shown that somatic mutation within a B cell clone can occur after isotype switching mediated by deletions within the C region locus. Thus, a region required for hypermutation may be present 3′ of this locus. The discovery of a second IgH enhancer element in this region (42) is intriguing in this regard, particularly in light of recent data suggesting that a region including the "downstream" enhancer element in the mouse κ locus is required for hypermutation of V_κ transgenes (25). Finally, two MAR elements have been mapped to either side of the intronic IgH enhancer (43), and our largest transgenic constructs contain only a truncated form of the 3′ MAR element. Whether promoter activity, enhancers, MAR regions, or other as yet undefined elements in IgH loci are requisite for V_H somatic hypermutation should be directly testable using the transgenic approach.

We thank Rick Coffee, Vijaya Kommineni, Ethel Derr, and Alexander Krasev for technical assistance.

This work was supported by grants from the National Institutes of Health (AI-23739) and the American Cancer Society (IM-557). During some of this work, T. Manser was a Pew Scholar in the Biomedical Sciences.

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Received for publication 28 August 1992 and in revised form 18 November 1992.

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