Brief Report

Spatial Separation of Parental Genomes in Preimplantation Mouse Embryos

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Abstract. We have used two different experimental approaches to demonstrate topological separation of parental genomes in preimplantation mouse embryos: mouse eggs fertilized with 5-bromodeoxyuridine (BrdU)-labeled sperm followed by detection of BrdU in early diploid embryos, and differential heterochromatin staining in mouse interspecific hybrid embryos. Separation of chromatin according to parental origin was preserved up to the four-cell embryo stage and then gradually disappeared. In F1 hybrid animals, genome separation was also observed in a proportion of somatic cells. Separate nuclear compartments during preimplantation development, when extreme chromatin remodelling occurs, and possibly in some differentiated cell types, may be associated with epigenetic reprogramming.

Key words: 5-bromodeoxyuridine • fluorescence in situ hybridization • mouse interspecific hybrids • nuclear architecture • preimplantation embryo

Introduction

An enormous body of data from classical genetics (Cattanach and Kirk, 1985), nuclear transplantation experiments (McGrath and Solter, 1984; Surani et al., 1986), and human imprinting disorders (Lalande, 1996; Hall, 1997) suggests that normal mammalian development requires the participation of both a maternal and a paternal genome. Opposing patterns of gene expression from maternally and paternally derived alleles of imprinted genes explain the importance of having both parental genomes for normal embryonic development (Fundele and Surani, 1994; Tilghman, 1999).

The one-cell embryo is formed from two very different sets of chromatin: the highly compact, transcriptionally totally inert sperm DNA, and the maternal egg chromatin. Dramatic chromatin remodeling and reprogramming of developmental programs occur in the early mammalian embryo, resetting the differential gametic marks into their functional forms. Ovulated oocytes appear to be globally undermethylated, whereas the sperm genome is relatively methylated. A genome-wide demethylation during preimplantation development leads to indistinguishable alleles at most gene loci, but not at those that are imprinted (Howlett and Reik, 1991; Olek and Walter, 1998). Although a net demethylation could be caused by a combination of undermethylated maternal and methylated paternal DNA (Monk et al., 1987; Sanford et al., 1987), accumulating experimental evidence suggests that overall changes in methylation levels during early development may be the sum of highly dynamic and, maybe, differential processes in parental genomes (Yoder et al., 1997; Rougier et al., 1998; Mayer et al., 2000).

The oocyte is stocked with maternal mRNAs and proteins that are required for the development of the one-cell embryo. In the mouse embryo, major activation of the zygotic genome begins at the two-cell stage (Schultz, 1986). However, minor gene transcription occurs in the late one-cell embryo on already replicated DNA. Both replication and transcription are initiated earlier in the male pronucleus, which is also less condensed than the female pronucleus (Bouniol-Baly et al., 1997). Reporter gene transfection experiments (Wiekowski et al., 1993) and BrUTP incorporation assays (Aoki et al., 1997) showed a greater transcriptional activity in the male pronucleus. Activation of the male pronucleus may depend on transient histone H4 hyperacetylation of paternal DNA (Adenot et al., 1997).

Sperm and egg chromatin exhibit extreme differences in methylation and structure. After breakdown of the pronu-

†The authors dedicate this paper to the memory of Wolfgang Mayer who died on August 19, 1999.

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clear envelopes, the maternal and paternal chromosomes form a single metaphase plate. However, despite extensive chromatin remodeling in the zygote, the unspecific germline differences are not completely erased before the embryo cleaves into two cells. It seems plausible to assume that the process of converting the complementary sets of maternal and paternal chromosomes into a specialized diploid somatic genome may be regulated in a parent-specific manner and occur in separate nuclear compartments. To test this hypothesis, we have used different in situ approaches demonstrating separation of chromatin according to parental origin in the fertilized egg and the early diploid mouse embryo.

Materials and Methods

Collection and Preparation of Mouse Preimplantation Embryos

Embryos for immunocytochemistry were derived from an intraspecific *Mus musculus* (MMU)[1] cross between (C57BL6 × C3H) F1, for simplicity termed B6C3F1 mice. For fluorescence in situ hybridization (FISH) experiments, F1 embryos were derived from an interspecific cross between female laboratory mice, B6C3F1, and European wild mice, *Mus spretus* (MSP; strain SMZ).

Embryos were collected from superovulated mice according to standard procedures (Hogan et al., 1994). Unfertilized oocytes from B6C3F1 mice were collected 18 h after human choriogonadotropin (hCG) injection. Fertilized one-cell embryos were prepared from superovulated females mated with untreated males at 22 and 30 h after hCG injection. Fertilization occurred ~12 h (± 1 h) after hCG treatment. Two-cell embryos were collected at 34 and 44 h after hCG injection. Four-cell embryos were collected 57 h after hCG. Eight-cell stages were flushed and collected at 62 h, and morulae at 90 h after hCG treatment.

All embryos were released from the oviducts or uteri by inserting a 30-gauge needle into the infundibulum of the oviduct and injecting 0.2 ml M2 flushing medium (Sigma Chemical Co.). One-cell embryos were washed thoroughly in a drop of M2 medium containing 0.65 mg/ml hyaluronidase and then several times in PBS to remove any maternal material. Two-cell embryos and more advanced stages were washed in PBS only. The collected embryos were incubated in a drop of hypotonic solution (50 mM KCl) for 2 h at 4°C. Each single embryo was transferred into a drop of ice-cold (4°C) fixative consisting of three parts methanol and one part glacial acetic acid, fixed for 40 min, and then transferred onto a clean microscope slide. The embryo on the slide was covered with one drop of a 1:1 mixture of methanol and acetic acid. After air-drying of the fixative, the preparations were stored at 4°C for up to several weeks.

Preparation of Fibroblast Nuclei

Somatic cell nuclei were prepared from a primary fibroblast culture established from peritoneum of an adult F1 hybrid animal. Monolayer cells were grown in DME supplemented with 10% FBS and antibiotics. Cells were detached from culture flasks by gentle trypsinization, pelleted, and resuspended in 50 mM KCl. After a very short (<1 min) hypotonic treatment, cells were fixed overnight by adding four volumes of a 3:1 mixture of methanol and acetic acid. Slides were prepared using the conventional drop-splash technique.

BrdU Labeling of the Paternal Genome

Male B6C3F1 mice received an initial BrdU pulse by injecting i.p., 2 mg BrdU (in 200 μl PBS) and were then supplied continuously with drinking water adjusted to pH 7.0 and containing 0.5 mg/ml BrdU. Since BrdU is sensitive to daylight and luminescence from most lamps, the water bottles were wrapped with tin foil and the BrdU-containing water was changed at least once a week. BrdU is incorporated in place of thymidine into the replicating DNA of mitotically dividing somatic and premeiotic cells. The cycle time to produce mature spermatozoa from BrdU-labeled premeiotic cells in mice is ~35 d. After continuous BrdU feeding for at least 5 wk, the BrdU-treated males were mated with superovulated B6C3F1 females. Under the experimental conditions chosen, the BrdU was not toxic and did not induce recognizable developmental abnormalities. Even after prolonged treatment (1 yr or longer), BrdU-treated males did not show obvious BrdU side effects and could still be used for matings. Some pregnancies resulting from matings between BrdU-treated males and normal females were allowed to go to term. The offspring produced and the litter size were normal.

Immunofluorescent Staining of BrdU-labeled DNA

BrdU-substituted DNA was visualized by a commercially available monoclonal anti-BrdU antibody (Boehringer Mannheim Corp.). In double-stranded DNA, the bromine atom is hidden in the phosphodiester backbone of the double helix and, therefore, not accessible to antibody molecules. Since the anti-BrdU antibody only recognizes its chromosomal epitopes if the DNA is in the single-stranded form, the embryo preparations were denatured in 70% formamide, 2× SSC for 1 min at 80°C and then dehydrated in an ice-cold ethanol series (70, 85, and 100%). After brief air-drying, the slides were incubated at 37°C with mouse anti-BrdU antibody, diluted 1:50 with PBS, in a humidified incubator for 30 min. The slides were then washed in PBS three times for 10 min each and incubated for 30 min with FITC-conjugated anti-mouse IgG (Dianova), appropriately diluted with PBS. After three further washes with PBS, the preparations were counterstained with 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI) in 2× SSC for 5 min. The slides were mounted in 90% glycerol, 0.1 M Tris-HCl, pH 8.0, and 2.3% 1,4-diazobicyclo-2,2,2-octane.

Fluorescence In Situ Hybridization

For FISH, the slides were treated with 100 μg/ml RNase A in 2× SSC at 37°C for 60 min and with 0.01% pepsin in 10 mM HCl at 37°C for 10 min, and then dehydrated in an ethanol series (70, 85, and 100%). Slides were denatured at 80°C in 70% formamide, 2× SSC, pH 7.0, and again dehydrated in an alcohol series. MSP genomic DNA was labeled by standard nick translation with biotin-16-dUTP and MMU genomic DNA with digoxigenin-11-dUTP (Boehringer Mannheim Corp.). 10 ng/μl each of biotinylated MSP and digoxigenated MMU DNA were coprecipitated with 500 ng/μl salmon sperm carrier DNA, and redissolved in 50% formamide, 10% dextran sulfate, 2× SSC. After 10 min denaturation at 70°C, 30 μl of hybridization mixture was applied to each slide and sealed under a coverslip. Slides were left to hybridize in a moist chamber at 37°C for 1–3 d. Slides were washed three times for 5 min in 50% formamide, 2× SSC at 42°C, and once for 5 min in 0.1× SSC at 65°C, and blocked with 4× SSC, 3% BSA, and 0.1% Tween 20 at 37°C for 30 min. Biotinylated MSP DNA was detected by FITC-avidin (Vector Laboratories) and digoxigenated MMU DNA by Cy3-conjugated antidigoxin antibody (Dianova). Chromosomes were counterstained and mounted, as described above.

Digital Imaging Microscopy

Images were taken with a Zeiss epifluorescence microscope equipped with a thermoelectronically cooled charge-coupled device camera (Photometrics CH250), which was controlled by an Apple Macintosh computer. Grayscale source images were captured separately with filter sets for FITC, Cy3, and DAPI. Grayscale images were pseudocolored and merged using ONCOR Image and Adobe Photoshop software. It is worth emphasizing that, although a digital imaging system was used, all signals described here were clearly visible by eye through the microscope.

Results

Localization of BrdU-labeled Paternal DNA in Early Mouse Embryos

The germ-cell line in male mice was labeled with the halogenated thymidine analogue BrdU, as described previously (Ito et al., 1988). This BrdU-substituted DNA was detected in embryos of the next generation by anti-BrdU

[1]*Abbreviations used in this paper:* BrdU, 5-bromodeoxyuridine; DAPI, 4',6-diamidino-2-phenylindole; FISH, fluorescence in situ hybridization; hCG, human choriogonadotropin; MMU, *Mus musculus*; MSP, *Mus spretus.*

immunofluorescence staining and served as a cytological marker for the paternal genome in interphase nuclei of the zygote and cleaving mouse embryo. Fig. 1 a shows a mouse egg upon fertilization. The condensed sperm nucleus contained BrdU in both DNA strands of the paternal chromosomes and, therefore, was heavily labeled with the anti-BrdU antibody, whereas the activated female pronucleus was devoid of label. Decondensation of the sperm chromatin was followed by formation of the male pronucleus.

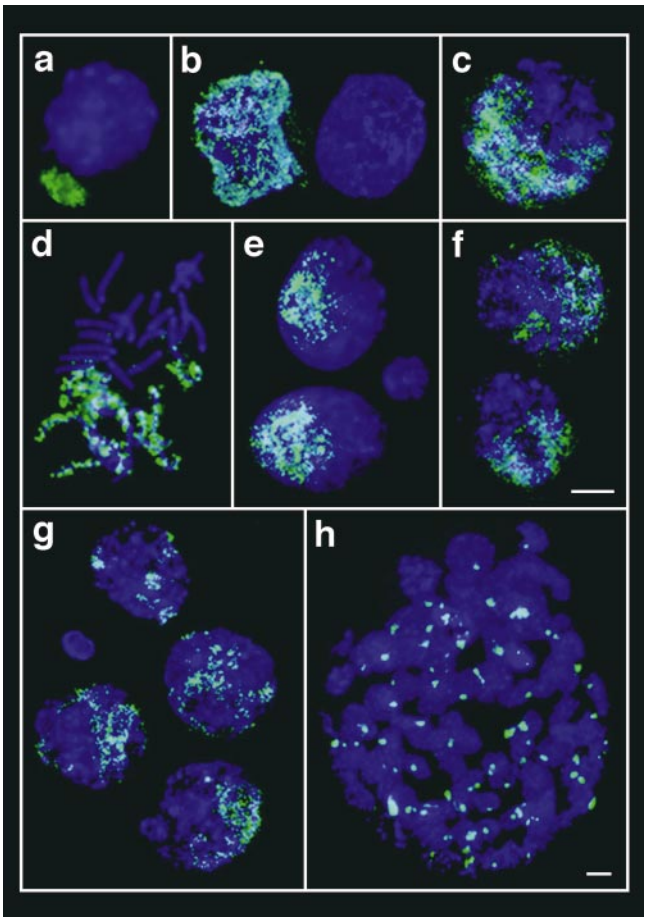

Figure 1. Distribution of paternal chromatin in early mouse embryos. BrdU-treated male mice were mated with untreated females and the resulting embryos stained with FITC-conjugated anti-BrdU antibody (green). Nuclei and chromosomes were counterstained with DAPI (blue). a, Highly condensed sperm nucleus and fertilized egg (3). Numbers in parentheses indicate the number of embryos analyzed. b, Male and female pronuclei at 10 h after fertilization (20). The somewhat larger male pronucleus shows a nearly uniform BrdU staining, indicating that the entire sperm DNA is substituted with BrdU. c, After nuclear envelope breakdown the two chromosome sets form a single diploid nucleus (2). d, First metaphase at 20 h after fertilization (5). e, Two-cell embryo during G_1 phase at 22 h (>10). The second polar body remains completely BrdU negative. f, Two-cell embryo during G_2 phase at 32 h (>10). The male chromatin occupies approximately half of the nuclear volume. g, Four-cell embryo and second polar body at 45 h after fertilization (10). At this point, only half of the paternal chromosomes are still labeled with BrdU. h, 32-cell embryo at 78 h (>5). The one or two BrdU-positive sperm DNA strands per nucleus are consistent with random strand-segregation mechanisms. Bars, 10 μm.

Both the male and female pronuclei swelled and became apposed in the center of the zygote. The male pronucleus exhibited a nearly uniform punctate BrdU staining (Fig. 1 b). Since no localized (partial) labeling of the male pronucleus was seen in >20 one-cell embryos analyzed at 10 and 18 h after fertilization, we conclude that the entire male genome was more or less uniformly substituted with BrdU. The smaller female pronuclei always remained BrdU negative, demonstrating the specificity of the technique. Following a very short G_2 phase of 1 h (Howlett, 1986) and breakdown of the pronuclear envelopes, the two chromosome sets remained completely separated, forming a single diploid nucleus (Fig. 1 c). Even in the absence of colcemid, highly condensed metaphase chromosomes could be observed at ~20–22 h after fertilization (Fig. 1 d). It is striking that the disruptive mitotic process did not lead to an intermingling of the two chromatin sets.

Two-cell embryos in G_1 (>10) and G_2 phase (>10) were prepared at 22 and 32 h after fertilization. All paternal chromosomes in these two-cell embryos were still labeled with BrdU, but due to the semiconservative DNA replication, in only one DNA strand. In all two-cell embryos analyzed, the paternal chromosomes were nonrandomly distributed throughout the entire nuclear volume. Usually each chromatin set occupied approximately one half of the nucleus (Fig. 1, e and f), which is consistent with the view that paternal and maternal chromosomes remained completely separated. As expected, the second polar body, which was still present on many embryo preparations (Fig. 1, e and g), was always BrdU negative. Four-cell embryos were prepared after the second embryo cleavage at ~45 h after fertilization. The fact that after two replication cycles only half of the paternal chromosomes contained BrdU (sperm DNA strands) rendered topographic analysis difficult. Nevertheless, most nuclei of four-cell embryos showed a highly localized distribution of BrdU label within the nuclear area (Fig. 1 g), indicating that the parental genomes were still separated at this point. Because of increasing loss of BrdU label with every successive replication cycle, the BrdU method is not suited for studying topological separation of paternal chromatin in more advanced preimplantation embryos or even in adult animals.

Segregation of the BrdU-labeled sperm chromosomes was followed up to the morula stage at 78 h after fertilization. Consistent with the results of Ito et al. (1988), we found a random distribution of the 40 sperm DNA strands into different parts of the embryo. In well-spread preparations (Fig. 1 h), ~50 distinct BrdU signals could be seen on a 32-cell embryo. This somewhat too high number (there were only 40 BrdU-positive sperm DNA strands) may be explained by the occurrence of sister chromatid exchanges during preceding interphases or be due to technical problems (split immunofluorescence signals).

Nonrandom Distribution of Paternal and Maternal Centromeres in Mouse Interspecific Hybrids

To rule out that the observed separation of parental genomes during early embryogenesis was a result of BrdU incorporation into sperm DNA, we used crosses between MMU and MSP, which diverged two to three million years ago (O'hUigin and Li, 1992). Differences in sequence and

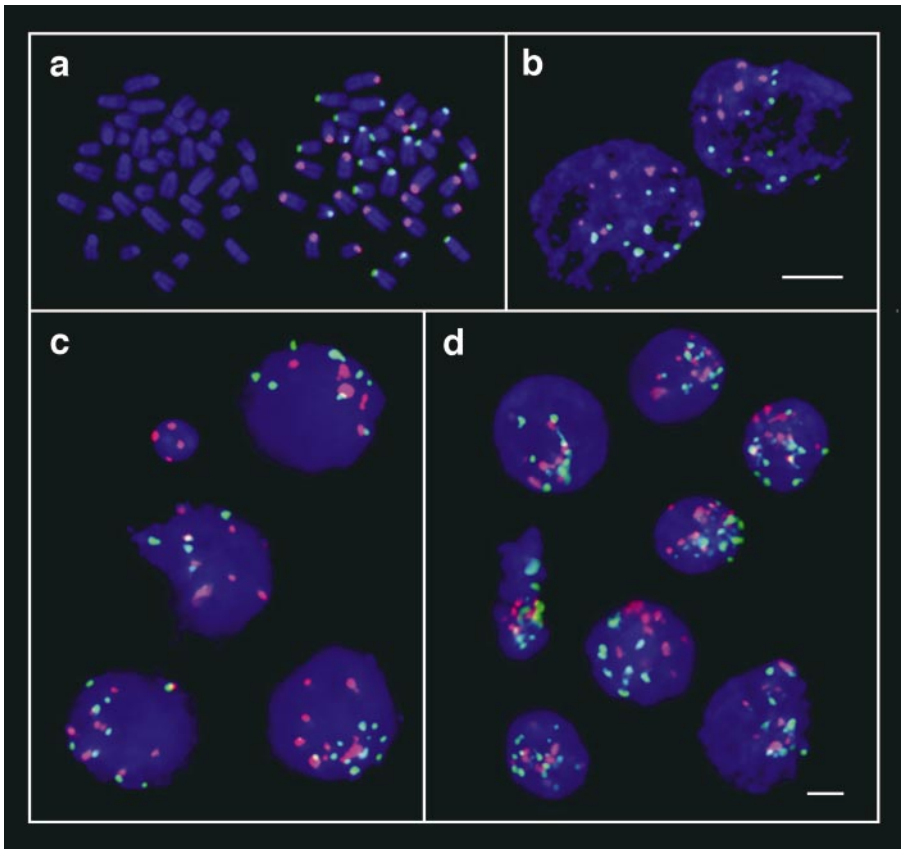

Figure 2. Distribution of paternal and maternal centromeres in early mouse embryos derived from matings between (untreated) MSP males and MMU females. Biotinylated MSP genomic DNA and digoxigenated MMU genomic DNA were hybridized together and detected with FITC-avidin and Cy3-conjugated antidigoxin antibody. Chromosomes and nuclei were counterstained with DAPI (blue). a, Well-spread first metaphase of MMU × MSP hybrid embryo. The left image shows DAPI staining and the right image comparative FISH of the same spread. The maternal MMU centromeres exhibit red FISH signals. The paternal centromeres are stained in green. b, Two-cell F1 embryo (>10). Numbers in parentheses indicate the number of embryos analyzed. Maternal and paternal centromere complements remain separated and together occupy approximately half of the nucleus. c, Four-cell embryo showing genome separation and centromere clustering (10). Note the absence of green (paternal centromere) fluorescence in the polar body. d, Eight-cell embryo (5). Separation of the two centromere sets is seen in some, but not all, cells. Bars, 10 μm.

copy number of centromeric satellite DNAs (Matsuda and Chapman, 1991) were used to identify maternal (MMU) and paternal (MSP) chromosomes in F1 hybrid embryos by FISH. By comparative hybridization of digoxigenated MMU genomic DNA and biotinylated MSP genomic DNA, the MMU (Fig. 2, red) and MSP centromeres (Fig. 2, green) were labeled in different colors during both metaphase and interphase. However, since nonrepetitive MMU and MSP sequences show a polymorphism rate of only ~1% (Takahashi and Ko, 1993), it was not possible to discriminate between maternal and paternal euchromatic chromosome arms by in situ methods (Fig. 2 a).

Consistent with our BrdU-labeling experiments, essentially all (>10) MMU × MSP two-cell embryos displayed a striking separation of maternal and paternal heterochromatin (Fig. 2 b). In addition, both the paternal and maternal centromeres were not evenly distributed throughout the entire nuclear volume, but clustered together in one half of the nucleus. This orientation of centromeres, which was also seen in DAPI-stained preparations of normal MMU embryos (data not shown), was reminiscent of the Rabl polarization of chromosomes (Rabl, 1885). Distribution of centromeres at one cell pole and, by extrapolation, of (long-arm) telomeres at the opposite cell pole may represent a passive relic of preceding mitosis. On the other hand, it may reflect an active chromosome arrangement to prevent intermingling of the two genomes. Centromere separation and clustering were still visible in most nuclei of four-cell embryos (Fig. 2 c), but in <50% of nuclei of eight-cell embryos (Fig. 2 d). The second polar body showed only MMU centromere staining and, thus, served as a hybridization control. In more advanced 16–64-cell embryos, only 5–10% of cells demonstrated a nonrandom (localized) distribution of the two centromere sets (data not shown).

To learn whether genome separation is maintained in differentiated cells, comparative genomic hybridization was performed on peritoneal fibroblasts of an adult animal. Similar to the situation in advanced embryo stages, relatively few (<10%) nuclei displayed clearly nonrandom heterochromatin staining patterns (Fig. 3 a). One possible interpretation would be that in at least some somatic cell types and/or cell-cycle stages the diploid chromosome complement may be separated in two haploid sets. Previous FISH studies on human fibroblasts and HeLa cells suggested that homologous chromosomes and, by extrapolation, the paternal and maternal complements, may be positioned on opposite sites of the prometaphase chromosome rosette (Nagele et al., 1995). However, mouse interspecific fibroblasts showed a more or less random distribution of maternal MMU and paternal MSP chromosomes around the (pro)metaphase rosette (Fig. 3 b). Since clear separation of the two haploid sets was never observed in >50 prometaphases analyzed, the nonrandom distribution of paternal and maternal centromeres in a proportion of interphase nuclei must arise through active chromosome (centromere) movements during interphase.

Discussion

By two independent technical approaches, BrdU labeling of sperm DNA and mouse interspecific hybrids, we have

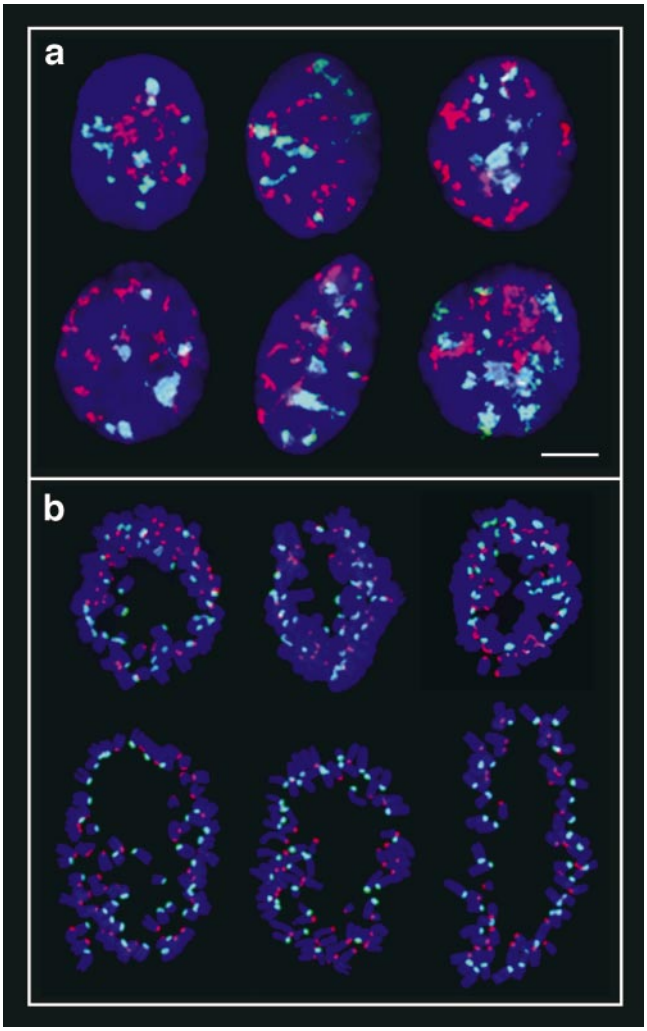

Figure 3. Distribution of paternal (green) and maternal (red) centromeres in somatic cells of MMU × MSP hybrid animal. a, Peritoneal fibroblast nuclei (>500) displaying spatial separation of paternal and maternal heterochromatin blocks. Numbers in parentheses indicate the number of cells analyzed. Bar, 10 μm. b, Random distribution of MMU and MSP chromosomes around representative prometaphase rosettes (>50).

shown topological separation of paternal and maternal chromatin in the early diploid mammalian embryo. Earlier autoradiographic experiments suggested a nonrandom localization of radioactively labeled sperm DNA strands in one- and two-cell mouse embryos (Odartchenko and Keneklis, 1973). In a conceptually related study, also using BrdU to label the paternal DNA, Ito et al. (1988) found random segregation of sperm DNA strands in the developing mouse embryo. However, they did not observe compartmentalization of parental chromatin. We feel this may be due to differences in preparative techniques and/or to the small number of early preimplantation embryos analyzed. Thus, up to now the general belief was that the autoradiographic results were experimental artifacts due to only partial labeling of the sperm genome and/or limited spatial resolution. Our study presents the first comprehensive analysis of the higher-order nuclear organization of parental chromatin during early mammalian development. Since we did not find regions of paternal pronuclei from BrdU-treated males that remained completely unlabeled, we exclude the formal possibility that localization of BrdU label in later embryonic stages reflects partial labeling of sperm DNA.

Topological separation of the parental genomes was preserved up to the four-cell embryo stage and then gradually disappeared or was no longer detectable with our in situ methods. A proportion (5–10%) of nuclei from more advanced embryos and even of adult tissues still showed striking centromere separation in mouse interspecific hybrids. Although genome separation seems to exist in at least some somatic cell types, it must be far less stable than in preimplantation embryos. It may be difficult to observe, because it is transient and/or modulated by the cell cycle. Since separation of haploid chromosome sets was not seen in prometaphase rosettes, nonrandom distribution of paternal and maternal interphase heterochromatin does not passively reflect mitotic chromosome arrangement. However, at present we cannot rule out that compartmentalization of MMU and MSP chromosomes in somatic cells may be a secondary (hybrid) effect. Association between heterochromatic blocks may be mediated by similar chemical properties of the chromatin in these chromosome regions, such as the high concentration of simple repeats. Pairing of regions with identical or closely related repeat DNAs may be preferred over those between more diverged regions (Haaf et al., 1986), explaining the formation of two separate heterochromatic compartments in mouse interspecific nuclei.

Accumulating experimental evidence suggests that higher-order nuclear organization may influence both normal developmental and pathological cellular processes and, hence, deserves to be studied in greater detail. Nuclear architecture is thought to provide a structural framework that may affect the accessibility of chromatin to regulatory factors and, thus, may serve an essential role in the regulation of gene expression beyond that at the single-gene level (Manuelidis and Borden, 1988; Haaf and Schmid, 1991). In situ methods are extremely powerful tools for elucidating the relationship between nuclear structure and function. Our experiments clearly demonstrate that paternal and maternal chromatin occupy separate nuclear entities at the time of preimplantation development when epigenetic chromatin remodelling (Mayer et al., 2000) and programming of the appropriate patterns of parent-specific developmental gene expression occur (Fundele and Surani, 1994). We argue in favor of the notion that genome separation during early mammalian development is functionally important, rather than a passive consequence of the different histories of sperm and egg chromation before fertilization. In addition, genome separation may serve a functional role in at least some differentiated cell types. While highly speculative, it is interesting to consider the possibility that disturbances in genome separation are not compatible with normal embryogenesis and are an important reason for early embryo loss, i.e., after in vitro fertilization, parthenogenesis, or mammalian cloning.

We thank Annie Orth and Francois Bonhomme (University of Montpellier II) for providing *M. spretus* mice.

Submitted: 9 November 1999
Revised: 6 January 2000
Accepted: 7 January 2000

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