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Absence of hypomethylation and LINE-1 amplification in a white × black rhinoceros hybrid

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Abstract

Genomic stress resulting from the interspecific hybridization of marsupials has been shown to lead to hypomethylation and transposable element over-amplification. Here we investigated both methylation status and transposable element (LINE-1) activity in an F1 hybrid between the black (*Diceros bicornis*) and white rhinoceros (*Ceratotherium simum*). Our data show that in this instance the hybrid genome was not characterised by gross hypomethylation and LINE-1 over-amplification thus extending previous investigations on eutherian mammals. These findings underscore observations that wide-scale genomic instability involving hypomethylation and mobile element release may be marsupial specific phenomena within Mammalia.

Introduction

O'Neill, O'Neill and Graves (1998, 2001) investigated differences between parental and hybrid genomes in marsupials and demonstrated that the hybrids displayed genomic hypomethylation. In addition, the KER-V endogenous retrovirus was amplified by 20% in a *Wallabia bicolor* × *Macropus rufogriseus* F1 hybrid relative to the parental species (O'Neill, O'Neill and Graves, 2002). The authors proposed that hybrid genomes face perturbations leading to the disruption of major epigenetic controls, such as methylation, thus allowing subsequent amplification of junk mobile elements (O'Neill et al., 1998, 2001; see comments in Kidwell & Lisch, 1998). This hypothesis was tested but could not be extended to Placentalia. No methylation differences were observed between the parental and F1 genomes of representatives of the Equidae, Muridae, Camelidae and Bovidae (Roemer et al., 1999; Robinson et al., 2000) suggesting that methylation release in hybrids was either marsupial specific (rather than a more widespread mammalian feature) or is more subtle in Placentalia and thus escapes detection. Surprisingly, O'Neill and colleagues' documentation of mobile elements over-amplification in marsupial hybrids (1998, 2001) has never been tested in placentals.

It is now well established that methylation constitutes a means of controlling transposable elements in a wide range of species (Yoder, Walsh & Bestor, 1997; Bourc'his & Bestor, 2004) and as a consequence the two patterns (TE amplification and hypomethylation) are expected to be correlated. Since Long Interspersed Nuclear Element One (LINE-1) (reviewed in Ostertag & Kazazian, 2001) is a major component of mammalian genomes (Bürton et al., 1986; Dörner & Päabo, 1995; Lander et al., 2001; Waters et al. 2004), we chose to focus on these elements in our investigation of a recently described black (*Diceros bicornis*, DBI) × white rhinoceros (*Cerathoterium simum*, CSI) F1 hybrid (HYB) of the Order Perissodactyla (Robinson et al., 2004). We found no evidence of

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hypomethylation nor of differential LINE-1 amplification in this unusual interspecific cross suggesting that the patterns evidenced by hybrid marsupials may be lineage specific, or are not present at the same levels in Placentalia.

Material and methods

DNA extraction and methylation status

Fibroblast cell lines were established from skin biopsies of a female white rhinoceros, a male black rhinoceros and a female interspecific hybrid. All individuals were unrelated (see Robinson et al., 2004 for details). The biopsy was taken from the hybrid at 4 years and 5 months of age. Biopsies from the black and white rhinos were obtained in the field from wild adults of unknown age.

The total genomic DNA fraction was extracted from cell pellets obtained from the cell lines and concentration checked with a spectrophotometer and visually on an agarose gel. Methylation status was examined by restriction enzyme analysis that involved independent overnight digestions of 10 μg of total genomic DNA from each rhinoceros using 10 units of *Hpa*II (Promega) and *Msp*I (Promega) according to manufacturer's instructions. Samples were subjected to electrophoresis in a 0.8% agarose gel and visualised under UV light.

Southern blot analysis

A genomic Southern blot was prepared from the *Hpa*II and *Msp*I gel (detailed above) by capillary transfer onto a Hybond-N+ membrane (Amersham) following manufacturer's recommendations. A second Southern blot was prepared by digesting to completion 10 μg of total genomic DNA from a male black rhinoceros, a female white rhinoceros and the female F1 hybrid with *Eco*RI (Promega) according to the manufacturer's instructions. The LINE-1 probe used in our study was generated via PCR of genomic DNA from the hybrid rhinoceros (Waters et al., 2004). This was subsequently [α-^{32}P]dCTP-labelled (Megaprime Labelling Kit, Amersham) and hybridized overnight (65°C) to the membranes in Church buffer. The blots were washed twice for 5 min at room temperature in 1× SSC; 0.1% SDS and then a further two times (15 min each) in 1× SSC; 0.1% SDS at 65°C. The membranes were exposed to a super resolution Cyclone storage phosphor screen for 6 h and then scanned with the Packard Cyclone Storage System.

FISH experiments

Two successive PCRs using degenerate primers were performed in order to synthesise a LINE-1 biotin-labelled probe from DNA obtained from each cell line as described in Waters et al. (2004). The same protocol was independently applied to produce a DIG-labelled probe from the black rhinoceros DNA. Metaphase chromosomes were prepared from each cell line and subsequently treated with pepsin (1%, in 50 mM HCl) and baked at 65°C for 1 h. They were then codenaturated with the probe (2 μl of the PCR II product in Dextran Sulphate 10%, Formamide 65%, 2× SSC) at 72°C for 2 min on a slide heater, and incubated overnight at 37°C. Slides were washed for 5 mins in 2× SSC at 72°C and briefly rinsed in 1× PBD (1% Tween 20 in 4× SSC). Biotin and DIG were detected with Streptavidin-Cy3 and fluorescein-Anti-DIG, respectively. Finally, the slides were washed successively in three changes of 1X PBD (2 min, RT) and mounted in Vectashield with DAPI. Each FISH experiment was conducted at least twice and a minimum of 10 metaphases were examined using the Genus software (Applied Imaging). We serially performed the following hybridization combinations: CSI probe onto CSI chromosomes, DBI onto DBI, HYB onto HYB, DBI onto CSI, CSI onto DBI, DBI onto HYB and CSI onto HYB. We also performed a double colour FISH of the biotin-labelled DBI and DIG-labelled CSI probes onto the hybrid's metaphase chromosomes.

Results and discussion

Analyses conducted to determine the methylation status of genomic DNA from the rhinoceros hybrid and parental species using the methylation-sensitive *Hpa*II and methylation-insensitive *Msp*I restriction enzymes revealed no gross differences in methylation status among them. In all instances

*Hpa*II partially digested all genomes, whereas the use of *Msp*I resulted in complete digestion (Figure 1(a)).

The PCR, FISH and the Southern Blot analyses all clearly show that rhinoceros genomes possess LINE-1 elements. The Southern blot containing *MspI* and *HpaII*-digested DNA from the black, white and hybrid rhinoceros was hybridized to a LINE-1 probe generated by PCR from the F1 hybrid (Figure 1(b)). This probe consisted of a 300 bp region of ORFII (encoding for the Reverse Transcriptase) from many different LINE-1 copies (Waters et al. 2004). There was no evidence of bands in the hybrid lanes that were not present in at least one of the parental species. However, there were two bands (~4.1 and 1.8 kb) shared by the white and hybrid rhinoceroses that were lacking from the black rhinoceros lanes (Figure 1(b)). There was also a band (3.5 kb) which was shared by the black and hybrid rhinoceroses that was absent from the white rhinoceros. The bands shared by all three rhinoceroses represent LINE-1 that were active before the divergence of these species. Bands shared by

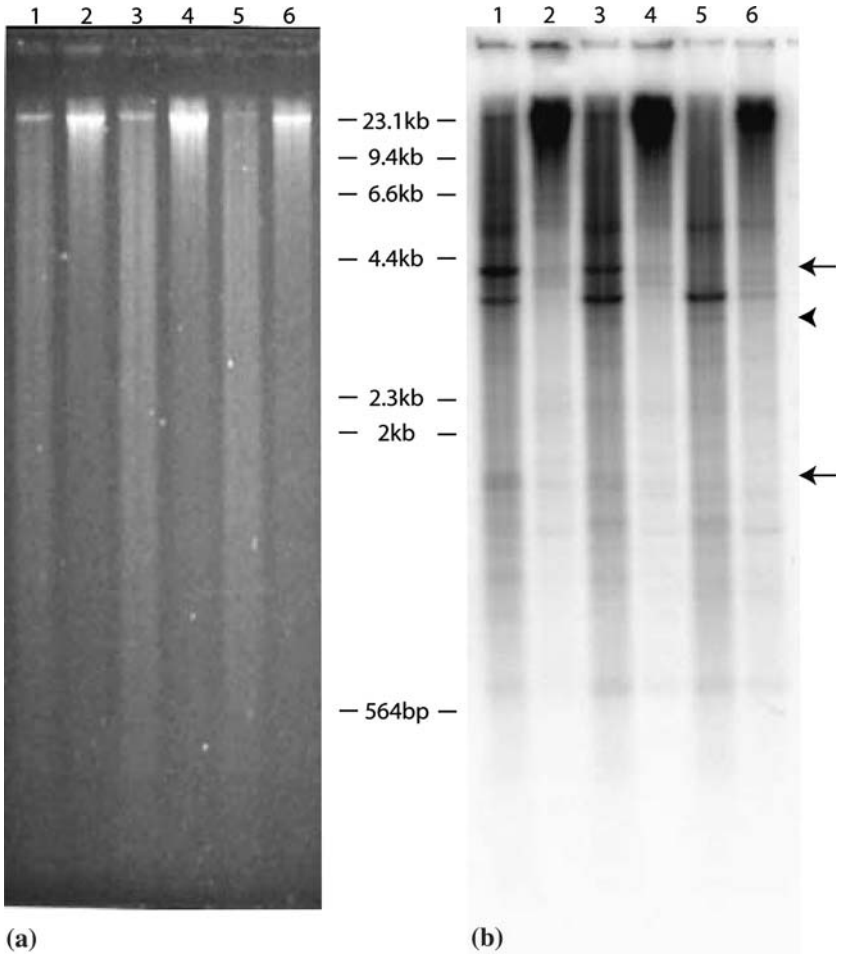

Figure 1. (a) Restriction enzyme analysis of genomic methylation status. Lanes 1 and 2 contain DNA from white rhinoceros digested with *Hpa*II and *Msp*I, respectively, lanes 3 and 4 contain DNA from F1 hybrid digested with *Hpa*II and *Msp*I, respectively, and lanes 5 and 6 contain DNA from the black rhinoceros digested with *Hpa*II and *Msp*I, respectively. All three rhinoceroses display the same restriction patterns indicating no release of methylation in the F1 hybrid. (b) Genomic Southern blot analysis of LINE-1: the Southern blot was obtained by capillary transfer from the gel in (a). Black arrows indicate bands shared by the white rhinoceros and the F1 hybrid. Arrowhead indicates a band shared by the black rhinoceros and F1 hybrid. All bands in one or both of the parents are also located in the hybrid lanes. There is no evidence of bands that are specific to the hybrid.

one parent and the hybrid represent LINE-1 that were active in only one lineage after the species diverged from each other. There are other examples of bands that are shared by the hybrid and one parent evident in our Southern analysis that can be visualised when the levels are darkened on the digital file. In no single instance were we able to detect a band in the hybrid that was not present in at least one of the parental species, clearly suggesting there is no differential release of L1 in the hybrid genome. A Southern blot containing *Eco*RI digested DNA from the three rhinoceroses similarly displayed a band that was present in the hybrid and white rhinoceros lanes but not the black rhinoceros (not shown). This Southern blot similarly failed to provide evidence of elevated L1 activity in the hybrid in that all the bands in the hybrid lane were shared by at least one of the parental species.

In addition, the absence of L1 activity in the hybrid was further supported by the FISH results since all hybridization patterns were the same irrespective of the probes and/or targets used. The two-coloured FISH onto the hybrid's chromosomes using both CSI and DBI probes showed no distributional bias (Figure 2). The detection of LINE-1 elements in all three rhinoceros genomes is consistent with the presence of LINE-1 elements in all therian genomes investigated thus far (Burton et al., 1986; Dörner & Pääbo 1995; Waters et al. 2004). Importantly, the interspecific rhinoceros hybrid genome displayed a similar pattern to the parental LINE-1 distributions and showed no detectable amplification (Figure 2). Although it could be argued that our technique is based on PCR, and consequently rare alleles may be underamplified during PCR rounds and thus not detected by FISH, over amplification of such alleles in the hybrid would have been detected in the HYB versus HYB experiment. This is based on the premise that these alleles, though rare in the parental species, would have become a major component of the hybrid genome. Such a phenomenon was not observed.

The homogeneous LINE-1 signal exhibited by these species (Figure 2) is in marked contrast to what is observed in human (Korenberg & Rykowsky, 1988) and rodents (Boyle et al., 1990). The fact that there was no banding-like pattern (characteristic of humans and rodents) suggests that LINE-1 elements in the Rhinocerotidae are not differentially accumulated in the AT-rich G-positive regions. This is in agreement with observations for another perissodactyl species, the pig (Thomsen & Miller, 1996), and also in other Placentalia genomes including Afrotheria and Xenarthra, but excluding Euarchontoglires (primates, rodents and lagomorphs; Waters et al., 2004). Only the X chromosomes were enriched for LINE-1 in comparison to the rest of the genome (Figure 2), which is consistent with Lyon's repeat hypothesis (Lyon, 1998) which suggests an involvement of LINE-1 in the X-inactivation process (Bailey et al., 2000; Hansen, 2003).

Our investigations confirm and extend previous studies that show interspecific hybrid genomes of the Placentalia are not grossly undermethylated, an observation that differs markedly from what has been reported in marsupials (O'Neill et al., 1998, 2001). Moreover, the absence of hypomethylation does not appear to depend on the evolutionary age of the hybridizing parental

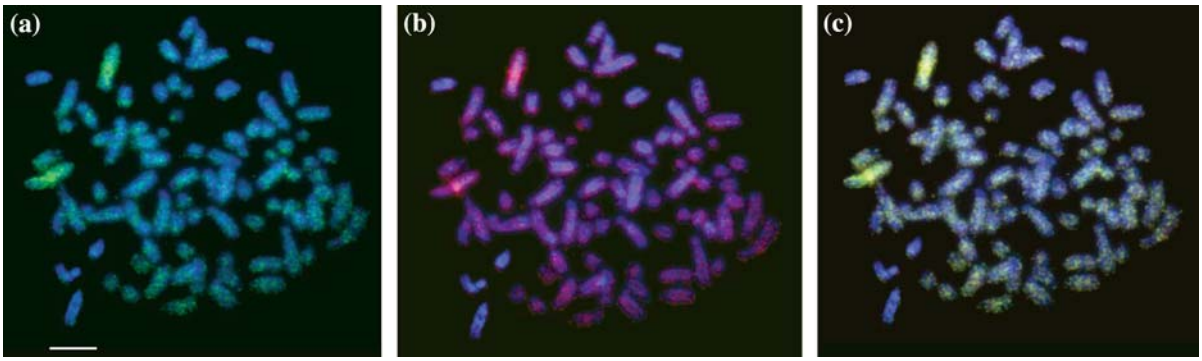

Figure 2. Two-colour FISH using biotin-labelled (a) black rhinoceros-derived and a DIG-labelled (b) white-rhinoceros-derived probes onto chromosomes of the F1 hybrid. (c) Shows the co-hybridization (yellow) of the biotin- (green) and DIG-labelled (red) probes on chromosomes of the F1. X chromosomes display the strongest signals. Bar represents 8 μm.

species given that case-studies are now available that span divergence dates from 2 to 20 myr (*Mus* spp.: <3 myr, Chevret et al., 2003, 2005; horse and donkey: 2–4 myr, Norman & Ashley, 2000, and references therein; blesbok and red hartebeest: ~6 myr, and sable and roan antelope: ~4 myr, calculated from Matthee & Robinson, 1999; African rhinoceroses: ~17 myr, Tougard et al. 2001, and references therein; dromedary and llama: ~20 myr, Montgelard et al., 1997). Although activation of other TEs cannot be excluded, this is the first study to examine LINE-1 dynamics in a mammalian hybrid and our data show that the genomic stress anticipated to result from an interspecific cross does not include the reactivation of LINE-1. As with methylation status, this contrasts with the situation observed in marsupial hybrids (O'Neill et al., 1998) which exhibit an amplification of mobile elements, specifically KERV-1 (which has sequence similarity to human endogenous retrovirus, HERV-K10).

In summary, our survey confirms that major wide-scale undermethylation and subsequent TE amplification in hybrid genomes could be a marsupial feature that is not found in Boreoeutheria (the two most derived eutherian clades; Murphy et al., 2001) or alternatively, does not occur at levels observed in marsupials. There is good evidence to show that TE activity (specifically LINE-1) is controlled by DNA methylation (Yoder et al., 1997; Bourc'his & Bestor, 2004) which is clearly supported by the accumulated data. Undermethylation and resultant TE amplification in marsupial hybrids on the one hand, and the apparent absence of hypomethymation and LINE-1 amplification in placental hybrids on the other, are simply opposing patterns consistent with the same phenomenon. In the absence of data from monotremes and other vertebrate hybrids such as those involving amphibians and birds, however, it is not possible to conclude which pattern constitutes the more derived condition.

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