A repeat DNA sequence from the Y chromosome in species of the genus *Microtus*

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Abstract

In most mammals, the Y chromosome is composed of a large amount of constitutive heterochromatin. In some *Microtus* species, this feature is also extended to the X chromosome, resulting in enlarged (giant) sex chromosomes. Several repeated DNA sequences have been described in the gonosomal heterochromatin of these species, indicating that it has heterogeneous and species-specific composition and distribution. We have cloned an AT-rich, 851-bp long, repeated DNA sequence specific for *M. cabreræ* Y chromosome heterochromatin. The analysis of other species of the genus *Microtus* indicated that this sequence is also located on the Y chromosome (male-specific) in three species (*M. agrestis*, *M. oeconomus* and *M. nivalis*), present on both Y and X chromosomes and on some autosomes in *M. arvalis* and absent in the genome of *M. guentheri*. Our data also suggest that the mechanism of heterochromatin amplification operating on the sex chromosomes could have been different in each species since the repeated sequences of the gonosomal heterochromatic blocks in *M. cabreræ* and *M. agrestis* are different. The absence of this sequence in the mouse genome indicates that its evolutionary origin could be recent. Future analysis of the species distribution, localization and sequence of this repeat DNA family in arvicolid rodent species could help to establish the unsolved phylogenetic relationships in this rodent group.

Introduction

Mammalian Y chromosome is small, largely heterochromatic and exceptionally gene poor. In fact, the majority of human and mouse Y chromosomes consist of repeated DNA sequences, located mainly in the heterochromatin of the long arm. Similarly, the Y chromosome of most species of the genus *Microtus* is mainly formed by constitutive heterochromatin. This feature is

extended also to the X chromosome in some species of this genus (*M. agrestis*, *M. cabreræ*, *M. chrotorrhinus*, *M. epiroticus* and *M. transcaspicus*), showing very enlarged (giant) sex chromosomes due to the presence of large blocks of constitutive heterochromatin (Modi 1987, Burgos *et al.* 1988b, 1988c, Singh *et al.* 2000).

Both cytogenetic and molecular analyses have demonstrated that the gonosomal constitutive heterochromatin in the genus *Microtus* is highly

heterogeneous (Burgos *et al.* 1988c, Modi *et al.* 2003, Marchal *et al.* 2003). In fact, several repeated DNA sequences located in the sex chromosomes have been described in this genus. The analysis of these sequences shows differences between sex chromosomes in different species and between X and Y chromosomes in the same species. Thus, some of them are present in the sex chromosomes of some species but absent in others, or they are located on the heterochromatin of the X chromosome but absent on the Y (Marchal *et al.* 2003).

The large number of different repeated DNA sequences described on the sex chromosomes of *Microtus* and their species-specific distribution suggest a natural predisposition of the sex chromosomes of these species to accumulate heterochromatin. Furthermore, the sequences included and amplified in the gonosomal heterochromatin have probably been randomly selected in each species (Burgos *et al.* 1990, Singh *et al.* 2000, Marchal *et al.* 2003, Modi *et al.* 2003).

In addition, several L1 and non-L1 retroposons with a preferential location in the heterochromatin of the sex chromosomes have been described in species of the genus *Microtus*. These elements also show species-specific presence and distribution (Kholodilov *et al.* 1993, Neitzel *et al.* 1998, Mayorov *et al.* 1999, Neitzel *et al.* 2002, Marchal *et al.* 2003).

Here we present the analysis and characterization of a repeated DNA sequence cloned from *M. cabreræ*. This sequence is exclusive of the heterochromatic block of the Y chromosome in *M. cabreræ* and three other species of the genus *Microtus* analysed, while in *M. arvalis* this sequence is located on both sex chromosomes and in some autosomes.

Materials and methods

Genomic DNA extraction

Male and female genomic DNA from several *Microtus* species was extracted following a standard phenol–chloroform procedure. The species analysed were: *M. cabreræ*, *M. agrestis*, *M. arvalis*, *M. nivalis*, *M. oeconomus* and *M. guentheri*. (No female DNA was available from *M. arvalis*, *M. oeconomus* and *M. guentheri*.)

Repeat DNA isolation, cloning and sequencing

Electrophoresis of *M. cabreræ* male genomic DNA digested with *Eco*RI showed, among others, an 851-bp prominent band. This band was eluted from the agarose gel and cloned into pGEM-T easy Vector (Promega) as described previously (Sánchez *et al.* 1996). White colonies were screened using as probe the same digoxigenin-labelled band. Four positive clones (McaY(851)-2, -15, -36 and -49) were completely sequenced using the CEQ DTSC Quick Star Kit from Beckman Coulter and sequence reactions were analysed in a Beckman Coulter automated sequencer (model CEQ 2000XL).

Southern blot

Genomic DNAs were digested with restriction endonucleases, gel electrophoresed in 1% agarose and blotted onto Nylon membranes (Amersham) according to Bullejos *et al.* (1997). Membranes were probed overnight at 65°C with the positive clone McaY(851)-49 labelled with digoxigenin. Alkaline phosphatase detection was carried out according to the supplier's recommendations (Roche).

Sequence analysis

Pairwise sequence alignments and multiple alignments were carried out with the program CLUSTAL W 1.6 (Thompson *et al.* 1994). Sequence homology searches were performed in GenBank using BLASTN 2.2.2 with default parameters (Altschul *et al.* 1997).

Chromosome preparation and fluorescent in-situ hybridization

Chromosome preparations from *M. nivalis* were obtained from bone marrow cells of a male individual according to Burgos *et al.* (1986). Permanent fibroblast cell lines from male individuals of *M. cabreræ*, *M. agrestis*, *M. arvalis*, *M. oeconomus* and *M. guentheri* were used to obtain chromosomes as described by Neitzel *et al.* (1998).

FISH was performed as was previously described for *M. cabreræ* SRY HMG box using probes PCR-labelled with biotin-16-dUTP (Roche) or

Spectrumorange-dUTP (Vysis) (Fernández *et al.* 2002).

Results and discussion

EcoRI digestion of genomic DNA from *Microtus cabreræ* showed, among others, a male-specific prominent satellite band of 851 bp. This band was purified from the agarose gel and ligated into pGEM-T vector. Four positive clones, named McaY(851)-2, -15, -36 and -49, were completely sequenced (Gene Bank accession numbers: AJ630462–AJ630465). These clones showed almost identical sequences (in fact, clones McaY(851)-15 and -36 have the same sequence), with the percentage of identity between clones ranging from 99.2 to 100%. The variability observed was restricted to one deletion in clone 49, which has 850 bp while the other clones have 851 bp, and to six nucleotide positions; in five of them two clones present the same nucleotide and in the other two a different one.

The consensus sequence has an AT content of 58.3%. Other repeated DNAs from species of the genus *Microtus* are also AT-rich. Thus, the AT content of MSAT-160, MSAT-21 and MSAT-2750 ranges between 58 and 64% (Modi 1992, 1993b, Ivanov & Modi 1996, Fernández *et al.* 2001, Shevchenko *et al.* 2002). The repeat DNA

sequence pMAHAE2, located exclusively on the gonosomal heterochromatin of the giant sex chromosomes from *M. agrestis*, has a high AT content (65%) (Kalscheuer *et al.* 1996). The heterochromatin of the sex chromosomes of *M. agrestis* is enriched also in L1 (pMAECO-14) and non-L1 (pMA11/3) retrotransposons which are 61% and 58% AT-rich, respectively (Neitzel *et al.* 1998, 2002).

The consensus sequence of McaY(851) does not show any relevant homology with sequences of the NCBI database, either repeats or single-copy sequences. Internal sequence analysis does not show any particular features, while translation of the sequence in the six possible frames showed no open reading frames (ORF), as all have multiple stop codons.

Southern blots of *M. cabreræ* male and female genomic DNA digested with *EcoRI* and probed with clone McaY(851)-49 revealed several hybridization bands only in male samples (Figure 1a). The most prominent band corresponds to the 851-bp fragment, and the others have estimated band sizes that range between 3 and 7 kb. To determine if this repeated sequence is arranged in tandem, we digested male genomic DNA with *PstI*, an endonuclease that cuts once on the sequence McaY(851), and with *PvuII* and *SmaI*, that do not cut (Figure 1b). Southern blot analysis of *PstI*-digested DNA showed two bands of 2.6 and 11.9 kb while the enzymes that do not cut on

Figure 1. (a) Southern blot of female (f) and male (m) genomic DNA from *Microtus cabreræ* digested with *EcoRI* and hybridized with McaY(851) repeated DNA sequence; (b) Southern blot of male genomic DNA from *Microtus cabreræ* digested with *PstI*, *PvuII* and *SmaI* hybridized with the same probe as in a; (c) Methylation analysis of the McaY(851) sequence by Southern hybridization on male DNA digested with the isosquizzomer *Sau3AI* and *MboI* and probed as in a.

McaY(851) showed only one band (5.9 kb with *PvuII* and 14.7 kb with *SmaI*). Hence, in conclusion, this sequence is not arrayed in tandem, it has flanking sequences that are not homologous and it could be part of a higher repeat unit.

Analysis of the methylation status of this sequence was preformed by cutting male genomic DNA with the isoschizomers *Sau3AI* and *MboI*, which recognize and cut the same nucleotide sequence (GATC) but have different sensibility to cytosine methylation. While *Sau3AI* does not cut when cytosines are methylated, *MboI* is insensitive and cuts. As the sequence McaY(851) has two target sites for these enzymes, three bands were observed in the Southern blot: the internal fragment, with 185 bp and two fragments of 550 bp and 450 bp corresponding to the ends of the sequence, which indicated that both flanking sequences present closed target sites for these enzymes (Figure 1c). In addition, the Southern blot result indicated that these sequences are not methylated, as the banding pattern was identical on male genomic DNA digested with both enzymes (Figure 1c).

Screening for the presence of this sequence in other *Microtus* species (*M. agrestis*, *M. arvalis*, *M. nivalis*, *M. oeconomus* and *M. guentheri*) and in *Mus musculus* was performed with genomic DNAs digested with *EcoRI* by high-stringency Southern blot hybridization. Only a single band was observed on male samples of *M. agrestis* (11 kb) and *M. nivalis* (6 kb), indicating that this repetitive DNA, as in *M. cabreræ*, is male-specific in these species (Figure 2). In *M. oeconomus* male DNA, there are several bands with sizes that range between 3.5 and 12.5 kb (Figure 2), and, although no female DNA was available for this species, FISH analysis indicated a male-specific location also in this species (see below). In *M. arvalis*, a smear from 3.5 to 7 kb was observed in male DNA (Figure 2); however, in this species, this sequence is not male-specific as was demonstrated by FISH (see below). Finally, no bands were observed on *M. guentheri* (male DNA) and mouse (male DNA), which suggests that the 851-bp repeated DNA sequence is absent from the genome of these two species. These differences in the Southern blot hybridization patterns indicate that the molecular organization of this sequence is not the same in all the species analysed.

To determine the chromosomal location of this sequence, we performed FISH on male metaphase spreads of the *Microtus* species analysed in this paper. In *M. cabreræ*, this sequence is located along the whole heterochromatic block of the Y chromosome although it is not homogeneously distributed (Figure 3a). In *M. agrestis*, this sequence is localized in three bands on the heterochromatic block of the Y chromosome: one proximal to the centromere, one interstitial and another in telomeric position (Figure 3b). In *M. oeconomus* and *M. nivalis*, the whole heterochromatic long arm of the Y chromosome is positive for this sequence (Figure 3d, e). In *M. arvalis*, this sequence is present on the heterochromatin of the Y chromosome, on the X chromosome and, at least, on one autosomal pair (Figure 3c). Hence, in *M. arvalis*, unlike in the other species analysed, this sequence is not Y-specific. No signals were observed in *M. guentheri* chromosomes (Figure 3f), according to Southern blot results, indicating that this sequence is completely absent on the genome of this species.

Different repeated DNA sequences have been described on the Y chromosome heterochromatin of *Microtus* species. MSAT-160, a satellite DNA located on the centromeres of most autosomes of *Microtus* species, is also located on the X and Y chromosomes (Modi *et al.* 2003). Particularly, this satellite is present in the centromere and heterochromatic block of the X chromosome in some species. Regarding the Y chromosome, MSAT-160 is distributed over the whole heterochromatic block in some *Microtus* species (e.g. *M. chrotorrhinus*), present only in some bands in other species (e.g. *M. cabreræ*), or completely absent from the Y chromosome in others (e.g. *M. agrestis*) (for a review, see Marchal *et al.* 2003). MSAT-2750 is present only on the heterochromatic blocks of both X and Y chromosomes of *M. chrotorrhinus* (Marchal *et al.* 2003, Modi *et al.* 2003). MSAT-21 is present on the X and Y heterochromatic blocks of *M. chrotorrhinus* and *M. rossiaemeridionalis*, as well as in some autosomal centromeres in the latter species (Marchal *et al.* 2003, Modi *et al.* 2003). In the same way, MS2, MS3 and MS4 repeated DNA sequences are located on the heterochromatin of both sex chromosomes in several *Microtus* species and absent in others, showing also an autosomal distribution in some of them

Figure 2. Southern blot of female (f) and male (m) genomic DNA digested with *Eco*RI from different species probed with McaY(851) repeated DNA sequence from *Microtus cabreræ*. M.agr: *M. agrestis*; M.niv: *M. nivalis*; M.oec: *M. oeconomus*; M.arv: *M. arvalis*; M.gue: *M. guentheri* and M.mus: *Mus musculus*.

(Marchal *et al.* 2003). Finally, the repeated DNA sequence pMAHAE2 from *M. agrestis* is present in the heterochromatic blocks of both sex chromosomes of this species (Kalscheuer *et al.* 1996).

Despite the large number of repeated DNA sequences studied in *Microtus* sex chromosomes, none of these sequences presented a preferential location for one of the sex chromosomes. Hence, McaY(851) is the first satellite DNA described in *Microtus* with a preferential distribution on the Y chromosome. This sequence is Y-specific in four species analysed, is located in both sex chromosomes and in some autosomes in *M. arvalis* and is completely absent in the genome of *M. guentheri*.

Y-specific repeated DNA sequences have been described in several mammals such as mouse (Platt & Dewey 1987), rat (Essers *et al.* 1995), and human, where two repeat DNA families account for 50–70% of the DNA content of the Y chromosome (Cooke *et al.* 1983). It has been suggested that the extremely large content of repeated sequences of the Y chromosome is a consequence of its permanent hemizygous state (Platt & Dewey 1987). Repeated DNA sequences are probably generated at high frequency in all chromosomes. They could be lost from autosomes and the X chromosome during meiotic recombination, while the absence of homologous pairing and

recombination during meiosis in the non-recombining region of the Y chromosome would protect them from elimination (Platt & Dewey 1987).

One of the mechanisms proposed for amplification of repeated DNA sequences is unequal crossing-over between homologous chromosomes at meiosis. However, this mechanism was not responsible for the amplification of McaY(851) on *Microtus* Y chromosome since this sequence is located exclusively inside the non-recombining region (NRY) of the Y chromosome. Furthermore, non-recombining status is extended to the whole Y chromosome in some of the species analysed (*M. cabreræ*, *M. arvalis*, *M. agrestis*, *M. guentheri*), since they present asynaptic sex chromosomes (Megías-Nogales *et al.* 2003). In fact, this amplification mechanism is not valid for the heterochromatic blocks of both X and Y chromosomes in *Microtus* because these regions never pair at meiosis, whatever the condition, synaptic or asynaptic, of the sex chromosomes (Singh *et al.* 2000, Megías-Nogales *et al.* 2003).

In *M. agrestis* the heterochromatic blocks of both sex chromosomes present sequence homology (Neitzel *et al.* 1998). Two mechanisms have been proposed to explain the amplification of the same sequences on the heterochromatin of X and Y sex chromosomes of *M. agrestis* (Singh *et al.* 2000). One is based on somatic unequal exchanges that take place in mitotic germ-line cells. The other is based on the repair of double-strand breaks, which are particularly frequent on asynapsed elements during meiosis. This repair of heterochromatic sequences could produce translocations of large fragments between heterochromatic blocks of the X and Y in this species. It is unlikely that these two mechanisms of amplification took place during the evolution of the sex chromosomes of *M. cabreræ*, since the composition of the heterochromatin of both sex chromosomes is different. In fact, differences in sequence composition between the X and Y heterochromatic blocks of *M. cabreræ* have been revealed by FISH with MSAT-160 satellite DNA (Fernández *et al.* 2001) or with *SRY* gene (Fernández *et al.* 2002), by X chromosome painting (Marchal *et al.* 2004) and in this study. Other amplification mechanisms, such as sister chromatid exchanges or replication slippage, could also explain the heterochromatin amplification on the

Figure 3. Fluorescent *in-situ* hybridization using McaY(851) satellite DNA sequence as probe on male metaphase spreads of several *Microtus* species: (a) *M. cabreræ*; (b) *M. agrestis*; (c) *M. arvalis*; (d) *M. oeconomus*; (e) *M. nivalis* and (f) *M. guentheri*. This sequence is located on the heterochromatin of the Y chromosome in all species, with the exception of *M. guentheri* (2f). In *M. arvalis*, it is also located on the X chromosome and in some autosomal pairs (arrows) (2c).

sex chromosomes of *Microtus*. These mechanisms could amplify different sequences in each gonosome, resulting in independent amplification of repeated sequences on the X and Y heterochromatin, as is the case of *M. cabreræ*. Whatever the mechanism, it seems that amplification of a repetitive sequence on sex heterochromatin was an independent event in each *Microtus* species. This has been demonstrated for MSAT-160 (Modi 1993a), and in this study where McaY(851), while it is scarcely amplified in *M. agrestis* Y chromosome, in *M. cabreræ* (and in the other species that possess it) it occupies almost the whole heterochromatic region.

The sequence McaY(851) is absent from mouse genome but exists in most *Microtus* species analysed. This suggests that it has a very recent evolutionary origin that probably preceded the origin of the asynaptic sex chromosomes in the genus *Microtus*, as it is present in species with synaptic and asynaptic sex chromosomes. Furthermore, it is probable that this sequence was present in the ancestor species of this rodent group since it is also present in *M. nivalis*, a species considered to be very ancient with a similar karyotype to the supposed primitive karyotype for arvicolid rodents (Burgos *et al.* 1988b).

Sequence acquisition and amplification have been proposed to play a role in the evolution of the mammalian Y chromosome (Eicher *et al.* 1989). The acquisition and differential amplification of the McaY(851) sequence in some *Microtus* Y chromosomes could be relevant for studying the evolutionary divergence of this chromosome in this genus. There are three possibilities for the ancestral situation of this sequence: (1) spread on the heterochromatin of the Y chromosome and later reduced (*M. agrestis*), lost in some species (*M. guentheri*) or transferred to X chromosome and autosomes in others (*M. arvalis*); (2) located in several positions of the karyotype, including Y chromosome (*M. arvalis*), then lost from the X chromosome and autosomes in some species, becoming Y specific (*M. cabreræ*, *M. nivalis*, *M. oeconomus*), and finally reduced on the Y chromosome (*M. agrestis*), or completely lost from the genome of others (*M. guentheri*) or (3) located exclusively on autosomes, and then transferred to the Y chromosome, amplified and finally lost from the rest of the genome. This situation has

occurred with two families of repeated DNA sequences located on human Y chromosome, which are absent from this chromosome on chimpanzee and gorilla, where they are exclusively autosomal (Kunkel & Smith 1982, Cooke *et al.* 1982).

McaY(851) in *M. cabreræ*, a species with XY sex reversal and with the *SRY* gene present in all fertile females (Burgos *et al.* 1988a), would become a very interesting and useful tool to determine the genetic sex of individuals in genetic population and zoological studies. Uniparental transmitted sequences, such as maternal mitochondrial genes and paternal Y-linked sequences, are useful in phylogenetic analysis (Lundrigan *et al.* 2002). In fact, mitochondrial cytochrome b and *SRY* gene have been used to construct molecular phylogenies in murid rodents, including some *Microtus* species (Martin *et al.* 2000, Lundrigan *et al.* 2002, Jaarola & Searle 2004). Analysis of the distribution, localization and sequence of McaY(851) in arvicolid rodents could help to establish the complex and unclear phylogenetic relationships in this mammalian group.

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