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.DJ) 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.DJ. 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. and either 1.5 or 2.3 kb (including the intronic enhancer and 5’ matrix attachment region [MAR]) flanking the 3’ side of V. 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. genes; and (b) among such transgenes, even those containing only 150 bp of natural V. 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.DJ. 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.2-JH4 region, a MAR, and the intronic enhancer. Collectively, the data suggest that sequences 5’ of the V. promoter are dispensable, a V. 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. 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. 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.
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\(\alpha\) gene and various amounts of natural 5' and 3' flanking DNA, the transgenic V\(\alpha\) and associated 5' flanking region could recombine with endogenous IgH DNA, leading to the expression of transgenic V\(\alpha\)-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\(\alpha\) gene is part of a functional H chain locus, on V\(\alpha\) 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\(\alpha\)DJ\(\alpha\) 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\(\alpha\), 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 x C57BL/6)F1 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 \(\mu\)g Ars-KLH in CFA. 1 wk later, the mice received three intraperitoneal injections of 50 \(\mu\)g 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-Ag14. The following lines were used in this study: X1 and X7 hybrid loci, 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 x C57BL/6)F1 female mice to A/J male mice, and the mice used in this study were obtained by backcrossing these founders to A/J mice.

Nucleotide Sequencing. Direct sequencing of Ig RNA was performed as described previously (4). pBluescript plasmid DNA was sequenced with \(\alpha\)-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 DyeDexoxy" kit (Applied Biosystems, Inc., Foster City, CA), and the automated sequencer (Applied Biosystems, Inc.), or the "fmole" PCR sequencing kit (Promega Biotec, Madison, WI).

Results

Experimental Strategy. Clues to the location of the cis-acting elements required for V\(\alpha\) hypermutation are provided by previous findings that: (a) mutations are observed at high frequency only within a region encompassing the rearranged V\(\alpha\) 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\(\alpha\) 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 multiplicity transgenomes containing an unmutated canonical V\(\alpha\)DJ\(\alpha\) gene, various amounts of natural 5' and 3' flanking sequence, and plasmid vector sequences. We previously demonstrated that in such mice a transgenic V\(\alpha\)DJ\(\alpha\) 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\(\alpha\) (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\(\alpha\) 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
no homology to transgenic or Ig DNA. The transgenic \( V_n \) 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 \( V_n \) appear identical to those observed in conventional IgH loci that encode IgG H chains. That is, the unrearranged \( J_n3 \) and \( J_n4 \) segments are present, followed by the matrix attachment region (MAR)/enhancer region, followed by a region containing switch DNA, and then the \( C_y \) exons (27; and A. M. Giusti and T. Manser, unpublished results). Canonical \( V_n \)-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 \( V_n \) 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 \( V_nDJ_n \) 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 \( V_n \) hypermutation. Further, since the transgenes in different lines of mice contain varying amounts of natural 5' and 3' sequence flanking the \( V_nDJ_n \), such an analysis would also allow an evaluation of the requirements for proximal \( V_n \) 5' and 3' flanking regions in the hypermutation process.

### Sequencing of \( V_n \) 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 \( V_n \) 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 \( V_n \) region cDNA followed by cloning and sequencing of the PCR product. The data in Fig. 2 show that the \( V_n \) genes contain numerous somatic mutations and many contain transgene-
type junctional nucleotides. Previous Southern blotting analyses have shown that in the hybridomas expressing \( V_n \) genes with transgene-type junctional nucleotides, these \( V_n \) genes are present in transgene-IgH hybrid loci (Fig. 1). In contrast, such analyses have shown that the canonical \( V_n \) 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 \( V_n \) 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 \( V_n \) 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 \( V_nD_j \) and immediate flanking regions present in the hybrid loci of the hybridomas X7-5D3 and X7-3C5, and \( \lambda \) 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 \( V_nD_j \) and flanking regions of these clones are shown in Fig. 3. The sequences within the \( V_n \) coding region of the clones agree with those obtained from RNA (Fig. 2), revealing many mu-

![Figure 2. Sequences in the \( V_n \) coding region of \( V_n \) mRNA expressed by transgenic hybridomas.](image)

The sequences are compared with the consensus sequence of unmutated canonical \( V_n \) genes (CANON) and the unmutated sequence of the 36-65 transgene \( V_n \) 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 (t), as are the location of CDRs and regions encoded by D and J2 gene segments.
mutations. Numerous mutations also exist in the DNA flanking the \( V_\alpha \) coding regions in these hybrid loci. Some of these mutations observed in the 3C5 and SD3 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-SD3 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 non-coding 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 \( V_\alpha \) coding region it is 2.0%, in the \( J_\alpha 2-J_\alpha 3 \) 3' flanking DNA it is 1.8%, in the \( J_\alpha 3-J_\alpha 4 \) region it is 0.7%, and in the DNA 3' of \( J_\alpha 4 \) 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 \( V_\alpha \) Genes Present in IgH Hybrid Loci Are Hyper-mutated, but Other Transgenic \( V_\alpha \) Genes Are Not. All of our hybridomas contain an expressed canonical \( V_\alpha \) 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 \( V_\alpha \) gene and on transgenic \( V_\alpha \) genes that are not part of functional H chain loci. PCR primer combinations that would allow amplification of all canonical \( V_\alphaDJ_\alpha \) 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 \( V_\alpha \) clones derived from hybridomas X7-SD3 (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 \( V_\alpha \) 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 \( V_\alpha \) 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 \( V_\alpha \) gene that is part of the transgene-IgH hybrid locus has undergone hypermutation. This conclusion is strongest in the case of X7-SD3, 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 \( V_\alpha \) 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 \( V_\alpha \) 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 \( V_\alpha \) 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 \( V_\alpha \) 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 \( V_\alpha \) 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 \( V_\alpha \) 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 \( V_\alpha \) 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 immunized 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.
Discussion

The B cell hypermutation process appears to act on canonical \( V_\alpha \) 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 Am-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 X7-3CS, 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 \( V_\alpha \) 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 \( V_\alpha \) coding se-

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**Figure 3.** Sequences of the \( V_\alpha \) 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). pBSKII vector sequence is double underlined, and the \( J_\alpha \) gene segments are single underlined. The 3' XbaI site used in cloning the SD3 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).
sequences 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<sub>n</sub> 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<sub>n</sub> 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<sub>n</sub> 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<sub>n</sub> 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<sub>n</sub> 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<sub>n</sub>s, while few or no mutations took place in the many other V<sub>n</sub>s 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<sub>n</sub> and V<sub>n</sub> 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 rearranged DNA sequences 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<sub>n</sub> 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<sub>n</sub> promoter is requisite for mutation.

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Figure 4. Sequences of the V, 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 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). The clone insert present in X7-5D3-9 appears to have resulted from artificial exchange of DNA between unmutated and mutated V, loci during PCR, since this insert lacks the cluster of mutations centered at codon 11. The recurrent G to C change in the J,2 region of the sequence 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\(_w\) subregions and appear to have resulted from artifactual exchange of DNA between mutated and unmutated canonical V\(_w\) loci during PCR.
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 VH region; (stippled boxes) V~ coding sequence, within coding sequence the D, J,2, and CDR.2 regions are indicated. The Vs-D and D-J,2 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 Vr-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 (Vr-D junctions of functional H chain RNA not complementary to probe); lane D, NX41-1 hybridoma (functional nucleotides of functional H chain RNA complementary to probe); lane E, spleen from a naive A/J mouse. Experimental: 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 (PR1H2 line), contains a transgene with extensive natural sequence flanking the 5' side of the 36-65 Vs gene but no IgH enhancer [A. M. Giusti and T. Manser, unpublished results]; lane 5, SI-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.

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 Vs 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 Vs 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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