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Mutation pattern of immunoglobulin transgenes is compatible with a model of somatic hypermutation in which targeting of the mutator is linked to the direction of DNA replication

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We have previously demonstrated that B lymphocyte specific somatic mutations are introduced into the variable regions of immunoglobulin κ transgenes in two independent transgenic mouse lines. The frequency, distribution and nature of these mutations strongly suggest that they arose as a result of the process of somatic hypermutation, which is responsible, in part, for affinity maturation during an immune response. Unexpectedly, in these multiple copy transgenic lines, many of the transgene copies showed no evidence of somatic mutation. This paradox was addressed by determining the sequence of each transgene copy in several B cell hybridomas derived from a mouse line carrying three copies of the κ transgene. It was found that the somatic hypermutation process in different B cells from the same mouse preferentially targets one, but not the same, transgene copy. We present a model, based on the pattern of this targeting, which links somatic hypermutation to the orientation of the Ig gene relative to the direction of DNA replication.

Key words: DNA replication/immunoglobulin genes/somatic hypermutation

Introduction

The diversity of the preimmune repertoire of the mouse is based on a germline encoded pool of gene segments and the way in which they are joined to give rise to immunoglobulin (Ig) variable (V) region sequences (reviewed in Engler and Storb, 1988). The important contribution of somatic hypermutation to the further diversification of these Ig V region sequences has been well documented (reviewed in Steele, 1991). The mechanism underlying somatic mutation, however, has remained elusive.

The finding that gene conversion generates the preimmune repertoire in the chicken (Reynaud *et al.*, 1985; Thompson and Neiman, 1987; Carlson *et al.*, 1990) has rekindled the notion that somatic gene conversion may be the mechanism responsible for Ig diversification during an immune response in mammals (Maizels, 1989). While this process appears to contribute to somatic hypermutation in the rabbit (Becker and Knight, 1990), the nature and distribution of the mutations in mouse and man are more suggestive of point mutations, perhaps arising during error-prone DNA replication of repair (Chien *et al.*, 1988).

The salient features of the somatic mutation process can be summarized as follows: (i) mutations are localized to the rearranged V(D)J region and its flanking introns and promoter upstream sequences, whether the Ig gene is productively or non-productively rearranged; (ii) somatic mutations are extremely rare in germline V genes; (iii) single base substitutions predominate; and (iv) ongoing rearrangement is not required for the introduction of mutations.

We have previously demonstrated that prerearranged κ transgenes in two independent transgenic mouse lines are properly targeted by the somatic hypermutation machinery of B cells (O'Brien *et al.*, 1987; Hackett *et al.*, 1990). During this analysis, it became evident that transgene copies adjacent to the mutated copies showed no evidence of somatic mutation. This observation is striking if one considers that the copies are otherwise identical in sequence, and that somatic mutations are thought to be introduced in a stepwise manner during a number of cell generations (Clarke *et al.*, 1985; Levy *et al.*, 1989). The evidence described below suggests that in addition to the targeting of Ig variable regions, there exists in the multiple copy transgenic system a higher order of targeting as evidenced by the preferential mutation of only one transgene copy in different B cells from the same mouse. We speculate that this effect may be a consequence of the favorable orientation of the targeted transgene copy relative to an origin of DNA replication.

Results

The presence of multiple copies of the κ transgene was advantageous to assess the V region specificity of mutator action (Hackett *et al.*, 1990; Rogerson *et al.*, 1991). We initially assumed that since every transgene is an identical copy of the others, if recognition sequences were involved in the targeting of the 'mutator', they should be present in all of the copies. The predicted outcome was that each copy would show evidence of somatic mutation in its V region. However, in two independent mouse lines, many κ transgenes were found that have not undergone somatic mutation (O'Brien *et al.*, 1987). This suggested that something in addition to DNA sequence information within the gene may be required to focus the mutational mechanism on Ig genes.

We addressed this question by examining a population of hybidomas derived from two mice of the same transgenic line (194-2) and determined which of the three κ transgene copies in the array had been targeted for somatic mutation. Unfortunately, the other mouse line previously analyzed carries 13 copies, and the ordered isolation of all of these is virtually impossible.

Genomic clones of transgene copies isolated from hybridoma 3A1 exhibit both mutated and non-mutated V regions

An indication that not all copies of the transgene array were mutated was evident during sequence analysis of transgene genomic clones (O'Brien *et al.*, 1987), particularly when we examined in more detail four transgene genomic clones from hybridoma 3A1. Two exhibited identical sequence differences from κ-MOPC167, the transgene, at nucleotide positions 390 (C → A), 838 (A → C), 942 (G → C) and 1031 (G → C). One of these DNA clones was sequenced across 3.7 kb, including the C region, and found to have only these four mutations (Hackett *et al.*, 1990). The second DNA clone was not sequenced as extensively, but both very likely represent the same transgene copy isolated twice. The remaining two DNA clones were found to be unmutated across 600 bp of V region and flanking intron sequence as well as 280 bp encompassing part of the C gene and 3′ untranslated region. This analysis suggested that not all copies of the transgene array are mutated.

Characterization of the transgene array at the integration site in mouse line 194-2

To study the mutation state of the various κ transgene copies, a more careful assessment of transgene copy number and configuration at the integration site was necessary (see Figure 1). The 20.5 kb plasmid had been linearized for microinjection at the *Eco*RI site. Two copies of the transgene were oriented head to tail, while a third was oriented tail to tail. We arbitrarily assigned a left to right orientation to the three copies (see Figure 1 and all subsequent figures).

By monitoring transfer efficiencies in Southern blots, we determined that the 33 kb *Kpn*I fragment exhibited twice the hybridization signal intensity of the 20.5 kb fragment using a Vκ167 probe, consistent with the deduced configuration of the array (data not shown). The large fragment also hybridized to a Cκ probe, but not a pUC18 probe. The 20.5 kb fragment hybridized to all probes: Vκ, Cκ and pUC18, consistent with the shown configuration. The Southern blot analysis revealed putative chromosomal DNA–transgene junctions which hybridized only to a pUC18 probe. We have also identified a fragment consistent with the head to tail junction between the left and middle copies by using a 3′κ enhancer (Meyer and Neuberger, 1989) probe (Hagman *et al.*, 1990), however the tail to tail junction between the middle and right copies has undergone a deletion which removed the *Eco*RI site and part of the junction of the middle and right transgene copies. We have been unable to identify and characterize the exact boundaries of this deletion because of the presence of repetitive DNA sequences adjacent to it, but we estimate that at least 2.5 kb are missing from the junction (i.e. ~1.2 kb from each trans-

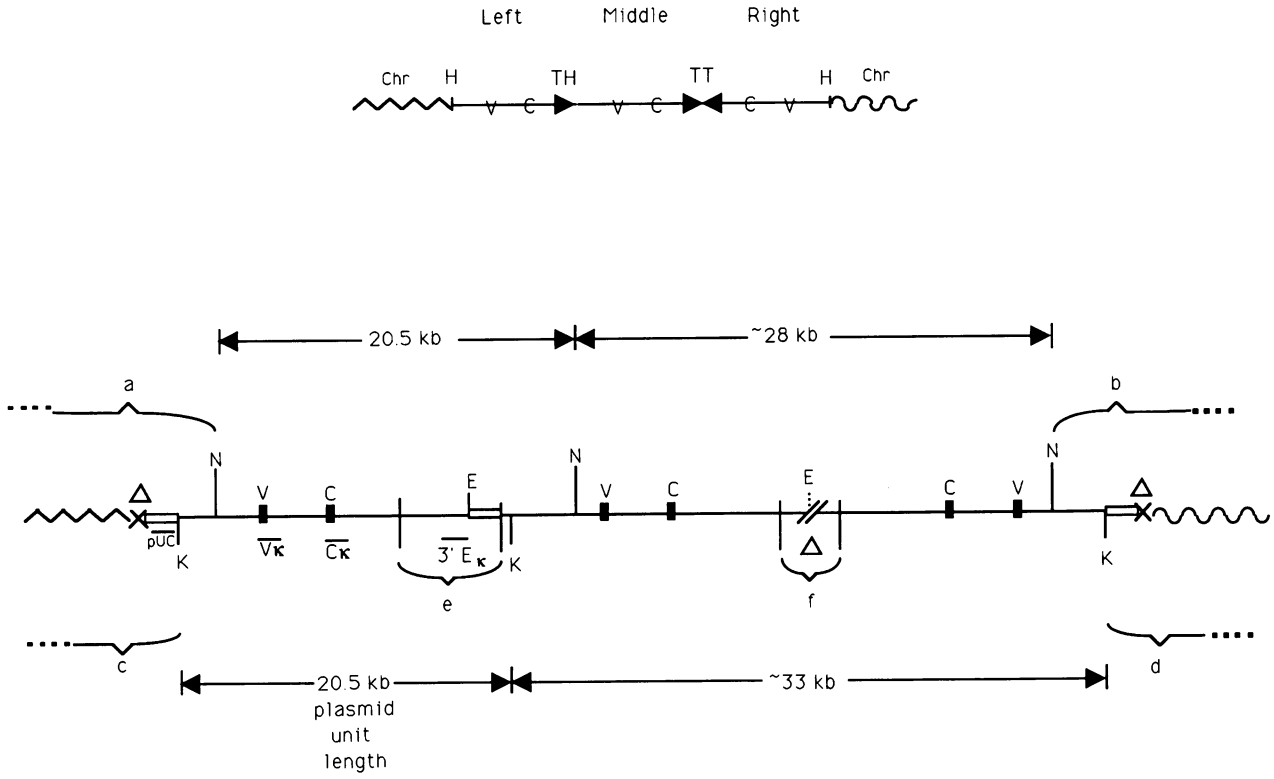

Fig. 1. Transgene arrangement at the integration site. At the top of the figure is a schematic diagram showing the orientation of each of the three transgene copies, integrated into chromosomal (Chr) DNA. Each plasmid copy is shown as an arrow, two of which are oriented head (H) to tail (T), while the third is oriented tail to tail. Individual transgene copies have been denoted left, middle, and right. A more detailed view of the integration site is shown below. The plasmid was linearized for microinjection at *Eco*RI (E) sites. Our evidence suggests that the *Eco*RI sites at the ends of the array have been destroyed (X), and that a small deletion (Δ) of pUC18 sequences in the left copy and larger deletion of pUC18 sequences has occurred in the right copy. Plasmid unit length fragments, and DNA fragments containing part of the middle and left copies are generated using either *Nhe*I (N) or *Kpn*I (K) restriction digests; probes used to characterize these fragments are shown (variable region, Vκ; constant region, Cκ′ 3′ κ enhancer, 3′Eκ). Fragments a–f are junction fragments. The chromosomal DNA–transgene junctions (a, b, c and d) hybridize only to pUC18 probes (pUC), a and b are 6.8 and 14 kb whereas c and d are 13 and 20 kb, however, we have not established at which end of the array they reside. The internal head to tail transgene junction fragment (e) has been identified using several restriction enzymes and the 3′ κ enhancer probe. The tail to tail junction fragment (f) has undergone a small deletion on both the middle and right copies.

gene tail section). The intact tail to tail *KpnI* fragment was expected to be 35.5 kb in size. However, we estimate the observed fragment size to be ~33 kb.

We have also determined by Southern blots and sequence analysis (see below) that the pUC18 sequences located at the chromosomal junctions are not intact. The left copy has lost ~80 bp of pUC18 sequence, whereas the right copy has lost both the bacterial origin of replication and the ampicillin resistance gene (~1.5 kb). However, it does contain a portion of the *lacZα* sequence. The middle copy appears to have completely intact pUC18 DNA.

Characteristics of the hybridomas analyzed

As assessed by ELISA assays, the five hybridomas in which somatic mutations were found either secrete IgM or IgG (Figure 3), consistent with the notion that somatic mutations are introduced independently of class switching (Rudikoff *et al.*, 1984; Manser, 1989; Shan *et al.*, 1990). All hybridomas except one (2G7) bind phosphorylcholine (PC), the original immunogen (see Materials and methods). Using appropriate oligonucleotides, it was determined by PCR that the PC negative hybridoma does not have a rearranged T15 V_H gene (data not shown), often associated with PC binding specificity. It is likely that the B cell giving rise to this hybridoma arose in response to some other antigen epitope.

Strategy for sequencing individual transgene copies from hybridomas generated from mouse line 194-2

By exploiting unique restriction sites within the transgene, we isolated the κ transgene copies from hybridoma DNA.

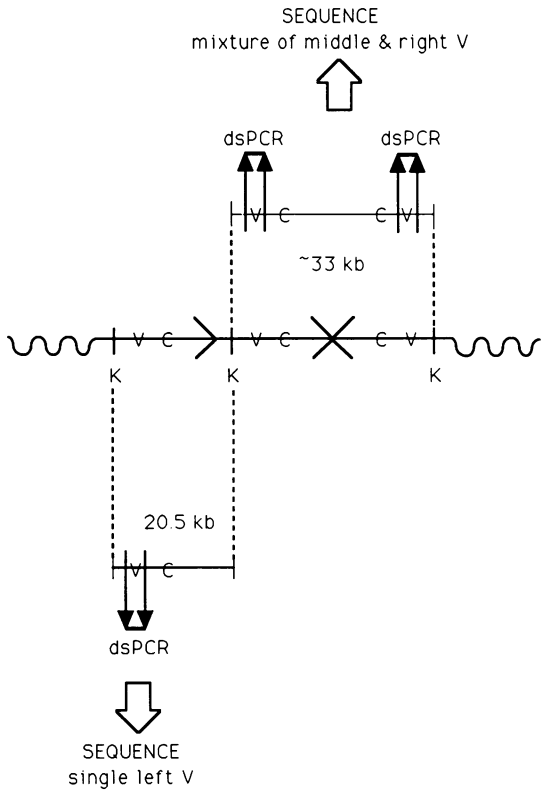

Fig. 2. Isolation of transgene variable regions and sequencing strategy. Genomic DNA is digested with *KpnI* (K) which cuts once within the transgene, and fragments are separated by electrophoresis. Agarose blocks containing transgene fragments are excised, and V regions amplified by the PCR (see Materials and methods). Double stranded PCR products are purified and sequenced directly or after cloning.

Since the restriction enzyme *KpnI* cuts once within the transgene, a restriction digest with this enzyme results in the release of the left transgene copy as a 20.5 kb unit length fragment, effectively separating it from the other two copies which migrate as a single 33 kb fragment (Figure 2). It should be emphasized that the former fragment carries a single Vκ167 segement, whereas the latter carries two Vκ167 segments, one from the middle transgene copy of the array and one from the right copy. Note that this procedure also results in the isolation of Cκ regions. After electrophoresis, agarose blocks containing these fragments were excised and, using appropriate oligonucleotides, the V and C regions of the transgenes were amplified by the polymerase chain reaction and either sequenced directly, or cloned before sequencing.

The V region of this transgene had been shown in the past to be somatically mutated at a very high frequency between nucleotide positions 369 and 1155 (Hackett *et al.*, 1990). Focusing the analysis on this region facilitates the search for new mutations in different hybridomas. In addition, we amplified the C region and surrounding sequences in an effort to confirm the V region specificity of any newly identified set of somatic mutations.

Variable region sequences from isolated transgene copies

Both hybridoma DNA and transgenic kidney DNA (from the mouse from which the hybridoma was generated) were subjected to the experimental protocol described above. The length of the amplified products detected was consistent with that predicted for the VJ segment of the transgene. Nucleotide sequencing revealed that amplification of a Vκ167−Jκ5 segment had occurred and was specific for the rearranged variable region of the transgene (rather than a related endogenous gene) since in all cases they shared the mutations present in the gene used for oocyte microinjection in the production of transgenic mice (see Materials and methods). Consistent with this notion, Southern blots showed that all five hybridomas had the endogenous Vκ167 gene in the germline configuration.

In all five hybridomas which we analyzed in detail, the PCR amplified V region of the left transgene copy was unmutated. The mixture of the middle and right copies generated two types of DNA clones, one set had no somatic mutations while a second set of clones carried all the somatic mutations identified for that hybridoma (Figure 3). For some of these nucleotide changes we determined directly that they were not present in kidney DNA, indicating that they are somatic mutations. The other changes also must be somatic, since each of them only occurred in one of the five hybridomas.

Constant region sequences from isolated transgene copies

We previously established that genomic DNA clones representing mutated transgene copies from hybridomas derived from mice carrying 13 and three copies of the Ig κ gene exhibited no somatic mutations in and around the Cκ gene segment, a region encompassing 2 kb of sequence (including the right copy of 3A1), confirming the V region specificity of the mutations observed (O'Brien *et al.*, 1987; Hackett *et al.*, 1990). Additional sequence analysis was done on the Cκ region from mutated and unmutated transgenes isolated from hybridomas presented herein: ~800 bp from

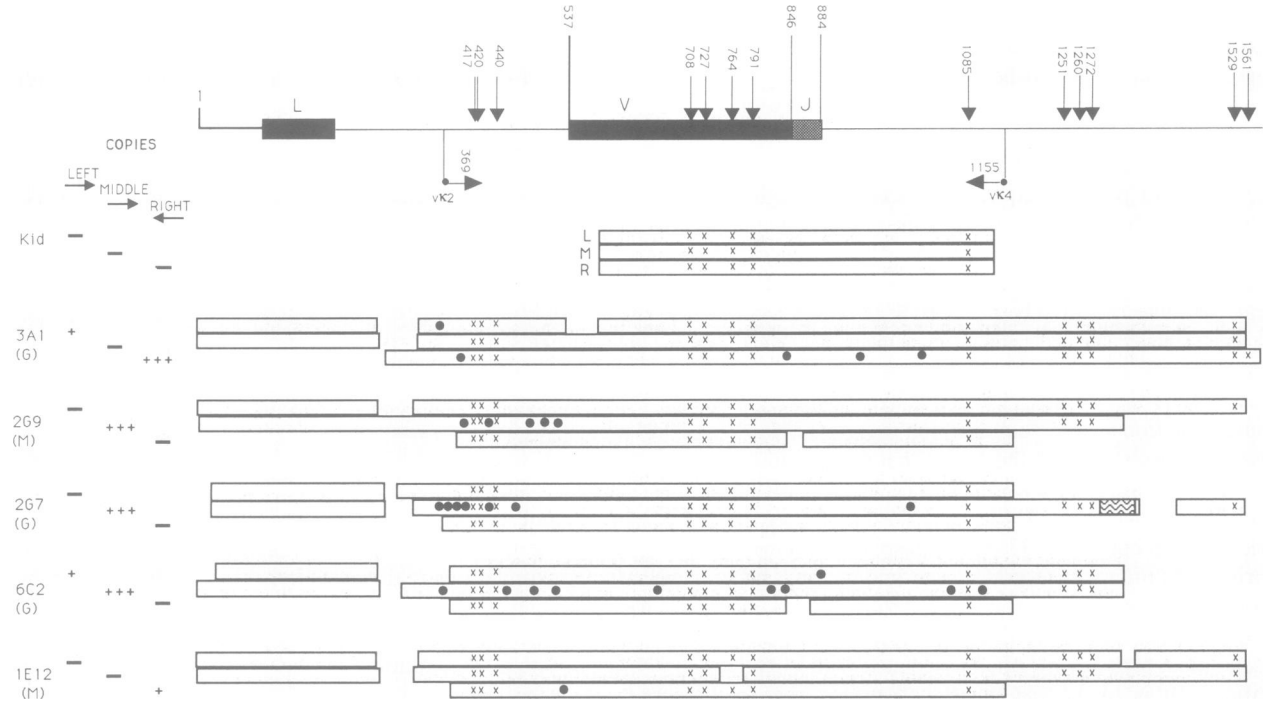

Fig. 3. Distribution of somatic mutations in the κ transgene array. A diagram of the κ transgene from positions 1−1565 (Selsing and Storb, 1981; Hackett *et al.*, 1990) is shown at the top of this figure. Depicted are the leader sequence (L), leader intron, variable (V) and joining (J) gene segments, and part of the major intron. Vertical arrows above the gene indicate the location of mutations already present in the gene (Hackett *et al.*, 1990) which serve as markers for the transgene. Shown below the gene are the two primers which were used for PCR. For each primer the position of the most 3′ nucleotide is indicated. The bottom part of the figure shows the sequencing results of transgenic variable regions isolated by PCR and plasmid rescue obtained for hybridomas 3A1, 2G9, 2G7, 6C2 and 1E12, which secrete either IgG(G) or IgM(M) antibodies and transgenic kidney (Kid). The extent of the sequence information for each copy, left (L), middle (M), or right (R) is indicated as a horizontal bar. The marker mutations are depicted as an X, while newly introduced somatic mutations are indicated as filled circles. The right transgene copy from hybridoma 3A1 was previously sequenced from position 262−3950 (Hackett *et al.*, 1990). Transgenes with multiple mutations are scored as +++, with single mutations as +, and those with no mutations across the region sequenced as −. The wavy area in the middle copy of 2G7 is a deletion of 49 bp (positions 1276−1324), whose origin is unclear, present in three independently rescued middle copy plasmids and proven by PCR to exist in the hybridoma (not shown). The single point mutations in the left copies of 3A1 and 6C2 were only identified in rescued plasmids, because these regions were either not PCR amplified (3A1), or the sequencing of the PCR product did not extend to that position (6C2).

the left and middle copies of all hybridomas, and ~ 600 bp from the right copies of 2G9 and 2G7. Again, no somatic mutations were found, consistent with a targeted mutator activity toward V regions (data not shown).

Isolation of transgene copies by plasmid rescue

Southern blots had revealed a deletion at the tail to tail junction in the array, in addition to a fairly extensive deletion of the 3′ pUC18 sequence of the right copy. The fact that all the hybridomas revealed in Southern blot analysis the expected 4.8 kb *Bam*HI transgene fragment encompassing the leader intron to ~1 kb downstream of the constant region (O'Brien *et al.*, 1987) suggested a certain level of internal integrity of all three transgene copies. The genomic clone isolated from hybridoma 3A1 which exhibited somatic mutations was sequenced almost in its entirety (3.7 kb) and showed no deletions (Hackett *et al.*, 1990). The non-mutated DNA clones of 3A1, while not sequenced as extensively, were also isolated as 4.8 kb fragments and appeared to be intact between the two *Bam*HI sites. Nevertheless, we extended the analysis to ascertain the integrity of the transgene copies over the longest DNA distance possible.

This was achieved by exploiting the fact that the microinjected gene used to generate this mouse line carried the entire pUC18 sequence on it. This allowed a plasmid rescue scheme based on ampicillin selection of bacteria carrying transgene copies. As a result of the deletion of pUC18 sequences in the right copy, this analysis only allows the rescue of the left and middle copies of the array. However, the left copy was precisely the one which needed to be further analyzed because of its apparent lack of mutations. Furthermore, the isolation of the middle copy allowed us to determine which one of the copies from the middle and right copy mixture was mutated.

Hybridoma DNA was digested with *Eco*RV and as shown in Figure 4, this results in the release of a 15.7 kb fragment which contains most of the middle transgene copy sequences in addition to a short segment of the left copy containing the 3′ κ enhancer. Two other fragments corresponding to the left and right transgene copies were also expected. Southern blot analysis using pUC18 probes reveals that all three fragments migrate so close to each other that they appear to migrate as one fragment. When electrophoresis is run for longer time periods, we resolve one of the three *Eco*RV fragments as a faster moving fragment with a diminished hybridization signal intensity with the pUC probe, suggesting it may correspond to the right transgene copy.

It is fortuitous that the *Eco*RV fragments of interest are of the same size, in that it normalized the rescue efficiency of the transgene copies. We identified rescued plasmids corresponding to the left and middle transgene copies. Restriction mapping of all of these plasmids and Southern blot analysis of the plasmids rescued from 3A1 demonstrated that the integrity of these copies was not compromised during integration beyond the deletional events we have characterized previously.

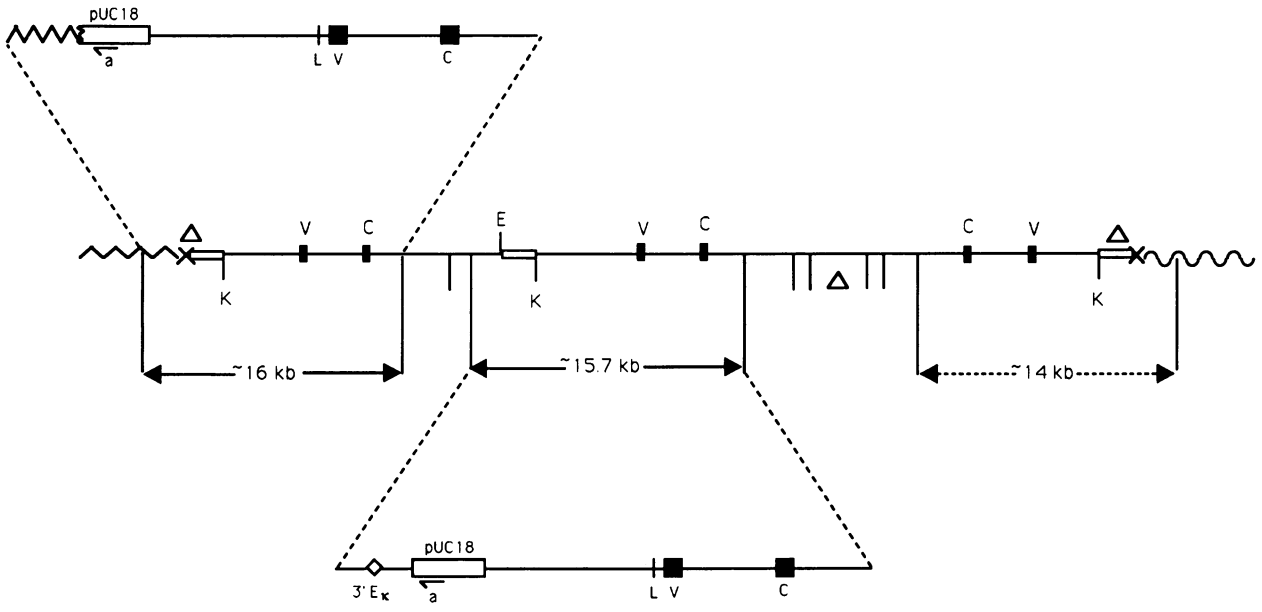

Fig. 4. Isolation of κ transgenes by plasmid rescue. The transgene array is depicted in diagrammatic form along with the *Eco*RV sites present in each copy and adjacent chromosomal sequences (vertical lines below the array). The longer lines define the *Eco*RV boundaries for each rescued plasmid. The rescued plasmids are shown in expanded form. The right copy plasmid could not be rescued because part of the pUC18 sequences including the ampicillin resistance and origin sequences have been deleted (see text). However, both the middle and left copies have been rescued and characterized (data not shown) by extensive restriction enzyme mapping and Southern blot analysis using probes for Vκ, cκ, 3'Eκ and the left chromosomal junction. Primer pUC18A (a) (5'd[CAGTGAGCGCAACGCAA]3') was used to sequence both the chromosomal–transgene junction of the left copy, and the internal transgene junction of the left and middle copies. The VJ intron and C regions were sequenced for both copies as described (Materials and methods). The chromosomal DNA adjacent to the left copy appears to be Y chromosome specific (see text). (Δ) denotes deletion events.

Mutation patterns in the rescued plasmids

The rescue of the transgene copies afforded us the opportunity to determine which of the copies in the mixture of the middle and the right ones, was mutated and to examine V and C region templates not obtained by DNA amplification. The absence of potential errors due to thermostable polymerase greatly simplified the sequencing task. Furthermore, we sequenced beyond the region originally examined in PCR products. Rescued plasmids corresponding to the left copies exhibited no somatic mutations in the portions of the V regions previously sequenced, confirming the sequencing results of PCR products obtained earlier from these V segments. However, upon additional sequencing, one mutation at position 355 (C → T) was found in 3A1, and one at position 891 (A → T) in 6C2 (Figure 3 and Table I).

Rescued plasmids corresponding to the middle copy showed mutations in three hybridomas (2G9, 2G7 and 6C2) and no mutations in two (3A1 and IE12) (Figure 3). In the latter, the previously identified mutations must therefore reside in the right copy. The three hybridomas with mutations in the middle copy showed every base substitution and the 1 bp deletion which had previously been identified by amplification of the V region mixture from the middle plus right copies.

Discussion

The three transgene copies are located at one integration site and are linked

The 194-2 mouse line has been carried for many generations (6 years) and the transgene array has always segregated as a single allele. Linkage of the three transgene copies comes from several lines of evidence: (i) the 33 kb *Kpn*I fragment contains two V regions, one exhibiting somatic mutations, while the other does not, as assessed by sequencing PCR products derived from the isolated fragment; (ii) restriction mapping followed by Southern blots have identified a head to tail junction, in which the 3' κ enhancer (intact only in the left copy), is linked to the pUC18 vector sequence from the middle copy; (iii) using pUC18 sequencing primers it was determined that rescued plasmids containing most of the middle copy transgene were linked to sequences from the 3' end of the left transgene copy (Figure 4), in that the junction sequence was identical to that obtained from the plasmid used for microinjection. The latter points demonstrate that the left copy is adjacent and linked to the middle copy, whereas the first point demonstrates that the middle copy is linked to the right copy.

We also determined the sequence for the chromosomal–transgene junction in rescued plasmids representing the left transgene copy. This sequence is apparently Y-chromosome specific (not shown). Breeding records of mouse line 194-2 showed that the transgene was only transmitted by and to males, confirming the integration into the Y chromosome. This proves directly, that the somatic hypermutation process is independent of *cis*-elements within the immunoglobulin gene loci located outside of the transgene sequence (O'Brien *et al.*, 1987).

Evidence that the mutations are somatic

All mutations so far detected in transgenic V regions are unique to the hybridoma in which they were found. This is highly significant since all hybridomas were derived from two individuals of the same mouse line. Mutations which might have arisen in the germline (perhaps during integration) would be expected to be shared by all hybridomas. This has not been the case. Furthermore, we have sequenced PCR

Table I. Newly introduced somatic mutations in κ transgenes[a]

Hybridoma[b]	Copy	Mutation		*P*
3A1	Left	C → T	355	<0.037
	Right	C → A	390	
		A → C	838	
		G → C	942	
		G → C	1031	
2G9	Middle	T → G	401	<0.012
		A → G	434	
		T → C	484	
		T → C	495	
		T → C	500	
2G7	Middle	G → A	381	<0.0014
		G → A	389	
		ΔA	402	
		A → T	411	
		A → G	439	
		A → T	459	
		T → A	1004	
6C2	Left	A → T	891	<0.00015
	Middle	C → T	366	
		A → G	455	
		A → G	488	
		A → C	499	
		G → A	673	
		T → G	814	
		A → T	843	
		G → A	1071	
		T → C	1111	
1E12	Right	G → A	510	n.s.

[a]See Figure 3.
P = probability that distribution of mutations arose by chance.
n.s. = not significant.

products obtained from transgenic kidney DNA, and although the sequence analysis did not extend into the leader intron, we demonstrated that 10 mutations present in hybridoma κ transgenes are absent in kidney (germline) DNA (Figure 3). The available evidence clearly indicates that all mutations reported herein arose in B cells and are not due to either cloning artifacts or errors by the thermostable polymerases (see Materials and methods).

Nature and impact of the mutations observed

Most of the mutations identified in the transgenes could not have been antigen selected. Of the 28 mutations which have been detected in the five hybridomas analyzed, only four fall in coding regions, and three of these are base substitutions which result in silent mutations. Only one mutation resulted in an amino acid replacement (hybridoma 6C2, position 843, Phe → Tyr). Thus, the preferential targeting of a single copy is unlikely to be due to antigen selection.

A summary of somatic mutations detected in the κ transgenes is shown in Figure 3 and Table I. All mutations detected (with two exceptions) appear to be point mutations. This is the first time in which analysis of somatic mutations in transgenes has revealed deletions: one nucleotide at position 402 and 49 nucleotides at position 1276–1324 in hybridoma 2G7. Whether the 49 bp deletion is related to the somatic hypermutation process is unknown. However, similar deletions have been found in the introns of endogenous mutated κ genes (Weber *et al.*, 1991a). No insertions have been observed in the areas we have sequenced. This analysis brings the total number of somatic point mutations observed in these κ transgenes to 61 (this paper and Hackett *et al.*, 1990) and consistent with the analysis of somatic mutations in endogenous Ig genes, both transitions and transversions are observed and there appears to be a greater than 2:1 bias toward transitions.

Different somatic mutation frequencies in different copies of the transgene array suggest a target preference

We assume that the presence or absence of mutations within the sequence examined is an adequate reflection of the overall state of mutation in any given transgene copy, because we have sequenced a window in the gene where the highest frequency of mutations had been observed previously (Hackett *et al.*, 1990 and reviews in Steele, 1991). It is clear that the presence of an array of transgene copies in mouse lines examined here and elsewhere (Durdik *et al.*, 1989; Gerstein *et al.*, 1990; Hackett *et al.*, 1990; Sharpe *et al.*, 1991) does not preclude a transgene from being mutated at apparently similar frequencies relative to endogenous genes. One may have expected that a mutator factor may be limiting and that in a multicopy mouse each transgene copy may be mutated at a frequency which is decreased from that of endogenous genes by a factor related to transgene copy number. Previously we had determined that many point mutations (12 in a 1.3 kb stretch in one, and 21 in 1.5 kb in another) were accumulated by at least one transgene copy in each of two B cells carrying 13 copies of a κ transgene (Hackett *et al.*, 1990). Based on the current analysis, the high somatic mutation frequencies observed for the mutated copy suggests that point mutations accumulate as a result of targeting of that copy, relative to the other copies which exhibit very low mutation frequencies or no evidence of mutation at all (the statistical analysis, Table I, shows that it is highly unlikely that the preferential targeting of one copy was due to chance).

Transcriptional status of the transgene copies

The κ167 gene is a functionally rearranged Ig light chain gene which contains all the transcriptional regulatory elements necessary for proper expression. It has been previously shown that κ167 transgenic mice express the transgene in a tissue specific manner indicating that the microinjected gene carries with it all the sequences necessary for B cell factors to activate the gene and regulate its transcription (Storb *et al.*, 1986). Transcriptional activation may be critical in that it may result in the accessibility of Ig genes to the mutator. When mRNAs of the hybridoma 3A1 were sequenced, co-expression of the single coding region mutation was detected along with the unmutated base, indicating that, in the hybridoma, at least two transgenes were being transcribed (O'Brien *et al.*, 1987). However, the transcriptional status of the transgenes at the time the mutator was active remains unknown. With this caveat in mind, it appears that lack of transcriptional activity is not the reason for lack or scarcity of mutations in two of the transgene copies.

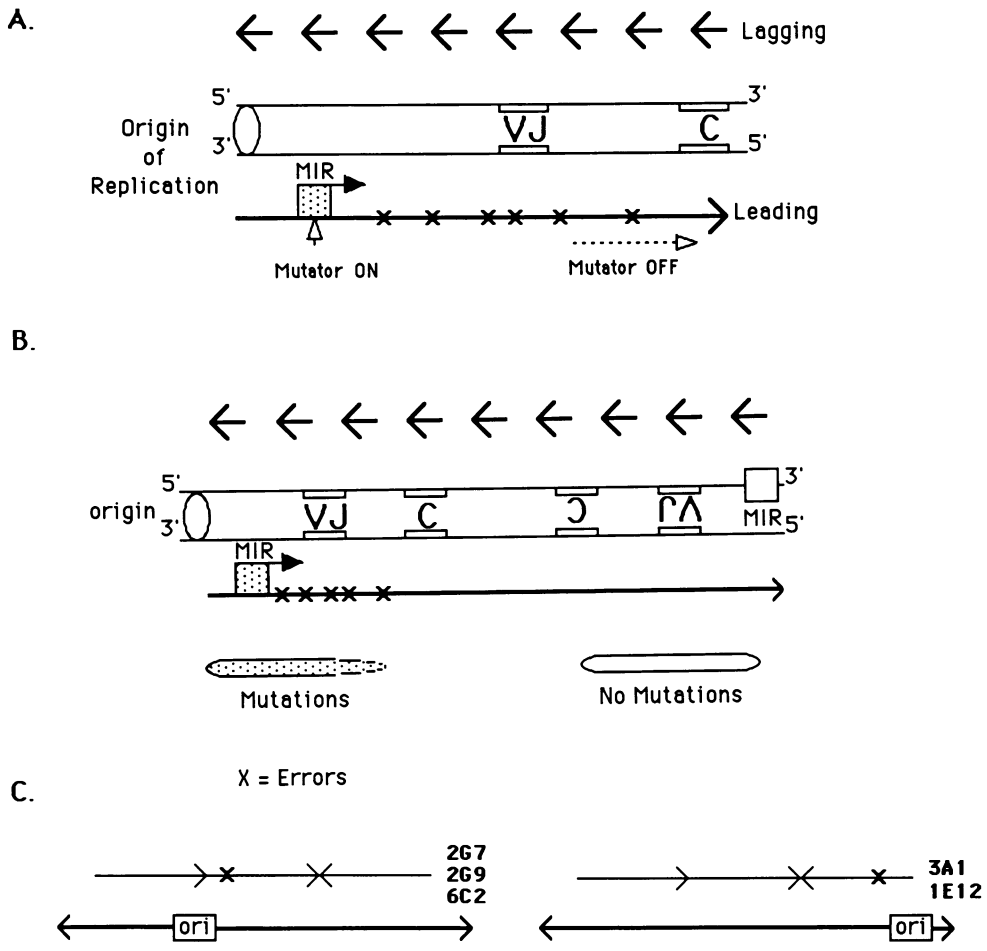

Fig. 5. Schematic representations of the model for somatic hypermutation. (**A**) Components of the model. The two lines in the middle represent the two parent DNA strands, and the lines above and below represent the newly synthesized strands. (**B**) Explanation of why in two transgene copies in opposite orientation only one may be mutated. (**C**) The left and right parts show the three transgene copies (thin arrows) and the postulated bidirectional origins of replication in the two types of hybridoma.

We also monitored the methylation status of a single *Hpa*II site located within the variable region of the transgenes in the various hybridomas examined (data not shown). It was determined that all three copies were completely undermethylated at this site in all five hybridomas. As with transcription, we do not know the methylation status at the time the mutator was active in the B cell. However, the unrearranged endogenous Vκ167 gene was hypermethylated at this site as expected for an inactive gene in a B cell, suggesting that the fusion process did not alter the immunoglobulin gene methylation pattern in general. Thus, the DNA methylation analysis further supports that there was no difference in gene activation between the highly mutated and unmutated transgene copies.

Proposed model for the somatic hypermutation of Ig genes

Several models have been presented to explain how somatic mutations are introduced in V regions of Ig genes: (i) site specific nicking of the DNA followed by repair via an error-prone DNA polymerase (Brenner and Milstein, 1966; Gearhart and Bogenhagen, 1983); (ii) somatic gene conversion (Baltimore, 1981; Maizels, 1989); (iii) homologous recombination of reverse transcribed Ig mRNA (Steele and Pollard, 1987); and (iv) V region DNA replication independent of chromosomal DNA replication (Manser, 1990). No direct evidence exists that supports any of these models. The models were formulated to explain one of the most striking features of the somatic hypermutation process: V region specificity. Model (ii), somatic gene conversion, while apparently responsible for Ig gene mutations in chicken (Reynaud *et al.*, 1985; Carlson *et al.*, 1990) and rabbit (Becker and Knight, 1990), is not likely for mouse and man (Chien *et al.*, 1988; Wysocki and Gefter, 1989). It is difficult to reconcile the third model with the fact that mutations have been found upstream of the transcriptional promoter (Both *et al.*, 1990; Lebecque and Gearhart, 1990), making it unlikely that transcripts are responsible for the targeting.

These models are framed in the context of what is observed for endogenous genes. However, we must now bring to bear the findings in the transgenic mouse system. In addition to V region specificity, there appears to be preferential targeting of a single transgene copy in a three copy array. The apparent targeting of one transgene copy for several cell generations requires that the mutator function remains somehow associated with only that copy. Models (i) and (iv) above are difficult to reconcile with that finding. Model (i) would require that the same copy continues to be a target for a nicking enzyme. There is no obvious way to maintain such targeting. Model (iv) likewise would require that mainly one V region continues to be a target for cell cycle independent replication. We wish to propose a model in which this

targeting is related to the maintenance of one specific origin of replication at the expense of other possible ones (Figure 5). Other models independent of replication can be envisioned, but will not be discussed here for the sake of brevity and because it is more difficult to envision a process of continued targeting of a specific region independent of transcription or replication. Transcriptional targeting of one preferred copy appears unlikely, because of the lack of evidence of differential transcription or chromatin activation in the three copies.

We propose a model in which somatic mutation is due to the temporary presence in mutating B cells of a mutator factor which suppresses the fidelity of replication. The factor interacts either with the leading strand replication complex, or with the lagging strand, but not both. For the sake of simplicity of presenting the model, we will assume that the mutator acts on the leading strand.

We further propose that the factor has a high affinity for a 'mutation initiation region' (MIR) located upstream of V gene transcriptional promoters. In order to direct mutations into the V gene, it is proposed that an origin of replication must be upstream of the MIR, that the factor has affinity only for the leading strand replication complex, and that it has directionality in its action towards the V gene, so that the factor will automatically be carried only into the V gene. Finally, the factor is proposed to have a relatively low processivity so that it dissociates from the replication complex within a certain distance from the MIR, but not at a defined site of release. The model further requires that all mutable V (and D ?; Roes *et al.*, 1989) genes have an upstream MIR, but that no other genes have this sequence. This mutator factor is only produced in mutating B cells (presumably memory B cell precursors, Kocks and Rajewsky, 1989; Linton *et al.*, 1989; McHeyzer-Williams *et al.*, 1991) during a few cell generations. The factor can only bind to MIRs in transcriptionally active chromatin; therefore, most unrearranged V genes are not mutated.

We will consider a number of findings relevant to replication and somatic hypermutation in light of this model. (i) Mammalian origins of replication are ~20–300 kb apart (Edenberg and Huberman, 1975). Their exact structures have not been defined (Stillman, 1989). Replication termini are apparently not well defined and, except for certain cases, like EBV (Gahn and Schildkraut, 1989), may occur randomly, wherever neighboring replication forks meet (Challberg and Kelly, 1989; Stillman, 1989). The frequency of origins of replication appears to increase around active genes (Brown *et al.*, 1987), including Ig genes (Calza *et al.*, 1984; Hatton and Schildkraut, 1990). The model of somatic mutation requires that in a rearranged Ig gene an origin of replication is located upstream of the MIR and the V gene and that no termination occurs before the middle of the JC intron. It remains to be determined if any origin of replication is compatible with hypermutation of V genes. Several different transgene integration sites have given rise to somatic mutants (O'Brien *et al.*, 1987; Durdik *et al.*, 1989; Hackett *et al.*, 1990; Gerstein *et al.*, 1990; Sharpe *et al.*, 1991). Thus, any origin may work or the transgenes carry their own functional origins (see below).

The number of active origins in differentiated cells is lower than that of potential origins (Edenberg and Huberman, 1975). In embryonic tissues a much higher frequency of origins is found. In the different B cell parents of the hybridomas described here, apparently different origins of replication were activated. We assume that the somatic mutation process is started after antigen stimulation of a resting, non-cycling B cell. Based on our findings and hypothesis, entry into S phase would occur from different origins in different cells, but during the several cell generations in which the somatic mutation process is ongoing (see below), the origins in a given cell around the active immunoglobulin gene would remain the same.

Thus in the cells where the middle copy is mutated, a bidirectional origin of replication is postulated to be located upstream of the middle copy V region, but downstream of the V region of the left copy presumably within a transgene sequence (Figure 5C) (see below). Termination would occur outside of both ends of the transgene array. This would allow mutations to arise in the V region of the middle copy, but not in the left or right copies, because the leading strand of replication does not access these V regions from their 5′ end.

In the cells with mutations in the right copy, an origin of replication is proposed to fire which is located upstream of the V region of the right copy, while the origin upstream of the middle copy remains silent. One termination site would have to occur to the left of the left copy.

Finally, the single point mutations found in the left copy in two of the hybridomas could be explained by the occasional firing of an origin of replication located to the left of the left copy and chain elongation through the left copy, before the replication fork from either of the two origins had reached the left V region. Alternatively, in 6C2 a single origin to the left of the transgene array could have given rise to the one point mutation in the left copy and the nine mutations in the middle copy, although the unequal frequency of mutations in the two copies makes this scenario less likely.

The postulated origin of replication between the left and the middle V must be located within transgene sequence because we have found by DNA sequencing that the left and middle copies are contiguous, without interspersed host DNA. The postulated origins at the right and left of the array could be located either within the transgenes or in flanking DNA. In the former case the origins could be in a DNA sequence shared by all three transgene copies, such as a region upstream of the V gene promoter and upstream of the postulated MIR (see Figure 5). Differential firing of the right or left origin could then not be explained by a sequence dependent control, but would be due to a more global control. Precedents have been found in other systems, where in oligomeric or multimeric genes only some of the potential origins fire (Schvartzman *et al.*, 1990; Hamlin *et al.*, 1991). On the other hand, the right and left flanking sequences within the Y chromosome are presumably unique in the region, and may be separately firing origins due to some sequence specific control.

(ii) The somatic hypermutation process is apparently ongoing over several cell generations with the result of steadily increasing numbers of point mutations (Griffiths *et al.*, 1984; Clarke *et al.*, 1985; Levy *et al.*, 1989). Thus, the proposed mechanism would continue to target the same copy in the transgenic situation, just as in endogenous genes.

(iii) It has been reported that a strand bias may be discerned in the point mutations observed in immunoglobulin genes (Golding *et al.*, 1987; Lebecque and Gearhart, 1990). This would support the postulate that the errors are only

introduced into one strand, due to affinity of the factor for either the leading or the lagging strand replication complex, but not both, assuming that they are of different composition (Roberts *et al.*, 1991; Bullock *et al.*, 1991).

(iv) Somatic point mutations have been seen as far as 200–600 bp upstream of the cap site in endogenous heavy chain genes (Kim *et al.*, 1981; Both *et al.*, 1990; Lebecque and Gearhart, 1990), but not at 4 kb upstream (Kim *et al.*, 1981). No extensive sequencing of mutated Vκ genes upstream of the promoter has been done. The κ transgene described in this paper contained 4 kb upstream, another one with demonstrated mutations had ~0.7 kb of upstream sequence (Sharpe *et al.*, 1991). Thus, the postulated MIR should be located no further than 0.7–4 kb upstream of Vκ promoters.

(v) The 3′ limit of the somatic mutations seems to be related to the distance from the V region. Thus, a Vκ gene rearranged with Jκ1 or Jκ2 has mutations in VJ and at decreasing frequencies in the 3′ J sequences, but not in the JC intron (Huppi and Weigert, pers. comm.; Huppi, 1984; Weber *et al.*, 1991b), whereas with our transgenes where the rearrangement is with Jκ5 the mutations extend to the middle of the Jκ–Cκ intron (this paper and Hackett *et al.*, 1990). The endogenous MOPC167 gene has a similar 3′ limit of intron mutations (Hackett *et al.*, 1990) and hybridomas using the same VJ combination also show mutations in the middle of the intron (Lebecque and Gearhart, 1990; Weber *et al.*, 1991a). Thus, the release of the mutator factor may be determined by the distance traveled from the MIR, and may be a function of limited processivity of factor binding. This would imply that the sparing of the C region from mutations may not be absolute; the occasional mutation tract may reach that far 3′ (Motoyama *et al.*, 1991). It would also be expected that a gradient of mutation frequencies exists, decreasing from upstream of the gene to 3′ of V. There are not enough data available to determine if a gradient exists between upstream and the V region. However, the frequency of mutations does seem to decrease between the VJ region and the 3′ J regions (Huppi, 1984; Weber *et al.*, 1991b) or the Jκ5–Cκ intron (Hackett *et al.*, 1990; Lebecque and Gearhart, 1990; Weber *et al.*, 1991a).

(vi) Somatic hypermutation seems to be a process which is in general restricted to rearranged immunoglobulin V genes (Pech *et al.*, 1981; Selsing and Storb, 1981; Gorski *et al.*, 1983). However, point mutations have been observed in the unrearranged Vλ genes of the λ myeloma MOPC315 (Weiss and Wu, 1987). It is possible that the unrearranged Vλ genes are in open chromatin within this relatively small immunoglobulin gene locus, comprising a total of ~200 kb (Storb *et al.*, 1989; Carson and Wu, 1989). It is conceivable that open chromatin is required to allow access of the mutator factor to the MIR and that germline V genes may be mutated if they happen to be in the vicinity of rearranged genes. Thus, the next germline Vκ or V_H gene upstream of a rearranged V gene (generally ~10 kb away) may perhaps be mutated sometime.

(vii) T lymphocytes appear never to change the sequence of their T cell receptor (TCR) genes. Thus, T cells may never produce the mutator factor, or TCR genes may not have a MIR sequence, or both. These questions are currently under investigation.

(viii) In endogenous Ig genes not necessarily all rearranged V genes (heavy and light chain) show evidence of somatic mutation (see Steele, 1991). Assuming that the mutator factor has access to all active V genes this may be a consequence of the differential firing of downstream versus upstream origins in different Ig genes.

The model we propose is compatible with the available data concerning somatic hypermutation of immunoglobulin genes. Many variations on the details of the model, especially the replication aspects, can be envisioned. In its present form the model makes a number of predictions which can be experimentally tested.

Materials and methods

Origin of the κ transgene

The κ transgene, derived from MOPC167, has been described (Storb *et al.*, 1986). The Vκ167 gene segment is present as a single germline V gene, therefore, the absence of related family members simplifies the analysis of mutations observed in this V gene. The myeloma κ gene differs from the germline sequence by 15 single base substitutions (another at position 847 is probably junctional) localized in and around the variable region. The pUC18 based plasmid pVC167 used for microinjection contains the rearranged κ gene with 4 kb of genomic sequence upstream of the leader and 9.5 kb downstream of the constant region (Storb *et al.*, 1986).

Generation of hybridomas

Mice transgenic for the rearranged κ gene of MOPC167 (pVC167) were immunized with phosphorylcholine (PC) coupled to keyhole limpet hemocyanin to increase the likelihood of isolating B cells that had undergone somatic mutation. Two immunization protocols were followed, one is described in detail in O'Brien *et al.* (1987), the alternative immunization procedure was that of Gearhart *et al.* (1981). It consisted of an intraperitoneal (i.p.) injection of PC-KLH (0.1 mg) in complete Freund's adjuvant. A month later a second, identical i.p. injection was given and after 3 days splenic cells were isolated and fused as described (Manz *et al.*, 1988). Hybridomas generated from both fusions were screened in an ELISA assay for Ig secretion, and PC–BSA binding (O'Brien *et al.*, 1987). Hybridomas 3A1, 2G9 and 1E12 from the first fusion were subcloned by limiting dilution, whereas hybridomas 2G7 and 6C2 from the second fusion were isolated as single colonies. Hybridomas were initially screened for mutated transgenes by a limited sequencing analysis, and those found to carry mutations were examined in more detail.

DNA isolation and PCR

Hybridoma and kidney DNA (30 μg) were digested with *Kpn*I; and fragments were separated by electrophoresis on 0.5% low melting agarose (FMC) at 4°C. Agarose blocks containing the 20.5 and 33 kb transgene fragments were excised from the gel and melted at 65°C. About 6% of this agarose-template mix was used in 100 μl PCR reactions (Saiki *et al.*, 1988) containing 50 mM KCl, 10 mM Tris–HCl pH 8.3 (room temperature), 0.01% (w/v) gelatin, 200 μM in each dNTP, 50–100 pmol of each primer, 1.5 mM $MgCl_2$, and 2.5–4 U of *Taq* polymerase (Beckman, Perkin-Elmer Cetus). When hybridoma DNA was amplified directly, i.e. without digestion and fractionation by electrophoresis, 0.5 μg of DNA was used. DNA amplification reactions were also carried out using 2.5 U of Vent DNA polymerase (New England Biolabs), 10 mM KCl, 10 mM ammonium sulfate, 20 mM Tris–HCl pH 8.8 (room temperature), 2 mM magnesium sulfate, 200 μM in each dNTP, 100 μg/ml BSA and 0.1% Triton X-100. The temperature cycling protocol was performed on the Twin block apparatus from ERICOMP and consisted of 33 cycles with denaturation steps at 94°C for 1.5 min, annealing steps at 65°C for 2 min and extension steps at 72°C for 3 min, with the following exceptions: during the first two cycles, denaturation was carried out for 5 min, while the final cycle had an extension time of 7 min. The primers used for amplification were Vκ2 (5′d[CTTAGAATTCCCAAAGCTGATGGCCCAGATGA]3′) and Vκ4 (5′d[ACGTCTAGAAGACCACGCTACCTG]3′) (Figure 3). PCR products were separated on 1% agarose gels, excised and electrophoresed into hydroxyapatite (HTP, Bio-Rad), and eluted from the HTP on G75 columns with sodium phosphate (modified from Tabak and Flavell, 1978).

Hybridization probes

The following probes were used: Cκ (Manz *et al.*, 1988); Vκ which corresponds to the ~500 bp *Msp*I–*Bam*HI fragment isolated from pVC167; 3′Eκ (Hagman *et al.*, 1990); pUC18, (the entire plasmid); *lacZ*, corresponds

to 144 bp between *Pvu*I and *Bam*HI isolated from pUC18; 5'Chr, a 304 bp PCR product obtained with primers 5'd(ATTCTCT-TGAGTACTTGGTG)3' and 5'd(GATTTTTCCCAGGAGATCTG) 3'.

Plasmid rescue

Hybridoma DNA (15 μg) was digested with *Eco*RV in a 50 μl volume, diluted to 100 μl with TE pH 8.0, and phenol/chloroform extracted. After ethanol precipitation, DNA was resuspended in TE pH 8.0 and 2 μg were ligated overnight at 4°C in a 100 μl volume, followed by phenol/chloroform extraction, ethanol precipitation and resuspension in 10 μl of TE buffer. This volume was microdialyzed by placing it on a Millipore membrane type VS, 0.025 μm overlaid on 500 ml of TE. About 4 μl of dialyzed DNA were mixed with 40 μl of competent DH10B bacterial cells (BRL, Grant *et al.*, 1990) and the mix electroporated in a 0.2 cm cuvette using the Bio-Rad Gene Pulser apparatus (settings: 25 μF, 2.5 kV, and 400 ohms). Plasmid DNA from ampicillin resistant colonies was isolated following standard procedures (Sambrook *et al.*, 1989).

Sequencing analysis

A substantial body of sequencing data in this study has been derived from PCR products obtained by amplifying DNA templates with Taq polymerase (Beckman and Cetus). Therefore the search for somatic mutations is complicated by the introduction of *Taq* errors during the amplification. Direct sequencing of double stranded PCR products yielded sequence information of below average quality, whereas sequencing cloned PCR products was very reliable. Therefore in all cases, to distinguish somatic mutations from *Taq* errors, we analyzed DNA clones obtained from at least two independent PCR reactions. In some cases we repeated DNA amplifications using Vent polymerase (NEB) which exhibits a much lower error frequency than *Taq*. PCR products were cloned into pBluescribe (Stratagene) using restriction sites *Eco*RI (located on Vκ2) and *Xba*I (located on Vκ4). Rescued plasmids were alkali denatured, mixed with primers, annealed and ethanol precipitated. Sequencing was performed using the Sequenase kit from US Biochemicals. Primers for VJ intron sequencing included: 5'd(GAGTGAAGGCTGAGGATGTG)3' (position 811): 5'd(GTTTCA-GCTCCAGCTTG)3' (position 868); 5'd(CTCCTCAGCTCCTGATC)3' (position 706); 5'd(GACCTGCAGGAGATGGAAAC)3' (position 602); 5'd(GTGATACATGCCTGTCAAGC)3' (position 275); in addition to primers previously detailed (Hackett *et al.*, 1990). Primers for C region sequencing included: Cκ1, 5'd(TCTCTGTCTGAAGCTTGAAACTGAA)3'; Cκ3, 5'd(ACAGAGATCTCAAGTGCAAAGACTC)3'; Cκ4, 5'd(GAC-ATCAATGTCAAGTG)3'; Cκ5, 5'd(ACATAACTGTTTACACA)3'.

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