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5-Methylcytosine-Rich Heterochromatin in the Indian Muntjac

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Abstract
Two 5-methylcytosine (5-MeC)-rich heterochromatic regions were demonstrated in metaphase chromosomes of the Indian muntjac by indirect immunofluorescence using a monoclonal anti-5-MeC antibody. The metaphases were obtained from diploid and triploid cell lines. A major region is located in the 'neck' of the 3;X fusion chromosome and can be detected after denaturation of the chromosomal DNA with UV-light irradiation for 1 h. It is located exactly at the border of the X chromosome and the translocated autosome 3. A minor region is found in the centromeric region of the free autosome 3 after denaturing the chromosomal DNA for 3 h or longer. The structure and possible function of the major hypermethylated region as barrier against spreading of the X-inactivation process into the autosome 3 is discussed.

The Indian muntjac *Muntiacus muntjak vaginalis* is a small deer with a remarkable karyotype. It has the lowest diploid chromosome number among all mammals (2n = 6♀, 7♂) [Wurster and Benirschke, 1970]. In contrast, the closely related Chinese muntjac *M. reevesi* possesses a chromosome number of 2n = 46, with exclusively telocentric chromosomes [Wurster and Benirschke, 1967]. The dramatic reduction of the chromosome number in the Indian muntjac is the result of repeated centromeric and tandem fusions [Hsu et al., 1975; Shi et al., 1980; Brinkley et al., 1984; Rattner, 1986; Lin et al., 1991; Yang et al., 1995; Latour et al., 1996]. The odd chromosome number (2n = 7) in the male Indian muntjac originated by a fusion of the extant autosome 3 and the original free X sex chromosome (designated as 3^X chromosome in the present study). A detailed molecular characterization of fusion sites in the chromosomes of the Indian muntjac has already been accomplished [Chi et al., 2005; Tsipouri et al., 2008].

Numerous previous and detailed cytogenetic analyses were conducted on the constitutive heterochromatin present in the genome of the Indian muntjac [e.g. Comings, 1971; Comings and Okada, 1971; Kato et al., 1974; Sharma and Dhaliwal, 1974; Carrano and Wolff, 1975; Kimura et al., 1980; Brinkley et al., 1984]. However, to the best of our knowledge, only a single investigation focused on the visualization of 5-methylcytosine (5-MeC)-rich heterochromatin in this species [Vasilikaki-Baker and Nishioka, 1983]. In that study, polyclonal anti-5-MeC antibodies, which were first produced by the group of Bernard F. Erlanger some decades ago [Erlanger and Beiser,

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1964; Erlanger et al., 1972], were applied to UV-irradiated and photooxidized metaphase chromosomes of a male muntjac. All chromosomes showed some degree of antibody binding along their length and at their centromeric regions, with a distinct immunofluorescence in the centromeric region of autosome 3 and the elongated centromeric 'neck' of the 3^X fusion chromosome. The banding pattern obtained by anti-5-MeC immunofluorescence was reverse to that obtained by quinacrine banding. However, the fluorescence signals recorded by Vasilikaki-Baker and Nishioka [1983] with polyclonal anti-5-MeC antibodies at the centromeric regions of muntjac chromosomes were of moderate intensity and reduced in contrast by the overall bright fluorescence along the chromosomes.

In the early 1990s, the first monoclonal antibodies against 5-MeC and other modified nucleosides were produced [Reynaud et al., 1992] and subsequently used in cytogenetics [Barbin et al., 1994; Miniou et al., 1994; Montpellier et al., 1994; Bernardino et al., 1996, 2000]. Antisera specific for 5-MeC were initially applied by the group of Orlando J. Miller to the chromosomes of several mammalian species, including human, chimpanzee, gorilla, cattle, mouse, and kangaroo rat [Miller et al., 1974; Schreck et al., 1974, 1977; Schnedl et al., 1975, 1976].

In the present study, the 5-MeC-rich heterochromatic chromosome regions in muntjac chromosomes were detected by indirect immunofluorescence using a highly specific monoclonal anti-5-MeC antibody.

Materials and Methods

Cell Lines and Culture Conditions

Four different cell lines derived from male muntjacs were available. The cell lines E7-KS and Mmv-E7 are diploid and derived from spontaneously transformed fibroblasts. The cell line Muntjac-♂ is also diploid, but derived from primary growing fibroblasts, whereas the cell line Muntjac-3n is triploid and originated from spontaneously transformed fibroblasts. The latter cell line was initially established in Denver, Colo., USA, and was kindly provided by Robert T. Johnson, Cambridge.

The cells were grown in DMEM (Life Technologies) supplemented with 10% (v/v) fetal bovine serum (Sigma), penicillin (100 U/ml), and streptomycin (100 µg/ml). Cells were subcultured using trypsin-EDTA detachment. For each subculture 1/10 of the volume of the parental cell suspension was transferred to a new flask. To each new flask 9/10 volumes of fresh medium were added.

Chromosome Preparation

Freshly fed and nearly confluent cell cultures were incubated with Colcemid (Gibco, 0.1 µg/ml) for 3 h at 37°C. Cells were detached with trypsin-EDTA solution, transferred to centrifuge tubes, and centrifuged at 1,400 rpm for 8 min, after which the supernatant was discarded. Hypotonic solution (0.075 M KCl, prewarmed to 37°C) was added to the cell pellet, gently mixed by pipetting, incubated for 12 min at 37°C, and centrifuged at 1,400 rpm for 8 min. The supernatant was discarded, and 1–2 ml of fixative solution (methanol:acetic acid, 3:1) was slowly added. The fixed cell suspensions were stored at 4°C or –20°C. Chromosome slides were prepared according to the conventional air-drying method and kept at room temperature for 24 h prior to chromosome banding or immunofluorescence.

Chromosome Banding Analyses

The demonstration of constitutive heterochromatin (C-bands) in the chromosomes followed the technique of Sumner [1972], and the detection of active nucleolus organizer regions (NORs) was performed according to Goodpasture and Bloom [1975] and Howell and Black [1980]. Photographs of high-quality C-bands and Ag-stained NORs were taken on an AxioImager A1 microscope (Zeiss) equipped with a thermoelectronically cooled charge-coupled device camera (Applied Spectral Imaging) using BandView software. In the karyotypes, the chromosomes were arranged according to the previous cytogenetic literature on this cervid species.

Detection of Hypermethylated Chromosome Regions

Hypermethylated DNA was detected by indirect immunofluorescence using a monoclonal antibody against 5-MeC. In double-stranded DNA, the methyl groups are hidden in the phosphodiester backbone of the double helix and not accessible to the antibody. The anti-5-MeC antibody recognizes and binds to its target only if the DNA is in the single-stranded configuration. Therefore, the slides with the chromosome preparations were immersed 1 cm below the level of a buffer solution (PBS) and denatured by UV-light irradiation for 1, 3 or 5 h at a distance of 10 cm from a UV lamp (254 nm). For indirect immunofluorescence, the slides were first incubated in a coplin jar for 1 h in blocking solution (PBS, with 0.3% BSA, 0.1% Tween) and then with 50 µl of a monoclonal mouse anti-5-MeC (primary) antibody (Imprint® monoclonal anti-5-methylcytosine antibody 33D3, Sigma-Aldrich) diluted 1:1,000 with the blocking solution in a humidified incubator at 37°C for 1 h. A non-siliconized coverslip (22 × 60 mm) was placed on the 50-µl drop to spread the anti-5-MeC antibody over the complete slide surface. Subsequently, the slides were washed twice in PBS (with 0.3% BSA) for 3 min each and then incubated with 70 µl of the secondary antibody (TRITC-conjugated rabbit anti-mouse IgG, Sigma-Aldrich) diluted 1:200 with PBS. The incubation conditions were as with the primary antibody. After 2 further washes with PBS for 3 min each, the chromosome preparations were mounted in Vectashield® mounting medium (Vectashield) with DAPI. Image analysis was performed with Zeiss epifluorescence microscopes equipped with thermoelectronically cooled charge-coupled device cameras (Applied Spectral Imaging) using easyFISH 1.2 software.

Results

In muntjac metaphases obtained from cell line E7-KS and UV-irradiated for only 1 h, the sole region showing a specific anti-5-MeC antibody labeling is located in the upper part of the well-known heterochromatic 'neck' in

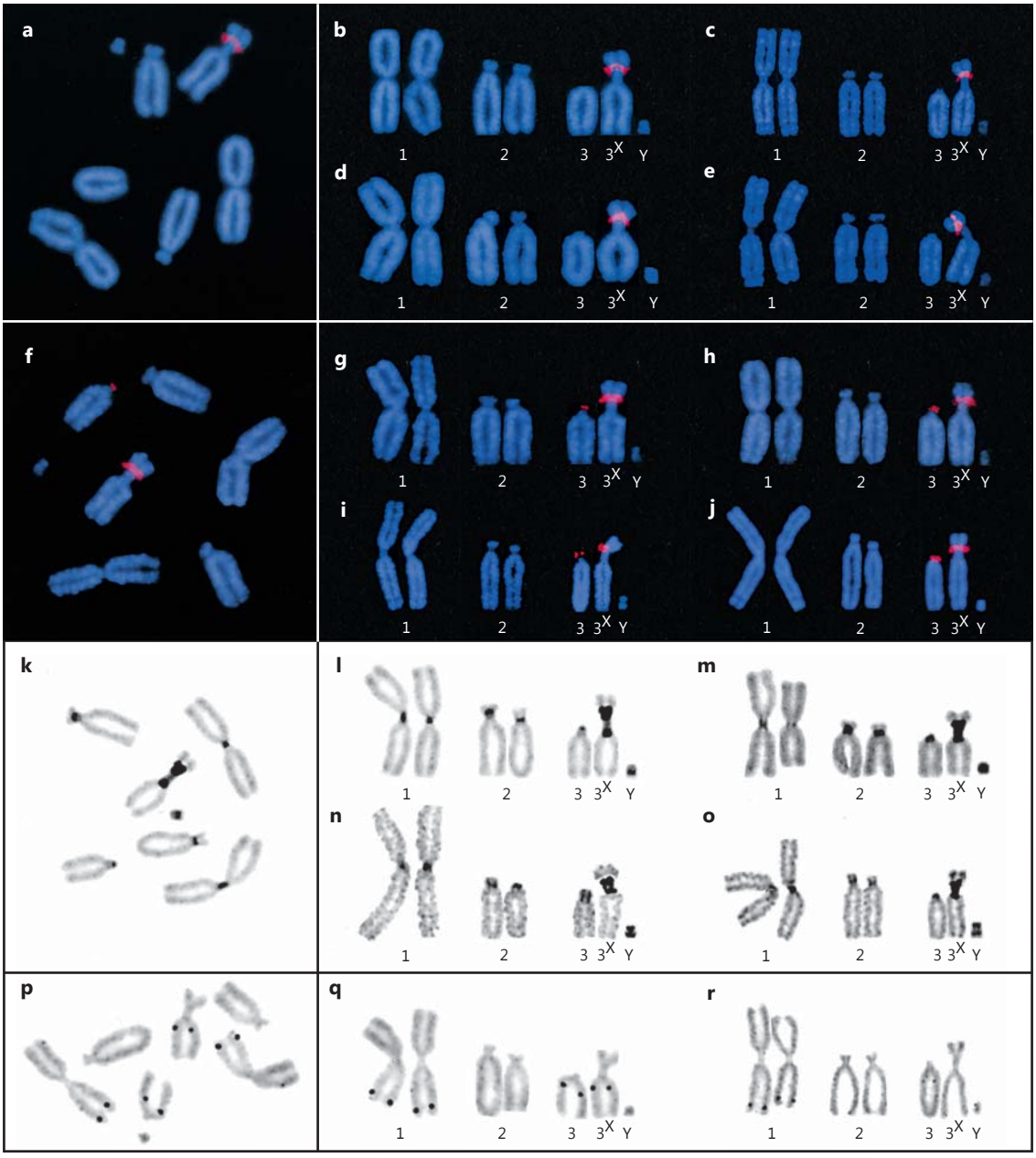

Fig. 1. Metaphases and karyotypes of the diploid cell line E7-KS from a male Indian muntjac. **a–j** Indirect immunofluorescence using a monoclonal antibody against 5-MeC. The 5-MeC-rich heterochromatic regions show bright red fluorescence signals, the chromosomes are stained blue with DAPI. The cells in **a–e** were UV-irradiated for only 1 h, those in **f–j** for 3 or 5 h. **k–o** C-banding of constitutive heterochromatin. **p–r** Ag-staining of active NORs.

the 3^X fusion chromosome. The fluorescence signal detected in this region is extremely bright with sharp boundaries. An example of such a metaphase and 4 different karyotypes are depicted in figure 1a–e. In none of the metaphases found in these preparations, the fluorescence signal did extent to the rest of the heterochromatic 'neck', and no further specific hypermethylated regions were detected in the other chromosomes.

In chromosome preparations denatured by UV-light irradiation for 3 or 5 h, hypermethylation is detected again in the upper part of the heterochromatic 'neck' in the 3^X fusion chromosome, and additionally in the het-

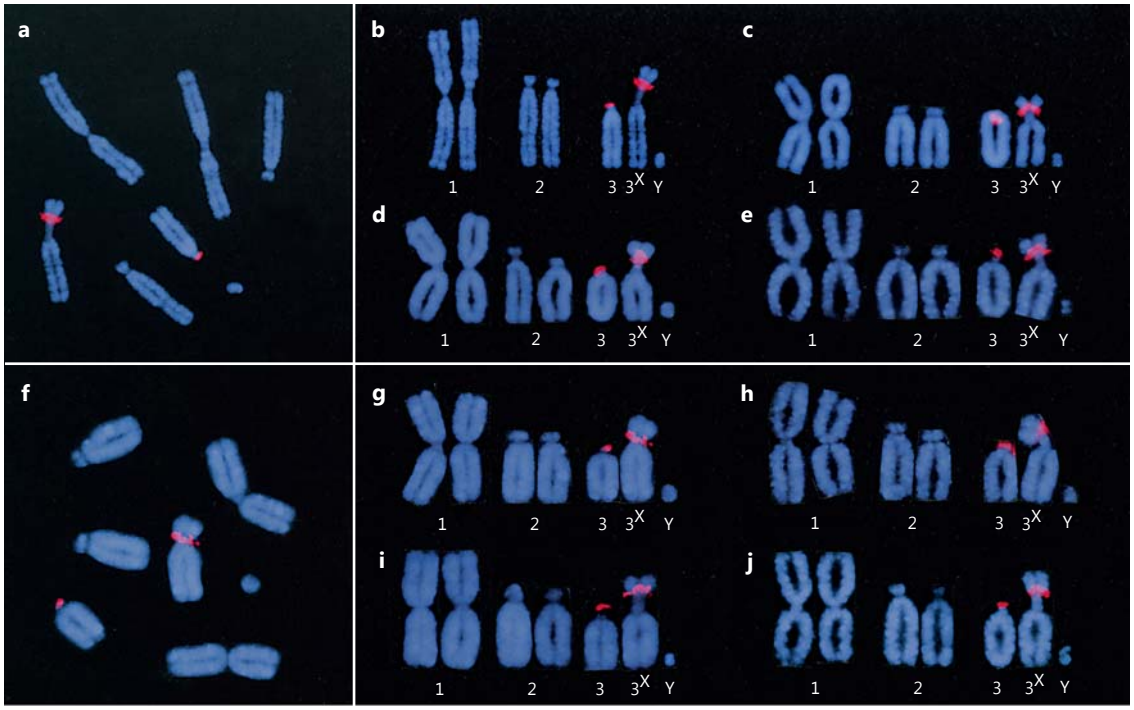

Fig. 2. Metaphases and karyotypes of the diploid cell lines Mmv-E7 (**a–e**) and Muntjac-♂ (**f–j**) from male Indian muntjacs showing indirect immunofluorescence using a monoclonal antibody against 5-MeC. The 5-MeC-rich heterochromatic regions show bright red fluorescence signals, the chromosomes are stained blue with DAPI. The cells were UV-irradiated for 3 or 5 h.

erochromatic centromeric region of the free autosome 3. This is exemplified in figure 1f–j. In the autosomes 1 and 2 as well as in the muntjac Y chromosome, 5-MeC-rich constitutive heterochromatin cannot be demonstrated.

C-banding of the metaphases yields the well-known heterochromatic patterns of muntjac chromosomes (fig. 1k–o). Autosomes 1 and 2, and the free autosome 3 contain discrete amounts of centromeric heterochromatin, the very small Y chromosome is completely heterochromatic. The 3^X chromosome is distinguished by the large heterochromatic 'neck', which extends from about the middle of the short arm down to the centromeric region.

Ag-staining shows that active NORs are present in the long arm telomeric region of autosomes 1 and in the proximal third of autosomes 3 (fig. 1p, q). Active NORs in autosomes 1 are found in all metaphases analyzed, whereas those in the autosomes 3 may be absent (fig. 1r). Neither the autosomes 2 nor the fused X chromosome or the Y chromosome bear active NORs.

Two further cell lines analyzed (Mmv-E7 and Muntjac-♂) are also diploid with no obvious chromosome rearrangements. Again, in preparations UV-irradiated for 3 h or longer, hypermethylation is present in the heterochromatic 'neck' of the 3^X fusion chromosome and in the heterochromatic centromeric region of the free autosome 3 (fig. 2). In none of the other heterochromatic regions, anti-5-MeC antibody labeling is detectable. No C-banding or Ag-staining was applied to these cell lines.

The fourth cell line examined (Muntjac-3n) was originally triploid, but during culture the Y chromosome was lost. Additionally, before triplication of the genome, the long arms of the 2 free autosomes 3 enlarged as result of a partial duplication or an insertion (fig. 3). The hypermethylation pattern recognizable after UV-light irradiation for 3 or 5 h is the same as in all other cell lines examined. It is restricted to the upper part of the neck in the 3^X fusion chromosome and to the centromeric heterochromatin in the free autosomes 3 (fig. 3a–c). Analysis of the C-banding patterns excludes any apparent reshufflings, deletions or duplications of the heterochromatic regions (fig. 3d–f).

In all 4 cell lines analyzed, most interphase cell nuclei exhibit the same number of brightly fluorescing anti-5-MeC antibody signals as found in the metaphase chromosomes. In some few nuclei, the fluorescing signals

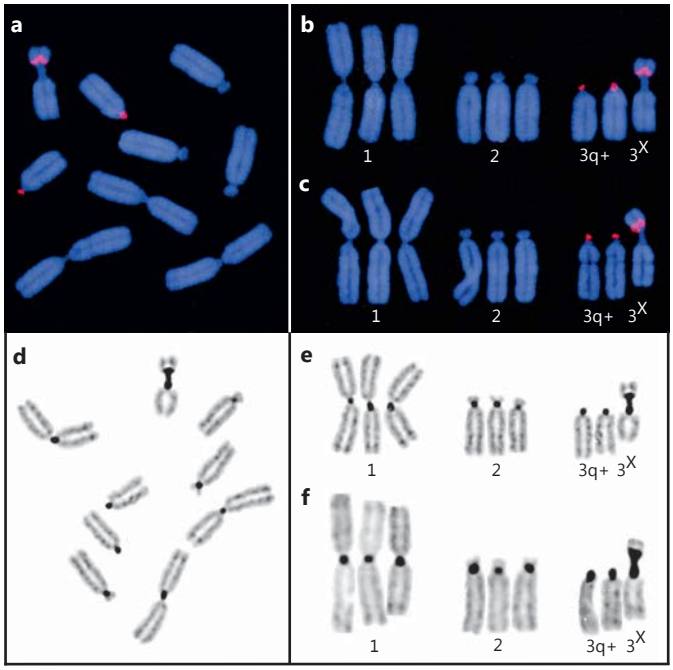

Fig. 3. Metaphases and karyotypes of the triploid cell line Muntjac-3n derived from fibroblasts of a male Indian muntjac. **a–c** Indirect immunofluorescence using a monoclonal antibody against 5-MeC. The 5-MeC-rich heterochromatic regions show bright red fluorescence signals, the chromosomes are stained blue with DAPI. The cells were UV-irradiated for at least 3 h. **d–f** C-banding of constitutive heterochromatin.

have a thread-like morphology of low contrast which indicates that the hypermethylated regions are decondensed and replicating (not shown).

No specific anti-5-MeC antibody labeling of euchromatic chromosome bands or larger euchromatic segments is observed by immunofluorescence microscopy. If occasionally present in some metaphases, these signals are most probably artifacts caused by insufficient washing of the chromosome preparations after incubation with the TRITC-conjugated secondary antibody.

It must be emphasized that the results obtained with the immunofluorescence experiments are reproducible irrespective of the fixation time of the cell suspensions (1–60 days), the temperature at which the fixed cell suspensions are kept (4°C, –20°C), the age of air-dried chromosome slides (1–12 months), and the incubation time with the primary and secondary antibodies (1–5 h). Crucial for the experiments, however, are the quality of the antibodies, the time of UV-light irradiation of chromosome preparations, and the washing procedures after incubation with the primary and secondary antibody.

Discussion

Like in primates, rodents, birds, and fishes [Miller et al., 1974; Schreck et al., 1974, 1977; Schnedl et al., 1975, 1976; Schmid et al., 2015a, b], in the Indian muntjac microscopically detectable 5-MeC-rich repetitive DNA sequences are restricted to a subset of the heterochromatic regions present in the karyotypes. The best known example is the human karyotype, in which visible hypermethylation of reiterated DNA is present exclusively in the centromeric and paracentromeric heterochromatin of the autosomes 1, 9, 15, and 16 as well as in the long arm heterochromatin of the Y chromosome [Schnedl et al., 1975]. There seems to be no general rule about the chromosomal locations of hypermethylated heterochromatic regions in vertebrate genomes. These very distinct fluorescent markers are species-specific and, therefore, quite useful for comparative cytogenetics in many vertebrate taxa. Even phylogenetically closely related species of birds, amphibians and fishes, with the same diploid number, chromosome morphology and C-banding, show different hypermethylation patterns [Schmid et al., 2015a, b].

The very heterogeneous chromosomal distribution of hypermethylated heterochromatic regions among vertebrate species is easily explained. The predominant components of constitutive heterochromatin are repetitive DNA sequences which are characterized by repetition of relatively long monomers (of a few hundred base pairs) over many megabases of DNA [for reviews, see Brutlag, 1980; Long and Dawid, 1980; Singer, 1982; Southern, 1984; Beridze, 1986]. It is not uncommon to find up to 25% of a genome made up of different repetitive DNA families [for review, see Lohe and Roberts, 1988]. Even among closely related species, reiterated DNAs usually differ in quantity, sequence and chromosomal location [Miklos, 1985]. Since they are not subjected to evolutionary pressure, sequence changes in repetitive DNAs can be accumulated and fixed in genomes very much faster than can the changes in functional (transcribed) DNA. Therefore, constitutive heterochromatin is heterogeneous within and between species [for review, see Verma, 1988]. This variability of heterochromatin is also characterized by extremely rapid changes of its hypermethylation patterns. Thus, it can be predicted that the amounts and locations of hypermethylated heterochromatic regions in the karyotypes of the Indian muntjac, the related Chinese muntjac *M. reevesi* and other more distant cervid species may be strikingly different.

In the Indian muntjac, hypermethylated heterochromatin is confined to the upper part of the 'neck' in the 3^X

fusion chromosome and to the centromeric region in the free autosome 3. This specific hypermethylation pattern is consistent in all cell lines analyzed and found irrespective of the degree of ploidy of the cells. The larger fluorescing signal in the 'neck' of the 3^X fusion chromosome is already detectable after denaturing the chromosomal DNA by UV-light irradiation for 1 h, whereas the demonstration of the distinctly smaller signal in the centromeric region of the free autosome 3 requires UV-light irradiation for at least 3 h or longer. The differing time of UV-light exposure is an indication for structural differences in the DNA of the 2 hypermethylated regions.

The molecular structure of the constitutive heterochromatin in Indian muntjac chromosomes was examined by Ueda et al. [1987] using restriction enzyme digestion, in situ hybridization with cloned DNA probes and distamycin A/DAPI fluorescence staining. They showed that the heterochromatic centromeric region of the free autosome 3 and the heterochromatic 'neck' in the 3^X chromosome are distinct from each other and different from the heterochromatic regions in the remaining chromosomes. A single band, located at the extreme end of the heterochromatin in the short arm of the 3^X chromosome, is characterized by bright distamycin A/DAPI fluorescence. A repetitive DNA probe, pMM3 (30 kb in length), derived by a double digest of genomic Indian muntjac DNA with the restriction enzymes *Hae*III and *Alu*I, was found to hybridize exclusively to this band. This is exactly the same site in the 3^X chromosome which in the present study turned out to be hypermethylated. It is conceivable that the pMM3 probe used by Ueda et al. [1987] is composed of 5-MeC-rich repetitive DNA and, therefore, responsible for the specific anti-5-MeC antibody labeling.

In another molecular cytogenetic approach, Cheng et al. [2009] generated 7 different non-satellite sequence elements from a 69-kb-long Indian muntjac BAC clone and mapped these probes by FISH to the chromosomes. Although all probes hybridized mainly to the heterochromatic centromeric regions, none was specific for the hypermethylated region in the short arm of the 3^X chromosome.

The fact that in the Indian muntjac 3^X chromosome a large hypermethylated heterochromatic region is located exactly at the border of the X chromosome and the translocated autosome 3 provokes interesting speculations about its possible function. In female mammalian cells carrying spontaneously originated aberrant X;autosome translocations, the X inactivation process can run over to the adjacent autosome causing detrimental genetic imbalances [for review, see White et al., 1998]. Thus, 5-MeC-rich repetitive DNA in the 'neck' region of the muntjac 3^X chromosome could act as an effective molecular barrier which prevents the spreading of X inactivation into the autosome in one of the two 3^X chromosomes in female cells. It is conceivable that the presence of this hypermethylated region in the X chromosome of the ancestral muntjac population was the prerequisite for the maintenance and evolutionary fixation of the 3^X chromosome once this fusion occurred in the founder individual. The immediate question that arises is whether in other known mammalian species with fixed X;autosome fusions or X;autosome translocations similar hypermethylated heterochromatic regions are present at the X-autosome chromatin border. This can be easily determined by examining their metaphases by indirect immunofluorescence with the highly specific monoclonal anti-5-MeC antibody.

Another interesting point worthwhile to examine is whether the X inactivation in female muntjac cells can spread into the adjacent chromosome 3 when the cell cultures are exposed to demethylating agents. Such agents are, for example, the cytosin analogs 5-azacytidine and 5-2′-azadeoxycytidine which cause extensive demethylation of the 5-MeC residues and reduce DNA methyltransferase activity in the cells [Jones and Taylor, 1980; Jones, 1985; Cheng, 1995].

Despite the above-mentioned possible function of the hypermethylated heterochromatic region in the 3^X chromosome of the Indian muntjac, it cannot be excluded that the peculiar chromosomal position of these repetitive DNA sequences has just randomly evolved without being essential for the X inactivation process operating in female cells.

Disclosure Statement

The authors have no conflicts of interest to declare.

References

- Barbin A, Montpellier C, Kokalj-Vocak N, Gibaud A, Niveleau A, et al: New sites of methylcytosine-rich DNA detected on metaphase chromosomes. Hum Genet 94:684–692 (1994).
- Beridze T (ed): Satellite DNA (Springer, Berlin 1986).
- Bernardino J, Lamoliatte E, Lombard M, Niveleau A, Malfoy N, et al: DNA methylation of the X chromosomes of the human female: an in situ semi-quantitative analysis. Chromosoma 104:528–535 (1996).
- Bernardino J, Lombard M, Niveleau A, Dutrillaux B: Common methylation characteristics of sex chromosomes in somatic and germ cells from mouse, lemur and human. Chromosome Res 8:513–525 (2000).

Brinkley BR, Valdivia MM, Tousson A, Brenner SL: Compound kinetochores of the Indian muntjac. Evolution by linear fusion of unit kinetochores. Chromosoma 91:1–11 (1984).

Brutlag DL: Molecular arrangement and evolution of heterochromatic DNA. Annu Rev Genet 14:121–144 (1980).

Carrano AV, Wolff S: Distribution of sister chromatid exchanges in the euchromatin and heterochromatin of the Indian muntjac. Chromosoma 53:361–369 (1975).

Cheng X: DNA modification by methyltransferases. Curr Opin Struct Biol 5:4–10 (1995).

Cheng YM, Li TS, Hsieh LJ, Hsu PC, Li YC, Lin CC: Complex genomic organization of Indian muntjac centromeric DNA. Chromosome Res 17:1051–1062 (2009).

Chi JX, Huang L, Nie W, Wang J, Su B, Yang F: Defining the orientation of the tandem fusions that occurred during the evolution of the Indian muntjac chromosomes by BAC mapping. Chromosoma 114:167–172 (2005).

Comings DE: Heterochromatin of the Indian muntjac. Replication, condensation, DNA ultracentrifugation, fluorescent and heterochromatin staining. Exp Cell Res 67:441–460 (1971).

Comings DE, Okada TA: Fine structure of the kinetochore in Indian muntjac. Exp Cell Res 67: 97–110 (1971).

Erlanger BF, Beiser SM: Antibodies specific for ribonucleosides and ribonucleotides and their reaction with DNA. Proc Natl Acad Sci USA 52:68–74 (1964).

Erlanger BF, Senitzer D, Miller OJ, Beiser SM: Nucleic acid-reactive antibodies specific for nucleosides and nucleotides. Acta Endocrinol Suppl (Copenh) 168:206–221 (1972).

Goodpasture C, Bloom SE: Visualization of nucleolar organizer regions in mammalian chromosomes using silver staining. Chromosoma 53:37–50 (1975).

Howell WM, Black DA: Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia 36:1014–1015 (1980).

Hsu TC, Pathak S, Chen TR: The possibility of latent centromeres and a proposed nomenclature system for total chromosome and whole arm translocations. Cytogenet Cell Genet 15: 41–49 (1975).

Jones PA: Effects of 5-azacytidine and its 2′-deoxyderivative on cell differentiation and DNA methylation. Pharmacol Ther 28:17–27 (1985).

Jones PA, Taylor SM: Cellular differentiation, cytidine analogs and DNA methylation. Cell 20: 85–93 (1980).

Kato H, Tsuchiya K, Yoshida TH: Constitutive heterochromatin of Indian muntjac chromosomes revealed by DNAse treatment and a C-banding technique. Can J Genet Cytol 16: 273–280 (1974).

Kimura S, Yamazaki K, Kato Y: Kinetics of DNA replication in the Indian muntjac chromosomes as studied by quantitative autoradiography. Chromosoma 77:309–323 (1980).

Latour DR, Vig BK, Finze EM, Paweletz N: The centromeres of the Indian muntjac: evidence for the existence of multiple centromeres? Mutat Res 356:187–195 (1996).

Lin CC, Sasi R, Fan YS, Chen ZQ: New evidence for tandem chromosome fusions in the karyotypic evolution of Asian muntjacs. Chromosoma 101:19–24 (1991).

Lohe A, Roberts P: Evolution of satellite DNA sequences in *Drosophila*, in Verma RS (ed): Heterochromatin. Molecular and Structural Aspects, pp 148–186 (Cambridge University Press, Cambridge 1988).

Long EO, Dawid IB: Repeated genes in eukaryotes. Ann Rev Biochem 49:727–764 (1980).

Miklos GLC: Localized highly repetitive DNA sequence in vertebrate and invertebrate genomes, in MacIntyre RJ (ed): Molecular Evolutionary Genetics, pp 241–321 (Plenum Press, New York 1985).

Miller OJ, Schnedl W, Allen J, Erlanger BF: 5-Methylcytosine localised in mammalian constitutive heterochromatin. Nature 251: 636–637 (1974).

Miniou P, Jeanpierre M, Blanquet V, Sibella V, Bonneau D, et al: Abnormal methylation pattern in constitutive and facultative (X inactive chromosome) heterochromatin of ICF patients. Hum Mol Genet 3:2093–2102 (1994).

Montpellier C, Burgeois CA, Kokalj-Vokac N, Muleris M, Niveleau A, et al: Detection of methylcytosine-rich heterochromatin on banded chromosomes. Application to cells with various status of DNA methylation. Cancer Genet Cytogenet 78:87–93 (1994).

Rattner JB: Organization within the mammalian kinetochore. Chromosoma 93:515–520 (1986).

Reynaud C, Bruno C, Boullanger P, Grange J, Barbesti S, Niveleau A: Monitoring of urinary excretion of modified nucleosides in cancer patients using a set of six monoclonal antibodies. Cancer Lett 61:255–262 (1992).

Schmid M, Smith J, Burt DW, Aken BL, Antin PB, et al: Third report on chicken genes and chromosomes 2015. Cytogenet Genome Res 145: 78–179 (2015a).

Schmid M, Steinlein C, Yano CF, Cioffi MB: Hypermethylated chromosome regions in nine fish species with heteromorphic sex chromosomes. Cytogenet Genome Res, Epub ahead of print (2015b).

Schnedl W, Dev VG, Tantravahi R, Miller DA, Erlanger BF, Miller OJ: 5-Methylcytosine in heterochromatic regions of chromosomes: chimpanzee and gorilla compared to the human. Chromosoma 52:59–66 (1975).

Schnedl W, Erlanger BF, Miller OJ: 5-Methylcytosine in heterochromatic regions of chromosomes in Bovidae. Hum Genet 31:21–26 (1976).

Schreck RR, Erlanger BF, Miller OJ: The use of antinucleoside antibodies to probe the organization of chromosomes denatured by ultraviolet irradiation. Exp Cell Res 88:31–39 (1974).

Schreck RR, Erlanger BF, Miller OJ: Binding of anti-nucleoside antibodies reveals different classes of DNA in the chromosomes of the kangaroo rat *(Dipodomys ordii)*. Exp Cell Res 108:403–411 (1977).

Sharma T, Dhaliwal MK: Relationship between patterns of late S DNA synthesis and C- and G-banding in muntjac chromosomes. Exp Cell Res 87:394–397 (1974).

Shi L, Ye Y, Duan X: Comparative cytogenetic studies on the red muntjac, Chinese muntjac, and their F1 hybrids. Cytogenet Cell Genet 26: 22–27 (1980).

Singer MF: Highly repeated sequences in mammalian genomes. Int Rev Cytol 76:67–112 (1982).

Southern EM: DNA sequences and chromosome structure. J Cell Sci Suppl 1:31–41 (1984).

Sumner AT: A simple technique for demonstrating centromeric heterochromatin. Exp Cell Res 75:304–306 (1972).

Tsipouri V, Schueler MG, Hu S, Dutra A: Comparative sequence analyses reveal sites of ancestral chromosomal fusions in the Indian muntjac genome. Genome Biol 9:R155 (2008).

Ueda T, Irie S, Kato Y: Longitudinal differentiation of metaphase chromosomes of Indian muntjac as studied by restriction enzyme digestion, in situ hybridization with cloned DNA probes and distamycin A plus DAPI fluorescence staining. Chromosoma 95:251–257 (1987).

Vasilikaki-Baker H, Nishioka Y: Immunological visualization of 5-methylcytosine-rich regions in Indian muntjac metaphase chromosomes. Exp Cell Res 147:226–230 (1983).

Verma RS (ed): Heterochromatin. Molecular and Structural Aspects (Cambridge University Press, Cambridge 1988).

White WM, Willard HF, Van Dyke DL, Wolff DJ: The spreading of X inactivation into autosomal material of an X;autosome translocation: evidence for a difference between autosomal and X-chromosomal DNA. Am J Hum Genet 63:20–28 (1998).

Wurster DH, Benirschke K: Chromosome studies in some deer, the springbok, and the pronghorn, with notes on placentation in deer. Cytologia 32:273–285 (1967).

Wurster DH, Benirschke K: Indian muntjac, *Muntiacus muntjak*: a deer with a low diploid chromosome number. Science 168:1364–1366 (1970).

Yang F, Carter NP, Shi L, Ferguson-Smith MA: A comparative study of karyotypes of muntjacs by chromosome painting. Chromosoma 103: 642–652 (1995).