**The EMBO Journal** Vol.16 No.13 pp.3987–3994, 1997

# Cells strongly expressing Igκ transgenes show clonal recruitment of hypermutation: a role for both MAR and the enhancers

**Beatriz Goyenechea, Norman Klix, José Yélamos, Gareth T.Williams, Andrew Riddell, Michael S.Neuberger[1] and César Milstein[1]**

Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

[1]Corresponding authors

B.Goyenechea and N.Klix contributed equally to this work

**The V regions of immunoglobulin κ transgenes are targets for hypermutation in germinal centre B cells. We show by use of modified transgenes that the recruitment of hypermutation is substantially impaired by deletion of the nuclear matrix attachment region (MAR) which flanks the intron-enhancer (Ei). Decreased mutation is also obtained if Ei, the core region of the κ3′-enhancer (E3′) or the E3′-flank are removed individually. A broad correlation between expression and mutation is indicated not only by the fact that the deletions affecting mutation also give reduced transgene expression, but especially by the finding that, within a single mouse, transgene mutation was considerably reduced in germinal centre B cells that poorly expressed the transgene as compared with strongly expressing cells. We also observed that the diminished mutation in transgenes carrying regulatory element deletions was manifested by an increased proportion of B cells in which the transgene had not been targeted at all for mutation rather than in the extent of mutation accumulation once targeted. Since mutations appear to be incorporated stepwise, the results point to a connection between transcription initiation and the clonal recruitment of hypermutation, with hypermutation being more fastidious than transcription in requiring the presence of a full complement of regulatory elements.**

*Keywords*: diversity/enhancers/hypermutation/immunoglobulin/MAR/somatic mutation

## Introduction

Somatic hypermutation is a major contributor to antibody affinity maturation. In germinal centre B cells, nucleotide substitutions are introduced into a region of several kilobases of DNA that includes the rearranged V gene segments of the immunoglobulin heavy and light chain loci. The mechanism of hypermutation is unknown, although several lines of evidence point to a linkage to transcription. Thus, the process reveals strand polarity, it is inhibited by the removal of *cis*-acting transcription enhancer elements and the mutation domain is located downstream of the promoter (see reviews by Neuberger and Milstein, 1995; Storb, 1996; Weill and Reynaud, 1996).

Experiments to date looking at the requirement for *cis*-acting elements have focused largely on the overall extent of mutation rather than differentiating between the frequency with which a gene is targeted for mutation and the extent of mutant accumulation once so targeted. We were therefore moved to extend our previous work on the immunoglobulin κ locus (Betz *et al.*, 1994), dissecting the contributions of the two κ enhancer regions and asking whether the portions necessary for transcription enhancement corresponded to the portions essential for hypermutation, paying special attention to the distinction between mutational targeting and mutation accumulation.

## Results

### *Removal of either MAR or Ei diminishes hypermutation*

In previous work, by comparing V region mutation in Lκ and LκΔ[Ei/MAR] transgenes, we showed that removal of a 688 bp region from the $J_κ$–$C_κ$ intron leads to a very substantial reduction in hypermutation (Betz *et al.*, 1994). This deletion takes out both the intron-enhancer (Ei), which has been shown in transfection assays to confer lymphoid-specific transcriptional enhancement (Picard and Schaffner, 1984; Queen and Stafford, 1984), and the MAR, which was identified as a nuclear matrix attachment region (Cockerill and Garrard, 1986) but which does not exhibit cell type specificity and which has not been shown to have any major role in transcriptional activation.

To determine whether it was the removal of Ei or of MAR that was responsible for the reduction in hypermutation, we prepared Lκ derivatives that were separately depleted of either element. Two constructs (differing in the precise location of the deletion endpoints) were made for each type of deletion (Figure 1) and multiple transgenic mouse lines established.

Hypermutation was assessed by PCR cloning and sequencing of the transgenic V genes from sorted germinal centre B cells obtained from the mouse Peyer's patches. Whilst there is significant founder-to-founder variation, it is notable that, compared with the Lκ controls, deletion of either Ei or MAR on their own is sufficient to give a dramatic drop in hypermutation (Table I). The effect of the MAR deletion, which in all four lines examined leads to mutation rates that are severely diminished or even undetectable above background, is more severe than that of Ei.

The diminished mutation of the LκΔ[Ei] and LκΔ[MAR] transgenes is largely attributable to a large decrease in the proportion of transgenic V gene clones that carry any mutations at all. This figure drops from a range of 65–75% in the Lκ control mice to 5–33% in the LκΔ[Ei] and

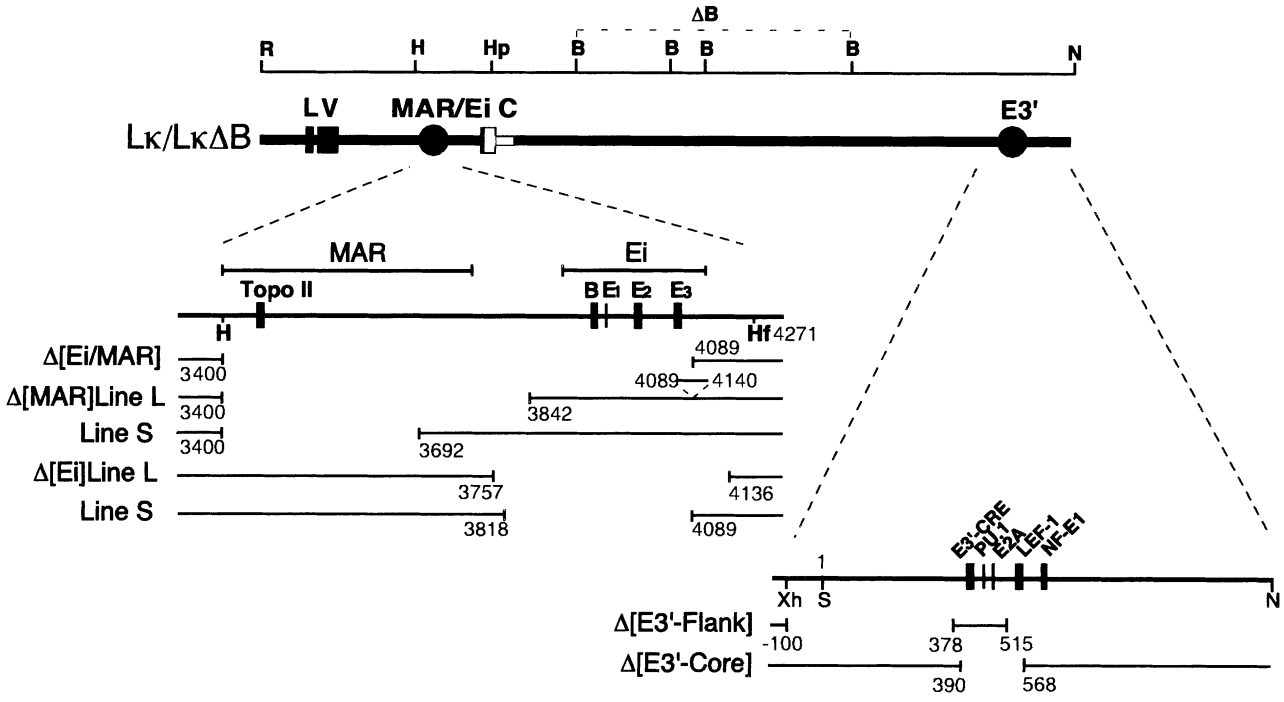


**Fig. 1.** The transgenes. The transgenes are all based on the ancestral constructs Lκ or LκΔB (both of which are good hypermutation targets) as described in Materials and methods; in LκΔB, the region between the *Bam*HI (B) sites downstream of $C_\kappa$ is deleted as indicated. Lκ is of mouse origin except for a small region (in white) including most of $C_\kappa$, which is of rat origin. The extent of the internal deletions in the different constructs is indicated with the numbering following Max *et al.* (1981) in the $J_\kappa$–$C_\kappa$ intron and Meyer and Neuberger (1989) around the 3′-enhancer. Other restriction sites are abbreviated H, *Hin*dIII; Hf, *Hin*fI (not all sites depicted); Hp, *Hpa*I; R, *Eco*RI; N, a *Not*I linker that has been inserted into an *Eco*RI site; S, *Sac*I; and X, *Xho*I. Various factor-binding sites within the enhancers and MAR are indicated.

LκΔ[MAR] animals. Furthermore, the few LκΔ[Ei] and LκΔ[MAR] transgene copies that do get targeted for mutation are still able to accumulate multiple nucleotide substitutions (Figure 2). If we restrict our analysis to mutated sequences, then the average number of substitutions accumulated in the mutated LκΔ[Ei] and LκΔ[MAR] V genes is only reduced ~2-fold compared with the number of substitutions accumulated on the parental Lκ transgene (Table I). These effects are specific to the transgenes themselves since they are observed in cells that otherwise retain their normal mutational capacity. Thus, sequence analysis of PCR-amplified clones derived from the 3′-flank of rearrangements of $V_HJ558$ family members to $J_H4$ reveals that LκΔ[Ei], LκΔ[MAR] and control mice are similar with respect to the accumulation of mutations in their endogenous heavy chain loci (8.7, 14 and 9.1 substitutions/kb respectively determined as described in Materials and methods).

Thus, both the Ei and, particularly, the MAR deletions effect a severe inhibition of the targeting of the transgene for hypermutation; it remains possible that, in addition, these same deletions also effect a small inhibition of mutation accumulation once the transgenic V gene has been targeted.

### *Removal of either E3′-core or E3′-flank diminishes hypermutation*

The E3′, which has also been shown to be important for mutational targeting (Betz *et al.*, 1994), is composed of a core enhancer region surrounded by a conserved flank, which can suppress the activity of the core in pre-B cell lines (Pongubala and Atchison, 1991; Meyer and Ireland, 1994). Transgenic mice were established to discriminate the relative roles of the core and flank in mutational targeting. Whilst again the different founder lines mutate to different extents, it is clear that deletion of either the core or the flank results in diminished targeting of hypermutation (Table I and Figure 2). As with the Ei and MAR deletions, whilst there is a decrease in the number of transgene copies that are targeted for mutation, those that are so targeted can still accumulate multiple nucleotide substitutions.

### *Deletions affect κ expression*

The expression of the various transgenes was monitored by flow cytometric analysis of splenic B cells using an anti-rat κ antibody, exploiting the fact that the $C_\kappa$ exon (but not the remainder) of the various transgenic constructs is of rat origin. Even amongst the B cells of a single transgenic mouse, there is heterogeneity with respect to transgene expression on the B-cell surface, with the transgenic κ being in competition with endogenous mouse κ in those cells in which κ gene expression is not allelically excluded (Figure 3). Whereas the Lκ construct expresses well in four independent lines with few spleen cells expressing endogenous mouse κ, this same dominance is not observed with the Lκ derivatives in which Ei, parts of E3′ or MAR have been removed (Table II). The level of transgene expression broadly correlates with the level of hypermutation, although the correlation is not a straightforward one.

In contrast to these flow cytometric results, however, no decrease in transgene expression effected by the Ei deletion is evident if expression is estimated by the

**Table I.** Mutation of the transgenes

| Mouse line | Clones | | Mutations total | Mutations/$10^3$ bp | |
|---|---|---|---|---|---|
| | All | Mutated | | All clones | Mutated clones |
| Lκ | | | | | |
| Line 3 | 76 | 53 | 267 | 12.4 | 20.0 |
| Line 6 | 73 | 54 | 262 | 12.7 | 17.2 |
| Line NG | 59 | 43 | 197 | 11.8 | 16.2 |
| Line WTM7 | 88 | 52 | 184 | 7.4 | 12.5 |
| LκΔ[Ei/MAR] | 75 | 10 | 16 | 0.8 | [a] |
| LκΔ[3′E] | 37 | 13 | 23 | 2.2 | 6.3 |
| LκΔ[MAR]S | | | | | |
| Line 1 | 42 | 2 | 2 | 0.2 | [a] |
| LκΔ[MAR]L | | | | | |
| Line 1 | 61 | 10 | 23 | 1.3 | 8.1 |
| Line 2 | 63 | 14 | 34 | 1.9 | 8.6 |
| Line 3 | 42 | 2 | 5 | 0.4 | [a] |
| LκΔ[Ei]S | | | | | |
| Line 1 | 75 | 19 | 48 | 2.3 | 9.0 |
| Line 2 | 60 | 20 | 49 | 3.0 | 8.7 |
| LκΔ[Ei]L | | | | | |
| Line 1 | 225 | 43 | 161 | 2.5 | 13.3 |
| LκΔ[E3′-Flank] | | | | | |
| Line 1 | 63 | 6 | 9 | 0.5 | [a] |
| Line 2 | 101 | 27 | 70 | 2.3 | 9.1 |
| LκΔ[E3′-Core] | | | | | |
| Line 1 | 98 | 25 | 41 | 1.5 | 5.8 |
| Line 2 | 42 | 13 | 19 | 1.7 | [a] |

[a]Too few mutated clones for meaningful calculation.
The Vκ segment of each transgene was cloned following PCR amplification from sorted germinal centre B cells. For each transgene, the table gives the total number of PCR clones sequenced, the number of those that carried one or more mutations within the V segment (282 bp), the total number of mutations identified and the mutation frequency. This frequency (point mutations per $10^3$ bp) is computed both with respect to all clones analysed and with respect to only those clones that carry mutations. For several transgenic lines, mutation was analysed in multiple individual animals. The variation in mutation rates between animals was lower than that found when comparing different mouse lines that carried the same transgene. The data for the Lκ, LκΔ[Ei/MAR] and LκΔ[3′E]transgenes are taken from Betz *et al.* (1994), González-Fernández and Milstein (1993), Yélamos *et al.* (1995) and Goyenechea and Milstein (1996). Only the unmodified Lκ transgenes in the NG and WTM7 mice are used in the computation.

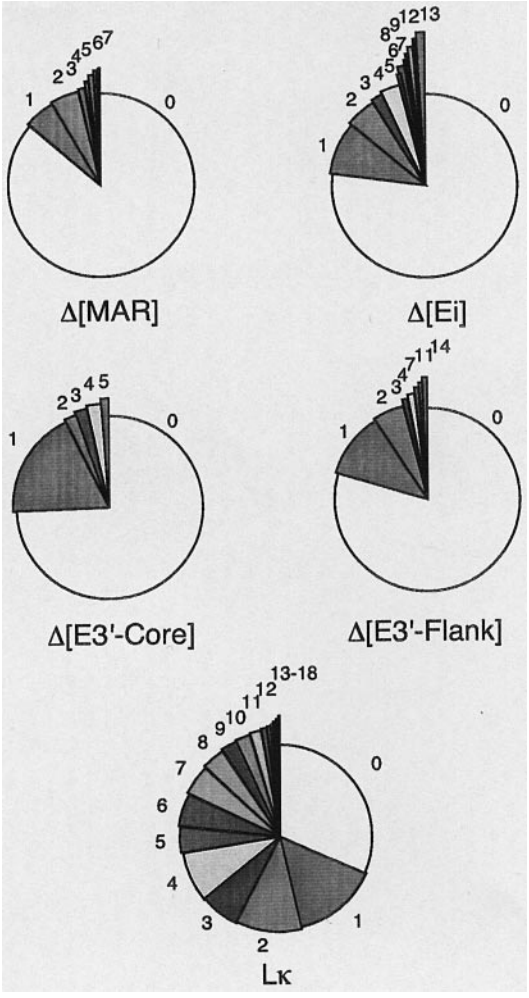


**Fig. 2.** Frequency distribution of clones with respect to the number of mutations they carry. In each pie chart, the size of each segment is a measure of the proportion of clones that carry the indicated number of mutations. For each type of transgenic construct (grouped simply as Lκ, Δ[MAR], Δ[Ei], Δ[E3′-Core] and Δ[E3′-Flank]), the results of the analysis of the individual animals presented in Table I are pooled together and presented as a single pie chart.

abundance of transgene mRNA in transgene-positive splenic B cell hybridomas (data not shown). This parallels previous observations with the LκΔ[Ei/MAR] transgene where the simultaneous removal of both Ei and MAR had little effect on the abundance of κ mRNA in hybridomas but led to a significant drop in transgene expression, as well as exclusion of endogenous κ expression as judged by flow-cytometric analysis of splenic B cells (Betz *et al.*, 1994; Meyer *et al.*, 1996). Thus, the flow-cytometric monitor of expression on the B-cell surface is a better correlate of hypermutability.

### *Most transgene mutations are found in those B cells strongly expressing the transgene*

Given this correlation between hypermutation and cell surface expression of the transgene, we wondered whether the targeting of transgene hypermutation in a particular mouse was more efficient in those cells which expressed the transgene highly than in those of the transgene-dull population. Fractionation of Peyer's patch B cells from LκΔ[Ei] and LκΔ[MAR] mice into populations that were either bright or dull for transgenic κ expression (TGκ$^{hi}$ and TGκ$^{lo}$) revealed that most mutated transgenes were found amongst the more brightly expressing cells (Figure 4). Although transgene hypermutation was much diminished in the transgene-dull population, the endogenous heavy chain locus was still at least as well mutated; the mutation frequency in the 3′-flank of $V_HJ558$ family member–$J_H4$ integrations was six substitutions/kb in the transgene-bright LκΔ[MAR] germinal centre B cell subpopulation and 16/kb in the transgene-dull subpopulation. Thus, the decreased efficiency of mutational targeting of the transgene in the transgene-dull subpopulation does not reflect any impairment of the hypermutation capability of the cells themselves; the somewhat increased mutation of the endogenous loci in these cells is a topic for further investigation.

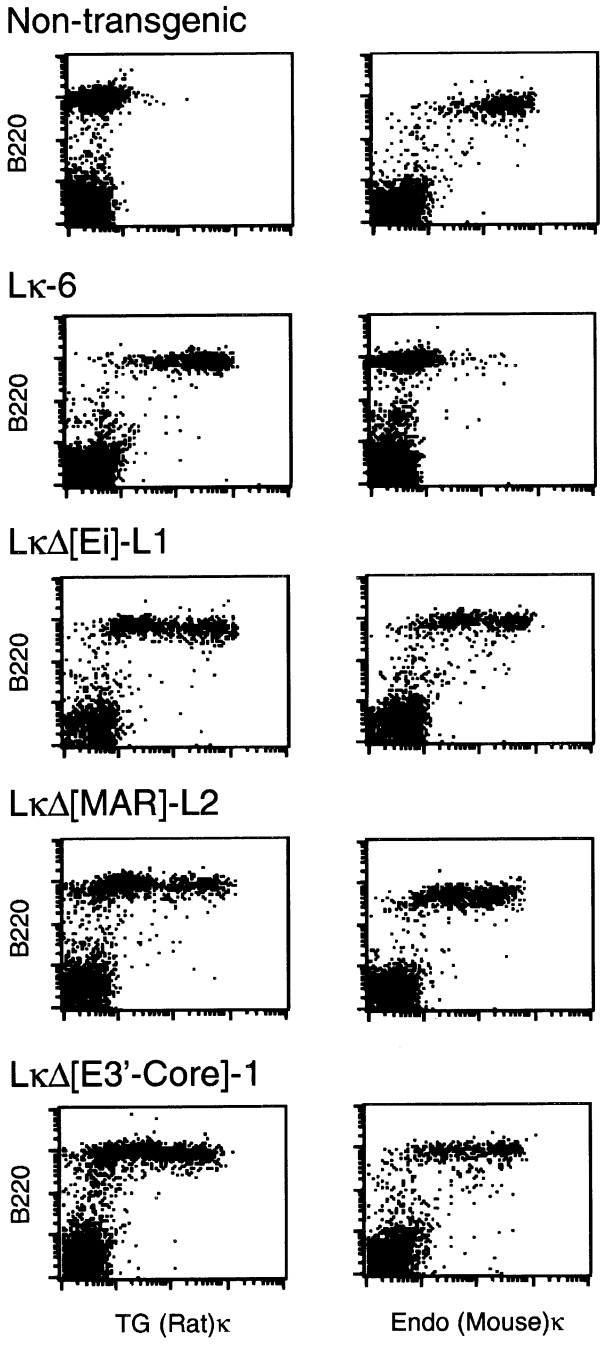


**Fig. 3.** Transgene expression. Expression was analysed by cytofluorimetric analysis staining of splenic B cells with phycoerythrin-conjugated anti-CD45R(B220) and either mouse (endogenous) or rat (transgenic) κ biotinylated mAbs and FITC-conjugated streptavidin.

**Table II.** Transgenic and endogenous κ chain expression

| Mouse line | Rat-κ | Mouse-κ | Mutations/ $10^3$ bp |
|---|---|---|---|
| Non-transgenic | 0 | 100 | – |
| Lκ | | | |
| Line 6 | 100 | 0 | 12.7 |
| Line NG | 95 | 5 | 11.8 |
| Line WTM7 | 94 | 11 | 7.4 |
| tLκΔ[Ei/MAR] | 55 | 41 | 0.8 |
| LκΔ[E3′] | 4 | 72 | 2.2 |
| LκΔ[MAR]S | | | |
| Line 1 | 8 | 91 | 0.2 |
| LκΔ[MAR]L | | | |
| Line 1 | 25 | 54 | 1.3 |
| Line 2 | 25 | 61 | 1.9 |
| Line 3 | 70 | 14 | 0.4 |
| LκΔ[Ei]S | | | |
| Line 1 | 59 | 42 | 2.3 |
| Line 2 | 70 | 29 | 3.0 |
| LκΔ[Ei]L | | | |
| Line 1 | 45 | 53 | 2.5 |
| LκΔ[E3′-Flank] | | | |
| Line 1 | 17 | 69 | 0.5 |
| LκΔ[E3′-Core] | | | |
| Line 1 | 28 | 42 | 1.5 |
| Line 2 | 4 | 96 | 1.7 |

Igκ expression on the surface of splenic B cells was determined by cytofluorimetry on at least two animals for each transgenic line. The values (mean κ fluorescence on $B220^+$ cells) are normalized with respect to Lκ6 transgenic and non-transgenic litter mates, giving these control lines values of 100 and 0 for transgenic/endogenous κ expression as indicated. Hypermutation frequencies, taken from Table I, are shown for comparison.

## Discussion

### *MAR and E3′-flank are essential for full hypermutation*

The results described here further link hypermutation and expression. Previous results have shown the importance of the Ei/MAR and E3′ regions for immunoglobulin κ hypermutation (Betz *et al.*, 1994). The dissection of the contributions of the core enhancers and their flanks reveals that the flanks are also essential for the proper recruitment of hypermutation. Indeed, the effect of the MAR deletion is even more pronounced than that of Ei removal.

The critical importance of the MAR was not anticipated. Whilst originally defined by an *in vitro* nuclear matrix binding assay (Cockerill and Garrard, 1986), no major functional importance for the MAR is apparent from a deletion analysis of the κ intronic enhancer performed using transfection into lymphoid cell lines (Queen and Stafford, 1984). This might, in part, reflect an inadequacy of transfection assays in revealing all sequences that support enhancer activity. Thus, the region flanking the core of the IgH intronic enhancer [which also possesses MAR activity (Cockerill *et al.*, 1987)] was found to be critical for the expression of IgH transgenes although dispensable for the activity of the enhancer in cell line transfection assays (Forrester *et al.*, 1994). An ability of Ei/MAR and E3′ to partially cross-substitute for each other in some functions, as suggested by *in vivo* gene targeting experiments (Gorman *et al.*, 1996; Xu *et al.*, 1996), might explain the fact that we can detect considerable residual κ transgene expression after deletion of the intronic MAR.

With regard to the requirement for the E3′ flanking region, this region (like that flanking the IgH intronic enhancer), whilst having no enhancer activity of its own, has been shown to be able to regulate the activity of a linked enhancer in appropriate cell types (Imler *et al.*, 1987; Scheuermann and Chen, 1989; Pongubala and Atchison, 1991; Meyer and Ireland, 1994). It will

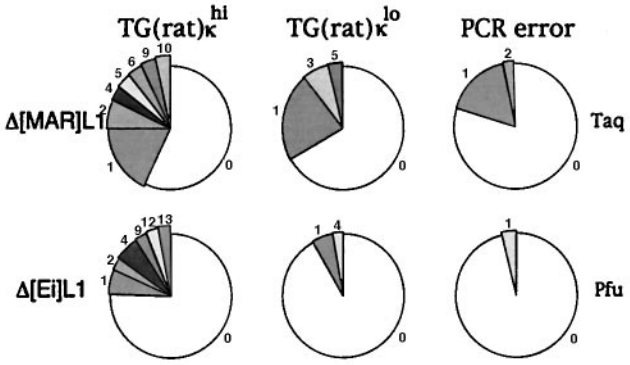


**Fig. 4.** Transgene hypermutation in germinal centre B cells that are bright or dull for transgene expression. Peyer's patches germinal centre B-cells [(CD45R)B220$^+$, PNA$^{hi}$] were fractionated further into subpopulations staining brightly (TG(rat)κ$^{hi}$) and dully (TG(rat)κ$^{lo}$) for rat κ; transgene hypermutation was analysed by PCR. Endogenous mutation of the region flanking the 3′ side of $V_H$J558 family member–$J_H$4 integrations was determined as described by Jolly *et al.* (1997) using DNA from the fractionated LκΔ[Ei] cell populations (see text). Amplification of the transgenic V gene was performed using *Taq* polymerase for LκΔ[MAR] and *Pfu* polymerase for LκΔ[Ei]. Pie charts are included to demonstrate the background error rate due to PCR alone, using these two polymerases as determined using cloned hybridoma controls.

obviously be interesting to ascertain whether the E3′ flanking region also displays MAR activity.

### *Correlation between hypermutation and transcription*

Tinkering with enhancer elements and their flanks gave rise to transgenes which showed significant founder-to-founder variation in behaviour; this is presumably due to position effects which are minimized when the transgenes contain their full complement of *cis*-regulatory elements. Nevertheless, the experiments reveal a broad correlation between the elements required for transgene hypermutation and those required for expression, with the removal of a single *cis*-element usually having a more dramatic effect on hypermutation than transcription.

This broad correlation between expression and mutation is particularly evident when expression is monitored by flow cytometric analysis of transgene expression on the surface of splenic B cells rather than by Northern blot analysis of mRNA accumulation in splenic hybridomas. Thus, whereas removal of Ei and/or MAR from the transgene significantly diminished κ expression in the flow cytometric assay, removal of Ei/MAR or MAR alone had relatively little effect on the accumulation of immunoglobulin κ mRNA in terminally differentiated plasma cells (Xu *et al.*, 1989; Meyer *et al.*, 1990, 1996; Betz *et al.*, 1994; data not shown). This contrast probably reflects that whereas Ei/MAR may regulate κ expression at the surface Ig$^+$ B cell stage of development, κ expression in terminally differentiated antibody-secreting cells may be mainly under the control of E3′ (Meyer *et al.*, 1990, 1996; Roque *et al.*, 1996).

A quite distinct pointer to the correlation between transcription and hypermutation came from analysing Lκ-derived transgenes which did not fully suppress endogenous κ rearrangement. Here we found that germinal centre B cells could be fractionated into a subpopulation that stained brightly and one that stained dully for transgene expression. Significantly more of the mutated transgenes were found in the brightly staining subpopulation than in the transgene-dull subpopulation, even though the hypermutation mechanism was fully effective (as judged by mutations at the endogenous IgH locus).

### *How might hypermutation be linked to transcription?*

The results throw new light on the link between hypermutation and transcription. The data suggest that the reduction in hypermutation is more in the targeting of the defective transgene copies for mutation rather than in the extent of mutation accumulation once targeted. Indeed, what seems to be affected is the probability of a particular transgene copy becoming committed to the hypermutation pathway. Reduced mutational targeting not only correlates broadly with reduced overall expression but, more significantly, those B cells within the germinal centres of a single mouse that stain brightly for transgene expression are more likely to have targeted the transgene for mutation.

Nevertheless, the correlation is not straightforward. In some transgenic mice (such as LκΔ[MAR] line L3), *cis*-element removal has relatively little effect on expression (possibly owing to integration position effects) but there is a substantial reduction in hypermutation. This, taken together with the fact that it is mutational targeting (rather than the accumulation of mutations once targeted) that is affected by *cis*-element removal, leads us to put forward a model in which, whilst mutational targeting is linked to transcription initiation, the recruitment of hypermutation is critically dependent on the precise nature and quality of the transcription initiation complex. Thus, removal of a *cis*-element is likely to affect the nature of the transcription initiation complex; the change in this complex could then differentially affect the recruitment of the hypermutation machinery as opposed to the recruitment of RNA polymerase II.

The dependence of hypermutation on the immunoglobulin enhancers as well as the fact that the mutation domain is located downstream of the promoter has led us and others to propose that some form of transcription-coupled repair (Hanawalt, 1995) could form the basis of hypermutation (Neuberger and Milstein, 1995; Peters and Storb, 1996). To account for the results presented here, we propose a model (Figure 5) in which a hypermutating priming factor (HPF), specific to hypermutating B cells, can be recruited during assembly of the immunoglobulin gene transcription initiation complex. HPF recruitment is viewed as being effected by the particular constellation of transcription factors forming the initiation complex but is not an all or nothing event. Most complete initiation or modified complexes will recruit HPF, but a small proportion may not. In contrast, incomplete initation complexes (which will form as a consequence of *cis*-regulatory element disruption and/or positional effects), whilst often able to bring in RNA polymerase, will be impaired though not wholly incompetent at HPF recruitment. The recruitment of HPF then leads to hypermutation on polymerase halting, possibly through the involvement of a specific error-prone repair, as discussed in the legend to Figure 5.

Many studies have indicated that, when V genes are subjected to hypermutation, relatively small numbers (e.g.

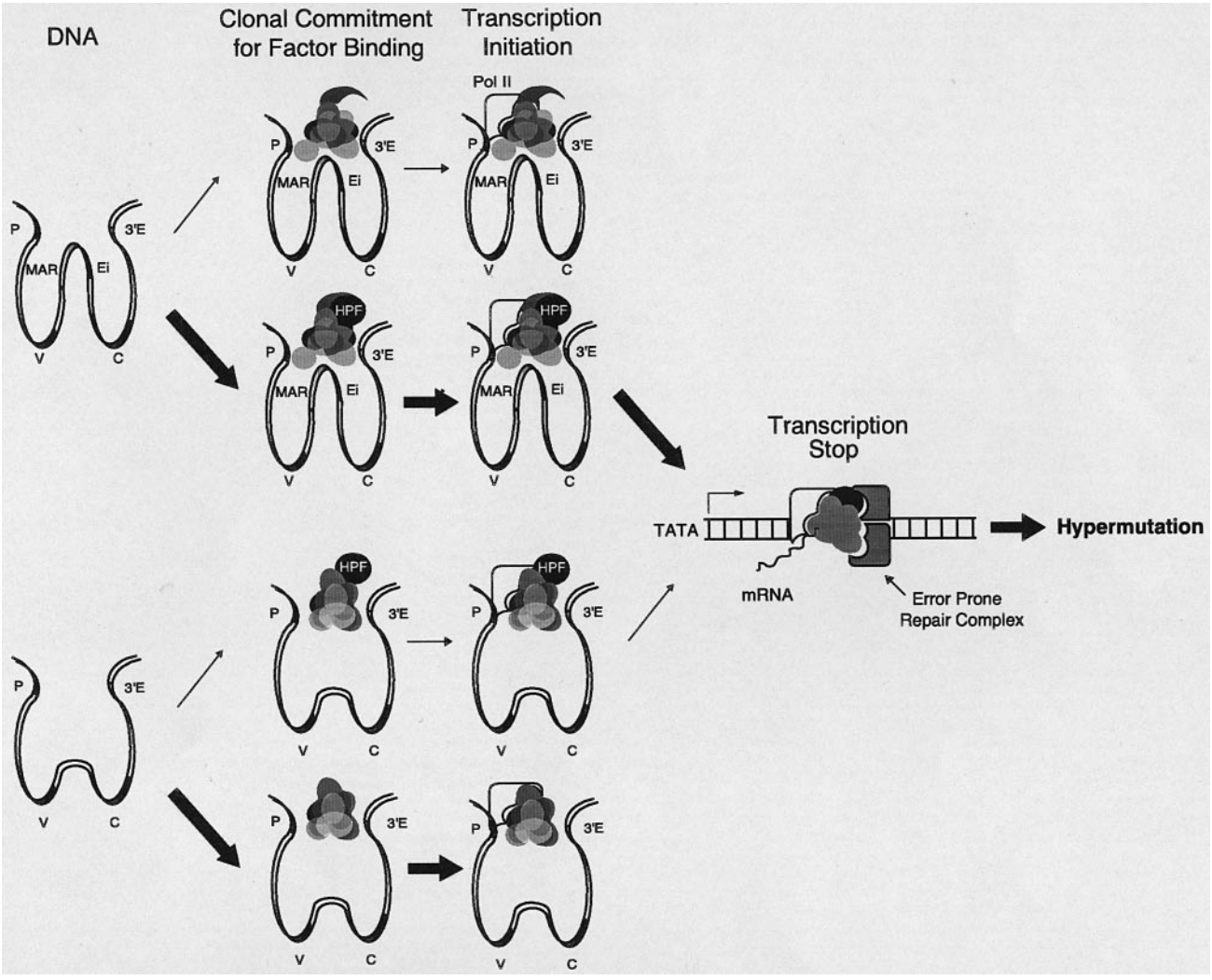


**Fig. 5.** Model for immunoglobulin gene hypermutation. Hypermutation is envisaged as being effected by the recruitment of hypermutating primer factor (HPF) to the RNA polymerase II transcription initiation complex. HPF recruitment is a stochastic event (illustrated with thick and thin arrows) which can be clonally maintained. Thus, a transgene retaining the full complement of transcription regulatory elements is likely (but not certain) to form a complete transcription initiation complex that will recruit HPF; in contrast, a transgene lacking Ei/MAR is more likely to form a partial transcription initiation complex which, whilst able to recruit RNA polymerase II, has a diminished probability of recruiting HPF. We envisage that HPF either accompanies the polymerase during transcription elongation or modifies the elongation complex with hypermutation ensuing, either because there is increased gratuitous polymerase stalling and subsequent transcription-coupled repair (as proposed by Peters and Storb, 1996) or (as illustrated here) because, on stalling, a specific error-prone repair complex is recruited.

one to four) mutations are introduced in a single cell cycle [see, for example, Clarke *et al.* (1985) as well as papers cited by Kepler and Perelson (1993)]; V genes that have accumulated large numbers of nucleotide substitutions have achieved this through being subjected to mutation in sequential cell cycles. Bearing this in mind, then the fact that the mice transgenic for the poorly targeted Lκ deletion derivatives nevertheless contain transgenic V genes carrying large numbers of nucleotide substitutions implies that, once a particular transgene is targeted for mutation, it might well remain marked as hypermutation-accessible through subsequent cell divisions. For these reasons, we propose that HPF recruitment manifests some clonal stability. The mechanism of maintaining such stability of association is, of course, a matter for speculation; however, it may be significant that there is substantial overlap between the elements that we find here to be required for full mutation and those that have been identified as playing a role in demethylation (Lichtenstein *et al.*, 1994).

The analysis suggests that there may be stochastically determined heterogeneity in the extent to which the immunoglobulin genes in different B-cell clones [or, indeed, different genes or transgene copies within the same cell (Rogerson *et al.*, 1991; Yélamos *et al.*, 1995)] are targeted for mutation. In both the Peyer's patch germinal centre B-cell population and the antigen-selected B cells from hyperimmune mice, a significant proportion of clones (~20%) have not targeted their endogenous immunoglobulin genes for mutation. This could reflect a strategy for maintaining a B-cell population in which one immunoglobulin locus has been targeted, but not the other, or for preserving an unmutated memory B-cell pool. Whilst these proposals are clearly speculative, our data do suggest that the *recruitment* of mutation is intimately linked to the *recruitment* of polymerase II transcription complexes, but with hypermutation being more fastidious in its requirement for the recruitment of a complete complex.

## Materials and methods

### *Transgenes and transgenic mice*

The transgenes are all directly based on Lκ (Sharpe *et al.*, 1991) except for LκΔ[Ei]Line L, LκΔ[MAR]Line S, LκΔ[E3′-Flank] and LκΔ[E3′-Core], which are based on the Lκ derivative LκΔB (Yélamos *et al.*, 1995). The long (L) and short (S) deletions of both Ei and MAR were created individually working with either (i) a *Hin*dIII–*Hpa*I subclone of Lκ in Bluescript and using PCR to create the specific deletions or (ii) *Sac*II–*Hpa*I subclones of LκΔ[Ei/MAR] in which the Ei/MAR has been deleted and replaced by a *Hin*dIII site (Betz *et al.*, 1994), and using PCR to reintroduce either Ei or MAR alone as *Hin*dIII fragments. The variant Ei/MAR subclones were then resected back into Lκ or LκΔB as indicated in the legend to Figure 1. Using the numbering of Max *et al.* (1981), which places the first A of the intronic *Hin*dIII site at position 3395, the sequences across the deletion borders are: ΔEiLineL [CTACTT$^{3757}$//$^{4136}$AAGGCC], using primers MAR1 (5′-TATTAAAAG-CTTAATGTATATTAATC-3′) and MAR2 (5′-TGACTCTTAAGTAGTT-TCAAGAGTT-3′); ΔEiLineS [CAATTC$^{3818}$/GAAGCT/$^{4089}$GAATTG], using the primers LuLu7 (5′-CCCTTGCTCCGCGGGAACCACTTT-CCTGAG-3′) and MJS195 (5′-CGGAAGCTTCGAATTGACATCATT-TTAAATTAAAAG-3′); ΔMARLineS [AAGCTT$^{3400}$//$^{3692}$TTGTGT], amplified with IE1 (5′-TTTATAAGCTTTTGTGTTTGACCC-3′), and RCKN127 (5′-IIIIIIGCGGCCGCGACTGWGGCACCTCCAG-3′) and ΔMARLineL [AAGCTT$^{3400}$/C/$^{3842}$GAAAGG], amplified with MSN196 (5′-CGGAAGCTTCGAAAGGCTGCTCATAATTCTA-3′); and MSN197 (5′-CGGAAGCTTAAGCCAGGGTCTGTATTTG-3′). This last construct also contains a direct tandem duplication of nucleotides 4089–4140 as a consequence of the insertion of the PCR fragment into LκΔ[Ei/MAR]; underlined letters represent nucleotides inserted at the deletion borders.

For LκΔ[E3′-Flank], the core region of E3′ (extending from $^{378}$GAG-TGT to GCCTGG$^{515}$, nucleotide numbering according to the sequence in Meyer and Neuberger, 1989) was PCR amplified with the oligonucleotides Dino1 (5′-TTATCTCGAGTGTCCCAGTGACCAA-3′) and Dino2 (5′-GGAGTGCGGCCGCCAGGCTGTTGGAGG-3′) as an *Xho*I–*Not*I fragment and substituted between the unique *Xho*I and *Not*I sites of LκΔB. For LκΔ[E3′-Core], the deletion was created on an *Xho*I–*Not*I subclone by religating between blunted *Nco*I and *Bsp*MI sites with the sequence across the deletion border being CCCATG$^{390}$//$^{568}$TACCCC.

Transgenic mice were established by microinjection to (C57BL/6×CBA)F1 zygotes. Founders (identified by tail blotting and serum enzyme-linked immunosorbent assay) were bred against F1s, and transgene copy numbers estimated by Southern blotting (10 copies, Δ[MAR]S1, Δ[E3′-Flank]2 and Δ[E3′-Core]1; eight copies, Δ[MAR]L2 and Δ[Ei]L1; six copies, Δ[MAR]L1 and Δ[E3′-Core]2; four copies, Δ[Ei]S1; three copies, Δ[Ei]S2; two copies, Δ[MAR]L3 and Δ[E3′-Flank]1).

### *Monitoring hypermutation*

Hypermutation was assessed by sequencing the transgenic $V_\kappa$Ox-1 after PCR amplification from PNA$^{hi}$B220$^+$ germinal centre B cells that had been sorted from pooled Peyer's patches of 4- to 6-month-old animals as previously described (Gónzalez-Fernández and Milstein, 1993). Hybridization with a transgene-specific oligonucleotide (Betz *et al.*, 1994) was used to confirm the transgenic origin of the cloned $V_\kappa$Ox-1 genes. Mutation of the endogenous IgH locus was monitored by determining the sequence of the region flanking the 3′ border of $J_H4$ rearrangements of $V_HJ558$ family members following PCR amplification as descibed elsewhere (Jolly *et al.*, 1997). Sorting of germinal centre B cells of LκΔ[Ei] and LκΔ[MAR] into subpopulations staining brightly and dully for transgenic (rat) κ was performed by pre-incubation with biotinylated anti-rat κ monoclonal antibody (R6.7/9.1; Springer *et al.*, 1982) in the presence of 2% mouse serum prior to washing and staining for peanut agglutinin (PNA) and CD45R(B220) as previously described (Gónzalez-Fernández and Milstein, 1993) but in the presence of Red670-conjugated streptavidin (Gibco).

### *Monitoring expression*

Flow cytometric assay of transgenic and endogenous mouse κ expression on splenic B cells was performed by staining with a biotinylated monoclonal antibody against either mouse (187.1; Yelton *et al.*, 1981) or rat κ chains followed by washing and incubation with phycoerythrin-conjugated RA3-6B2(Gibco) anti-CD45R(B220) antibody and fluorescein isothiocyanate (FITC)–streptavidin.

## Acknowledgements

We thank John Jarvis and Sarah Davies for derivation and testing of the transgenic mice. N.K. is a graduate student of the Institute for Biology II, University of Freiburg and supported by an EC training grant. We are grateful to the National Foundation for Cancer Research and the Association for International Cancer Research (for grant support to C.M.) and the Howard Hughes Medical Institute (for an International Research Scholar's award to M.S.N.).

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*Received on February 24, 1997; revised on March 26, 1997*