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

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Abstract. The longitudinal differentiation of metaphase chromosomes of the Indian muntjac was studied by digestion with restriction enzymes, in situ hybridization with cloned DNA probes and distamycin A plus DAPI (4'-6-diamidino-2-phenylindole) fluorescence staining. The centromeric regions of chromosomes 3 and 3+X of a male Indian muntjac cell line were distinct from each other and different from those of other chromosomes. Digestion with a combination of EcoRI* and Sau3A revealed a pattern corresponding to that of C-banding. Digestion with Alul, EcoRII or Rsal yielded a band specific to the centromeric region only in chromosomes 3 and 3+X. Furthermore, HinfI digestion yielded only a band at the centromeric region of chromosome 3, whereas DA-DAPI staining revealed a single band limited to the extreme end of the C-band heterochromatin of the short arm of 3+X. These results suggest that centromeres of Indian muntjac chromosomes contain at least four different types of repetitive DNA. Such diversity in heterochromatin was also confirmed by in situ hybridization using specific DNA probes isolated and cloned from highly repetitive DNA families. Heterozygosity between chromosome homologs was revealed by restriction enzyme banding. Evidence is presented for the presence of nucleolus organizer regions (NORs) on the long arm of chromosome 1 as well as on the secondary constrictions of 3 and 3+X.

Introduction

The chromosomes of the Indian muntjac, Muntiacus muntjak, are considered to be one of the most suitable materials for cytogenetic analysis of heterochromatin, because of several characteristic features: (1) low chromosome numbers (female, 6; male, 7); (2) large size; (3) presence of extraordinarily large centromeric heterochromatin on chromosome 3+X; (4) prominent secondary constrictions on chromosomes 3 and 3+X.

Different aspects of the heterochromatin of the Indian muntjac have been investigated, e.g., various banding patterns (Comings 1971; Kato et al. 1974; Sharma and Dhalwal 1974), sister chromatid exchanges (Carrano and Wolff 1975), and fluorescence by anti-kinetochore antiserum (Brinkley et al. 1984), and DNA replication patterns (Comings 1971; Kimura et al. 1980).

The conventional C-banding procedure has been routinely used to identify constitutive heterochromatic regions in the chromosomes of mammalian species (Iksu and Arrighi 1971). To study the more detailed structure of C-band positive regions, several banding techniques have been developed. One is the fluorescence staining technique specific for AT or GC base pairs (Schweizer 1981). Another approach involves the digestion of fixed chromosomes with restriction endonucleases specific for a given DNA sequence, and subsequent staining with the nonspecific fluorescent dye, propidium iodide (PI) (Lima-de-Faria et al. 1980; Miller et al. 1983; Ueda et al. 1983). High resolution in situ hybridization using biotin-labeled DNA probes derived from highly repetitive DNA families is also a powerful approach for distinguishing slight regional differences in the distribution of a given sequence of C-band heterochromatin (Manuelidis et al. 1982).

Thus far, such different approaches have been used singly. In the present study, we combined three methods in the hope that such an approach might provide new information on the detailed structural organization of the heterochromatin of the Indian muntjac.

Materials and methods

Cell cultures. Primary cultures of cartilage fibroblast cells were prepared from a newborn male Indian muntjac. The cells were grown in Ham’s F12 medium supplemented with 10% fetal calf serum and were cloned. One of these clones, the Mm1-M-1 (male) was used throughout the present study.

Chromosome preparations. Chromosome preparations were made according to the conventional air-drying method. The cells were exposed to 0.1 μg·ml Colcemid for 30 min, treated with hypotonic 1% sodium citrate for 15 min and fixed with three changes of 3:1 methanol:acetic acid. The cell suspension was dropped on glass slides and dried at room temperature.

Triple fluorescent staining with chromomycin A₃, distamycin A and 4'-6-diamidino-2-phenylindole (DAPI). A modification of the DA-DAPI staining method (Schweizer 1980) was used, which reveals the chromomycin A₃ R-bands in

Abbreviations: DA distamycin A; DAPI 4'-6-diamidino-2-phenylindole; NOR(s) nucleolus organizer region(s); PBS phosphate-buffered saline; PI propidium iodide
addition to DA-DAPI bands on the same chromosome specimen. The fixed chromosomes were stained first with 0.5 mg/ml chromomycin A₃ (Sigma) in the presence of 2.5 mM MgCl₂ for 30 min, and rinsed with McIlvaine’s buffer, pH 6.8 (0.15 M phosphate-citrate). The rinsed chromosomes were further stained with 1.0 mg/ml distamycin A (Sigma) for 20 min, washed briefly with the same buffer, and finally stained with 1.0 µg/ml DAPI (Sigma). The preparations were mounted in a 1:1 mixture of glycerol and McIlvaine’s buffer. For microscopic observations and photography, a Zeiss fluorescence microscope equipped with vertical illuminator 100 and HBO 50 mercury lamp was used. By applying two different excitation wavelengths, both DA-DAPI bands and chromomycin A₃ R-bands could be observed on the same metaphase chromosomes. Excitation filters used were BP365/41 for DAPI fluorescence, and BP436/8 for chromomycin A₃.

**DNA preparation.** Cells were incubated with proteinase K and DNA was extracted with phenol and treated with ribonuclease as described by Blin and Stafford (1976). The average length of DNA thus obtained was greater than 50 kb.

**Restriction enzyme digestion and agarose gel electrophoresis of DNA.** Restriction enzymes were obtained from Bethesda Research Laboratories (EcoRI, HhaI), Boehringer Mannheim (MspI, HpaII) and TOYOBO Co. (AluI, BamHI, EcoRI, HaeIII, HinfI, RsaI, Sau3A, Thal). EcoRI* (AATT) digestion was performed by lowering the ionic strength or increasing the glycerol concentration during digestion with EcoRI (Polisky et al. 1975). DNA samples digested with restriction endonucleases were subjected to electrophoresis through 1.4% agarose in 80 mM Tris-phosphate and 8 mM EDTA, pH 8.0, on a horizontal submerged gel at 70 V for 6-8 h. Gels were stained with 0.5 µg/ml ethidium bromide and photographed with Kodak Royal-X pan film.

**Restriction enzyme-induced banding.** About 30 units of restriction enzyme dissolved in 30 µl of appropriate buffer were applied to the air-dried chromosomes. The slides were mounted with coverslips and incubated in a moist chamber at 37°C (60°C for Thal) for 4-20 h. At the end of digestion, the coverslip was removed and the slide was rinsed in distilled water, treated with 100 µg/ml ribonuclease A for 1 h, and stained with 10 µg/ml PI for 15 min. Control slides were treated in the same way, except that the enzyme was omitted from the reaction mixture.

**Isolation and cloning of highly repetitive DNA probes.** Deproteinized muntjac DNA was doubly digested with HaeIII and AluI and the large 30 kb fragment was isolated by centrifugation in a 10% 40% sucrose density gradient. The purified DNA was inserted into the Smal site of plasmid vector pUC 19 by blunt-end ligation. The recombinant DNA was used to transform *Escherichia coli* K12 JM83 strain. The inserted DNAs were analysed by electrophoresis on a 0.6% agarose gel.

In a similar manner, we also cloned two different repetitive DNA fragments. The recombinant clone pMM 1 was derived from a BamHI digest of unfractionated muntjac DNA and has an insert size of 0.8 kb. Clone pMM 2 was derived from a HaeIII digest and has an insert size of 1.1 kb. The 0.8 kb insert of clone pMM 1 is identical to the satellite IA DNA fragment isolated by Bogenberger et al. (1982).

**Chromosome hybridization.** Chromosome spreads were treated with 100 µg/ml of RNase A in 2 x SSC for 1 h at 37°C, denatured with 70% formamide in 2 x SSC at 70°C for 3 min, dehydrated in a graded ethanol series starting from 70% and air dried. (1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate) DNA probes, labeled with biotin-dUTP (Bio-11-dUTP, Enzo-Biochem) by nick translation (Manuelidis et al. 1982), were dissolved in the hybridization buffer (10% dextran sulfate, 4 x SSC, 40% formamide, 0.3 mg/ml sonicated salmon sperm DNA, 1 x Denhardt’s solution, 10 mM sodium phosphate, pH 6.8) at 1 ng/ml, denatured at 70°C for 5 min and stored at 4°C. Approximately 30 µl of the probe solution was applied to each slide. Hybridization was achieved by incubating the slides with a coverslip at 37°C in a moist chamber overnight. The slides were washed extensively in 2 x SSC, in 0.5 x SSC (2 x 5 min), equilibrated in phosphate-buffered saline (PBS), and treated with 10% normal goat serum for 30 min at 37°C for blocking. The slides were then treated sequentially with the goat antibiotin antibody (ENZO Biochem, Inc. 1:50 dilution), biotinylated rabbit anti-goat IgG (Vector Lab., 1:50 dilution), and finally with avidin peroxidase conjugates (ABC reagent, Vector Lab.). Each treatment was done at 37°C for 12 h. The slides were washed extensively with PBS between treatments.

Color development was achieved by dipping the slides in a freshly prepared solution of 0.5 mg/ml 3,3’-diaminobenzidine (DAB, Sigma) in 10 mM Tris-HCl, pH 7.6, containing 0.01% H₂O₂. For hybridization using the pMM 3 probe, cobalt chloride was added to the DAB solution at the final concentration of 0.02% before adding H₂O₂ to enhance the reaction (Adams 1981).

**Chromosome numbering.** The chromosome numbering system for the Indian muntjac of Mainguy et al. (1975) was adopted in the present study. The karyotype of this species consists of metacentric (chromosome 1), subtelocentric (chromosome 2), telocentric (chromosome 3) and sex chromosomes. The male karyotype consists of seven chromosomes, and includes a single X fused to the end of one of the chromosome 3 pair (3+X chromosome).

**Results**

**Susceptibility of DNA and metaphase chromosomes of the Indian muntjac to restriction endonucleases which recognize C and G bases**

We treated the fixed metaphase chromosomes with several restriction endonucleases whose base sequence targets are composed of G and C bases (HaeIII, HpaII, MspI, HhaI, Thal), followed by staining with the non-specific fluorochrome PI. Whereas chromosomes were uniformly stained along their axes after digestion with the latter four enzymes, HaeIII produced characteristic banding which was apparently identical to that of G-banding (Fig. 1). This result is consistent with a previous report (Lima-de-Faria et al. 1980).

According to the report of Miller et al. (1983), the size of extractable DNA segments is 100 200 bp, whereas fragments longer than 1 kb remain in the fixed chromatin. HaeIII (GGCC) digestion of the deproteinized muntjac DNA produced broadly distributed DNA fragments of from less than 3 kb down to 0.2 kb (Fig. 2). With the other