Characterization of the heterochromatic chromosome regions in sheep

Jose Luis Fernández-García, Margarita Martínez-Trancón*, Araceli Rabasco, and Jose Angel Padilla

Departamento de Zootecnia, Genética y Mejora Animal, Facultad de Veterinaria, Universidad de Extremadura, Avda, de la Universidad s/n, 10071 Cáceres, Spain

(Received 7 November 1997, accepted 29 January 1998)

In order to elucidate the structural chromosome organization of the heterochromatic regions in sheep, we have used C-banding, silver-staining, sequential CDD technique and restriction endonuclease banding. By these banding techniques we obtained four fractions of repetitive DNA, the autosomal fractions A and B, the C fraction in the X chromosome, and the D fraction in the Y chromosome. Silver staining revealed active nucleolus organizer regions (NOR’s) on the telomeric GC-rich areas of chromosomes 1, 2, 3, 4, and 25 which were digested with HaeIII restriction endonuclease.

INTRODUCTION

Constitutive heterochromatin regions of metaphase chromosomes have been shown by standard C-banding techniques (Sumner, 1972). Not all C-bands correspond to heterochromatin (Catalá et al., 1981) and C-banding cannot differentiate between heterochromatin comprising one or several families of repetitive DNA (Miller et al., 1983).

In various species of bovids, the centromeric constitutive heterochromatin of the autosomes has been detected by using chromomycin A3 (Mayr et al., 1985). Chromomycin A3 (CMA3) is a specific fluorochrome that reveals GC-rich DNA chromosomal regions (Sahar and Latt, 1980). Molecular studies confirm that ovine constitutive heterochromatin harbours GC-rich DNA and comprises two satellite DNA fractions, satellite I and II (Kurnit et al., 1978).

The effects of the digestion with restriction endonucleases of type II on fixed chromosomes showed the existence of regions resistant to these enzymes, which led to differential banding patterns (Babu, 1988). The action of a restriction endonuclease depends on the amount of targets at a given chromosomal area: when the number of targets is high, short DNA fragments (less than 1 kb of length) are extracted during treatment, while low frequencies of recognition sites produce long DNA fragments (more than 1 kb of length) which are retained by chromosomal proteins. The observation of regions with different staining intensity points to distinct sensitivity to enzyme digestion, even showing areas that have been completely extracted (gaps) (Bianchi et al., 1990).

The present work elucidates the structural chromosome organization of the heterochromatic regions in Ovis aries L. by C-, sequential CDD-, Nucleolus Organizer Regions (NORs), and restriction endonuclease banding (RE).

MATERIALS AND METHODS

Cultures of heparinized peripheral blood of seven male and 10 female Merino sheep were set up and metaphase chromosomes prepared by standard methods. All air-dried slides were stored at –20°C until restriction endonuclease treatment.

C-bands were obtained by the technique of Sumner (1972). The slides were stained by two procedures: either with 5% Giemsa solution or with acridine orange in McIlvaine buffer. NORs were stained following the method of Howell and Black (1980). Some preparations were simultaneously stained for R-bands and QFH-bands by CMA3/DA/DAPI (CDD), according to Schweizer (1980).

Other slides were treated with different restriction enzymes (AluI, DdeI, HaeIII, RsaI, HindIII, BamHI, and EcoRI) by the procedure described by Mezzanotte et al. (1983) and Miller et al. (1983). For restriction endonuclease TaqI we used the procedures described by Marchi and Mezzanotte (1988) and Tagarro et al. (1991): the enzyme was dissolved in the assay buffer specified by the supplier and the preparations were incubated overnight in a moist chamber at 60°C and 37°C, respectively. Control slides were treated without enzymes. Treated slides were stained with 5% Giemsa (Gurr) in Sörensen buffer for 10 min. Moreover, with the aim to verify the digestion effects on chromosomes, preparations were also stained with a solution of ethidium bromide (5 µg/ml) in distilled water.

The chromosomes were studied in a Zeiss Axioskop mi-
croscope equipped with 50-W mercury lamp, excitation filters G365 and G435 for DA/DAPI and CMA3, respectively, and BP546 for ethidium bromide and in a Nikon Labophot-2 microscope for Giemsa staining. Tmax 100 film (Kodak) was used for fluorescence and Imagelink HQ (Kodak) was used for light microscopy.

RESULTS

The studied animals possessed the expected sheep karyotype (2n = 54). After C-banding, the centromeric regions of metacentric, X and Y chromosomes showed negative C-bands, though small amounts of constitutive heterochromatin could occasionally be observed in their centromeric regions, probably as result of technical difficulties. However, in the acrocentric autosomes these regions appeared intensely stained after C-banding treatment independently of the stain used, Giemsa or acridine orange (results not shown).

The sequential counterstain CDD method showed R and QPH complementary banding, but a better definition on all sheep chromosomes was obtained by CMA3 (Fig. 1). The CMA3-stained metacentric and acrocentric and the X chromosome showed a fluorescent band in the centromeric region. A weak DA/DAPI positive band was only detected in the short arm of the X chromosome (Fig. 3a, c) and a small positive CMA3 band can be seen in the short arm of the Y chromosome (Fig. 3b, b').

In the middle of the positive fluorescent material of some elongated acrocentric chromosomes we observed the existence of a thin band with a slight decrease in the intensity of fluorescence (Fig. 1, inset). Such differential response to chromomycin probably points to the existence at the centromere of a different heterochromatin fraction with a lower GC concentration.

Restriction endonuclease digestion produced five RE-banding patterns in the heterochromatic regions of ovine fixed chromosomes (Table 1). The digestion of metacentric and X chromosomes with AluI and DdeI endonucleases (which targets AG/CT and C/TNAG sequences, respectively) resulted in homogeneous weak stain and no differentiation of heterochromatic C-band areas (Figs. 2a and 3a, d). These chromosomes were extensively and evenly digested by these endonucleases, suggesting the existence of many AluI or DdeI targets equally distributed. AluI induced an intense digestion located in the short arm of the Y chromosome, probably the CMA3 positive region (c' and b' in Fig. 3b). However, on acrocentric autosomes we have found two types of effects: (i) some acrocentric chromosomes remained faintly stained after the treatment, as the results above mentioned, (ii) the heterochromatic region of other acrocentric chromosomes remained well-stained (C-banding) after digestion with this enzyme, suggesting a great absence of AluI target DNA-sequences. Both findings indicated the existence of differences in composition of repetitive DNA in this chromosomal area. In fact, we observed

![Fig. 1. CMA3 staining of ovine metaphase. The inset shows chromosome 17. The arrowhead indicates a thin band of decreased fluorescence within the positive fluorescent region. Scale line: 10 µm.](image)

Table 1. Effects produced in heterochromatin with C-banding, CMA₃-staining and RE treatment of fixed chromosomes

<table>
<thead>
<tr>
<th>Chromosome Morphology</th>
<th>Location</th>
<th>Tsy1-gap</th>
<th>AluI-DdeI-C-banding</th>
<th>AluI-gap</th>
<th>HaeIII-Real-Hinfl-C-banding</th>
<th>HaeIII-Real-Hinfl-gap</th>
<th>C-banding</th>
<th>CMA₃</th>
<th>DNA fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrocentric</td>
<td>centromeric</td>
<td>+</td>
<td>±</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>paracentromeric</td>
<td>+</td>
<td>±</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>B*</td>
</tr>
<tr>
<td>Metacentric</td>
<td>centromeric</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>±</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>p-arm</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>C-like</td>
</tr>
<tr>
<td>X</td>
<td>centromeric</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>±</td>
<td>±</td>
<td>–</td>
<td>B-like</td>
</tr>
<tr>
<td></td>
<td>p-arm</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>C</td>
</tr>
<tr>
<td>Y</td>
<td>p-arm</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>D</td>
</tr>
</tbody>
</table>

Negative sign (–) indicates no effects, positive sign (+) presence of effects and (±) the result non-regular or less intense digestion or staining respectively. B* indicates a modified fraction of non AluI and DdeI target in heterochromatic DNA of the acrocentric autosomes.
interindivial differences in the number of chromosomes with centromeric regions resistant to enzymatic action. Because of the absence of differential banding on the chromosomal arms, we could not identify these chromosomes.

TaqI (which targets T/CGA sequences) induced centromeric gaps on autosomes (Fig. 2b) corresponding in size and location to those of CMA3 bands. However, a distinct structural kind of heterochromatin was found in the short arm of the X chromosome, since this area stayed slightly affected after the treatment (Fig. 3a, g).

The digestion with HaeIII (GG/CC) brought about the formation of G-like bands (Fig. 2c), which permitted us to identify each chromosome pair, following Ansari et al. (1994) recommendations for O. aries L. The centromeric regions of metacentric chromosomes remained totally unstained after the treatment. This finding suggested that a large number of HaeIII targets were present in these areas, given that they were completely cleaved (gaps). On acrocentric autosomes, HaeIII induced small and faintly stained centromeric bands appearing as a lightly stained dot located at the top of chromatids. Below this band there was a thin negatively stained region (gap), named by us paracentromeric region. Both effects corresponded in size and location to that of C-bands. This combination of residual C-banding
with a centromeric gap was detected in all acrocentric autosomes. The centromeric region of the X chromosome remained almost completely digested by this endonuclease. However, in the X-p arm we observed a resistant HaeIII band (Fig. 3a, e), which probably corresponds to the previously found DA/DAPI positive band. On the other hand, the Y chromosome showed a differential digestion (a slight gap) on an arm (Fig. 3b, d’) that could belong to the previously observed CMA3 positively stained region.

The Ag-NOR areas were at the telomeric regions previously banded as chromomycin-bright bands of the autosomes 1, 2, 3, 4, and 25 (Fig. 4), though not all NOR-bearing chromosomes showed conspicuous black bodies after treatment. These telomeric regions were extensively digested by HaeIII (gaps), as might be foreseen since the nucleolar organizer regions were localized in CMA3 positive bands (rich in G-C bp).

Although RsaI (GT/AC) caused similar effects to those of HaeIII digestion, fewer distinct bands were obtained. This made chromosome identification confusing. However, the short arm of the X chromosome was more resistant to RsaI digestion than HaeIII digestion (Fig. 3a, e, respectively). HinFI (GA/NTC) produced similar effects to HaeIII and RsaI digestion, but positive CMA3 centromeric regions of the metacentric chromosome were faintly digested (gaps were difficult to see). Occasionally, gaps were observed on centromeric regions of some acrocentric chromosomes but not clearer than the same region digested by HaeIII or RsaI. BamHI (GGATCC) and EcoRI (G/AATTC) endonucleases did not induce any significant reaction or banding patterns.

**DISCUSSION**

The C-banding results are according to those obtained by Buckland and Evans (1978) in ovine chromosomes. CMA3 and DA/DAPI bind preferentially to GC- and AT-rich DNA regions, respectively, so it can be concluded that the bright chromosomal regions comprise these sorts of DNA. According to Mayr et al. (1985), the centromeric regions of ovine autosomes contain GC-rich heterochromatin. However, we have found a heterochromatic fraction with lower GC concentration, which divides the positive fluorescent material of some elongated acrocentric chromosomes. According to Mayr et al. (1985 and 1987) in phylogenetically related species such as ibex (*Capra ibex* L.), chamois (*Rupicapra rupicapra* L.) and goat (*Capra hircus* L.).

By using RE-, CDD-, and C-banding we found that sheep constitutive heterochromatin comprises four DNA fractions:
A, B, C, and D. The chromosomal location and properties of each DNA fraction are given in Table 1. Probably the A and B fractions correspond to satellites I and II, respectively (Kurnit et al., 1978), because they share similar positions in centromeric regions of sheep autosomes. More recently the results obtained by Burkin et al. (1986) by FISH suggest a loss or replacement of satellite I of metacentric chromosomes (formed by ancient Robertsonian translocation), this fact is in agreement with the detection of A fraction only in acrocentric chromosomes. The existence of a DNA C-fraction located in the p-arm of the X chromosome is supported by its differential digestion with TaqI endonuclease and by the presence of a faintly positive DAPI band in this region (c and g in Fig. 3a). This fact agrees with Hayes et al. (1991) that an important fraction of the p-arm is euchromatin. Generally, in centromeric region with Hayes et al. (1991) that an important fraction of the band in this region (c and g in Fig. 3a). This fact agrees with the idea that the chromatin of the NOR regions presents a nonnucleosomal structure (Derenzini et al., 1987), facilitating digestion by restriction endonucleases.

The D fraction was an extensively AluI-digested region in the short arm of the Y chromosome, which would imply a repetitive DNA fraction rich in AG/CT targets. The existence of an Alu repetitive DNA cluster in the short arm of the Y chromosome, was found by FISH using a bovine probe containing Alu-like repetitive DNA elements (Rajcan-Separovic and Sabour, 1993).

On the other hand, some restriction endonucleases have shown their ability to detect NORs which contain moderately repetitive DNA sequences. It is known that DNA sequences at NORs are rich in GC and they are usually marked by means of silver staining methods, as can be attained following the work of Mayr et al. (1985). HaeIII shows capacity to digest NORs inducing intense gaps in these regions of eukaryotic chromosomes (Padilla et al., 1993). This fact agrees with the idea that the chromatin of the NOR regions presents a nonnucleosomal structure (Derenzini et al., 1987), facilitating digestion by restriction endonucleases.

Intraspecific chromosome polymorphisms produced in acrocentric autosomes after digestion with AluI and DdeI suggested that these endonucleases did not affect some fractions of repetitive DNA that remained darkly stained. Intraspecific AluI polymorphisms have been observed in species such as man (Babu et al., 1988) and brown trout (Sanchez et al., 1990). This kind of AluI- and DdeI-resistant heterochromatin probably belongs to a fraction of DNA specific (B*) of acrocentric autosomes of sheep, since the centromeric regions of metacentric chromosomes were always sensitive to these restriction endonucleases (B). The large amount of AluI targets found along sheep chromosomes is according to the result obtained by Richardson et al. (1986), in the sense that the Alu-like sequences represent the single major family of interspersed DNA sequences in the bovine genome. However, our results and those obtained by FISH using two bovine AluI-like repetitive DNA probes (Rajcan-Separovic and Sabour, 1993), could suggest the existence of a repetitive DNA family with a very low frequency of AluI targets in the heterochromatic regions of acrocentric sheep chromosomes. Like Rajcan-Separovic and Sabour (1993), we think that this finding is necessary to understand more completely the significance of the distribution of the AluI and non AluI target sequences of the repetitive DNA of the sheep genome.

Restriction endonucleases like HaeIII also showed heterogeneity between metacentric and acrocentric autosomes. Bianchi et al. (1990) by using the same endonuclease observed similar effects in acrocentric autosomes, compared with the metacentric autosomes of the moose.

The variation in the C-banded material or repetitive DNA in the karyotype is a phenomenon observed in a great variety of animal species (Babu et al., 1988; Bianchi et al., 1990). DNA amplification is the most common explanation for this variation; however, in sheep heterochromatic variation arose by two different ways. In the one hand, the mechanism involved in the centric fusion appeared associated with loss of heterochromatin (A-fraction). On the other hand, a resistant AluI and DdeI fraction (B*) could well be a result of variable amplification of repetitive DNA fractions, taking place during evolution (Bianchi et al., 1985).

REFERENCES


