Replication of the heterogeneous heterochromatin of the sex chromosomes of *Microtus cabrerae*

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Abstract. We used bromosubstitution to investigate the mode of replication of different types of heterochromatin located in the sex chromosomes of *Microtus cabrerae*. Our results clearly show that, although the heterochromatin is late replicating, the replication timing of different types of constitutive heterochromatin is related to their banding properties: R-type heterochromatin replicates before G-type heterochromatin, and this replication is asynchronous in both sex chromosomes. Furthermore, the late replication behaviour of the inactive X may spread to its constitutive heterochromatin. In some cells, a region of the constitutive heterochromatin of the late replicating X spontaneously switches to early replication, which may be related to transcriptional activity. Replication behaviour of the constitutive heterochromatin in the Y chromosome is similar to that of the late replicating X.
Key words. Constitutive heterochromatin; *Microtus cabrerae;* replication; bromosubstitution; sex chromosomes.

The process of DNA replication occurs in two phases, the 'early replication period', in which R-bands replicate, and the 'late replication period', in which G-bands replicate [2–4]. Both constitutive and facultative heterochromatin are late replicating [5, 6].

We studied the late replication pattern in the sex chromosomes of *Microtus cabrerae*, a species in which extensive heterochromatin occupies the entire short arm and one third of the long arm of the X chromosome and nearly the whole long arm of the Y chromosome. This heterochromatin is very heterogeneous, as shown by its response to G- and C-banding techniques and fluorochrome staining. Four different types of heterochromatin are located together on the X chromosome, and two on the Y chromosome. The staining properties of these different types of constitutive heterochromatin were described elsewhere [7]. We were interested in discovering whether base composition or banding behaviour of the constitutive heterochromatin was related to functional aspects such as replication timing.

Since constitutive heterochromatin in the X chromosome of *M. cabrerae* is located in a chromosome which may be subject to inactivation, we also investigated whether location on the late replicating X might affect the timing of replication of a particular type or types of heterochromatin.

Materials and methods

Bone marrow cells from two females and one male of *Microtus cabrerae* were bromosubstituted as follows: Cells from each individual were divided into four samples and cultured in 4 × 10 ml RPMI 1640 supplemented with 20% FCS and ConA (Sigma) at the concentration recommended by the supplier for each batch, over 92 h at 37 °C in an atmosphere of 5% CO_2. To investigate the late replication pattern, 10 mM bromodeoxyuridine (BrdU) was added to each culture 4 h, 3 h, 2.5 h and 1 h before harvest, and colchicine was added 15 min before harvest to a final concentration of 1 µg per ml. Chromosomes were prepared in accordance with our usual method [8]. Slides were stained with bisbenzimide for 15 min, then rinsed in distilled water. Three drops of Ca^{++}- and Mg^{++}-free PBS were dropped onto the slides, which were covered with a coverslip and incubated for 10 min at 60 °C under a black light lamp located 5 cm above the slides. Finally, the slides were washed in tap water, then in distilled water, and then stained with Giemsa.

Results and discussion

Pairs of sex chromosomes were classified according to the extent of replicated bands before continuous labelling with BrdU. This was used to determine the order in which bands replicate: dark bands appearing in nearly wholly bromosubstituted chromosomes replicate first, followed by progressively later replicating bands in less bromosubstituted chromosomes. Elongation of the bromosubstituted segment was always observed, which was assumed to occur during preparatory processes, as demonstrated by scanning electron microscopy studies [9]. This makes the heterochromatic short arm almost as long as the long arm.

For the nomenclature of the X chromosomes bands we followed criteria published previously [7] (fig. 1 a).

Replication of the euchromatic portion of the X chromosomes. As in all species of mammals studied up to now, the inactive X chromosome of *M. cabrerae* is late replicating (fig. 1 b–f). It starts to replicate at band q23 when ERX has fully replicated its euchromatic R-bands (fig. 1 b), then bands q31, q33 and q35 replicate apparently synchronously, although the existence of a few cells in which only q23 and q31 were replicated (fig. 1 c) suggests that q31 replicates shortly before q33 and q35. Some variability was found at this level, as some cells had finished ERX replication by the time the R bands of the LRX had replicated (fig. 1 e), while other cells showed the same replication pattern in both X chromosomes (fig. 1 d). Hence, our results demonstrate a variable rate of replication of the LRX, as euchromatin replication may end either with or after replication of the ERX.

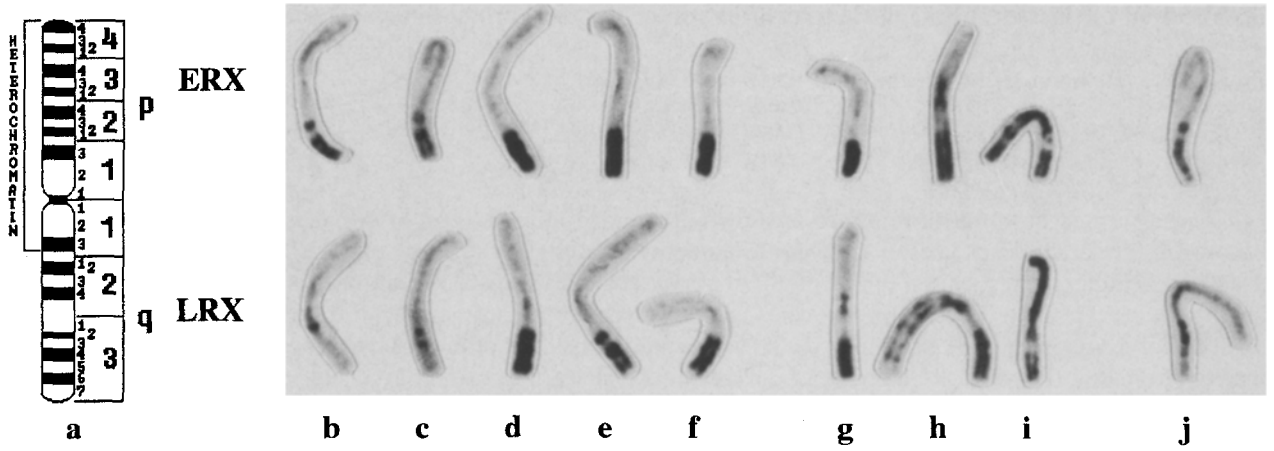

Figure 1. *a* Nomenclature of the bands of the X chromosome of *M. cabrerae*. *b–f* Mode of replication of the euchromatic portion of the X chromosome of *M. cabrerae*. ERX: early replicating X; LRX: late replicating X. *g–i* Mode of replication of the heterochromatic portion of the X chromosome of *M. cabrerae*. X chromosomes in the same column come from the same cell. *j* Note the switch to early replication of the pericentromeric region and p12–q12 bands on the LRX.

Variable replication rates of the inactive X chromosome were also noted in human lymphocytes [10], and was considered to be responsible for the asynchronous and independent replication of the LRX. In any case, replication of the euchromatic portion of both X chromosomes was usually complete before any bands of the heterochromatic portion had begun to replicate.

Replication of the heterochromatic portion of the X chromosomes. As expected, the constitutive heterochromatin of the X chromosomes of *M. cabrerae* replicated very late. Clear differences in the time of replication of different types of heterochromatin were also demonstrated. p12 and q12 are the first bands to replicate (fig. 1 g). The whole heterochromatic region of the X chromosome, with the exception of the centromere, stains positively with DAPI [7], demonstrating its AT-richness, but although AT-rich euchromatin stains positively with trypsin G-banding, the AT-rich heterochromatin of the p12 and q12 bands stained negatively. Thus, the R-type AT-rich constitutive heterochromatin of the p1 and q1 regions is the earliest late replicating heterochromatin. Next, the remaining AT-rich R-type constitutive heterochromatin located at regions p2, p3 and p4, replicated (fig. 1 h), followed by the AT-rich G-type constitutive heterochromatin. The final region to be replicated is the boundary between euchromatin and heterochromatin (q13) (fig. 1 i). Thus, R-type euchromatin is 'early/early' replicating, and G-type euchromatin is 'early/late' replicating, while R-type heterochromatin is 'late/early' replicating and G-type heterochromatin is 'late/late' replicating.

Differences in the time of replication between the constitutive heterochromatin of the two X chromosomes were detected. The bands p12 and q12 replicated earliest in one of the two X chromosomes, followed by the remaining R-C-bands of the same chromosome and the p12 and q12 bands of the other X chromosome. Then the remaining G-C-bands of both X chromosomes replicated. Band q13, located at the boundary between the euchromatin and the heterochromatin, is the latest replicating portion in both X chromosomes (fig. 1 g–i). No differences between the heterochromatin of the active and inactive X chromosomes were noticed with respect to the replication timing of the constitutive heterochromatin of the X chromosomes in *Microtus agrestis* [11]. Thus, although *M. cabrerae* and *M. agrestis* are closely related, and have sex chromosomes with similar characteristics (i.e., with large heterochromatic segments), the replication behaviour of their sex-chromosome-located constitutive heterochromatin is quite different. Furthermore, heterogeneity such as has been demonstrated in the constitutive heterochromatin of *M. cabrerae* was not found in *M. agrestis*, and whereas this chromatin in *M. cabrerae* is AT-rich, in *M. agrestis* it is GC-rich [12]. These facts suggest that it is the mechanism of accumulation of this type of chromatin in specific chromosomes, which was inherited from a common ancestor rather than the heterochromatic segments of their sex chromosomes per se. This heterochromatin then accumulated after the divergence of both species.

In some cells, bands p12 and q12 replicated before euchromatin replication had finished, showing a spontaneous switching to early replication, which was always detected on the LRX (fig. 1 j). This variable replication timing may be related to transcriptional activity as it is correlated with variable methylation [15].

Replication of the Y chromosome. The replication pattern of the Y chromosome provides no additional information on the replication mode of different types of constitutive heterochromatin. Its R-type constitutive heterochromatin, although late replicating, replicates before its G-type constitutive heterochromatin (fig. 2). Thus, in this chromosome, as in the X chromosome, the staining properties of different types of heterochromatin are related to its replication timing. Replication of the Y chromo-

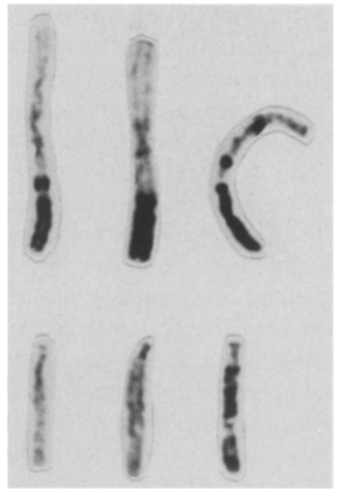

Figure 2. Replication mode of the Y chromosome of *M. cabrerae*. Sex chromosomes in the same column come from the same cell.

some does not start until the euchromatic portion of the X chromosome has replicated (fig. 2). Using 5-azacytidine treatment it has been shown that the euchromatic and heterochromatic portions of the X chromosome of *M. agrestis* may be influenced by two independent regulatory sites that control the timing of replication [11]. Our results suggest that replication of the Y chromosome may be controlled by a regulatory site functionally similar to that which may control replication of the heterochromatic portion of the LRX in XX individuals, as the Y chromosome starts its replication later than the X, but completes replication sooner.

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Microclimatic origin of inhaled air affects olfactory navigation of homing pigeons

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Abstract. Earlier experiments led to the conclusion that homing pigeons are able to deduce positional information from atmospheric odours they perceive at the site of release. Here we show that this ability is significantly reduced, if the inhaled air does not originate from the free airspace in an open landscape but from a close-to-the-ground level of a forest or a maize field.
Key words. Orientation; navigation; homing; pigeons; olfaction; microclimate; atmospheric trace compounds.

Many experimental findings strongly suggest that the homing of pigeons from unfamiliar areas is substantially based on positional information deduced from the local atmosphere by olfaction [1, 2]. However, nothing is known on the chemical nature of the trace gases apparently involved and on their distribution in the airspace. A small analytical step to approach this problem is to ask whether every volume of air at a given location contains the same quality and amount of navigational information irrespective of its immediate environment. Does it make a difference whether the birds inhale and smell ambient air at the ground or at a higher altitude, in a closed forest or over open grassland, inside or outside a building, etc.?

Here we report on experiments that aimed to ask for possible differences in initial orientation as depending on the pigeons' preceding exposure to air from inside a forest versus air from open land, and on exposure to air from the ground of a maize field versus air from above open land.

Materials and methods

The forest experiments. Pigeons were transported from the loft to the release area on the top of a van in two