BIOLOGY OF REPRODUCTION (2012) **86**(3):88, 1–10
Published online before print 23 November 2011.
DOI 10.1095/biolreprod.111.095273

Retroelement Demethylation Associated with Abnormal Placentation in *Mus musculus* × *Mus caroli* Hybrids[1]

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ABSTRACT

The proper functioning of the placenta requires specific patterns of methylation and the appropriate regulation of retroelements, some of which have been co-opted by the genome for placental-specific gene expression. Our inquiry was initiated to determine the causes of the placental defects observed in crosses between two species of mouse, *Mus musculus* and *Mus caroli*. *M. musculus* × *M. caroli* fetuses are rarely carried to term, possibly as a result of genomic incompatibility in the placenta. Taking into account that placental dysplasia is observed in *Peromyscus* and other *Mus* hybrids, and that endogenous retroviruses are expressed in the placental transcriptome, we hypothesized that these placental defects could result, in part, from failure of the genome defense mechanism, DNA methylation, to regulate the expression of retroelements. Hybrid *M. musculus* × *M. caroli* embryos were produced by artificial insemination, and dysplastic placentas were subjected to microarray and methylation screens. Aberrant overexpression of an X-linked *Mus* retroelement in these hybrid placentas is consistent with local demethylation of this retroelement, concomitant with genome instability, disruption of gene regulatory pathways, and dysgenesis. We propose that the placenta is a specific site of control that is disrupted by demethylation and retroelement activation in interspecific hybridization that occur as a result of species incompatibility of methylation machinery. To our knowledge, the present data provide the first report of retroelement activation linked to decreased methylation in a eutherian hybrid system.

DNA methylation, epigenetics, evolution, genomics, hybrid dysgenesis, mVL30, placenta, retrovirus

INTRODUCTION

Mus musculus × *Mus caroli* hybrids are rarely carried to term, with most embryos dying early [1]. The hybrid embryos are growth retarded in comparison to both parental species at all stages of development [1], suggestive of early and intrinsic genomic incompatibility [2]. The incompatibility appears to result from within the placental-embryonic barrier, because transfer of *M. caroli* × *M. caroli* embryos to the uterus of *M. musculus* females also failed to survive [2]. Genomic incompatibility in eutherian hybrids can manifest as disruption of normal development, such as the dysmorphic placental phenotypes observed in *Peromyscus* hybrids [3], *M. musculus* × *Mus spretus* hybrids [4], and equid hybrids [5]. Hybrid genomic instability may also manifest as chromosome deletions, amplifications, or structural rearrangements, such as those reported in a *M. musculus* × *M. caroli* hybrid [6]. Moreover, chromosome rearrangements observed in a marsupial hybrid were accompanied by global genome hypomethylation, resulting in amplification of a retrovirus [7]. Differentially methylated retroelements were also found in an interspecific *M. musculus* × *M. caroli* hybrid and were shown to surround a locus on chromosome 10 responsible for double minute chromosome formation and oncogene amplification in the karyotypes [8, 9].

The observation of abnormal phenotypes, chromosome abnormalities, and dysgenesis in hybrids is not surprising if associated with decreased global methylation patterns and/or site-specific demethylation of retroelements. Indeed, 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 (for review, see [10]), an increased rate of chromosomal instability, gene rearrangements and tumor susceptibility [11, 12], as well as retroelement activation [13].

The placenta, formed at midgestation [14], plays a major role throughout pregnancy, including ion and nutrient exchange, waste disposal, and synthesis of steroid and peptide hormones for fetal growth. Despite common physiological functions among organisms, the structure of the placenta is species specific [15]; thus, the placenta is a likely source of genomic conflict accounting for hybrid dysgenesis. Genomic conflict arising from opposing forces for nutrient acquisition occurs at the maternal-fetal interface (i.e., the placenta) and involves interactions between maternal and fetal genes and proteins [16]. Chuong et al. [17] recently demonstrated that within rodent species, just over 20% of genes specifically expressed in the mature placenta are rapidly evolving, with a subset (13.5%) of the placental proteins experiencing positive selection. Moreover, the study showed that genes highly expressed in the mature placenta evolve significantly faster than genes expressed earlier in placental development, further supporting the theory of genomic conflict in the evolution of placental genes [17]. Within *Peromyscus* hybrids, genomic conflict manifested as a loss of imprinting, possibly due to divergent epigenetic pathways involved in gene silencing [18–20].

Genes, such as those involved in mature placental development and genomic imprinting, are targets of genomic conflict. However, retroelements have been demonstrated to facilitate placental-specific gene expression and, thus, may also be potential targets of conflict. Indeed, conflict over time has led to the evolution of retrovirus accessory genes and innate

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Received: 31 July 2011.
First decision: 26 August 2011.
Accepted: 14 November 2011.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

immunity mechanisms in mammalian species [21] as well as a retroviral envelope gene that is an active participant in genetic conflict in *Drosophila* [22]. Placental-specific regulation of adjacent genes by endogenous retroviruses has also been demonstrated [23–29], and in fact, many long terminal repeat (LTR) promoters are active primarily in the placenta [30] (for review, see [31]), with several endogenous retroviruses co-opted for regulation of preimplantation placental growth and differentiation.

Well-controlled physiological mechanisms, including appropriate establishment of DNA methylation patterns, must be in place in the placenta to regulate retroviral expression and permit normal development [32]. Thus, methylation may also be a target of genomic conflict in the placenta. However, placental defects observed in Bovidae, Equidae, Muridae, and Camelidae hybrids were not linked to genome-scale methylation defects as assayed by restriction enzyme analyses [33, 34]. Likewise, interspecific hybrids between *Peromyscus* species do not show whole-genome changes in methylation using this same molecular method (O'Neill, unpublished observations). Rather, *Peromyscus* hybrids exhibit imprinted gene expression perturbations associated with allele-specific hypomethylation [18, 19], indicating methylation changes may be locus specific in hybrid dysgenesis [4, 35, 36].

We recently demonstrated that differential methylation in an interspecific *M. musculus* × *M. caroli* hybrid targeted retroelement sequences [8] rather than the genome in toto. However, that study was limited to cell lines derived from a hybrid individual and, thus, was not able to address the hypothesis that any morphological defect, such as those observed in the placenta, is associated with methylation perturbations. Given the observations that retroelements are targeted by methylation changes in hybrids [7, 8, 37] and that retroelements are involved in species-specific placental expression patterns [29, 38] and are subject to control by methylation [38, 39], we set out to determine whether demethylation in hybrid placentas targets retroelements in an in vivo model. Presented herein is, to our knowledge, the first direct evidence for an association of retroelement demethylation in eutherian hybrid dysgenesis and genome instability.

MATERIALS AND METHODS

Artificial Insemination

Production of interspecific hybrids using *M. musculus* females (CD1; Charles River Laboratories) and *M. caroli* males (The Jackson Laboratory) was performed as described previously [1] with modifications. All animal care and use protocols were approved and followed the active Institutional Animal Care and Use Committee protocol at University of Connecticut. Approximately 25 day-old *M. musculus* females were given a hormonal treatment of 5 IU (0.1 ml) of equine chorionic gonadotropin administered via intraperitoneal injection for superovulation. Human chorionic gonadotropin was then administered 48 h later at a dose of 5 IU (0.1 ml) injected intraperitoneally. To inseminate the female, the sperm suspension was drawn into a 1-ml, disposable tuberculin syringe fitted with a 4-cm, 22-gauge blunted needle bent 2 cm from its tip at an angle of 110°. The cervix was then penetrated with the blunted needle. Following injection of no more than 50 μl of sperm suspension, the vagina was plugged with one cotton wool ball soaked in isotonic saline. Sperm were collected from finely chopped cauda epididymides from dead *M. musculus* and *M. caroli* males. The males were killed by CO_2 asphyxiation. The sperm from the dissected males were allowed to swim clear in 0.2 ml of PBS (pH 7.2) containing 0.3% bovine serum albumin. The sperm sample was then diluted 25- to 50-fold in 10% formol saline, and the sperm concentration was determined using a hemocytometer counting chamber. The amount used to inseminate one female ranged from 2×10^6 to 5×10^6 sperm. Hybrid embryos were recovered from females (killed by CO_2 asphyxiation) by flushing with PBS. The embryonic and placental tissue was established for fibroblast cell culture and/or snap-frozen in liquid nitrogen for nucleic acid extractions.

Cell Culture and Karyotype Analysis

Primary fibroblast cell lines were established for karyotype analysis by aseptically mincing the tissue in PBS with a scalpel and/or digestion with collagenase at a final concentration of 40 U/ml at 37°C for a minimum of 1 h to a maximum of overnight. Disaggregated cells were rinsed with PBS and maintained at 37°C and 5% CO_2 in Dulbecco modified Eagle medium with penicillin-streptomycin (20 U/ml and 20 μg/ml, respectively), L-glutamine (1.46 mg/ml), fungizone (1 μg/ml), and mercaptoethanol (0.1 mM) supplemented with 10%–20% fetal bovine serum (Life Technologies). Confluent cell cultures were harvested, and slides were prepared per standard protocols. Briefly, colcemid was added to a final concentration of 0.1 μg/ml at 37°C for 1–2 h, and cells were trypsinized and treated with 0.075 M KCl at 37°C for 15–20 min, prefixed, and fixed with 3:1 methanol:acetic acid (modified Carnoy). Cells were dropped onto acetone-cleaned slides, air-dried overnight, dehydrated, and stored at −20°C. G-banding and fluorescence in situ hybridization were performed as described previously [8, 9].

High-Throughput Bisulfite Sequencing and Data Analysis of Mouse VL30-1

Five-hundred nanograms of genomic DNA from the hybrid 6H1 and the *M. musculus* embryo 6E3 were bisulfite treated following the Zymo EZ DNA Methylation Kit protocol. Bisulfite-treated DNA primers (methylation-specific primers) were designed to amplify a 221 bp of mouse (m) VL30-1 using MethPrimer [40]. The PCR product was subsequently cleaned using the QIAquick PCR purification kit (Qiagen) with the following primers: forward, 5′-GAAAGTGTGGTTTAGTTTTTATGAT; reverse, 5′-ACTAATCT AATCCTTCAAATCATTTC. Clean PCR products were quantified using a PicoGreen assay from Invitrogen as per the manufacturer's protocols. Library preparation for Roche/454 sequencing was done using 500 ng of PCR product with a GS Rapid Library Kit from Roche Applied Sciences. The sample was processed as per GS Rapid Library protocol from Roche Applied Sciences using GS Rapid Library MID adaptors with modifications as per 454 Application Brief 001-2010 (http://engencore.sc.edu/wp-content/uploads/APP001-2010_AmpliconSequencingTipsforGSFLXTitaniumv2.pdf). The libraries were pooled and titrated as per Roche Applied Sciences GS Titanium SV emPCR (Lib-L) to optimize the molecule copy: bead ratio (CPB) for large-volume sequencing. Once the correct CPB was determined, a full-scale emPCR reaction was prepared using a GS Titanium SV emPCR (Lib-L) kit to obtain template-enriched beads for sequencing. Enriched beads were then loaded on a single lane of an eight-region GS Titanium PicoTiterPlate and sequenced using a GS Titanium Sequencing Kit XLR70 with 200 cycles on a Roche/454 Pyrosequencer. The raw image data were then fully processed on a computer cluster running Roche/454 data analysis processing software. Visualization and quality control of DNA methylation data from bisulfite deep sequencing was performed using BiQ Analyzer [41].

Microarray Screening

Briefly, total RNA (100 ng) was processed and hybridized to an Affymetrix Mouse 430A 2.0 GeneChip array. The cDNA synthesis, in vitro transcription, labeling (two-cycle eukaryotic target labeling), and fragmentation to produce the oligonucleotide probes were performed as per the manufacturer's protocol. Hybridization was performed using the GeneChip Hybridization Oven 640 for 21 h. The chips were washed in a GeneChip Fluidics Station 450 (Affymetrix) and the results visualized with a Gene Array scanner using the Affymetrix software. The placental array experiments included duplicate samples and replicate hybridizations for each of the two hybrids, 6H1 and 6H2, for the *M. musculus* embryo 6E3, and for the *M. caroli* embryo CE4. The Affymetrix data were analyzed using DNA-Chip Analyzer (dChip; www.dchip.org). Changes in gene expression were considered if the values met the filtering criteria of a 20% or greater positive hybridization (P) in the arrays used (sample hybridizations). The *P*-value for expression differences is set to a default of less than 0.05 in the dChip software. The National Institutes of Health (NIH) Database for Annotation, Visualization, and Integrated Discovery (DAVID) [42] functional annotation tool was then used to highlight the most relevant gene ontology (GO) terms associated with the dChip-generated list of genes significantly differentially regulated in the hybrid placentas in comparison to the *M. musculus* × *M. musculus* placentas. High classification stringency analysis in DAVID was performed only on transcripts that were greater than 2.5-fold up- or down-regulated in the hybrid placentas in comparison to the *M. musculus* × *M. musculus* placentas. Functional annotation clustering was statistically measured by the Fisher exact test. The functions of the misregulated hybrid placental genes were determined using the GO biological process annotation using the PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System [42].

Quantitative PCR

Real-time PCR analysis of mVL30-1 (forward, 5′-actactgcctgagacttcacctac; reverse, 5′-cccgccttccgcttaccc), *Dnmt1* (forward, 5′-cctagttccgtggctacgaggagaa; reverse, 5′-tctctctcctctgcagccgactca), *Dnmt3a* (forward, 5′-gccgaattgtgtcttggtg gatgaca; reverse, 5′-cctggtggaatgcactgcagaagga), *Dnmt3b* (forward, 5′-ttcagt gaccagtcctcagacacgaa; reverse, 5′-tcagaaggctggagacctccctctt), and beta-actin (forward, 5′-acacccgccaccagttcg; reverse, 5′-cgatggaggggaatacagcc) was performed. iQ SYBR Green Supermix (Bio-Rad) was used to amplify cDNA from the hybrids (6H1 and 6H2), *M. musculus* (6E3), and *M. caroli* (CE4). Each reaction contained 7.5 μl of iQ SYBR Green Supermix, 6.0 μl of water, 0.5 μl each of forward and reverse primers (100 ng/μl), and 0.5 μl of cDNA. Reactions were done in triplicate in 96-well plates and run on the Bio-Rad iCycler Optical Module instrument. PCR was carried out using the following steps: Initial denaturation was performed at 94°C for 3 min, followed by 40 cycles for mVL30-1 and 45 cycles for the *dnmt*s of 94°C for 30 sec, annealing temperature (58°C) for 30 sec, 72°C for 30 sec, and real-time data collection at 80°C for 10 sec). A melt-curve analysis followed amplification (55°C–95°C, +0.5°C per cycle). Relative quantification was based on the relative expression of the target gene (mVL30-1 or *dnmt* genes) versus the reference gene (beta-actin). Resultant values were standardized using the relative expression ratio mathematical model [43]. Significant differences ($P < 0.05$) in relative expression ratios between samples were determined by *t*-test analysis.

Northern Blot Analysis

Ten micrograms of placental RNA from hybrid (6H1 and 6H2), *M. musculus* × *M. musculus* (6E3), and *M. caroli* (CE4) were electrophoresed in a 6% formaldehyde, 1.2% agarose gel and transferred to Hybond membrane (Amersham) in 10× SSC (1× SSC: 0.15 M sodium chloride and 0.015 M sodium citrate) as per the manufacturer's instructions. Hybridization at 65°C was performed overnight in 1 mM ethylenediaminetetra-acetic acid, 0.5 M Na_2HPO_4, and 7% SDS, followed by washing at 65°C with 2× SSC and 0.1% SDS or 1× SSC and 0.1% SDS. Autoradiography was performed at −80°C overnight using Kodak x-ray film.

RESULTS

M. musculus × M. caroli *Hybrid Embryos Have an Apparently Normal Karyotype Despite Morphological Abnormalities*

Because *M. musculus* and *M. caroli* will not naturally mate, artificial insemination of CD1 females with *M. caroli* sperm was used to produce interspecific hybrids. CD1 and *M. caroli* were used for these crosses to recapitulate previous research [1, 9, 37, 44]. In all, 188 females were inseminated with sperm from *M. caroli* and 30 females with sperm from *M. musculus* (artificial insemination control), with a resultant pregnancy rate of 5.8% and 30%, respectively. Few *M. musculus* × *M. caroli* hybrids were expected to proceed to term, because the majority usually result in resorption between 9 and 15 days of pregnancy [45] and the postimplantation mortality rate is high (97%) for these crosses [1]. The reverse cross (*M. caroli* × *M. musculus*) and F2 generations were not attempted, because prior research found that the few hybrids successfully produced were sterile and that *M. caroli* blastocysts died when transferred to the *M. musculus* uterus [1, 2, 46, 47]. Two hybrid embryos (6H1 and 6H2) were retrieved at 13 days of gestation from one CD1 female (CD1F6-4) inseminated with sperm from a *M. caroli* male (CM6-5) during artificial insemination experiment 6 (AI6). An additional three hybrid embryos (11H1, 11H3, and 11_7H1) were retrieved at 13 days of gestation from two CD1 females (CD1F11-2 and CD1F11-4) inseminated with sperm from *M. caroli* males (CM11-6 and CM11-7, respectively) during artificial insemination experiment 11 (AI11). CD1 × CD1 age-matched embryos (6E3, 6E2, 11E1, and 11E2) were also collected using artificial insemination for AI6 and AI11.

The hybrid embryos were morphologically evaluated as growth retarded and grossly abnormal in comparison to the CD1 × CD1 age-matched controls. Hybrid placentas were hypoplastic, with an apparent lack of labyrinth vasculature, in comparison to the CD1 × CD1 controls (Fig. 1). The hybrids 6H1 and 6H2 were male with a 2n = 40 karyotype, and 11H1 and 11H3 were female with a 2n = 40 karyotype. *M. musculus* and *M. caroli* chromosomes are G-band identical, with the exception of the amount of centromeric heterochromatin; therefore, fluorescence in situ hybridization with a *M. musculus* probe specific to the major satellite centromere array, pMSAT6 [48], was used to demonstrate that hybrids carried an equal contribution of chromosomes from each parent (data not shown), in contrast to previously produced hybrids [6, 44].

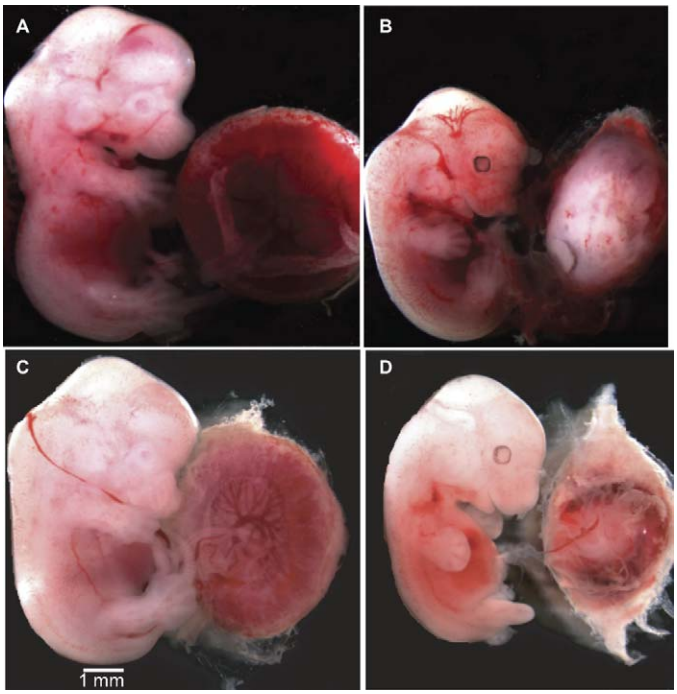

FIG. 1. Hybrid and normal embryos and placentas produced by artificial insemination. The *M. musculus* × *M. caroli* hybrid 6H1 (**B**) and 6H2 (**D**) embryos and placentas (13 days of gestation) are shown in comparison to age-matched *M. musculus* × *M. musculus* embryos and placentas (**A** and **C**). Hybrid embryos are growth retarded, with hypoplastic placentas, in comparison to the controls.

Retroviral and Methyltransferase Genes Are Aberrantly Expressed in the Placentas of M. musculus × M. caroli *Hybrids*

The biological response of the placenta to interspecific hybridization was examined via gene expression profiling on Affymetrix arrays. Gene expression patterns in the hybrids 6H1 and 6H2 were compared to the age-matched and artificial insemination control sample 6E3 (CD1) and a paternal species control (*M. caroli*). Present calls (i.e., positive hybridization) for placental mRNA from CD1, *M. caroli*, and hybrids averaged 58.8%, 57.04%, and 57.5%, respectively, indicating robust validity for detecting mRNA gene expression differences between these species and their hybrids on the Affymetrix platform.

The Affymetrix data across all datasets were analyzed using DNA-Chip Analyzer for gene expression changes specific to the hybrid placentas. To test our hypothesis that demethylation in hybrid placentas affects retroelements, we reasoned that the retroelements targeted for demethylation should also exhibit

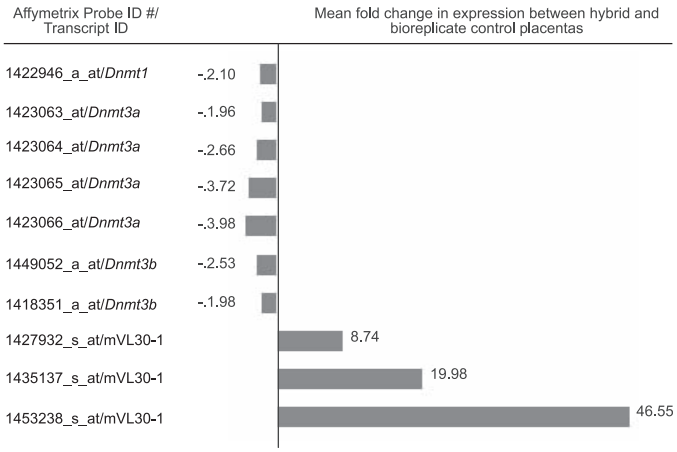

FIG. 2. DNA methyltransferase and retroviral gene expression in hybrid placentas. Microarray analysis shows an increase in the expression of an X-linked mouse retroelement, mVL30, and a decrease in expression of DNA methyltransferase genes in *M. musculus* × *M. caroli* hybrid placentas in comparison to bioreplicate control placentas (*M. musculus* and *M. caroli*).

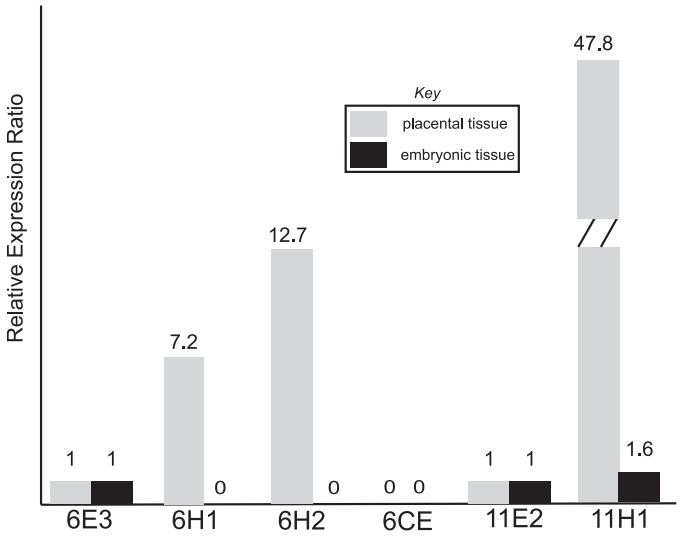

FIG. 3. Expression of mVL30-1 in hybrid embryos and placentas by qRT-PCR. Hybrid (6H1, 6H2, and 11H1) placentas show a statistically significant ($P < 0.05$) increase in expression of mVL30-1 that is restricted to the placenta (gray fill); expression in the hybrid embryos (black fill) is decreased or not significantly different. Expression ratios are relative to the experimental and species control placental tissue (6CE, 6E3, or 11E2).

expression changes [49]. In addition to global gene expression changes (see below), we analyzed changes in gene expression for the transcripts of DNA methyltransferase genes *Dnmt1, Dnmt3a,* and *Dnmt3b* and for the 87 probe sets contained on the Affymetrix array that were associated with the terms ''retroelement'' and/or ''retrovirus.'' Gene expression changes for one retroelement and one retrovirus were the only transcripts within this probe set that exhibited a fold-change of greater than 1.2. Up-regulation of *M. musculus* endogenous defective Murine leukemia virus LI-12 (MlvLI-12) truncated pol/envelope fusion protein mRNA and three different transcripts of *M. musculus* mVL30-1 retroelement mRNA were coincident with down-regulated expression of DNA methyltransferase genes. The highest up-regulation was observed for MlvLI-12 (LOC433762), at greater than 50-fold; however, this record was subsequently removed by RefSeq staff from the National Center for Biotechnology Information database and, thus, was not further analyzed. X-linked mVL30-1 transcripts were up-regulated in expression, whereas expression of the DNA methyltransferases, *Dnmt1*, *Dnmt3a*, and *Dntm3b,* were down regulated in the *M. musculus* × *M. caroli* hybrid placentas versus the control samples (6E3, *M. caroli*) (Fig. 2).

Validation and quantification of Affymetrix expression were performed for mVL30-1 (AF486451.1) and the methyltransferase genes *Dnmt1* (MGI:94912), *Dnmt3a* (MGI:1261827), and *Dnmt3b* (MGI:1261819). Quantitative RT-PCR (qRT-PCR) results showed a statistically significant ($P < 0.05$) increase in the expression of mVL30-1 in the hybrid placentas 6H1 and 6H2 relative to the *M. musculus* × *M. musculus* (6E3) placenta (Fig. 3). Minimal expression of mVL30-1 was detectable in *M. caroli* by RT-PCR or qRT-PCR, likely due to sequence divergence for the primer sequences given that relative expression levels of mVL30-1 were similar for *M. musculus* and *M. caroli* as determined by Gene Chip and Northern blot analysis (Supplemental Fig. S1; all Supplemental Data are available online at www.biolreprod.org). The qRT-PCR evaluation of mVL30-1 expression showed the aberrant overexpression of mVL30-1 was restricted to the placental tissue, with significantly decreased expression levels found in the embryonic tissue (Fig. 3). In addition, qRT-PCR showed a statistically significant ($P < 0.05$) decrease in expression of *Dnmt1*, *Dnmt3a*, and *Dnmt3b* in the hybrid placentas relative to the control placentas, with the decreased expression again limited to the placental tissue (Fig. 4). Although the fold-changes in expression levels vary between the two technical procedures, Affymetrix (Fig. 2) and qRT-PCR (Figs. 3 and 4), the qRT-PCR confirmed the Affymetrix expression array data. The placental-specific, abnormal expression patterns for mVL30-1, *Dnmt1, Dnmt3a,* and *Dnmt3b* in *M. musculus* × *M. caroli* hybrids was confirmed by qRT-PCR on hybrid and control RNAs obtained from another litter, AI11 (11H1 and 11E2) (Fig. 3). A statistically significant overexpression of mVL30-1 restricted to the placental tissue was observed for the hybrid embryo 11H1, with a statistically significant ($P < 0.05$) decrease in expression of *Dnmt1* and *Dnmt3b* in the hybrid placenta relative to the control placenta. Thus, an increase in expression of the maternally derived, X-linked mVL30 was found in male hybrids as well as in female hybrids, specific to the placenta.

A recent report demonstrated overexpression of X-linked genes in sterile F1 hybrid male mice in a *M. musculus* × *M. domesticus* hybrid system during spermatogenesis [50]. To determine whether the mVL30 retroelement was overexpressed simply because it resides on the X chromosome, we assayed for gene expression changes in the placenta by chromosome in each hybrid. We found no significant difference between the X chromosome and the autosomes (Supplemental Fig. S2), indicating that X-linked overexpression either is not present in this hybrid system or is a phenomenon restricted to spermatogenesis.

The methylation status of the retrovirus mVL30-1 was assayed in a hybrid placenta and a control placenta by treating the genomic DNA with bisulfite followed by amplification of a 221-bp product of mVL30-1 using methylation-specific primers. The methylation-specific mVL30-1 products were deep-sequenced via 454 pryosequencing and subsequently analyzed against the CpG sites and methylation status of bisulfite-treated and untreated samples. A statistically significant ($P < 0.01$) difference was found in the methylation status

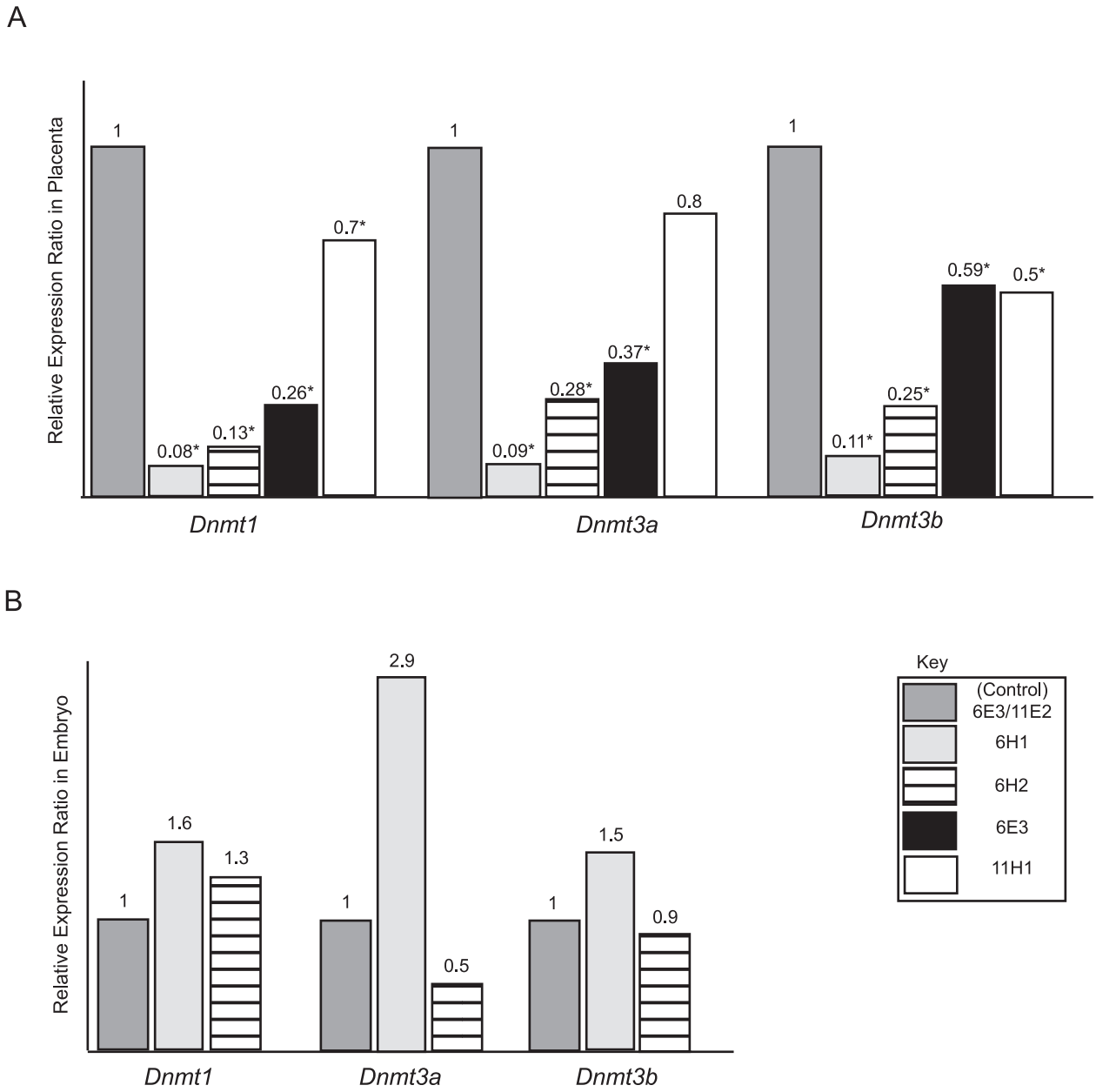

FIG. 4. DNA methyltransferase gene expression in hybrid embryos and placentas by qRT-PCR. A statistically significant (*$P < 0.05$) decreased expression of *Dnmt1*, *Dnmt3a*, and *Dnmt3b* in hybrid (6H1 and 6H2) placentas relative to the experimental and maternal species control placenta (6E3) was found, as was a statistically significant (*$P < 0.05$) decreased expression for *Dnmt1* and *Dnmt3b* in the 11H1 hybrid placenta relative to the experimental and maternal species control placenta (11E2). The relative expression values for the maternal species control placentas (6E3 and 11E2) are normalized to one and, thus, are presented together in a single column. Decreased DNA methyltransferase gene expression is limited to the placental tissues (**A**), because no evidence of significant, consistent expression changes for *Dnmt1*, *Dnmt3a*, or *Dnmt3b* was found in embryonic tissue from the AI6 hybrids in comparison to the control embryo (**B**).

of mVL30-1 between the hybrid and the control placenta across all CpGs (Fig. 5).

The Biological Significance of an Aberrantly Expressed Retroelement in the Placentas of M. musculus × M. caroli *Hybrids*

It is evident from the Affymetrix expression array analyses that although overexpression of mVL30-1 occurred, no one single gene or pathway was affected in the *M. musculus* × *M. caroli* hybrids. More than 1000 transcripts were differentially expressed by a fold-change of 2.5 or greater in the hybrid placentas in comparison to the pooled replicates of *M. musculus* and *M. caroli* placentas, with 347 being more than 5-fold different and 118 being more than 10-fold different (Supplemental Table S1). Although many pathways are disrupted in the hybrid placentas, the gene expression patterns observed further support the finding that genes involved in normal development of the placenta are affected.

The NIH DAVID [42] functional annotation tool was used to highlight the most relevant GO terms in the dChip-generated list of misregulated transcripts (2100 Affy IDs) within the hybrid placentas in comparison to the control placentas. Annotations were based on comparison of hybrid placental transcripts to only the pooled biological replicates of *M. musculus* × *M. musculus* placentas and not *M. caroli* placentas

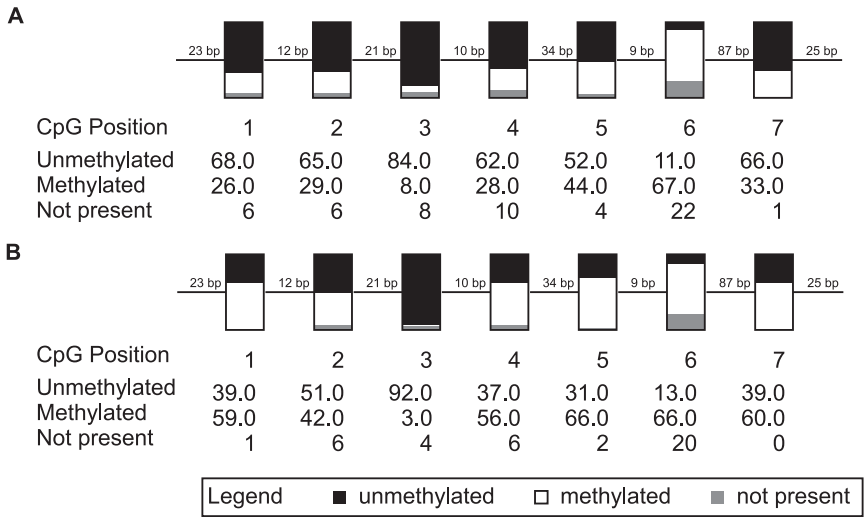

FIG. 5. Methylation analysis of mVL30-1 in hybrid and control placentas. BiQ Analyzer methylation data show a statistically significant ($P < 0.01$) difference in the methylation status of mVL30-1 between the hybrid placenta 11H1 (**A**) and the control placenta 11E1 (**B**).

to lessen the impact of specifies-specific single-nucleotide polymorphisms on our data analyses (the DAVID method uses species-specific gene and protein identifiers and does not include *M. caroli* in the population background genome sets). Functional annotation clustering showed that misregulated hybrid placental transcripts (Supplemental Table S1) were highly associated (gene enrichment scores > 2.0; $P < 0.05$) with the terms listed in Table 1. As shown in Table 1, the functional group that contained most of the differentially expressed genes was one associated with cellular components of the membrane, suggestive of placental tissue developmental defects as a cause, because the placental structure is largely membranous [51, 52]. The clustering of misregulated hybrid placental transcripts into Functional Group 6 in Table 1 (biological processes associated with blood vessel and vasculature development) is consistent with the morphological finding of lack of vascularization observed in the hybrid placentas (Fig. 1). Concomitantly, a high functional annotation enrichment score (7.42) was seen for the protein domain classification of cadherins (Table 1). Cadherins are transmembrane proteins that play important roles in cell adhesion within epithelial, neuronal, and placental tissue [53, 54] and, when aberrantly expressed, result in placental layer formation and vascularization defects [55]. Cadherins are dependent on calcium ions to function; calcium-binding protein domains (Table 1, Functional Group 13) and 140 genes that function in calcium binding (Supplemental Table S2) are misregulated in the hybrid placentas. Frels et al. [2] proposed an immune response, with the trophoblast as the site of rejection, as the primary cause of the high mortality of *M. musculus* × *M. caroli* hybrid embryos. The GO biological process annotation within the PANTHER Classification System [42] lends support to this

TABLE 1. NIH DAVID functional annotation clustering of aberrantly expressed *M. musculus* × *M. caroli* hybrid placental transcripts.*

Functional group no.	Term with highest percentage within functional group category	Functional group enrichment score	Count	Percentage of misregulated transcripts
1	Extracellular region	8.42	386	19.31
2	Cadherin	7.42	28	1.40
3	Polysaccharide binding	4.77	27	1.35
4	Membrane part	4.65	593	29.66
5	Insulin-like growth factor binding	4.44	11	0.55
6	Blood vessel development	3.91	54	2.70
7	Serine-type endopeptidase inhibitor activity	3.71	24	1.20
8	Multicellular organismal development	3.59	319	15.96
9	Regulation of bone remodeling	3.47	13	0.65
10	Enzyme inhibitor activity	3.02	39	1.95
11	Regulation of body fluid levels	2.99	23	1.15
12	Tissue remodeling	2.95	31	1.55
13	EGF_CA	2.92	24	1.20
14	Proteinase inhibitor I1, Kazal	2.79	12	0.60
15	Cell cycle phase	2.76	50	2.50
16	Cell death	2.50	118	5.90
17	Regulation of transferase activity	2.40	37	1.85
18	Endoribonuclease activity	2.39	10	0.50
19	Phosphate metabolic process	2.34	120	6.00
20	Immunoglobulin C1-set	2.34	16	0.80

* The most relevant Gene Ontology terms in the dChip-generated list of misregulated transcripts within the hybrid placentae in comparison to the control placentae (gene enrichment scores >2.0, $P < 0.05$). The functional group that contained most of the differentially expressed genes was one associated with cellular components of the membrane, suggestive of a cause of placental tissue developmental defects. The clustering of misregulated hybrid placental transcripts into Functional Group 6 is consistent with the morphological finding of lack of vascularization observed in the hybrid placentae.

proposal, with statistically significant values for GO terms associated with placental development as discussed above and with 163 GO terms associated with immunity and defense (Supplemental Table S3).

Whereas VL30 sequences are not included in the GO Annotations, downstream effects of mVL30-1 overexpression can be inferred using NIH DAVID. Synthesis of mVL30 RNA is induced by pituitary-derived hormones and is most specifically expressed in steroidogenic cells of the ovary, testis, adrenal cortex, and placenta [56]. The normal role of mVL30 RNA is as a regulatory RNA and is causally involved in the steroidogenic pathway [57, 58]. The annotation of misregulated hybrid placental transcripts indicates the steroid hormone metabolism pathway is disrupted, with 61 steroid metabolism genes identified by the GO biological process annotation (Supplemental Table S3). Levels of mVL30-1 RNA are also elevated in transformed cells and in cells undergoing rapid proliferation [59]; the pool of misregulated hybrid placental genes shows enrichment for genes involved in the biological process of mitosis and apoptosis (Table 1).

The VL30 retroelement RNA transfection also promotes metastasis [58, 60, 61]. The DAVID analysis suggests a tumorigenic potential for the hybrid cells: The highest enrichment score (8.42) for the misregulated hybrid placental genes is for those terms associated with the extracellular region or space (Table 1), the extracellular matrix receptor interaction pathway is disrupted in the hybrid placentas (data not shown), and 56 misregulated hybrid placental genes biologically function in oncogenesis (Supplemental Table S3). This disruption of the extracellular matrix pathway and enrichment for genes in the extracellular region is intriguing considering that disruption of cell-extracellular matrix junctions is associated with the ability of tumor cells for uncontrolled growth [62, 63]. Interestingly, a similar set of genes were also found to be misregulated in *Peromyscus* hybrid placentas [64].

DISCUSSION

We hypothesized that placental defects observed in *Peromyscus* and other *Mus* hybrids could be due, in part, to failure of the genome defense mechanism (i.e., DNA methylation) to regulate the expression of retroelements in the placenta. Gene expression analyses were limited to the 87 probe sets representing retroelements/retroviruses on the Affymetrix GeneChip used herein; however, these were subsequently narrowed to three probe sets for the retroelement mVL30 based on filtering criteria and fold-expression change delimiters. Although our initial screening using Affymetrix arrays provided a significant technological limitation due to low coverage for target retroelements, we have successfully identified and confirmed a specific retroelement that is demethylated and overexpressed in hybrid placental tissues. To our knowledge, this is the first report of hybridization-induced targeted disruption of methylation of a retroelement resulting in its overexpression in the placenta of eutherian hybrids. The placental trophectoderm is less methylated in normal mouse blastocysts than is the inner cell mass [65]; however, despite the lower overall DNA methylation levels, this epigenetic mark is required for proper development. For example, demethylation leads to smaller placentas having a reduced labyrinth layer, with the most pronounced effect at 12 and 13 days of gestation [66]. These macroscopic placental defects resulting from demethylation are reminiscent of the *M. musculus* × *M. caroli* placentas described herein and, thus, led to our proposal that the placenta is a specific site of control disrupted by demethylation and retroelement activation in interspecific hybridization. Although limited in the retroelements that can be assayed given the use of predesigned gene chips in the present study, we found a significant expression change in mVL30-1 in hybrid placentas. Detectable expression of mVL30-1 is not observed in embryonic tissues in *M. musculus* until after 9 days of gestation, with a dramatic up-regulation of expression at 15 days in extraembryonic tissues [67]. The expression profile of this element in the developing embryo suggests the overexpression observed in the *M. musculus* × *M. caroli* hybrids is not simply a delay in expression but, rather, aberrant overexpression. Examination of the expression profile and hypomethylation status of this retroelement, as well as those not represented on the Affymetrix array, at multiple embryonic stages of development would provide interesting and valuable information regarding placental formation and genetic conflict in hybrids. However, the low success rate for production of these hybrids significantly limits the use of this model system for such analyses.

The VL30 elements, which encode for a novel species of RNA [68], are a class of sequences that structurally resemble eukaryotic retroviral proviruses and retrotransposons [59]. More specifically, mVL30-1 contains the canonical features of endogenous retroviruses, with two LTRs surrounding an internal region averaging 5 kb [67]. However, mVL30-1 within the murine genome contains multiple stop codons and little resemblance to the protein-coding regions of their retroviral counterparts (i.e., *gag, pol,* and *env*). Although nonfunctional as a retrovirus, mVL30-1 is by no means inactive, because mVL30-1 RNA is capable of hitchhiking in virions produced by other viruses and has been known to promote metastasis after cross-species transmission through active viral transmission from a packaging cell line [61]. Moreover, mVL30-1 is an active noncoding RNA, functioning to induce gene expression in steroidogenic cells by binding to polypyrimidine tract-binding protein-associated splicing factor (PSF); the formation of the PSF-mVL30 RNA complex leads to induction of genes otherwise repressed by PSF [58].

Our gene expression analysis shows that gene pathways associated with mVL30 regulation, such as steroidogenesis [56–58] and proliferation [58, 60, 61, 69], were disrupted in the placentas of the hybrids produced in the present study (Table 1 and Supplemental Table S3). The pathogenic potential of endogenous retroviruses has been shown in other steroid-sensitive tissues, such as infertility due to recombinational activity of an endogenous retrovirus in male germ cells and a link between endogenous retroviruses, mammary tumorigenesis, and hormonal stimulation [15]. Thus, the pathological role of mVL30-1 in oncogenesis and transformation is well established and lends support to up-regulated transcriptional activation of mVL30-1 as a hypothetical candidate for the pathology observed in the *M. musculus* × *M. caroli* hybrids studied herein.

Epigenetic mechanisms, such as small RNAs, long noncoding RNAs (lncRNAs), DNA methylation, and chromatin modification, are host-defense mechanisms that have evolved across various systems to silence transposable elements. How mVL30 has been targeted for demethylation in the absence of other detectable retroelement changes is unknown. Although no small RNAs for mVL30 are known, an incompatibility of piwi-interacting RNAs (piRNAs) likely is not a causative factor in the induction of mVL30 activity. In *Drosophila,* hybrid dysgenesis results from an incompatibility between maternally deposited piRNAs and paternally inherited target transposable elements [70] in a hybrid genome. However, in our case, the maternally derived copy of

mVL30 (*M. musculus*-derived) is overexpressed; thus, any oocyte-deposited piRNAs would contain *M. musculus* copies of mVL30 and would effectively target these for silencing. It is possible that a pool of piRNAs is inherited from the paternal genome [71], but thus far, no mechanism for piRNA delivery through the male germ line has been reported (for review, see [72]). Alternatively, another class of small RNAs, such as crasiRNAs (centromere repeat associated short interacting RNAs; [73]), may be involved in the silencing process that has been compromised in this hybrid system, resulting in a failure to form a small RNA-mVL30 duplex, targeting the mVL30 DNA for methylation or resident histones for modification. Furthermore, epigenetic modification of mVL30-1 may also result from transcription of lncRNAs. Mouse major satellite transcripts, which may occur in varied amounts in these two species and likely are divergent based on the specificity of pMSAT6 [48], produce lncRNAs that associate with Small Ubiquitin-like Modifier (SUMO)-modified HP1 proteins [74]. Lastly, it has been demonstrated that treatment with the demethylating agent 5-azacytidine up-regulates the expression of mVL30 RNAs by as much as 50-fold [75]. However, a recent study showed that expression of VL30 elements can be induced by the formation of phosphoacetylated histone proteins at VL30 LTRs, indicating that the suppression of VL30 elements, at least in fibroblasts, is also under the control of dual-modified histone marks [76]. Thus, regulation of mVL30 is complex and varied, depending on the tissue examined.

Given the technical limitations of our hybrid system, we cannot assay whether mVL30-1 up-regulation is causative or associative in the pathology observed in these hybrid placentas, but we propose that the demethylation of mVL30-1 causes an overexpression of mVL30-1 RNA, which leads to an increase in PSF-mVL30 RNA complexes. Such a reduction in free PSF protein would lead to the increased expression of genes otherwise repressed by this protein [58]—namely, those in the pathways observed during the present study. Evaluation of GO process biological, molecular, and cellular annotations of the misregulated transcripts identified in the hybrid placentas indicates an overall theme in which the highest fold-enrichment is for those transcripts involved with protein binding in the membrane during the developmental process of placental formation. The functional classifications of the misregulated hybrid placental genes suggest an overall failure of normal placental development and oncogenic potential. Using the MGI Mammalian Phenotype Browser [77], 5.5% (98) of the misregulated hybrid placental genes with an associated Gene ID (1758) were classified as associated with a mutant phenotype related to placental development. The mammalian phenotype term does not necessarily imply that mutations in the gene cause the phenotype, nor is it feasible to demonstrate direct evidence of preferential alteration of genes with resultant placental phenotypes (the Mammalian Phenotype Browser has 45 082 genotypes with 214 651 annotations). However, these genes are strong candidates for validation by quantitative techniques as further proof of their role in the hybrid dysgenesis observed in our *M. musculus* × *M. caroli* embryos.

What mechanisms underlie the incompatibility between the *M. musculus* and *M. caroli* genomes in our hybrids that trigger demethylation? We propose an ''evolving'' Dobzhansky-Muller model in which incompatibilities between the X and autosomes [78–80] can arise through a variety of mechanisms, including interactions among heterochromatin, small RNAs, and epigenetically defined repeat regions, as opposed to a simple incompatibility between two specific loci [81]. Under this model, an *M. caroli* epigenetic control mechanism, such as

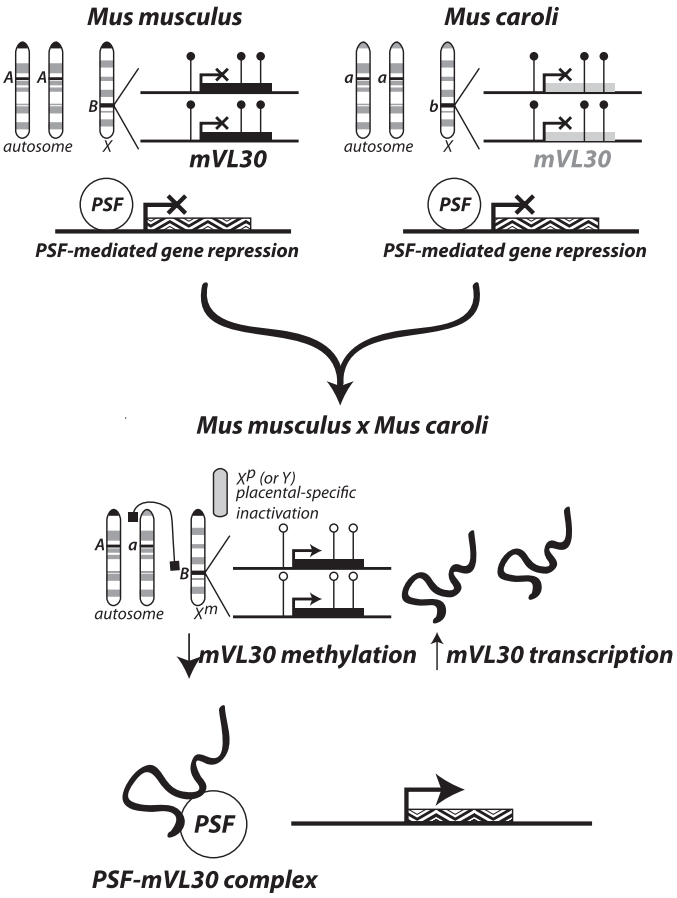

FIG. 6. Model of mVL30 activation and subsequent gene derepression. In each species (**top**), the X-linked mVL30 retrovirus is regulated via methylation under the control of an unknown, presumably autosomal locus. Genetic drift has led to divergence of both the control locus and target mVL30 (A and B in *M. musculus*; a and b in *M. caroli*). In each species, lack of active transcription for mVL30 frees PSF to repress gene expression of target genes (i.e., steroidogenic pathway). Upon interspecific hybridization, the control region is underdominant in the hemizygous state (Aa), cannot properly methylate, and thus repress the transcription of the X-linked, *M. musculus*-derived mVL30 (B), exemplifying the classic Dobzhansky-Muller incompatibility. In the placenta, only maternal X-linked expression is found due to trophoblast-specific paternal X inactivation. Increased expression of mVL30 leads to an increase in mVL30-PSF complexes and releases genes for expression that would otherwise fall under PSF regulation.

DNA methylation (a), affects *M. caroli* mVL30-1 RNA (repeat region b) via epigenetic modifications but cannot interact properly with *M. musculus* mVL30-1 (repeat region B) in the hemizygous state in the interspecific hybrid (Fig. 6). This type of locus incompatibility has been demonstrated to be responsible for male sterility in other *M. musculus* hybrids [82] and, more recently, has been identified as the mammalian speciation gene, *Prdm9* [83]. The loss of epigenetic silencing of mVL30 in the most divergent organ, the placenta, would lead to further gene derepression (e.g., increased binding to PSF and downstream deregulation of PSF-targeted genes) and subsequent placental failure. Moreover, this effect would be most acute in the placenta, because mVL30 is X-linked. In males, only the maternally expressed mVL30 is inherited, in which control via DNA methylation is lost due to underdominance in the hemizygous state; in females, placental-specific paternal X-inactivation [84] leads to maternal expression of mVL30, again via loss of DNA methylation

due to hemizygosity of an unknown control region (presumably autosomal). Specific patterns of methylation in mammalian placentas have been implicated in both the evolution and function of this organ [85] and, as such, can also provide an explanation for the placental defects observed in our *M. musculus* × *M. caroli* hybrids. Not surprisingly, the placenta is thus a prime location for the expression of parent-offspring genomic conflict and, in this case, incompatibility due to the 5–7 million years of divergence between *M. musculus* and *M. caroli*. Given the similarities observed between the PSF-regulated gene networks misregulated in our hybrid system and those genes affected in *Peromyscus* hybrids [64], gene activation caused by increased retroviral activity as a result of epigenetic incompatibilities in the hybrid state may be a general feature of placental failure in eutherian hybrids.

The complexity of hybrid dysgenesis in eutherians and other model systems is not a new discussion, but as yet, it is an incomplete one [81, 86]. Our data add to the accumulating evidence that interspecific hybridization is associated with activation of transposable elements, and to our knowledge, this is the first report of retroelement activation linked directly to decreased methylation as a result of interspecific eutherian hybridization. A functional genomics approach revealed that the pathology observed in *M. musculus* × *M. caroli* hybrids is concomitant with overexpression of the murine retroelement mVL30-1, an X-linked, noncoding RNA involved in gene regulation. The activation of mVL30-1 was a direct result of its undermethylation in the hybrid placentas, possibly due to a Dobzhansky-Muller incompatibility between an epigenetic control locus (autosomal) and the X-linked retroelement. A recent analysis of expression and methylation of Long INterspersed Element 1 (LINE-1) and endogenous retroelements in cancer cell lines has demonstrated a specificity of expression changes and methylation changes dependent on both the retroelement and the level of DNA methylation [49]. Thus, both small and large changes in methylation levels for a particular element can lead to changes in gene expression. We propose that the placenta is a specific site of control that is disrupted by demethylation and retroelement activation in interspecific hybridization and occurs as a result of X-autosome incompatibility of methylation machinery, noncoding retroelements, and target transcription factors and placental genes during hybrid embryo development.

ACKNOWLEDGMENT

Thanks to Brad Whaley for assistance with artificial insemination and Mike O'Neill for critical comments on the manuscript.

REFERENCES

1. West JD, Frels WI, Papaioannou VE, Karr JP, Chapman VM. Development of interspecific hybrids of *Mus*. J Embryol Exp Morphol 1977; 41:233–243.
2. Frels WI, Rossant J, Chapman VM. Intrinsic and extrinsic factors affecting the viability of *Mus caroli* × *M. musculus* hybrid embryos. J Reprod Fertil 1980; 59:387–392.
3. Rogers JF, Dawson WD. Fetal and placental size in a *Peromyscus* species cross. J Reprod Fertil 1970; 21:255–262.
4. Zechner U, Reule M, Orth A, Bonhomme F, Strack B, Guenet, Hameister H, Fundele R. An X-chromosome linked locus contributes to abnormal placental development in mouse interspecific hybrid. Nat Genet 1996; 12: 398–403.
5. Allen WR, Short RV. Interspecific and extraspecific pregnancies in equids: anything goes. J Hered 1997; 88:384–392.
6. Graves JA. Gene amplification in a mouse embryo? Double minutes in cell lines independently derived from a *Mus musculus* × *M. caroli* fetus. Chromosoma 1984; 89:138–142.
7. O'Neill RJ, O'Neill MJ, Graves JA. Undermethylation associated with retroelement activation and chromosome remodeling in an interspecific mammalian hybrid. Nature 1998; 393:68–72.
8. Brown JD, Golden D, O'Neill RJ. Methylation perturbations in retroelements within the genome of a *Mus* interspecific hybrid correlate with double minute chromosome formation. Genomics 2008; 91:267–273.
9. Brown JD, Strbuncelj M, Giardina C, O'Neill RJ. Interspecific hybridization induced amplification of Mdm2 on double minutes in a *Mus* hybrid. Cytogenet Genome Res 2002; 98:184–188.
10. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet 2003; 33(suppl):245–254.
11. Eden A, Gaudet F, Waghmare A, Jaenisch R. Chromosomal instability and tumors promoted by DNA hypomethylation. Science 2003; 300:455.
12. Gaudet F, Hodgson JG, Eden A, Jackson-Grusby L, Dausman J, Gray JW, Leonhardt H, Jaenisch R. Induction of tumors in mice by genomic hypomethylation. Science 2003; 300:489–492.
13. Bourc'his D, Bestor TH. Meiotic catastrophe and retrotransposon reactivation in male germ cells lacking Dnmt3L. Nature 2004; 431:96–99.
14. Hemberger M. Epigenetic landscape required for placental development. Cell Mol Life Sci 2007; 64:2422–2436.
15. Prudhomme S, Bonnaud B, Mallet F. Endogenous retroviruses and animal reproduction. Cytogenet Genome Res 2005; 110:353–364.
16. Haig D. Placental growth hormone-related proteins and prolactin-related proteins. Placenta 2008; 29(suppl A):S36–S41.
17. Chuong EB, Tong W, Hoekstra HE. Maternal-fetal conflict: rapidly evolving proteins in the rodent placenta. Mol Biol Evol 2010; 27: 1221–1225.
18. Vrana PB, Fossella JA, Matteson P, del Rio T, O'Neill MJ, Tilghman SM. Genetic and epigenetic incompatibilities underlie hybrid dysgenesis in *Peromyscus*. Nat Genet 2000; 25:120–124.
19. Vrana PB, Guan XJ, Ingram RS, Tilghman SM. Genomic imprinting is disrupted in interspecific *Peromyscus* hybrids. Nat Genet 1998; 20:362–365.
20. Wiley CD, Matundan HH, Duselis AR, Isaacs AT, Vrana PB. Patterns of hybrid loss of imprinting reveal tissue- and cluster-specific regulation. PLoS ONE 2008; 3:e3572.
21. Katzourakis A, Gifford RJ, Tristem M, Gilbert MT, Pybus OG. Macroevolution of complex retroviruses. Science 2009; 325:1512.
22. Malik HS, Henikoff S. Positive selection of Iris, a retroviral envelope-derived host gene in *Drosophila melanogaster*. PLoS Genet 2005; 1:e44.
23. Dunlap KA, Palmarini M, Spencer TE. Ovine endogenous betaretroviruses (enJSRVs) and placental morphogenesis. Placenta 2006; 27(suppl A): S135–S140.
24. Dupressoir A, Marceau G, Vernochet C, Benit L, Kanellopoulos C, Sapin V, Heidmann T. Syncytin-A and syncytin-B, two fusogenic placenta-specific murine envelope genes of retroviral origin conserved in Muridae. Proc Natl Acad Sci U S A 2005; 102:725–730.
25. Mi S, Lee X, Li X, Veldman GM, Finnerty H, Racie L, LaVallie E, Tang XY, Edouard P, Howes S, Keith JC Jr, McCoy JM. Syncytin is a captive retroviral envelope protein involved in human placental morphogenesis. Nature 2000; 403:785–789.
26. Muir A, Lever A, Moffett A. Expression and functions of human endogenous retroviruses in the placenta: an update. Placenta 2004; 25(suppl A):S16–S25.
27. Rote NS, Chakrabarti S, Stetzer BP. The role of human endogenous retroviruses in trophoblast differentiation and placental development. Placenta 2004; 25:673–683.
28. Ono R, Nakamura K, Inoue K, Naruse M, Usami T, Wakisaka-Saito N, Hino T, Suzuki-Migishima R, Ogonuki N, Miki H, Kohda T, Ogura A, et al. Deletion of Peg10, an imprinted gene acquired from a retrotransposon, causes early embryonic lethality. Nat Genet 2006; 38:101–106.
29. Chang-Yeh A, Mold DE, Brilliant MH, Huang RC. The mouse intracisternal A particle-promoted placental gene retrotransposition is mouse-strain-specific. Proc Natl Acad Sci U S A 1993; 90:292–296.
30. Chang-Yeh A, Mold DE, Huang RC. Identification of a novel murine IAP-promoted placenta-expressed gene. Nucleic Acids Res 1991; 19: 3667–3672.
31. Medstrand P, van de Lagemaat LN, Dunn CA, Landry JR, Svenback D, Mager DL. Impact of transposable elements on the evolution of mammalian gene regulation. Cytogenet Genome Res 2005; 110:342–352.
32. Reiss D, Zhang Y, Mager DL. Widely variable endogenous retroviral methylation levels in human placenta. Nucleic Acids Res 2007; 35: 4743–4754.
33. Robinson TJ, Wittekindt O, Pasantes JJ, Modi WS, Schempp W, Morris-Rosendahl DJ. Stable methylation patterns in interspecific antelope hybrids and the characterization and localization of a satellite fraction in the Alcelaphini and Hippotragini. Chromosome Res 2000; 8:635–643.
34. Roemer I, Grutzner F, Winking H, Haaf T, Orth A, Skidmore L, Antczak

D, Fundele R. Genome evolution. Global methylation in eutherian hybrids. Nature 1999; 401:131–132.
35. Hemberger MC, Pearsall RS, Zechner U, Orth A, Otto S, Ruschendorf F, Fundele R, Elliott R. Genetic dissection of X-linked interspecific hybrid placental dysplasia in congenic mouse strains. Genetics 1999; 153:383–390.
36. Matsuda Y, Chapman VM. Analysis of sex-chromosome aneuploidy in interspecific backcross progeny between the laboratory mouse strain C57BL/6 and *Mus spretus*. Cytogenet Cell Genet 1992; 60:74–78.
37. Brown JD, Golden D, O'Neill RJ. Methylation perturbations in retroelements within the genome of a Mus interspecific hybrid correlate with double minute chromosome formation. Genomics 2007; 91:267–273.
38. Rawn SM, Cross JC. The evolution, regulation, and function of placenta-specific genes. Annu Rev Cell Dev Biol 2008; 24:159–181.
39. Gimenez J, Montgiraud C, Oriol G, Pichon J-P, Ruel K, Tsatsaris V, Gerbaud P, Frendo J-L, Evain-Brion D, Mallet F. Comparative methylation of ERVWE1/syncytin-1 and other human endogenous retrovirus LTRs in placenta tissues. DNA Res 2009; 16:195–211.
40. Li LC, Dahiya R. MethPrimer: designing primers for methylation PCRs. Bioinformatics 2002; 18:1427–1431.
41. Bock C, Reither S, Mikeska T, Paulsen M, Walter J, Lengauer T. BiQ Analyzer: visualization and quality control for DNA methylation data from bisulfite sequencing. Bioinformatics 2005; 21:4067–4068.
42. Dennis G, Sherman B, Hosack D, Yang J, Gao W, Lane HC, Lempicki R. DAVID: Database for Annotation, Visualization, and Integrated Discovery. Genome Biol 2003;4:P3.
43. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001; 29:e45.
44. Chapman VM, Shows TB. Somatic cell genetic evidence for X-chromosome linkage of three enzymes in the mouse. Nature 1976; 259:665–667.
45. Rossant J, Croy BA, Clark DA, Chapman VM. Interspecific hybrids and chimeras in mice. J Exp Zool 1983; 228:223–233.
46. Rossant J, Frels WI. Interspecific chimeras in mammals: successful production of live chimeras between *Mus musculus* and *Mus caroli*. Science 1980; 208:419–421.
47. West JD, Frels WI, Chapman VM. *Mus musculus* × *Mus caroli* hybrids: mouse mules. J Hered 1978; 69:321–326.
48. Siracusa LD, Chapman VM, Bennett KL, Hastie ND, Pietras DF, Rossant J. Use of repetitive DNA sequences to distinguish *Mus musculus* and *Mus caroli* cells by in situ hybridization. J Embryol Exp Morphol 1983; 73: 163–178.
49. Goering W, Ribarska T, Schulz WA. Selective changes of retroelement expression in human prostate cancer. Carcinogenesis 2011; 32:1484–1492.
50. Good JM, Giger T, Dean MD, Nachman MW. Widespread over-expression of the X chromosome in sterile F_1 hybrid mice. PLoS Genet 2010; 6:e1001148.
51. Georgiades P, Ferguson-Smith AC, Burton GJ. Comparative developmental anatomy of the murine and human definitive placentae. Placenta 2002; 23:3–19.
52. Hernandez-Verdun D. Morphogenesis of the syncytium in the mouse placenta. Ultrastructural study. Cell Tissue Res 1974; 148:381–396.
53. Ivanov DB, Philippova MP, Tkachuk VA. Structure and functions of classical cadherins. Biochemistry (Mosc) 2001; 66:1174–1186.
54. Redies C, Heyder J, Kohoutek T, Staes K, Van Roy F. Expression of protocadherin-1 (Pcdh1) during mouse development. Dev Dyn 2008; 237: 2496–2505.
55. Rampon C, Bouillot S, Climescu-Haulica A, Prandini MH, Cand F, Vandenbrouck Y, Huber P. Protocadherin 12 deficiency alters morphogenesis and transcriptional profile of the placenta. Physiol Genomics 2008; 34:193–204.
56. Schiff R, Itin A, Keshet E. Transcriptional activation of mouse retrotransposons in vivo: specific expression in steroidogenic cells in response to trophic hormones. Genes Dev 1991; 5:521–532.
57. Shapiro JA, von Sternberg R. Why repetitive DNA is essential to genome function. Biol Rev Camb Philos Soc 2005; 80:227–250.
58. Song X, Sui A, Garen A. Binding of mouse VL30 retrotransposon RNA to PSF protein induces genes repressed by PSF: effects on steroidogenesis and oncogenesis. Proc Natl Acad Sci U S A 2004; 101:621–626.
59. Keshet E, Itin A. Patterns of genomic distribution and sequence heterogeneity of a murine ''retrovirus-like'' multigene family. J Virol 1982; 43:50–58.
60. Song X, Sun Y, Garen A. Roles of PSF protein and VL30 RNA in reversible gene regulation. Proc Natl Acad Sci U S A 2005; 102: 12189–12193.
61. Song X, Wang B, Bromberg M, Hu Z, Konigsberg W, Garen A. Retroviral-mediated transmission of a mouse VL30 RNA to human melanoma cells promotes metastasis in an immunodeficient mouse model. Proc Natl Acad Sci U S A 2002; 99:6269–6273.
62. Birchmeier W, Behrens J. Cadherin expression in carcinomas: role in the formation of cell junctions and the prevention of invasiveness. Biochim Biophys Acta 1994; 1198:11–26.
63. Sobel G, Szabo I, Paska C, Kiss A, Kovalszky I, Kadar A, Paulin F, Schaff Z. Changes of cell adhesion and extracellular matrix (ECM) components in cervical intraepithelial neoplasia. Pathol Oncol Res 2005; 11:26–31.
64. Duselis A, Qu X, Obergfell C, Mack JA, O'Neill MJ, O'Neill RJ, Vrana P. Cell cycle and extra-cellular matrix gene expression changes during hybrid placental development. Reprod Fertil Dev 2007; 19:695–708.
65. Santos F, Hendrich B, Reik W, Dean W. Dynamic reprogramming of DNA methylation in the early mouse embryo. Dev Biol 2002; 241:172–182.
66. Vlahovic M, Bulic-Jakus F, Juric-Lekic G, Fucic A, Maric S, Serman D. Changes in the placenta and in the rat embryo caused by the demethylating agent 5-azacytidine. Int J Dev Biol 1999; 43:843–846.
67. French NS, Norton JD. Structure and functional properties of mouse VL30 retrotransposons. Biochim Biophys Acta 1997; 1352:33–47.
68. Howk RS, Troxler DH, Lowy D, Duesberg PH, Scolnick EM. Identification of a 30S RNA with properties of a defective type C virus in murine cells. J Virol 1978; 25:115–123.
69. Bromberg ME, Konigsberg WH, Madison JF, Pawashe A, Garen A. Tissue factor promotes melanoma metastasis by a pathway independent of blood coagulation. Proc Natl Acad Sci U S A 1995; 92:8205–8209.
70. Brennecke J, Aravin AA, Stark A, Dus M, Kellis M, Sachidanandam R, Hannon GJ. Discrete small RNA-generating loci as master regulators of transposon activity in *Drosophila*. Cell 2007; 128:1089–1103.
71. Grandjean V, Gounon P, Wagner N, Martin L, Wagner KD, Bernex F, Cuzin F, Rassoulzadegan M. The miR-124-Sox9 paramutation: RNA-mediated epigenetic control of embryonic and adult growth. Development 2009; 136:3647–3655.
72. Bourc'his D, Voinnet O. A small-RNA perspective on gametogenesis, fertilization, and early zygotic development. Science 2010; 330:617–622.
73. Carone DM, Longo MS, Ferreri GC, Hall L, Harris M, Shook N, Bulazel KV, Carone BR, Obergfell C, O'Neill MJ, O'Neill RJ. A new class of retroviral and satellite encoded small RNAs emanates from mammalian centromeres. Chromosoma 2009; 118:113–125.
74. Maison C, Bailly D, Roche D, de Oca RM, Probst AV, Vassias I, Dingli F, Lombard B, Loew D, Quivy J-P, Almouzni G. SUMOylation promotes de novo targeting of HP1α to pericentric heterochromatin. Nat Genet 2011; 43:220–227.
75. Tzavaras T, Eftaxia S, Tavoulari S, Hatzi P, Angelidis C. Factors influencing the expression of endogenous reverse transcriptases and viral-like 30 elements in mouse NIH3T3 cells. Int J Oncol 2003; 23:1237–1243.
76. Brunmeir R, Lagger S, Simboeck E, Sawicka A, Egger G, Hagelkruys A, Zhang Y, Matthias P, Miller WJ, Seiser C. Epigenetic regulation of a murine retrotransposon by a dual histone modification mark. PLoS Genet 2010; 6:e1000927.
77. Blake JA, Bult CJ, Kadin JA, Richardson JE, Eppig JT. The Mouse Genome Database (MGD): premier model organism resource for mammalian genomics and genetics. Nucleic Acids Res 2011; 39: D842–D848.
78. Bateson W. Heredity and variation in modern lights In: Seward AC (ed.), Darwin and Modern Science. Cambridge, UK: Cambridge University Press; 1909;85–101.
79. Dobzhansky T. Genetic nature of species differences. American Naturalist 1937; 71:404–420.
80. Muller HJ. Isolating mechanisms, evolution and temperature. Biol Symp 1942; 6:71–125.
81. Brown JD, O'Neill RJ. Chromosomes, conflict, and epigenetics: chromosomal speciation revisited. Annu Rev Genomics Hum Genet 2010; 11:291–316.
82. Forejt J. Hybrid sterility in the mouse. Trends Genet 1996; 12:412–417.
83. Mihola O, Trachtulec Z, Vlcek C, Schimenti JC, Forejt J. A mouse speciation gene encodes a meiotic histone H3 methyltransferase. Science 2009; 323:373–375.
84. Takagi N, Sasaki M. Preferential inactivation of the paternally derived X chromosome in the extraembryonic membranes of the mouse. Nature 1975; 256:640–642.
85. Ng HK, Novakovic B, Hiendleder S, Craig JM, Roberts CT, Saffery R. Distinct patterns of gene-specific methylation in mammalian placentas: implications for placental evolution and function. Placenta 2010; 31: 259–268.
86. Michalak P. Epigenetic, transposon and small RNA determinants of hybrid dysfunctions. Heredity 2009; 102:45–50.