

Methylation perturbations in retroelements within the genome of a *Mus* interspecific hybrid correlate with double minute chromosome formation

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Abstract

A reduction in the DNA modification of cytosine methylation has been linked directly to chromosome rearrangements concomitant with retroelement amplification in several marsupial hybrid genomes. While phenotypes observed for interspecific eutherian hybrids are suggestive of methylation perturbations and retroelement instability, no link between retroelements, DNA methylation, and chromosome instability has yet been identified. Previous studies in eutherian hybrids, however, have been limited to a gross examination of methylation using methylation-sensitive restriction enzyme analysis or focused on single-copy genes and/or have avoided examination of repetitive DNA. Methylation changes and retroelements are proposed as mechanisms for double minute chromosome formation and oncogene amplification, both present in the genome of a *Mus* hybrid model, thus making it an ideal system to evaluate methylation status more closely. We have used the PCR-based methodologies methylation-sensitive amplicon subtraction (MS-AS) and methylation-sensitive representational difference analysis (MS-RDA) to detect differentially methylated sequences between three complex genomes and to isolate methylation perturbations in a *Mus musculus* × *Mus caroli* hybrid. This novel application of MS-AS and MS-RDA resulted in the isolation of differentially methylated retroelements surrounding the locus on Chromosome 10 responsible for double minute chromosome formation within this interspecific eutherian hybrid.

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Three cell lines derived from a grossly normal interspecific murine hybrid were reported to all contain double minute chromosomes [1], later determined by fluorescence *in situ* hybridization to be of the same chromosomal origin, Chromosome 10 [2]. Each of these cell lines exhibits amplification and associated overexpression of the paternal copy of the same gene (*Mdm2*) on the double minutes. Both the cytogenetic manifestation of DNA amplification as double minute chromosomes and the transcriptional upregulation of *Mdm2* are causally linked to CpG methylation in the genome, the former through the action

of retroelements [3] and the latter through the *p53* gene pathway [4]. Mutations in the genes and/or proteins involved in the establishment and maintenance of methylation patterns during mouse development result in embryonic lethality, loss of imprinting, and behavioral abnormalities (reviewed in [5]); an increased rate of chromosomal instability, gene rearrangements, and tumor susceptibility [6,7]; as well as retroelement activation manifesting as meiotic failure [8].

While perturbations to methylation were identified globally within hybrid marsupial genomes [9], genome-scale methylation patterns were reportedly normal in eutherian hybrids between antelope species [10] and in Equidae, Muridae, and Camelidae hybrids [11], as assayed by restriction enzyme analyses. Interspecific hybrids of *Peromyscus* also do not show whole-genome changes in methylation using this same method (R.J. O'Neill et al., unpublished observations), yet they exhibit imprinted gene expression perturbations associated with allele-specific hypomethylation [12,13]. In addition, restriction enzyme analysis using isoschizomeric enzymes (*MspI* and *HpaII*) allows for identification of large-scale methylation modifications; however,

Abbreviations: LINE, long interspersed nuclear element; PCR, polymerase chain reaction; MS-AS, methylation-sensitive amplicon subtraction; MS-RDA, methylation-sensitive representational difference analysis; ERV, endogenous retrovirus; ORF, open reading frame; LTR, long terminal repeat; SINE, short interspersed nuclear element; MIR, moderate interspersed repeat; MaLR, mammalian apparent LTR-retrotransposon; SNP, single-nucleotide polymorphism; UTR, untranslated region.

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it does not provide the resolution needed to detect small-scale changes [14]. Subtle changes in genome methylation may be the underlying cause of the loss of imprinting observed in the *Peromyscus* hybrids, the placental dysplasia and sterility reported in closely related *Mus* hybrids [15–17], and the genetic instability observed in our *Mus* hybrid [2].

A comparative analysis was performed to assess the ability of PCR-based genome scanning methods (MS-AS, methylation-sensitive amplicon subtraction, and MS-RDA, methylation-sensitive representational difference analysis) to detect sequences carrying unique demethylated CpGs in an interspecific *Mus musculus* × *Mus caroli* hybrid genome. MS-AS and MS-RDA are two methods that were designed to identify changes in methylation of unknown genes in complex genomes rather than limiting the evaluation to known genes [18–21]. We have successfully adapted the MS-AS and MS-RDA procedures to examine the differences between three genomes: two parental species and an interspecific hybrid. Both procedures produced a library of hybrid-specific demethylated sequences enriched in LINE-1 elements that map to Chromosome 10, adjacent to the locus derived in the double minute chromosomes. This is the first direct evidence of the involvement of retroelement demethylation in eutherian hybrid dysgenesis and genome instability.

Results and discussion

MS-AS and MS-RDA of three complex genomes

MS-RDA, a modification of representational difference analysis [22], is based on using a representation of unmethylated sites in the genomes to be compared [23]. The representations of the genomes are prepared by digesting the genomes with a methylation-sensitive restriction enzyme (*HpaII*) that targets approximately 10–20% of methylatable CpGs, and then the ratios of the genomes are optimized to allow the detection of methylation differences at the resolution of a single copy per diploid genome. Representations of the genomes (tester and driver) are amplified and enriched by PCR after ligation of the digested sequences to an oligonucleotide adaptor. Multiple cycles of subtractive hybridization between the tester and the driver samples are possible by switching the adaptors used in each round of subtraction. Traditional subtractive hybridization removes sequences common to the driver and tester and allows for the enrichment of unique double-stranded target tester sequences by PCR. This modification isolates uniquely demethylated target sequences in the tester genome. The MS-AS procedure differs from MS-RDA in that digested representations of the driver and tester genomes undergo only one round of competitive hybridization [24]. Two rounds of selective PCR amplification of the subtracted hybridization product are performed using adaptor-specific primers. The significant difference between MS-AS and MS-RDA is that MS-AS does not rely on multiple rounds of adaptor switching and amplification, thereby reducing the potential for spurious products and/or a lower yield. MS-AS is, however, limited by restriction enzyme choice based on adaptor design. The identification of demethylated sequences in a hybrid genome is a novel application of both the MS-AS and MS-RDA techniques.

After two rounds of subtractive hybridization, a range of difference products from 50 to 600 bp (MS-RDA) and from 50 to 500 bp (MS-AS) was cloned and sequenced. Clones (average insert size of 156 bp for MS-RDA and 160 bp for MS-AS) were initially categorized as a retroelement, an unannotated sequence from the mouse genome (typically identified on a single BAC clone in GenBank), or unidentified in GenBank. Within the pool of sequences isolated by MS-RDA, 66.6% (22/33 total clones) carried homology to LINE-1, long terminal repeat (LTR), or SINE sequences; 24.2% (8/33) were identified as nonrepetitive BAC clone sequences; and 9% (3/33) were unidentifiable by sequence searches to BLASTN, BLASTX, and tBLASTX. The pool of sequences isolated by MS-AS was reduced to only those clones containing homology to retroelements (12.7%, 6/47 total clones). This refinement was implemented specifically in the current analysis as it has previously been documented that hypomethylation can lead to instability of these repetitive types of sequences [25] and because a high percentage of these types of sequences had been isolated by MS-RDA in this hybrid system. The remainder of the sequences isolated by MS-AS, including clones that contained simple sequence repeats of (GGGAGA)_n, (AC)_n, or rDNA (12, 2, and 5/47 total clones, respectively) and nonrepetitive BAC clone sequences, were put aside for future studies.

The identities of the retroelement pools of sequences from MS-RDA (22 clones) and MS-AS (6 clones) were compared to one another (Table 1). Of these, there are three SINEs, one MIR, and two mouse B elements (B1 and B3), while three sequences (MS-RDA11, MS-RDA14, and MS-AS23) were homologous to LTRs of different retroelements (ERV-K, ERV-1, and MaLR, respectively) [26] and thus did not share identity with one another. The remainder of the MS-RDA and MS-AS clones (22 clones) were derived from LINE sequences; however, various portions of a *M. musculus* LINE were represented by a subset of sequences, homologous to the first, second, and third monomers of the 5' UTR, ORF2, and 3' UTR (Fig. 1). Classification and location of the clones in the LINE sequence were based on SNPs (Table 1 and Fig. 1). The percent identity of the clones to the LINE sequence shown in Fig. 1 were 66–96% spanning the 5' UTR, 93% in ORF2, and 88–100% for clones mapping to the 3' UTR.

Table 1
Retroelement identity of clones isolated by MS-RDA and MS-AS from a *Mus musculus* × *Mus caroli* hybrid genome

Identity to retroelement	Clone name
L1_MM (M29324)	MS-RDA: 13, 15, 16, 21, 22, 24, 25, 28, 31, 33, 35, 45 MS-AS: 24-M17, 25, 32
L1_MM_F (X57795)	MS-RDA: 6, 12, 20, 26, 32, 40 MS-AS: 37
LTR	MS-RDA: 11, 14 MS-AS: 23
B1	MS-AS: 13-M6
B3	MS-RDA: 38
MIR	MS-RDA: 29

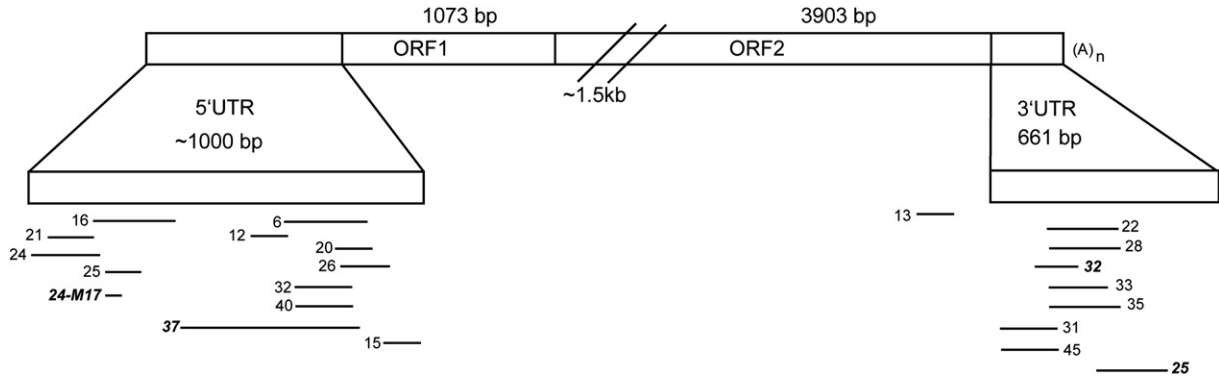


Fig. 1. Schematic of MS-RDA and MS-AS (bold italics) clones aligned to a *M. musculus* LINE-1 element.

Methylation status of retroelements

Several LINES isolated by both MS-AS and MS-RDA (MS-RDA 6, 12, 16; MS-AS 24-M17, 37) and the LTRs (MS-RDA 11) were analyzed for methylation status in genomic DNA from both the parental species and the hybrid genomes via Southern analysis with *MspI* (CCGG recognition sequence) and its isoschizomer, *HpaII*, whose endonuclease activity is hindered by CpG methylation (Figs. 2 and 3 and Supplementary Figs. 1A and B). For each separate sequence analyzed (six total), probe sequences hybridized to multiple fragments that were clearly hypomethylated in the hybrid compared to normal *M. musculus* and *M. caroli* DNA.

Figs. 2 and 3 show Southern hybridization of LINE-1 sequences (MS-RDA 16 and MS-AS 24-M17, respectively) to DNA from *M. musculus*, *M. caroli*, and the hybrid-derived cell lines B3, B5, and C3. Hypomethylation of the LINE-1s and the LTR sequences specific to hybrids is evident as multiple restriction fragments in the lanes of *HpaII*-digested hybrid DNA (B3, B5, C3) that are not present in the *HpaII* lanes of the parental DNA (closed arrowheads). Additional restriction fragments found in the *HpaII*-digested DNA in the hybrids that are not present in any parental digests (*MspI* or *HpaII*) (open arrowheads) are likely indicative of larger fragments that have

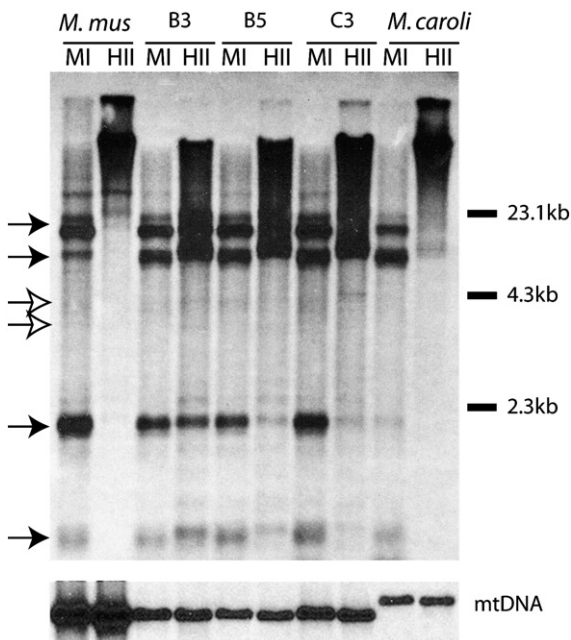


Fig. 2. Southern analysis of genomic DNA isolated from *M. musculus*; *M. musculus* × *M. caroli* hybrid-derived cell lines B3, B5, and C3; and *M. caroli* digested with *MspI* (MI) and *HpaII* (HII) and probed with MS-RDA clone 16. Bottom Southern analysis is the same blot probed with mitochondrial DNA (mtDNA). Closed arrowheads indicate *MspI* fragments that have undergone hypomethylation specific to the hybrid genomes as evidenced by the presence of corresponding bands in the *HpaII* digestion. Open arrowheads indicate *HpaII* restriction fragments unique to the hybrid genomes.

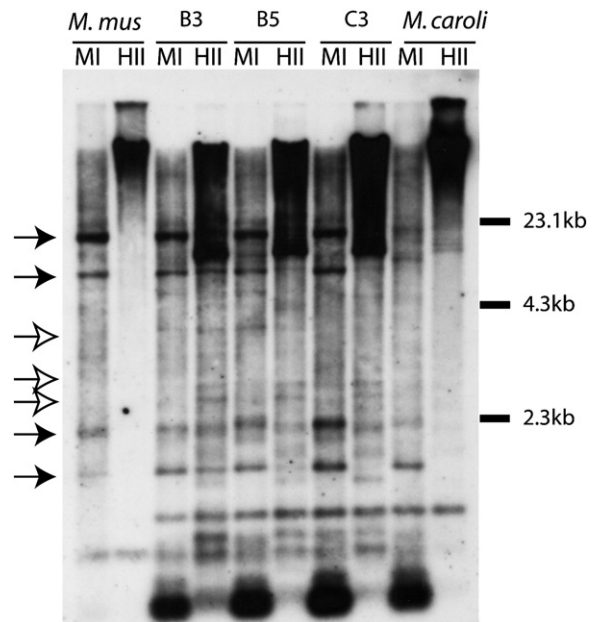


Fig. 3. Southern analysis of genomic DNA isolated from *M. musculus*; *M. musculus* × *M. caroli* hybrid-derived cell lines B3, B5, and C3; and *M. caroli* digested with *MspI* (MI) and *HpaII* (HII) (blot and control shown in Fig. 2) and probed with MS-AS clone 24-M17. Closed arrowheads indicate *MspI* fragments that have undergone hypomethylation specific to the hybrid genomes as evidenced by the presence of corresponding bands in the *HpaII* digestion. Open arrowheads indicate *HpaII* restriction fragments unique to the hybrid genomes.

undergone demethylation at only one of the CpGs bounding the restriction fragment.

All three hybrid cell lines show hypomethylation of retroelement sequences; however, amplification of these retroelement sequences is not detectable in these Southern analyses. The three cell lines derived from the *M. musculus* × *M. caroli* hybrid were previously shown to have different X-inactivation patterns [1,27], indicating derivation from different cell populations. However, all three cell lines share the same restriction fragment patterns for the LINE-1 probes (Figs. 2 and 3), indicating they share the same demethylated CpGs at the same sites. In addition, all three cell lines were previously shown to have amplification of the same paternally inherited oncogene, *Mdm2*. This suggests that the demethylation of these target sequences occurred very early in embryogenesis, prior to the establishment of X inactivation.

The Southern hybridization patterns observed for all MS-RDA and MS-AS retroelement clones were identical, suggesting these sequences all reside on a large fragment that was a preferential target for demethylation in the hybrid genome. While the parents of this cross were not obtainable for this study, 10 unrelated individuals of each species examined for these methylation differences showed no variation in methylation at these sites (data not shown). Due to a lack of distinguishing restriction fragment length polymorphisms for these target sequences between *M. musculus* and *M. caroli*, it is not possible to determine unequivocally the parental origin of the

demethylated retroelement sequences. However, the level of SNPs within these sequences compared to the mouse genome database [28] indicates *M. caroli* as the likely origin of these sequences.

The target of retroelement demethylation maps to chromosome 10

A potential preferential target for demethylation within this hybrid genome lies on Chromosome 10, as it was previously reported that the hybrid cell lines exhibited instability of a portion of the *M. caroli* copy of Chromosome 10, specifically *Mdm2* at 10qD2 (117,091,898–117,113,709, mm8 Build 36, Feb 2006) [2]. MS-RDA and MS-AS clones were evaluated for sequence similarity to annotated BACs on *M. musculus* Chromosome 10. Each LINE and SINE clone isolated by MS-RDA and MS-AS from the hybrid genome showed significant sequence identity (82–100% identity) to BAC clones on Chromosome 10 spanning 10qD2 via BLASTN (Fig. 4). MS-RDA clones 11 (LTR), 38 (B3A), 29 (MIR), and 36, and three clones isolated by MS-AS (30, 33, 34) that did not contain LINE-1 sequences, shared significant sequence identity only to *M. musculus* Chromosome 10 BAC clones (>93% identity). Two clones containing nonrepetitive Chromosome 10 sequence, MS-RDA clone 36 and MS-AS clone 33, were shown to be hypomethylated in the hybrid cell lines compared to normal *M. musculus* and *M. caroli* DNA (Supplementary Figs. 1C and D) and lie adjacent to the retroelement-containing clones (Fig. 4).

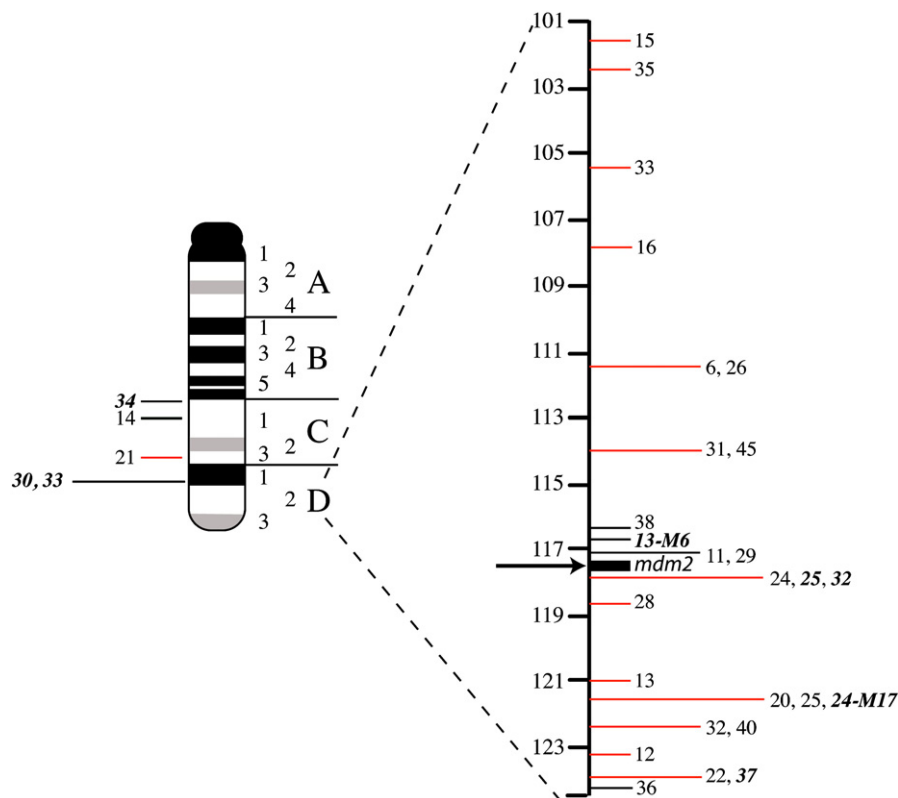


Fig. 4. Map of Chromosome 10D region indicating the relative locations of demethylated retroelements identified by MS-RDA/MS-AS and *Mdm2* (arrow). The length of each bar to the right of the map is proportional to the number of clones that are located within 10 kb of one another. The number indicates an MS-RDA clone, while MS-AS clones are in bold italic. Red lines are the locations of LINES. The scale bar spans from 101,460,000 to 124,475,000 bp, with a marker every 200,000,000 bp. The ideogram of Chromosome 10 is shown on the left.

There are greater than 100,000 copies of LINE-1s in the mouse genome, constituting approximately 10% of the mouse genome [29]. A random sampling of LINE sequences within any *Mus* genome should comprise LINE sequences from every chromosome in the genome; however, every LINE isolated from the hybrid genome by MS-AS and MS-RDA carries significant sequence identity restricted to Chromosome 10 sequences. The LINE-1 sequence-containing clones and the nonrepetitive Chromosome 10 sequence-containing clones are hypomethylated in the hybrid cell lines, showing definitively that this locus, adjacent to *Mdm2*, was the preferential target for demethylation in the hybrid genome.

Conclusions

The results of the current MS-RDA and MS-AS experiments in this *M. musculus* × *M. caroli* hybrid genome support our previous proposal that site-specific reduction in CpG methylation targets retroelements within mammalian hybrids and is the first demonstration of retroelement demethylation in a eutherian system. Concomitantly, hypomethylation of these elements is likely responsible for the instability leading to the amplification of a the *Mdm2* oncogene from Chromosome 10 on double minute chromosomes in this hybrid genome [2].

The mechanism responsible for the reduction of methylation in hybrids is as yet unknown but we postulate that it could be the result of interspecies incompatibility of the methylation machinery factors. It has been proposed that the loss of genomic imprinting in *Peromyscus* interspecific hybrids may be the result of rapid divergence of elements involved in epigenetic gene regulation [30]. DNA methyltransferases that epigenetically mark imprinting control regions also methylate repetitive elements (reviewed in [31,32]). The DNA methyltransferases *Dnmt3a* and *Dnmt3l* are required for the differential timing of demethylation and *de novo* methylation of repeated elements, as well as the differentially methylated regions of several imprinted loci, during germ cell differentiation and postfertilization (reviewed in [33]). Species specificity of these proteins and/or recognition sequences could result in a breakdown of sequence-specific methylation patterns in the hybrid genome. The mechanism by which specific targeting of these proteins to retroelements occurs is unknown, although the involvement of tissue-specific small RNA pools, such as piRNAs, has been suggested [32]. Thus, the incompatibility in establishing epigenetic marks at certain loci in hybrids, whether they are imprinted genes or retroelements, may be the result of aberrant control of the proteins involved in DNA methylation. Alternatively, the incompatibility may lie in species-specific targeting mechanisms, such as species-specific piRNA pools, required for retroelement-specific hypermethylation after fertilization.

Studies of human double minutes have shown that hypomethylation of CpG islands is associated with double minute (DM) formation [34]. The stability and structure of DMs are highly variable within one tumor type and across tumor types [35] and are a result of the most frequent manifestation of genome instability, amplification [36]. In cancer cells, LINEs are located at the sites of chromosome breakage [37] and fragile sites

[38], which are both implicated in the mechanism of DM formation [36,39]. The nature of DNA on DMs is different within and between cell types and yet all DMs are amplified DNA sequences, which suggests a common mechanism of formation. We propose a role for demethylation and retroelements in that demethylation of retroelements leads to genome instability and repeated chromosome breakage–fusion. Amplification via DMs has been shown to result from chromosome breaks followed by the bridge–breakage–fusion cycle [36]. LINE-1s, the type of sequence found to be demethylated in the *M. musculus* × *M. caroli* hybrid genome, are implicated in somatic mutations and disruption of tumor suppressor genes [40,41] and observed near translocation breakpoints or sites of chromosome breakage [37,42]. A direct mechanism leading to chromosomal instability is the loss of methylation. However, an indirect mechanism correlating with chromosomal instability is LINE-1 hypomethylation [43,44], and of particular interest is the suggestion that retroelements have been involved in the formation of double minute chromosomes [4]. In addition, the host methylation machinery may preferentially recognize retroelements and be, in some circumstances, the centers from which methylation spreads [37], giving support to our proposal that the hypomethylation and concomitant double minutes observed in our hybrids could have arisen as a result of defective recognition of methylation machinery due to species incompatibility. This study reveals the utility of MS-RDA and MS-AS to isolate differentially methylated sequences from a hybrid genome and a more general importance of our hybrid model system as an experimental model for the analysis of double minute formation and retroelement activity.

Methods

DNA isolation

Cell lines B3, B5, and C3 were graciously provided by G. Martin and J. Graves. Cell lines were independently derived from a 16.5-day *M. musculus* × *M. caroli* hybrid embryo [27]. Cells were maintained at 37 °C, 5% CO₂ in Dulbecco's modification of Eagle's medium with penicillin–streptomycin (20 units/20 µg/ml), L-glutamine (1.46 mg/ml), and fungizone (1 µg/ml), supplemented with 10% fetal bovine serum (Life Technologies). Cell pellets were harvested from confluent cell cultures and DNA was prepared as per standard phenol extraction protocol [45].

MS-RDA

Methylation-sensitive representational difference analysis was performed as described [23], with modifications. DNAs for the driver sample (an equimolar amount of the two parental DNA types) and the tester sample (the hybrid DNA) were digested with the methylation-sensitive enzyme *HpaII* at 10 units/µg of DNA in an overnight reaction at 37 °C. Micron-100-cleaned samples were ligated to annealed *RHpa24/RHpa11* adaptors [23] at 16 °C overnight. All adaptors were annealed by heating 8 µg of adaptor24 with 4 µg of adaptor11 at 70 °C for 2 min followed by cooling at 0.75 °C per minute for 60 min. PCR was performed in 5 (tester) to 10 (driver) duplicate reactions on 50 ng of the R-ligation using *RHpa24* as a primer to generate tester and driver amplicons. Cycles were 72 °C for 5 min; 94 °C for 1 min, 68 °C for 30 s, 72 °C for 2 min × 20 cycles; 72 °C for 10 min. R adaptors were removed from cleaned, pooled tester and driver amplicons by digestion with 10 units/µg *HpaII* at 37 °C overnight. The digested tester amplicons were ligated to annealed *JHpa24/JHpa11* adaptors at 16 °C overnight. Subtractive hybridization was performed

with 500 ng of the tester J ligation and 20 µg of each parental amplicon (post removal of R adaptor). Driver and tester samples were combined, extracted once with phenol/chloroform/isoamyl alcohol, and cleaned once with chloroform/isoamyl alcohol. The aqueous layer was precipitated with ice-cold ethanol and ammonium acetate to 2.5 M, washed with 70% ethanol, air dried, and resuspended in 3× EE buffer (30 mM Epps/3 mM EDTA). The sample was denatured at 98 °C and reannealed in the presence of NaCl at 67 °C for 20–40 h. Tris–EDTA was added to the hybridization upon completion, followed by duplicate primary PCRs using 1/20 of the subtractive hybridization product and *JHpa24* as a primer to enrich for the tester sequences. Cycle conditions were 72 °C for 5 min; addition of primer; 94 °C for 1 min, 68 °C for 30 s, 72 °C for 2 min × 10 cycles; 72 °C for 10 min. Mung-bean nuclease digestion (20 units NEB) was performed to digest single-stranded DNA. Difference product 1 (DP1) was generated by duplicates of secondary PCR using *JHpa24* as primer with cycles at 94 °C for 1 min, 68 °C for 30 s, 72 °C for 2 min × 18 cycles; 72 °C for 10 min. J adaptors were removed from cleaned, pooled DP1 by digestion with *HpaII* at 37 °C overnight. DP1 was then ligated to annealed *NHpa* adaptors at 16 °C overnight. A second subtractive hybridization at 67 °C for 20–40 h was completed on phenol-extracted (as above) tester (100 ng of N ligation) and 20 µg of each parental driver (post R adaptor digestion). Difference product 2 was generated by primary PCR, mung-bean nuclease digestion, and secondary PCR as above using *NHpa24* as the primer.

MS-AS

Methylation-sensitive amplicon subtraction was performed as described [24], with modifications. Tester (hybrid) and driver (combined parental DNA) were digested with excess *HpaII*, cleaned, and ligated to annealed *RHpa24/RHpa11* adaptors. Ligated DNA was amplified by PCR using *RHpa24* as primer and cycled as per the MS-RDA protocol (see above). Cleaned, pooled driver and tester were digested with *HpaII* overnight at 37 °C followed by end filling with *Pfu* and dGTP and dCTP. Five hundred nanograms of tester sample was ligated to R1/RB and L1/LB adaptors in separate reactions. Two subtractive hybridization reactions (R1/RB and L1/LB) were set up with 50 ng of cleaned tester and 1 µg of each cleaned driver in 200 mM Hepes, pH 8.3, 2 M NaCl, 0.08 mM EDTA, pH 8.0, 40% PEG8000. Denaturation was for 2.5 min at 98 °C followed by reannealing at 68 °C for 8 h. The samples were then combined without denaturing, with freshly denatured driver added, and allowed to hybridize at 68 °C for 14–22 h. Hybridization was stopped with the addition of 68 °C 20 mM Hepes, pH 8.3, 50 mM NaCl, 0.2 mM EDTA, pH 8.0. Two rounds of PCR were done to amplify selectively the tester subtractive hybridization product using LP1 primer in the first round with cycle conditions of 72 °C for 5 min; addition of primer; 94 °C for 30 s, 66 °C for 30 s, 72 °C for 1.5 min × 40; 72 °C for 5 min. The second round of PCR included cleaned amplicon from the first-round PCR and the primers LP2 and RP2 with cycles as for the first-round PCR with the exception of annealing temperature (68 °C).

Subcloning and sequencing

Difference products were cloned into the pGEM-T Easy vector (Promega) and transformed into TOP10 competent cells (Invitrogen). Insert-positive clones were sequenced using an ABI 377 or ABI 3130 (Applied Biosystems). Clone sequence management, alignments, and contig development were performed in Vector NTI (Invitrogen). Homology searches were performed with the GenBank BLAST suite of programs, CENSOR [46], RepeatMasker [47], and BLAT [28,48]. GenBank accession numbers for the MS-RDA and MS-AS clones are as listed in Supplementary Table 1.

Southern analysis

Genomic DNA from hybrid and parental cell lines was digested overnight with *MspI* and *HpaII*, electrophoresed in a 0.8% agarose gel, acid nicked, and denatured. Southern blots were prepared by transferring the DNA to Hybond N+ membrane (Amersham) as per the manufacturer's instructions. Hybridization of radioactively labeled probes was at 65 °C overnight in 1 mM EDTA/0.5 M Na₂HPO₄/7% SDS followed by washing in 65 °C 0.5× SSC/0.1% SDS. Autoradiography was at –80 °C overnight using Kodak X-ray film.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ygeno.2007.12.001](https://doi.org/10.1016/j.ygeno.2007.12.001).

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