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Rapid methods for the analysis of immunoglobulin gene hypermutation: application to transgenic and gene targeted mice

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ABSTRACT

Hypermutation of immunoglobulin genes is a key process in antibody diversification. Little is known about the mechanism, but the availability of rapid facile assays for monitoring immunoglobulin hypermutation would greatly aid the development of culture systems for hypermutating B cells as well as the screening for individuals deficient in the process. Here we describe two such assays. The first exploits the non-randomness of hypermutation. The existence of a mutational hotspot in the Ser31 codon of a transgenic immunoglobulin V gene allowed us to use PCR to detect transgene hypermutation and identify cell populations in which this mutation had occurred. For animals that do not carry immunoglobulin transgenes, we exploited the fact that hypermutation extends into the region flanking the 3′-side of the rearranged J segments. We show that PCR amplification of the 3′-flank of VDJ_H rearrangements that involve members of the abundantly-used V_HJ558 family provides a large database of mutations where the germline counterpart is unequivocally known. This assay was particularly useful for analysing endogenous immunoglobulin gene hypermutation in several mouse strains. As a rapid assay for monitoring mutation in the J_H flanking region, we show that one can exploit the fact that, following denaturation/renaturation, the PCR amplified J_H flanking region DNA from germinal centre B cells yields mismatched heteroduplexes which can be quantified in a filter binding assay using the bacterial mismatch repair protein MutS [Wagner *et al.* (1995) *Nucleic Acids Res.* 23, 3944–3948]. Such assays enabled us, by example, to show that antibody hypermutation proceeds in the absence of the p53 tumour suppressor gene product.

INTRODUCTION

Localised hypermutation of immunoglobulin V genes plays a key role in antibody diversification, contributing to the affinity maturation of antibodies in mouse and man as well as to the generation of the primary antibody repertoire in sheep. The hypermutation is targeted to a 1–2 kb region of DNA that includes the V gene exon with nucleotide substitutions being introduced at a rate of ~10^{-3} mutations per base pair per cell cycle. The process is restricted to a narrow window of B cell development and takes place in germinal centres. Its mechanism is unknown, although transgenic studies have identified some of the *cis*-acting sequences necessary for its recruitment to the V gene (reviewed in 1–4).

Hypermutation in antigen-specific responses has classically been analysed by cloning the expressed V genes from sorted (or immortalised) antigen-specific B cells and comparing the sequences of these V genes to those of their presumed germline counterparts. More recently, larger databases of mutations have been obtained by PCR cloning the expressed immunoglobulin V genes (or transgenes) from total sorted germinal centre B cells. Whilst such studies have provided valuable information on the extent and distribution of immunoglobulin hypermutation, the process of cloning and sequencing large numbers of V genes is laborious when simply used to quantify the extent of V gene hypermutation. Sensitive methods of quantification that allow a rapid throughput of a large number of samples could greatly facilitate the development of culture systems for B cells performing hypermutation. Such methods could also prove of use in screening for individuals deficient in antibody hypermutation.

Here, we describe methods that can be used in transgenic and non-transgenic mice to rapidly quantify immunoglobulin hypermutation and to enrich for hypermutated sequences.

MATERIALS AND METHODS

Transgenes and mouse lines

Transgenic mice were maintained by breeding with (C57BL/6×CBA) F1s. The Lκ6 mice carry six copies of the Lκ transgene (Fig. 1), which comprises a functional mouse $V_κOx1$-$J_κ5$ rearrangement linked to rat $C_κ$, and the κ intronic and 3′ enhancers which are both required for efficient hypermutation (5,6). This light chain is characteristic of antibodies produced by BALB/c mice in the primary response to the hapten 2-phenyl-5-oxazolone (7). The LκΔI, LκΔE and LκTr mice carry two to five copies of transgenes derived from Lκ by deleting sequences around the intronic enhancer, in the intronic enhancer, or by transposing the intronic enhancer, respectively. All of these alterations cause the V gene to be targeted less efficiently for

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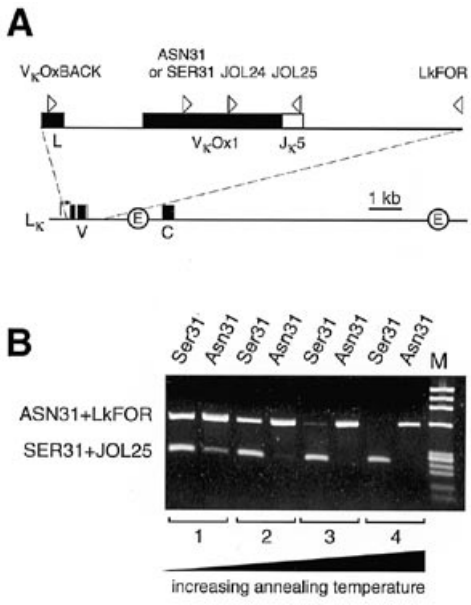

Figure 1. Hotspot-specific PCR. (**A**) Priming positions of κ oligonucleotides. The map of Lκ is shown together with a blow-up of the V region. The transcription start site is depicted by an arrow and the enhancers by an encircled E. The oligonucleotides prime from nucleotide positions 41 (VκOxBACK), 327 (ASN31/SER31), 422 (JOL24, which was 5′-biotinylated), 528 (JOL25, which was 5′-biotinylated) and 859 (LkFOR), with nucleotide position 1 being at the initiator ATG codon. (**B**) Optimisation of primer annealing temperatures to achieve specific amplification using *Taq* DNA polymerase. Plasmids containing V_κOx1 genes with wild-type (Ser) or mutated (Asn) codons at position 31 were used as templates. The primers used are indicated on the left. The ASN31 and SER31 reactions were carried out separately, then combined for gel electrophoresis. Annealing temperatures for SER31+JOL25 reactions were (1) 63°C, (2) 65°C, (3) 67°C and (4) 69°C. Annealing temperatures for ASN31+LkFOR reactions were (1) 58°C, (2) 61°C, (3) 64°C and (4) 67°C. Thirty-five PCR cycles were used.

mutation than it is in Lκ transgenes and will be described in detail elsewhere (Goyenechea,B., Klix,N., Yelamos,J., Williams,G., Riddell,A., Neuberger,M.S. and Milstein,C., submitted; Klix,N., Jolly,C., Brüggemann,M., Williams,G. and Neuberger,M.S., in preparation). The p53- and CD22-deficient mice have been described elsewhere (8,9) and were kindly provided by Isabelle Lavenir and Theresa O'Keefe (MRC Laboratory of Molecular Biology, Cambridge), respectively.

Extraction of genomic DNA

Cell suspensions from mouse thymus, spleen and Peyer's patches were made by forcing through a cell sieve in culture medium. Germinal centre B cells ($B220^+PNA^{hi}$) and non-germinal centre B cells ($B220^+PNA^{lo}$) were purified by flow cytometry from Peyer's patch cell suspensions (10). DNA was extracted from cells that had been lysed by freeze-thawing in water (~10 µl/10^5 cells) and incubated with proteinase K (100 µg/ml; Boehringer Mannheim) in *Pfu* DNA polymerase PCR buffer (Stratagene) at 56°C for 1 h. The proteinase K was then inactivated by heating at 95°C for 30 min.

Monitoring hypermutation by DNA sequencing

Transgenic V sequences were amplified using 'touchdown' PCR (11), cloned and sequenced as previously described (10), except that *Pfu* DNA polymerase (Stratagene) was used. Clones were confirmed to contain transgenic (not endogenous) V_κOx1 sequences by oligonucleotide hybridisation (6). For monitoring mutation at the endogenous IgH locus, a 1.2 kb segment from the 3′-flank of rearranged endogenous V_H genes was amplified using a primer complementary to a sequence common to FR3 of most V_HJ558 family members (GGA ATT CGC CTG ACA TCT GAG GAC TCT GC) together with a primer complementary to a sequence located at the 3′-end of the IgH intronic enhancer (GAC TAG TCC TCT CCA GTT TCG GCT GAA TCC). M13 plaques were positively selected for J_H4 rearrangements by hybridisation with oligonucleotide JH4 (TAT GCT ATG GAC TAC TGG) and negatively selected by hybridisation with oligonucleotide JH3 (CCT GGT TTG CTT ACT GG). Sequencing reactions were primed with oligonucleotide JH4 to facilitate alignment and mutation identification.

Asn31-specific PCR

Reaction mixtures (45 µl) contained *Taq* PCR buffer (Promega), 0.2 mM dNTPs, 0.2 µM each of LkFOR (11) and either ASN31 (GCG GCA TGC CAG CTC AAG TGT AAA) or SER31 (GCG GCA TGC CAG CTC AAG TGT AAG) oligonucleotides and genomic DNA corresponding to ~10^4 cells. The last three 3′ bases in the ASN31 and SER31 oligonucleotides were joined by phosphorothioate linkages to prevent degradation by 3′-exonucleases (12,13). Since the modification step that produces the phosphorothioate linkages is very efficient (12 and data not shown), the primers were used without purification. After overlaying the reaction mixtures with oil and heating to 90°C, *Taq* DNA polymerase (1.25 U; Promega) in 5 µl 1× *Taq* PCR buffer was added ('hot start') and 45 (unless otherwise stated) thermal cycles performed [94°C for 30 s, 67°C (unless otherwise stated) for 30 s, 72°C for 40 s]. Reactions to control for transgene abundance used the LkFOR/VkOXBACK primers (10). Reaction products were detected, following electrophoresis, by Southern blotting with a V_κOx1 probe.

We found that *Taq* DNA polymerase works better than *Pfu* and *Vent* (New England Biolabs) DNA polymerases in discriminating between wild-type and mutant templates (data not shown). The reason for this is not clear but it was not necessarily due to 3′-exonuclease activity in the *Pfu* and *Vent* DNA polymerases because the primers used were exonuclease resistant (see above) and because engineered exo$^-$ *Vent* DNA polymerase (14) produced results no different from wild-type *Vent* (data not shown).

In some cases, a prior amplification of transgenic sequences was performed using LkFOR and VkOxBACK primers for 20 cycles of PCR with *Pfu* DNA polymerase (94°C for 1 min, 60°C for 1 min, 72°C for 2 min). The products were purified using a Qiaquick PCR purification kit (Qiagen) into a volume of 100 µl TE buffer (15). Ten µl of the primary PCR products were then used as template for the secondary ASN31 or SER31 PCR reactions (25 cycles, unless otherwise indicated) which were essentially as in the previous paragraph.

MutS-binding assays

The biotinylated DNA substrates for the MutS binding assays were generated by PCR. For analysis of transgenes, a 155 bp segment of V_κOx1/J_κ5 was amplified using 'touchdown' PCR (11), *Pfu* DNA polymerase and a maximum of 30 PCR cycles, with primers JOL24 (biotin-CTT CTG CAG TCC CTG CTC

GCT TCA) and JOL25 (biotin-GCT CAA GCT TGG TCC CAG CAC CGA).

For the analysis of endogenous genes, sequences in the J_H1–J_H2 spacer region were amplified under similar conditions using primers JOL34 (biotin-GGA ATT CTA CTG GTA CTT CGA TGT CTG G) and JOL35 (biotin-CAG GCA GCT AGC CTC TGA CTG). However with $p53^{-/-}$ mice, polymorphisms in this region interfered with the MutS assay (data not shown); therefore sequences 3′ to J_H4, in a region where we have not detected polymorphism, were amplified with primers JOL40 (biotin-GTG AAT TCT ATG CTA TGG ACT ACT GG) and JOL29 (AGA TGT GGA GAT AAT CTG TCC).

After purification using a Qiaquick kit (Qiagen), the PCR products in 10 mM Tris–HCl, pH 7.4, 0.1M NaCl were denatured/renatured by heating to 100°C for 3 min, then 75°C for 90 min before chilling to 4°C. For MutS assays (16), a sheet of nitrocellulose (0.45 µm pore size, Schleicher and Schuell) and two sheets of underlying Whatman 3MM paper were pre-soaked in MutS buffer (16) for 5 min and assembled in a slot blotter (Stratagene). After removing excess liquid by suction, slots were incubated with MutS protein (20 µl at 25 ng/µl; Genecheck Inc., Fort Collins, CO) for 20 min, blocked with 200 µl 3% BSA/MutS buffer for 30 min and the solution pulled through the slots by suction. Aliquots (20 µl) of the heteroduplexed PCR products in 3% BSA/MutS buffer were then added to the slots and incubated with the immobilised MutS for 20 min. After five washes with 150 µl MutS buffer each, horseradish peroxidase-conjugated streptavidin [100 µl (Pierce) diluted 1:4000 in 3% BSA/MutS buffer] was added to the washed slots and left for 20 min. After washing as before, the nitrocellulose was removed from the slot apparatus, washed five more times with 50 ml MutS buffer and the MutS-bound DNA detected by enhanced chemiluminescence (ECL, Amersham).

For control blots, aliquots (10 µl) of the heteroduplexed PCR products diluted in MutS buffer were added to 400 µl 20× SSC (15), blotted directly onto nitrocellulose and fixed by cross-linking with 120 mJ of UV light in a Stratalinker (Stratagene). After blocking with 5% (w/v) skim milk powder in PBS for 1 h, and washing 4×5 min with PBS, the bound biotin–DNA was detected using horseradish peroxidase conjugated streptavidin and ECL.

Enrichment for mutated DNA using MutS

Self-heteroduplexed DNA amplified from germinal centre B cells by 28 PCR cycles with JOL24 and JOL25 primers was used in an immobilised MutS assay. The nitrocellulose with the MutS-bound DNA was excised, immersed in 10 µl 1× *Eco*RI digestion buffer (New England Biolabs) containing 100 µg/ml proteinase K and overlaid with mineral oil (Sigma). After incubation at 55°C for 10 min, proteinase K was heat inactivated at 95°C and the DNA renatured at 75°C for 90 min before chilling to 4°C. Following centrifugation at 16 000 *g* for 10 min, eluted DNA (2 µl aliquots of the supernatant) was further amplified using seven PCR cycles with JOL24/JOL25. The Qiaquick purified product was cloned into M13mp19 for sequencing. Sequencing of the same region of DNA directly amplified by 35 PCR cycles was used as a control.

RESULTS

Transgene mutation identified by hotspot-specific PCR

The Lκ transgene efficiently hypermutates in germinal centre B cells with the second base of the serine-31 codon of the transgenic V region being a mutational hotspot (5,10,17). Thus, in ~10% of all mutated Lκ transgenes, a transition mutation has altered the Ser31 codon from AGT to AAT (Asn) (18). Since *Taq* DNA polymerase is sensitive to 3′-terminal A:G mismatches in primers (19) we reasoned that a PCR primer specific for the Asn31 mutation might be used to give a PCR product only when the transgene was mutated at this hotspot. Sense primers ending with either a Ser31 G (SER31) or Asn31 A (ASN31) were synthesized. The last three 3′-bases in these oligonucleotides were joined by phosphorothioate bonds to prevent their degradation by 3′-exonucleases (12,13) which could lead to priming from the wrong templates. These SER31 or ASN31 oligonucleotides were then paired with downstream antisense oligonucleotides and used to amplify the $V_κOx1$ gene from plasmid templates that included either the wild-type $V_κOx1$ or a mutated derivative that contained an Asn codon at position 31 (Fig. 1A). At appropriately stringent annealing temperatures, the ASN31 oligonucleotide would only prime PCR amplification from the mutant and not the wild-type template; a complementary discrimination was achieved with the SER31 oligonucleotide (Fig. 1B). A homology length of 18 bases was optimal for both SER31 and ASN31 primers. Shorter primers gave a much narrower range of annealing temperatures in which wild-type and mutant templates could be discriminated, whereas longer primers could not discriminate at annealing temperatures below 72°C (data not shown).

To test whether hotspot-specific PCR could correctly detect transgene mutation in genomic mouse DNA (as opposed to cloned plasmid samples), we prepared DNA from thymus, spleen and Peyer's patch of Lκ transgenic mice. For each DNA sample, separate PCR reactions were set up to amplify both mutated (ASN31 and LκFOR primers) and total (VκOxBACK and LκFOR primers) Lκ transgenes. Equal aliquots of the two reactions were then pooled, run together in agarose gels and the products detected by blotting (Fig. 2A). The DNA samples from Peyer's patches (which contain many highly mutated germinal centre B cells, 10) gave a strong band in the ASN31 PCR reactions. Spleen, which contains relatively few mutated (memory) B cells gave weak and rather variable ASN31 signals and thymus, which is not expected to contain mutated B cells, gave barely detectable ASN31 products (Fig. 2A). Germinal centre B cells stain brightly with peanut agglutinin and therefore, as predicted, ASN1 PCR bands were obtained from PNA^{hi} (but not the PNA^{lo}) B cell sub populations from Peyer's patches.

Deleting or modifying intronic sequences in Lκ-derived constructs can reduce their efficiency as targets for hypermutation (5,6; Goyenechea *et al.*, submitted; Klix *et al.*, in preparation). As expected, such poorly mutating transgenes gave much weaker signals in ASN31 PCR reactions (Fig. 2B).

Hotspot-specific PCR selectively amplifies mutated sequences

These results indicate that PCR with the ASN31 oligonucleotide should selectively amplify mutated Lκ transgenes. To confirm this, ASN31 and SER31 products were amplified in parallel from

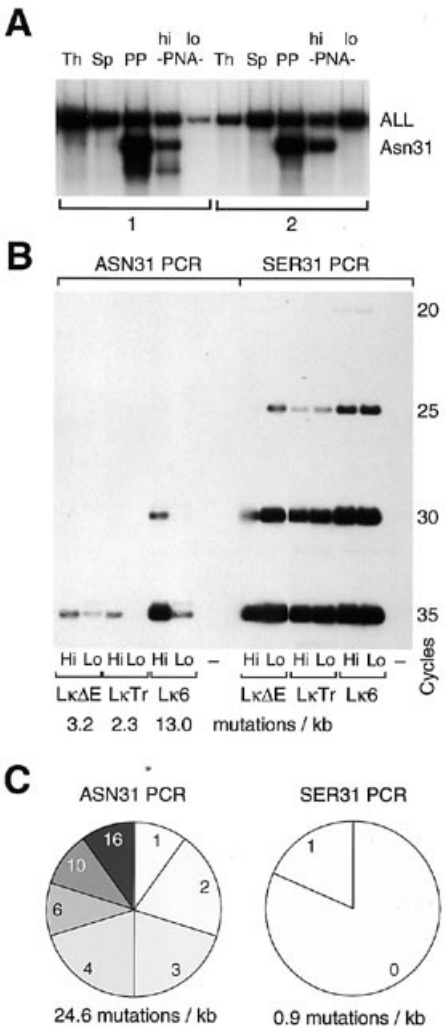

Figure 2. Transgene mutation detected by hotspot-specific PCR. (**A**) PCR with the ASN31 oligonucleotide is specific for mutated B cells. PCR amplification was performed either to detect all VκOx1 transgenes, mutated or not (ALL, using LκFOR and VκOXBACK primers, 25 cycles) or just to detect mutated transgenes (Asn31, using ASN31 and VκOXBACK primers, 45 cycles). The ALL and ASN31 reactions were carried out separately, then combined for gel electrophoresis. The template DNA was from different tissues of a pair of Lκ6 litter mates: thymus (Th), spleen (Sp), total Peyer's patch (PP), Peyer's patch germinal centre B cells (PNA^{hi}) or Peyer's patch non-germinal centre B cells (PNA^{lo}). (**B**) The ratio of ASN31 product to SER31 product in nested PCR correlates with the transgene mutation rate. Following a primary PCR (VκOxBACK/LκFOR primers), a nested PCR was performed using ASN31 and JOL25 or SER31 and JOL25, and aliquots run out on the gel after the indicated number of cycles. Template DNA was from sorted germinal centre (PNA^{hi}) or non-germinal centre (PNA^{lo}) Peyer's patch B cells of the indicated transgenic mice. The mutation rates of the transgenes in germinal centre B cells is given at the bottom (6; Goyenechea *et al.*, submitted; Klix *et al.*, in preparation). (**C**) PCR with ASN31 selectively amplifies mutated sequences from Lκ6 total unsorted Peyer's patch DNA. Pie slices represent the frequency of clones in the nested PCR populations that have the number of mutations indicated. The overall mutation rate is given below each chart. Ten ASN31 clones and 11 SER31 clones were sequenced (207 bp per clone).

total Peyer's patch DNA of Lκ6 mice, cloned into M13 and sequenced. In an unfractionated Peyer's patch cell sample, only 10–15% of the total cell population (i.e. the $PNA^{hi}B220^{+}$ fraction) should carry mutated immunoglobulin genes (see ref. 10). Thus, the SER31 PCR clones carried few mutations, but the ASN31 clones were heavily mutated (Fig. 2C). Even when an annealing temperature of 55°C was used with the ASN31 primer (which can cause some mispriming from Ser31 templates—see Fig. 1B), the sequences amplified from Lκ6 total Peyer's patch DNA were mostly mutated (data not shown). Thus, the ASN31 primer was selective for mutated sequences.

Transgene mutation identified by a MutS-binding assay

The hotspot-specific PCR worked for the detection of transgene mutation but, for more general use, we wished to devise an assay that did not depend on the existence of a dominant mutational hotspot. To this end, we exploited the ability of the bacterial MutS protein to recognise DNA mismatches (16). A 155 bp sequence of the transgenic VκOx gene (extending across CDR3 and Jκ5) was amplified from tissues using *Pfu* DNA polymerase and 5′-biotinylated primers (JOL24 and JOL25, Fig. 1A). The purified PCR product was denatured and renatured such that mutations in the original DNA template would lead to the creation of mismatched heteroduplexes. The amount of such duplexes was then measured by binding them to filter-immobilised MutS using the assay of Wagner *et al.* (16). The bound DNA was quantified using streptavidin-conjugated horseradish peroxidase and enhanced chemiluminescence (ECL). ECL detection of aliquots of the heteroduplex preparation that had been directly bound to filters allowed variation in DNA concentration to be controlled (Fig. 3A).

Using this assay, transgene mutation was readily detectable in DNA extracted from the Peyer's patches but not from the thymus of Lκ6 mice. The level of mutation appeared much higher in the PNA^{hi} (germinal centre) rather than PNA^{lo} Peyer's patch B cells, and mutation was only weakly detectable in spleen (Fig. 3A).

The reduced level of transgene mutation in LκΔI, LκTr and LκΔE transgenic mice was still reproducibly sufficient to give a signal in the MutS-binding assay (compare the signals obtained using DNA amplified from sorted PNA^{hi} versus PNA^{lo} B cells; Fig. 3B).

Enrichment for mutated sequences using immobilised MutS

The DNA bound to the filter-immobilised MutS should be enriched in mismatched duplexes and therefore in mutated V gene sequences. To confirm this, transgene DNA that had been PCR amplified from germinal centre B cells of the weakly mutating LκTr mouse was eluted from nitrocellulose-immobilised MutS and subjected to an extra seven cycles of PCR to convert it to homoduplexes before cloning into M13. Sequencing of these clones revealed that they were indeed much more mutated than comparable LκTr sequences cloned without MutS enrichment (Fig. 3C).

Sequence analysis of endogenous immunoglobulin hypermutation

We felt it should be possible to extrapolate the MutS assay to endogenous immunoglobulin sequences. However, whereas the V gene itself is the natural target of hypermutation, different B cells will express different rearranged V genes. This heterogeneity would greatly complicate the analysis. We thought we might be able to exploit the fact that the hypermutation domain extends into

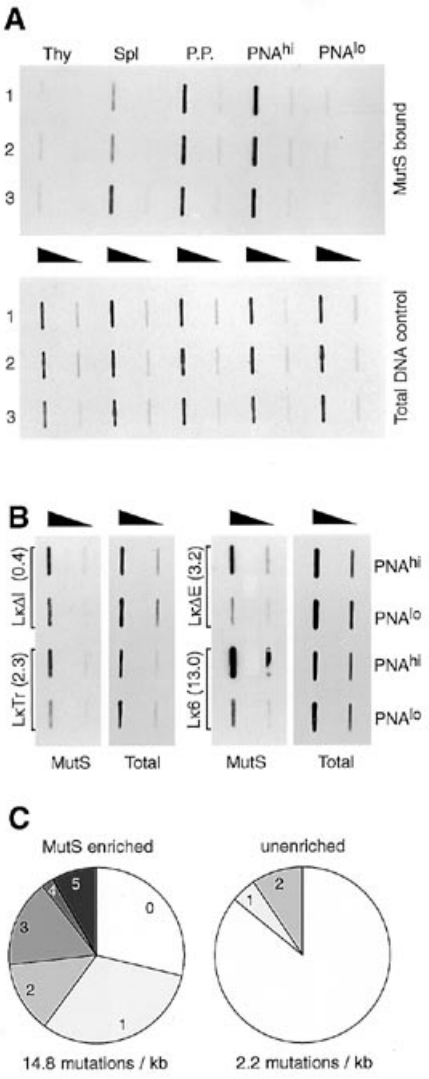

Figure 3. Transgene mutation identified by a MutS-binding assay. The transgenic V region was PCR amplified (biotinylated JOL24/JOL25) from lymphoid tissues, subjected to denaturation/renaturation in order to create heteroduplexes and aliquots incubated with MutS immobilised on a filter. The mismatched heteroduplexes bound to MutS were revealed by ECL. As a control for the total DNA abundance, aliquots of the renatured PCR products were also directly bound to a filter and revealed by ECL. (**A**) The top panel shows a MutS binding assay using heteroduplexes derived from different tissues of three Lκ6 litter mates (monitored at 10^{-1} and 50^{-1} dilutions, as indicated by wedges). The DNA abundance control is in the bottom panel. (**B**) MutS binding assay and DNA abundance control (monitored at 6^{-1} and 18^{-1} dilutions) to detect transgene mutation in germinal centre (PNA^{hi}) versus non-germinal centre (PNA^{lo}) B cells from mice carrying Lκ-derived transgenes (as indicated). The mutation rates (mutations/kb) of the transgenes in germinal centre B cells is given top left (6; Goyenechea *et al*., submitted; Klix *et al*., in preparation). (**C**) DNA amplified from LκTr germinal centre B cells that had been subjected to denaturation/renaturation and incubated with immobilised MutS, was eluted from the MutS, subjected to further PCR amplification (final total of 35 cycles) and cloned into M13. The pie charts indicate the mutation frequency found in 38 MutS-enriched clones, as well as in 22 unenriched LκTr controls.

the J-C intron which flanks the 3′-border of all rearranged V segments (10,20,21) To this end, rearranged V_H sequences were PCR amplified from sorted germinal centre B cells using a primer homologous to a conserved sequence in framework three of the

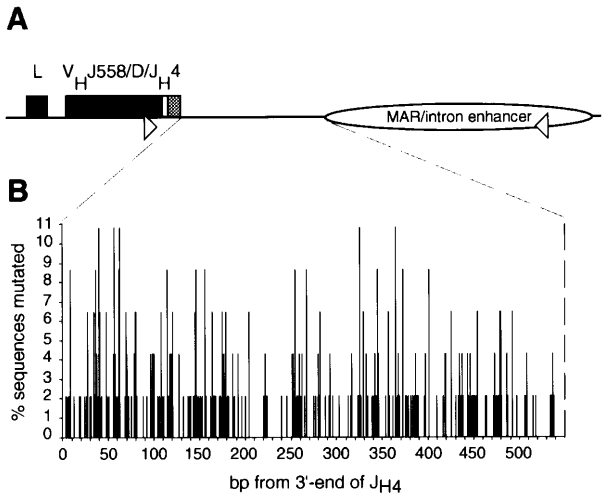

Figure 4. Mutation of the region flanking rearranged endogenous J_H4 genes determined by sequencing. (**A**) Map of a V(D)J_H4 rearrangement showing the positions of PCR primers used. The sense primer binds to a conserved sequence in FR3 of V_HJ558 genes, and the antisense primer binds 1098 bp 3′ to J_H4. (**B**) Histogram of mutations. 78% of all sequenced clones were mutated, and only mutated clones (46) are represented by the graph. Sequences were amplified from PNA^{hi} Peyer's patch B cells and pooled from several different lines of mice.

V_HJ558 family and an antisense primer complementary to the IgH intron enhancer (Fig. 4A). Clones that contained V to J_H4 rearrangements were selected by oligonucleotide hybridisation and sequence data from immediately 3′ of J_H4 obtained. This region was generally heavily mutated in germinal centre B cells isolated from several mouse lines (Fig. 4B).

The advantage of this sequencing approach over sequencing V-regions themselves is that the problem of uncertainty over definitively identifying the germline counterpart of the putative somatically mutated genes is avoided. Any one mouse can at most only carry two germline variants of the J_H4-flank sequenced, and in analysing mice bred into F1 backgrounds, we were readily able to identify such allelic variation and eliminate it from our databases.

Hypermutation in p53- and CD22-deficient mice measured using J_H flanks

CD22-deficiency is known to affect antigen receptor signalling and p53-deficiency affects DNA repair pathways, but neither has much effect upon the number of PNA^{hi} B cells in Peyer's patches (8,9,22 and unpublished data). Thus it was possible to use the above sequencing strategy to determine the effect of these gene deficiencies upon hypermutation. The results showed that immunoglobulin genes in CD22- and p53-deficient mice undergo hypermutation (Fig. 5A), and the pattern of mutations is apparently unaffected (not shown).

To develop a rapid MutS-based assay for screening gene-targeted mice, biotin-labelled PCR fragments were produced by priming with oligonucleotide pairs that were either situated within J_H1 and 168 nt 3′ of J_H1, or within J_H4 and 225 nt 3′ of J_H4. Even though this PCR strategy was not selective for rearranged immunoglobulin genes, extensive mutation in the products from germinal centre B cells relative to those from non-germinal centre

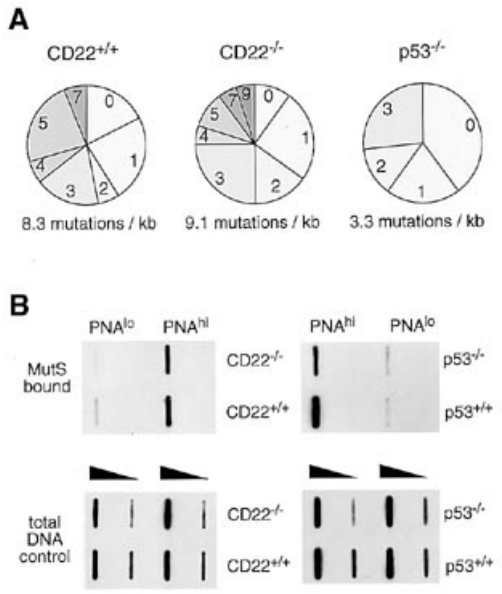

Figure 5. Hypermutation in CD22- and p53-deficient mice. (**A**) Mutation detected in the 3′ region (307 bp) flanking $V_HJ558/D/J_H4$ rearrangements in germinal centre B cells as described in the legend to Figure 4. The pie charts indicate the mutation frequency found in 18 $CD22^{+/+}$ clones, 20 $CD22^{-/-}$ clones and 15 $p53^{-/-}$ clones obtained from the Peyer's patches of a single mouse in each case. (**B**) Detection of mutation in J_H flanking regions by MutS-based assay. DNA was amplified from Peyer's patch PNA^{hi} or PNA^{lo} B cells and heteroduplexes were assayed at 6^1 and 18^{-1} dilutions.

B cells was readily detectable by MutS-based assay of wild-type and mutant mice (Fig. 5B).

Interestingly, both sequencing and the MutS-based assay suggest that immunoglobulin mutation in the p53-deficient mice occurs at a reduced rate (Fig. 5). Donehower *et al.* (8) noted that the $p53^{-/-}$ line used here suffers from a subtle immunodeficiency that is not a result of reduced lymphocyte numbers, and perturbed hypermutation might produce such a phenotype. However, since this mouse line was maintained by crossing homozygous knockout mice, littermate wild-type controls have not been available, and it is not yet clear whether the reduced hypermutation is a direct effect of p53-deficiency, or due to more trivial reasons. This obviously warrants further investigation.

DISCUSSION

We have here described two rapid assays for monitoring immunoglobulin hypermutation that do not entail DNA cloning and sequencing. One assay exploits the presence of a well characterised mutational hotspot in the $V_\kappa Ox$-1 gene by using PCR with the most 3′-base of one of the primers being complementary to the mutation hotspot. This assay rests on the assumption that DNA polymerases will only poorly extend primers with a 3′-mismatch. The other protocol involves the denaturation/renaturation of PCR-amplified immunoglobulin V gene sequences and exploits the ability of immobilised MutS protein to specifically bind mismatched DNA heteroduplexes (16).

How does the performance of the two assays compare? The hotspot PCR protocol has the advantage of being very easy to carry out, but obviously requires the presence of a major mutational hotspot in the DNA segment amplified. Furthermore, the PCR conditions are critical. 'Hot-start' and an annealing temperature above a critical threshold must be used. The hot start can be greatly simplified by the use of Amplitaq Gold™ (not shown), which is inactive until heated at 95°C for ~15 min, making the procedure suitable for a 96-well assay format from DNA extraction up to gel loading.

The immobilised MutS assay, whilst slightly more laborious than the hotspot assay, does not depend upon the presence of a mutational hotspot in the amplified DNA segment and, requiring fewer PCR cycles, is less prone to problems of PCR artefact or contamination. Care must, however, be taken to ensure that germline variation in the target sequence (allelic polymorphism) does not produce false positive results. Amplification of DNA from, for instance, thymus or PNA^{lo} B cells is an essential control for germline or PCR-generated diversity.

When the MutS assay indicates mutation in a PCR product, those mutations can be characterised by eluting the DNA and cloning it (Fig. 3C). This enrichment can be useful for analysing the pattern of mutation in poorly mutating sequences, but caution needs to be exercised in this analysis. We found that C to T transitions can be over represented by this enrichment (not shown), presumably because MutS has highest affinity for T:G mismatches (see ref. 16).

In order to analyse mutation of endogenous (as opposed to transgenic) immunoglobulin sequences, we used the region flanking the 3′-border of rearranged J_H segments, reasoning that this would allow mutation to be assayed without focusing on an individual V gene segment. Sequences immediately 3′ to rearranged J elements mutate as frequently as the rearranged V elements themselves (20,21,23). J_H4 segments rearranged to the large V_HJ558 family were readily amplified from germinal centre B cells, and thus provided a good target sequence for measuring and analysing hypermutation (Fig. 4).

Sequence analysis of mutated J_H4 flanks has not identified a strong hypermutation hotspot yet (Fig. 4B). This suggested that for rapid analyses, J_H flanks might constitute more suitable substrates for a MutS-based, rather than the hotspot protocol. Indeed, fragments amplified from J_H1 or J_H4 3′-flanks without any selection for rearranged alleles were useful substrates for rapid MutS-based mutation screening (Fig. 5B). J_H-flanks alone were amplified, because denaturing/renaturing PCR products that included the V(D)J sequences themselves would have produced spurious mismatches as a result of the imprecision of V(D)J recombination. If selectivity in the MutS-based assay for rearranged alleles (and thus increased assay sensitivity) were required it could easily be achieved by a primary amplification based on rearranged V genes (e.g., as in Fig. 4A), followed by a nested amplification of the J_H-flank alone.

To demonstrate the usefulness of mutation assays based on J_H-flanks, we showed that CD22- and p53-deficient mice hypermutate their immunoglobulin genes (Fig. 5). Whereas CD22 affects signalling in B cells (9,22,24), we show here that its absence does not affect hypermutation. With regard to p53, it has long been thought that hypermutation may be initiated by DNA strand breaks (25), and it is known that p53 is required for the normal response of cells to such breaks (reviewed in ref. 26). Furthermore, a DNA repair abnormality specific for rearranged V genes in germinal centre B cells has been reported (27). However, our data suggests that if DNA strand breaks are

required for hypermutation, they can be resolved without a p53-induced G1 checkpoint.

A major goal of hypermutation research is the identification of the proteins involved. This goal may be advanced by the development of culture systems in which hypermutation can be induced, or by the identification of individuals or gene-targeted mice in which germinal centres are formed, but where hypermutation is inactivated. The MutS-based assay described here is sufficiently sensitive for transgenes that mutate at very low rates. This sensitivity may be enough to detect mutation in *in vitro* culture systems and the assay is simple enough to enable screening of many humans or gene-targeted mice. Since the number of mouse gene-knockouts with obvious potential to directly affect immunoglobulin hypermutation is currently small, they are probably better analysed by the sequencing of rearranged J_H4-flanks than by the rapid, but less informative MutS-based assay.

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