Human minisatellite MS32 (D1S8) displays somatic but not germline instability in transgenic mice

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Human minisatellite MS32 (D1S8) shows instability both in the germline and, at much lower levels, in somatic DNA. To investigate factors that influence somatic and germline mutation, large cosmid-based constructs containing MS32 were introduced into mice, bred to homozygosity and tested for instability in blood and sperm. Analysis of single copy and multicopy transgenic lines revealed somatic mutants occurring at a frequency comparable with that seen in man. As in humans, these mutants arose mainly by simple intra-allelic duplications and deletions. In contrast, analysis of sperm DNA from four different transgenic lines showed no trace of the complex recombination-based germline instability seen in man, even using PCR-based approaches capable of detecting very rare mutants. These data provide further evidence that germline and somatic mutation at human minisatellite MS32 occur via distinct pathways, that a major barrier exists to the transfer of germline instability from humans to mice and that the mouse germline appears to be protected from mitotic instability of the type seen in blood.

INTRODUCTION

Minisatellites provide the most informative system for analysing processes of tandem repeat turnover in the human genome. Extreme diversity in repeat copy number and allelic structure, coupled with germline mutation rates as high as 15% per gamete (1), facilitates analysis of mutation processes. De novo mutants can be detected in pedigrees (1,2) and more efficiently by direct analysis of sperm DNA using single molecule PCR approaches which allow detailed characterization of the mutational behaviour of individual alleles (3–7). Structural analysis of the interspersion patterns of variant repeats within mutant alleles (3,4,6–8) has shown that germline instability is a recombination-based process most likely occurring at meiosis and frequently involving complex gene conversion-like transfers of repeat units between alleles (5). For some loci instability is polar, being largely restricted to one end of the repeat array and suggesting a role of flanking DNA in modulating instability. Direct evidence for flanking elements that regulate mutation has come from minisatellite MS32 (D1S8), where a single G→C base transversion at a site termed O1, 48 bp upstream of the unstable end of the array, is strongly associated with, and probably directly causes, a major suppression of germline instability in cis. The O1C variant does not, however, prevent an allele from acting as a donor of repeats during conversion (5,9).

Human minisatellites can also show somatic instability, though at much lower levels than seen in sperm (5). MS32 rare somatic mutants have been recovered from blood DNA by physical enrichment of abnormal length molecules and have been shown to arise through a simple non-polar process of intra-allelic duplication or deletion of repeat unit blocks. This process is completely distinct from the complex polar conversion events occurring in the germline and further occurs at a normal rate at an O1C-linked allele showing profound suppression of germinal instability (5).

Further analysis of minisatellite instability, including the developmental timing of mutation events, the functional dissection of cis-acting regulators of germline instability and the exploration of mutation processes in the female germline would be greatly facilitated by an animal model of human minisatellite instability. Transgenic mice carrying human minisatellite MS32 have therefore been generated. Initial experiments used a construct consisting of the repeat array plus 200 bp of 5′ and 3′ flanking human DNA, including the O1G variant associated with unstable alleles. Pronuclear injection into mouse embryos yielded both single copy and multicopy transgenes (10).

The multicopy transgenes showed substantial instability in mouse pedigrees (10). Further analysis of one multicopy integrant showed that instability was directly attributable to a large inverted repeat created by tail-to-tail joining of MS32 transgene units and that mutational disruption of the palindromic junction was sufficient to stabilize the transgene (11). Multicopy transgene instability does not therefore provide a model of human minisatellite mutation.

Analysis of instability at single copy integrants was hampered by their frequent rearrangement during insertion into the mouse genome. Investigation of one intact single copy integrant both in pedigrees and by small-pool PCR (SP-PCR; 4) analysis of transgenic sperm DNA showed no sign of germline instability (10). However, mice homozygous for this MS32 transgene could not be derived, preventing analysis of germline events arising from the inter-allelic interactions that occur in man. Furthermore, and in common with several other transgenic lines, this MS32 transgene has integrated into mouse ρ-satellite DNA, which may be an inappropriate genomic location for supporting minisatellite instability. Other explanations for the failure to transfer repeat

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instability from humans to mice include insufficient human flanking DNA in the construct required to drive mutation and the possibility of human/mouse incompatibility in repeat instability processes, though the mouse genome does contain endogenous minisatellites structurally similar to their human counterparts (unpublished data).

Issues of hemizygosity, integration site and inadequate flanking human DNA have therefore been addressed by creating a series of transgenic mice carrying a much larger cosmid-based construct containing an MS32 allele of known mutational instability in human sperm. In addition, mutation analysis in transgenic mice was extended to both somatic and germline instability using single molecule PCR methods capable of detecting even very rare mutant molecules (5), to determine whether somatic and germline instability can be transferred from humans to mice.

RESULTS

Construction of a large MS32 transgene

The original genomic clone of MS32 contained limited human DNA flanking the repeat array (12). A cosmid contig extending 60 kb around MS32 was therefore generated. From this contig, clone cMS32.28, containing 18 kb of human DNA upstream of the unstable end of the repeat array plus 10 kb of downstream flanking DNA, was fully sequenced to facilitate further manipulation (data not shown). This cosmid also contained a 100 repeat (2900 bp) MS32 allele of unknown mutational competence which was too large for the efficient detection of mutants by single molecule PCR (3). This allele, together with 3345 bp of 5′ flanking DNA and 200 bp of 3′ flanking DNA, was therefore excised from cMS32.28 and replaced with a corresponding DNA fragment PCR amplified from a tested semen donor (Fig. 1). The 4.3 kb SacII–AgeI fragment containing the MS32 allele was ligated to cosmid cMS32.28 DNA similarly digested with SacII plus AgeI to create cMS32.28.R, in which the uncharacterized MS32 allele and immediate flanking DNA in cMS32.28 was replaced by an allele of known mutational instability, without disruption of flanking DNA. Digestion of cMS32.28.R with Psp1406I yielded a 29 kb human DNA fragment which was used to generate transgenic mice. Primers 32–18R and 32+10R used for the characterization of mouse DNA flanking the transgenes are shown. Hatched box, MS32 allele; grey box, cosmid vector.

Structure and copy number of MS32 transgenes

Four transgenic founders, A–D, were identified by Southern blot analysis using MS32. Further Southern analysis confirmed the complete integrity of all transgene units present in these founders, with human DNA extending 18 kb 5′ and 10 kb 3′ of each tandem repeat array. Integrity was further verified for at least one transgene at each site of integration by successful long PCR amplification of 5 kb of overlapping stretches of flanking human DNA, using primer pairs staged at appropriate intervals, both on the founder mice and on sperm DNA from the human donor (data not shown). PCR amplification using primers closely flanking the minisatellite array showed that all transgene units contained a 29 repeat allele identical in length to the allele in the human donor. Further analysis of allele structure using four-state MVR-PCR (minisatellite variant repeat mapping by PCR) to determine the order of variant repeats within the repeat array (13) showed that the structure of all transgenic minisatellite arrays was identical to that of the progenitor allele (Fig. 1). Finally, transgene copy numbers were estimated both by dosage of Southern blot signals and by single molecule PCR analysis of the MS32 repeat arrays in extreme dilutions of mouse genomic DNA (4). Transgene C was single copy and the others multicopy, with copy numbers ranging from five to 18 (Table 1).

Genomic location of MS32 transgenes B and C

Transgenes B and C were lowest in copy number and were therefore selected for most subsequent analyses. The genomic location of these two transgenes was determined using vectorette PCR to isolate mouse genomic DNA immediately flanking the ends of the construct (14). Sequence analysis of vectorette PCR products from transgene B showed that it was flanked on the 5′-side by a unique sequence mouse DNA. Similarly, the single copy transgene C was flanked on the 3′-side by unique sequence mouse DNA and on the 5′-side by an incomplete LINE-1 element preceded by single copy DNA. Polymorphisms in flanking mouse DNA between strain C57Bl/6J and Mus spretus were identified and used to type mice from the EUCIB backcross (15). This
enabled transgenes B and C to be assigned to mouse chromosome 11, 37 cM from the centromere, and chromosome 14, 40 cM from the centromere, respectively. Both transgenes had therefore integrated into interstitial locations and not into centromere-proximal γ-satellite DNA.

### Table 1. Summary of sperm and blood mutation frequencies in humans and transgenic mice

<table>
<thead>
<tr>
<th></th>
<th>Human donor</th>
<th>Human allele A</th>
<th>Human allele B</th>
<th>Human allele C</th>
<th>Transgene A</th>
<th>Transgene B</th>
<th>Transgene C</th>
<th>Transgene D</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. repeats</td>
<td>29</td>
<td>63</td>
<td>42</td>
<td>38</td>
<td>29</td>
<td>29</td>
<td>29</td>
<td>29</td>
</tr>
<tr>
<td>Copy no.</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>-7</td>
<td>-5</td>
<td>1</td>
<td>-18</td>
</tr>
<tr>
<td>Frequency of mutants (×10^3)</td>
<td>460 (93%)</td>
<td>230 (83%)</td>
<td>180 (72%)</td>
<td>3.4 (84%)</td>
<td>≤12 (0/25 400)</td>
<td>≤0.75 (0/400 000)</td>
<td>≤0.06 (0/5 000 000)</td>
<td>≤5.5 (0/55 000)</td>
</tr>
<tr>
<td>Sperm</td>
<td>Blood</td>
<td></td>
<td></td>
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The frequency of different mutants involving the gain or loss of 3–20 repeats, a range fully scorable in all analyses, is given per amplifiable progenitor molecule, together with the percentage of changes involving gains of repeats. Frequencies are based upon the amount of genomic DNA surveyed, assuming 3 pg DNA/haploid genome and a single molecule amplification efficiency of 84% for MS32 alleles (4). Blood from the human donor was not available for analysis. The human allele C is linked to the O1C variant; sperm but not blood mutation is suppressed for this allele (5). Transgene A and D sperm mutation rates were determined by SP-PCR of sperm DNA. Transgene B and C sperm mutation rates were determined by SESP-PCR of sperm DNA. Only gain mutants were surveyed for the transgene sperm mutation rate, given the strong bias toward gains in the human donor. The upper 95% confidence limits for transgene sperm mutation rates are given, together with the number of mutant molecules detected and the number of sperm progenitor molecules scanned. Blood frequencies of different mutants are given, after eliminating repeat isolates due to somatic mosaicism. Data on human alleles A–C are taken from Jeffreys and Neumann (5). ND, not determined.

**Breeding to homozygosity of MS32 transgenes A–D**

Crosses between hemizygous mice positive for transgenes A–D were used to generate homozygous male mice for mutational analyses. Offspring were typed by MS32 Southern blot hybridization and dosage used to identify putative homozygous males; homozygosity was subsequently confirmed by test mating with transgene-negative females and demonstrating 100% transmission to at least 12 resulting progeny. All four transgenic lines were successfully bred to homozygosity.

**Mutational analysis of MS32 transgenes in blood**

Somatic instability was analysed in blood from transgene B and transgene C homozygotes. Given the single copy nature of transgene C, it was necessary to pool blood from six homozygous littermates to obtain sufficient DNA for mutation analysis. Mutants were detected by size-enrichment followed by small-pool PCR (SESP-PCR, 5). This technique involves digesting genomic DNA with MboI, which cleaves near the MS32 repeat array, followed by agarose gel electrophoresis and recovery of size fractions depleted in 29 repeat progenitor molecules and enriched in any abnormal length mutant molecules from nine to 49 repeats long. Adjacent size fractions were pooled and tested by SP-PCR for the presence of mutant molecules in addition to residual contaminating progenitor molecules (Fig. 2). SESP-PCR analysis of 2 × 10^6 and 3.5 × 10^6 progenitor molecules from lines B and C respectively revealed nine somatic mutants from line B and 16 from line C. In all cases the mutants had Southern blot signal intensities expected for PCR products derived from single molecules; no additional PCR products were seen on prolonged autoradiography. Furthermore, the mutant alleles had the correct length given the size range of the fractions from which they originated, confirming that these are true mutants and not PCR artefacts, which do occasionally arise in SP-PCR but usually result in major deletions (Fig. 2A; 5).

Transgene B and C show similar levels of somatic instability, with a mean frequency of blood mutants per progenitor molecule of 0.5 × 10^-5. This frequency may be a slight underestimate, since mutants showing gains or losses of one to two repeats will have been partly or completely discarded in the progenitor allele size fractions (5). However, this limitation also applies to SESP-PCR analysis of MS32 mutation in human blood DNA (5). The level of transgene instability seen in mouse blood is similar to that seen in human blood (Table 1; 5).
The structural basis of somatic instability

To determine the nature of the structural rearrangements occurring in these transgene blood mutants, all 25 mutant alleles were gel purified and analysed by four-state MVR-PCR (13) to determine the distribution of the repeat unit types (E, e, Y and y) along the repeat array. Comparison with the progenitor allele showed that almost all somatic mutation events at transgenes B and C involved either a simple duplication or a perfect deletion of blocks of repeat units (Fig. 3). Only two mutants showed evidence of more complex events. Mutant B-2 appeared to have arisen by addition of 10 repeats (EYEEEEEEE) of unknown origin, probably via a multistep mechanism. Mutant C-1 involved a 13 repeat duplication (eYeeEeYYEYY) followed by a 6 repeat deletion in the central 13 repeat triplate (eYee-----YY).

Previous analyses of the fidelity of single molecule PCR on minisatellites (3,4) suggest that these structurally complex size-validated mutants were authentic rather than due to Taq polymerase errors accumulated during PCR amplification.

In human blood somatic mutation at MS32 involves simple, strictly intra-allelic reduplications and deletions of blocks of repeats located apparently at random along the repeat array and with no evidence for a bias towards gain rather than loss of repeats (5). The blood mutants detected at these transgenes appear very similar to their human counterparts. Most events are simple, with no evidence of mutational polarity, as seen in human sperm. The possibility of inter-allelic exchanges cannot be tested, given the homozygous nature of the transgenes. There is no bias towards gain in size, but rather a tendency towards deletion, although this is not statistically significant. As with humans, mutation tends to involve large repeat copy number changes compared with sperm mutation; thus, 73% of human sperm mutants involve the gain or loss of five repeats or less, compared with only 26% of the transgene somatic mutants and 50% of human blood mutants (4,5).

Not all transgenic blood mutants were different. There were two instances of multiple isolates of the same mutant structure (Fig. 3, mutants B-3 and C-3), indicating somatic mosaicism. Eliminating these repeat isolates does not significantly alter the frequency of different mutants of 0.5 x 10^-5 for lines B and C. The detection of such somatic mosaicism further validates the SESP-PCR recovery of mutant alleles.

Germline mutation rate of MS32 transgenes A–D

The 29 repeat progenitor MS32 allele shows a 0.9% mutation rate in human sperm as measured by SP-PCR. The same technique was therefore applied to sperm DNA from homozygotes from all four transgenic lines. Despite scanning 25 000–55 000 amplifiable progenitor molecules per line, no gain of size mutants typical of human sperm were detected and only a few deletion events, which could have arisen as PCR artefacts, as is known to occur in SP-PCR (5). All four transgenic lines therefore showed at least a 40-fold reduction in the sperm gain mutation rate compared with man (Table 1).

To determine whether these transgenes do show sperm instability but at a very low rate, SESP-PCR was used to analyse sperm DNA from mice homozygous for transgenes B and C previously characterized for somatic mutation (Fig. 2). Scanning 4 x 10^5 and 5 x 10^6 molecules of transgenes B and C respectively revealed no potential mutant MS32 molecules showing size gains and Southern blot signal intensities compatible with single molecule PCR products. This indicated a reduction in the rate of sperm instability for transgenes B and C of at least 600- and 7500-fold respectively compared with man (Table 1).

DISCUSSION

Somatic instability at MS32 transgenes

SESP-PCR has sufficient sensitivity to allow even very low levels of tandem repeat instability to be detected and quantitated and currently provides the only method for detecting minisatellite mutation in the soma. MS32 displays somatic (blood) instability not only in humans but also in transgenic mice, with a spectrum of mutations similar to that seen in human blood. Mutation rates are 2- to 3-fold lower than their human counterparts, although this minor discrepancy might arise through allele length effects, with
the 29 repeat allele in transgenic mice being shorter than any of the MS32 alleles so far tested in human blood. Instability per progenitor molecule appears to be independent of transgene copy number or position of integration in the mouse genome, suggesting that the 28 kb of human DNA flanking MS32 contains all necessary information required to establish somatic instability. Little is known about processes of somatic mutation in humans and only one tissue, blood, has so far been analysed. Indirect evidence suggests that human somatic instability more likely involves intra-allelic mitotic recombination rather than replication slippage (5), but it is not known whether instability is an intrinsic property of the repeat array itself or whether instead it is modulated by flanking DNA, as occurs with germline mutation. The ability to transfer somatic instability to transgenic mice makes it possible to explore the influence or otherwise of flanking DNA on somatic mutation, to investigate instability in different tissues to determine whether the frequency of mutation is linked to cell proliferation and to define genetic, age-related and environmental factors that affect somatic instability.

Germline stability of MS32 transgenes

The original short MS32 constructs used to generate transgenic mice failed to show instability in transgenic sperm DNA (10,16). However, problems of transgene rearrangement, the tendency to integrate into \( \gamma \)-satellite DNA and the difficulty of breeding the transgenes to homozygosity prior to sperm mutation analysis greatly hampered the analysis of potential meiotic instability. The use of cosmid-based constructs has circumvented all of these problems. None of the transgenes showed any evidence of rearrangement either of the MS32 repeat array or of the flanking human DNA. The two mapped transgenes were located interstitially and not in centromeric DNA. All four transgenes could be homozygotes, indicating that integration had not induced recessive lethal deficiencies in the mouse genome. Despite these improvements, plus the inclusion of much larger amounts of human flanking DNA, none of the transgenes showed any signs of sperm instability, despite using very sensitive SESP-PCR to screen a total of \( 6 \times 10^9 \) progenitor molecules for mutants. Thus, somatic instability can be replicated in mice with rates within 3-fold of humans, whereas germline instability is massively suppressed. These data are consistent with evidence that somatic and germlinal instability in man occur by completely distinct processes (4,5). It also appears that mouse transgenic sperm fail to show not only meiotic-type instability, but also pre-meiotic instability of the type seen in human and transgenic mouse blood. This is reminiscent of the relative lack of simple mutation events found in human sperm, even at an allele carrying the O1C variant, where the sperm mutation rate is suppressed 110-fold (5), and suggests that the pre-meiotic phases of male germline development may be relatively protected from mitotic instability. The failure to transfer germline minisatellite instability to transgenic mice contrasts with recent reports of transgenic mice containing expanded trinucleotide repeat loci which display high levels of germline instability (17–19). These lines tend to show fairly consistent mutation rates irrespective of transgene location in the mouse genome, suggesting only modest position effects on repeat instability. The difference between the ability to transfer triplet and minisatellite instability to mice provides evidence for fundamental differences in the mechanisms of germline DNA instability between these two classes of repeat loci (20). Alternatively, it remains possible that triplet repeat transgenes may be mutating in mice by processes different from those involved in driving triplet expansions in humans.

The reasons for failing to transfer human minisatellite germline instability to mice remain unclear. The suppression appears to be complete, irrespective of integration site. It remains possible that an unknown and critical point mutation in the unsurveyed DNA flanking the minisatellite array could have been introduced when generating the cMS32.28.R cosmid, even though the overall structure of cMS32.28.R was correct and despite the use of long PCR to minimize the risk of base misincorporation. (24). Another possible reason for mutation suppression is that these large constructs still contain insufficient DNA to provide all the cis-acting elements necessary to support meiotic instability, despite evidence from the flanking stabilizing O1C variant pointing to an important role for DNA immediately flanking the unstable end of the MS32 repeat array (5). A similar difficulty in moving meiotic recombination hotspots within the yeast genome suggests that hotspot activity may be controlled by long-range chromatin effects rather than local DNA sequence (21). Such long range effects can only be tested for MS32 by generating even larger transgene constructs based on PACs, BACs or YACs. A third possible reason for the lack of sperm mutation is that transgene homozygosity might lead to suppression of meiotic instability. True MS32 homozygotes are rare in human populations. However, preliminary SP-PCR analysis of sperm from an individual homozygous for an O1G-linked MS32 allele suggests that homozygosity in this individual does not extinguish minisatellite instability, with the germline mutation rate observed falling within the normal range for minisatellite MS32 (K.Tamaki, and A.J.Jeffreys, unpublished data). Finally, there may exist a mouse/human barrier specific either for minisatellite MS32 or more generally for any human minisatellite introduced into mice. Such a barrier could result from incompatibilities preventing mouse trans-acting factors from recognizing cis-acting elements near MS32. Alternatively, the mouse genome might not process minisatellite DNA at meiosis in the same way as in humans. To resolve these questions will require the analysis of other human minisatelites in transgenic mice and the definition of tandem repeat instability processes at endogenous mouse minisatellite loci (22).

MATERIALS AND METHODS

Construction of a large transgene and generation of transgenic lines

Cosmid cMS32.28 was isolated from a human cosmid library constructed with the vector pAVCV007 (23). Sonicated cMS32.28 DNA was shotgun cloned into pBluescriptII SK+ and the human insert sequenced in its entirety on both strands using single-stranded templates plus finishing with PCR amplified double-stranded phagemid inserts (A.J.Jeffreys and J.Murray, unpublished data).

The smaller MS32 allele from the sperm donor was amplified by long PCR (24–26) from 50 ng sperm DNA in a 7 \( \mu \)l PCR reaction using the buffer described previously (5) plus 0.4 \( \mu \)M primer MS32–5F, 0.4 \( \mu \)M primer MS32+5F, 6% glycerol, 0.12 M Tris base, 0.07 U/\( \mu \)l Taq polymerase (Advanced Bio-technologies), 0.0035 U/\( \mu \)l Pfu DNA polymerase (Stratagene)
Amplification was carried out using a GeneAmp PCR system 9600 thermal cycler (Perkin Elmer Cetus) at 96 °C for 15 s, 68 °C for 15 min for 38 cycles. Primers were MS32–5′F (5′-CAGTGCTTGGCACATAATGAGC-3′) and MS32+5F (5′-GCCAGAAGGTCTAGTGAGAT-3′). The 10.3 kb PCR fragment was cleaved with SacII plus AgeI and ligated to cosmID cMS32.28 whose minisatellite had been removed by digestion with these two restriction enzymes; both enzymes cleave only once in cMS32.28 (Fig. 1). The cosmID was recloned using standard methodology to yield clone cMS32.28.R.

For transgenesis, cMS32.28.R DNA was cleaved with Clal plus Psp1406I (Fig. 1) and the 29 kb human DNA fragment purified on a NACS column (27). Approximately 300 copies of the Clal–Psp1406I fragment were microinjected by standard techniques into the male pronuclei of one cell embryos of C57BL/6J × CBA/ca F2 genotype. Embryos surviving to the two cell stage were transferred into recipient female mice. Mouse transgenic for MS32 were identified by Southern blot analysis of tail DNA (16). Four founder mice were bred with C57BL/6J × CBA/ca F2 mice and transgene-positive F2 mice were crossed to yield potential homozygotes. Such animals were selected by comparison of MS32 Southern blot signal intensities (11) and test bred with wild-type mice. True homozygotes were subsequently used for mutational analysis.

**Characterization of mouse DNA flanking the transgenes**

Mouse DNA flanking transgenes B and C was isolated by vectorette PCR (14) using outwardly directed primers located close to the 5′ and 3′ extremes of the construct (primers 32+10IR, 5′-TGAATCTAGGTATGACATTATGTC-3′) and 32–28R, 5′-TTGCCCTTGTTGCCAGGGTTG-3′ (Fig. 1). Vectorette PCR products from transgenic mouse DNA were sequenced and primer pairs designed for amplification of mouse DNA. For the 5′ flanking region of transgene B, primers TGB-AF (5′- GCAGATCTTAGGTCATCTGC-3′) and TGB-BR (5′-AACATTTCCCTATGTGGAGG-3′) were used to amplify a 324 bp segment of DNA from both C57BL/6J and 370 bp fragment from M. spretus; this length polymorphism arises from STR within the amplicon. For the 3′ flanking region of transgene C, primers TGC-AR (5′-TCACTACAGCCATCGCTGGA-3′) and TGC-IF (5′-AGTGCTGAGGGCTTTGAA-3′) were used to amplify a 628 bp DNA fragment from both C57BL/6J and M. spretus and to identify a DraI RFLP within this amplicon that distinguishes these two species. Fifty mice from the EUCIB backcross were typed for each C57BL/6J/ACC-3 transgene B, primers TGC-AF (5′-TGCAAGGGTGAA TCACAA TGCCTC-3′) and TGC-BR (5′-GCCAGAAGGTGTTACTGGTGAGA T-3′) 5′ close to the 5′ end of transgene C, primers TGC-BF (5′-AGTGCTGAGGGCTTTGAA T-3′) and 32–18R, 5′- GCAGA TTACTAGGTCTCA TGCC-3′ for 15 min for 38 cycles. Primers were MS32–5F (5′-GCCAGAAGGTGTTACTGGTGAGA T-3′) and 32+5F (5′-GCCAGAAGGTGTTACTGGTGAGA T-3′). The cosmid was recloned digestion with these two restriction enzymes; both enzymes cleave only once in cMS32.28 (Fig. 1). The cosmid was recloned plus I–Psp1406I fragment were microinjected by standard methodology to yield clone cMS32.28.R.

All subsequent SESP-PCR manipulations, including size fractionation, recovery, quantitation, detection, purification and mapping of MS32 mutants, were carried out as previously described (5,13). For both transgenes the size fractionation performed would have detected mutants that gain or lose 3–20 repeats. Changes of one repeat and an unknown portion of two repeat changes would have been discarded in fractions rich in progenitor allele molecules.

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