The Chromosomes of the New World Flying Squirrels (*Glaucomys volans* and *Glaucomys sabrinus*) with Special Reference to Autosomal Heterochromatin

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There are two species of New World flying squirrels included in the genus *Glaucomys*: *G. sabrinus*, the northern flying squirrel, and *G. volans*, the southern flying squirrel.

During a study of local rodents around Hanover, New Hampshire, several flying squirrels were caught and their chromosomes were analyzed. The occurrence, in this region, of both species allowed a comparative study of their respective karyotypes. Since the fossil record of flying squirrels is scant and the early history of this group highly speculative, new insight was hoped to be gained into their taxonomic relationship. Their identical karyotypes, in the presence of only minor morphologic differences, raises the question of whether their separation into two species is justified.

During this study, analysis of the karyotypes revealed a striking fuzziness of some of the chromosomes (Fig. 1). Autoradiography showed these segments to be late replicating. While all animals of the two species studied possessed heterochromatic blocs, the distribution of these segments on the autosomes varied; two different labelling patterns were found among adult animals of the same species. Other expressions of heterochromatin included the presence of large chromocenters in interphase nuclei and, cytologically, absence of pairing and crossing-over at meiosis.

Material and methods

In this study, two males and one female of the southern flying squirrel, *Glaucomys volans*, were live trapped in and around Hanover, New Hampshire. Four males of this species were obtained from commercial animal dealers in Florida. The single male *Glaucomys sabrinus* used was live trapped in Plainfield, New Hampshire.

Chromosome preparations were obtained from explant cultures of skin and subcutaneous tissues as described by Basrur *et al.* (1963) and/or from trypsinized kidney cell cultures using a modified procedure originally described by Rappaport (1956). All cells were fixed in glacial acetic acid and methyl alcohol (1:3); slide

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preparations were then air- or flame-dried and stained with aceto-orcein or carbol fuchsin.

Autoradiographic analyses were made according to the methods previously described (Low and Benirschke 1969). Six hours prior to interruption, cultures were exposed to ^3H -thymidine. Following fixation and staining, chromosome spreads were photographed. After exposure for 7–14 days, suitably labeled mitoses were rephotographed and karyotypes were prepared of labeled and unlabeled chromosomes.

Chromocenters

Chromocenters were studied in cultures from kidney, skin and diaphragm. Some of the preparations came from our chromosome studies and had, therefore, undergone subculturing by trypsinization and been submitted to hypotonic treatment before fixation. Other preparations stemmed from cultures directly grown and harvested on coverslips, without hypotonic treatment. The stains used were carbol-fuchsin and Giemsa, with comparable results. These stains did not allow differentiation between nucleoli and chromocenters. Labelling of interphase nuclei showed the vast majority of these dense chromatin bodies to be chromocenters by virtue of their incorporation of ^3H -thymidine.

Meiosis

Fresh testicular material was used for the study of meiotic behavior of heterochromatin. It was obtained from 6 *Glaucomys volans* and one *Glaucomys sabrinus*. Ford's method (1969), applied to the first 5 animals, gave good results for the study of first prophase stages, up to pachytene, while Meredith's method (1969), used on two *Glaucomys volans*, allowed visualization of the entire first prophase, including diakinesis, and of second division metaphases.

Results and discussion

Karyotypically, *G. volans* and *G. sabrinus* appear to be identical (Fig. 2). With a diploid number of 48, FN 80, the chromosome complement is composed of 30 metacentric (or submetacentric) and 16 acrocentric autosomes. These findings are in disagreement with those of Nadler and Sutton (1967), who reported a karyotype for *G. volans* containing a total of 28 meta- and submetacentric and 20 acrocentric chromosomes, FN 74. Moreover, they reported a difference in the centromere position of the sex chromosomes or a pair of autosomes in *G. sabrinus*, which our karyotypes did not substantiate. The X and Y chromosomes appear to be medium- and small-sized submetacentrics, respectively. In the male, the X chromosome was determined on morphological grounds (absence of pairing with any of the other medium-sized submetacentrics). The Y chromosome stood out, in over 50% of all cells examined, as a single, small, late replicating chromosome. In the two female animals, about 25% of the cells showed a single, late-labelling chromosome, which resembled in size and shape the unpaired chromosome designated as X in the male karyotype.

Phenotypically, the two species are similar; the northern flying squirrel, how-

ever, is slightly larger and heavier. Minor differences in pelage are also present, particularly on the pigmentation pattern of the belly fur. Weigl (1970) has compared the electrophoretic mobilities of the blood proteins of the two species,

Fig. 1. Mitotic spread of a female *Glaucomys volans*. The long arms of the two largest chromosome pairs show segments of marked fuzziness. Kidney epithelial cell culture. Carbofuchsin stain.

Fig. 2. Karyotypes of *Glaucomys sabrinus* (G.s.: upper rows) and *Glaucomys volans* (G.v.: lower rows). The two karyotypes appear identical.

especially the hemoglobins, and found them to be the same within species and consistently different between species. Of perhaps somewhat less significance as an indicator of species difference but advanced by Weigl (1970) as one of the parameters

favoring the theory of separate migrations of *G. sabrinus* and *G. volans* into North America, is the difference in their ecto-parasites: fleas carried by *G. sabrinus* are present also on Palearctic squirrels, while *G. volans* generally supports an entirely different flea fauna. Perhaps the most significant phenotypic characteristic distinguishing *G. sabrinus* from *G. volans* is the markedly different bacula of the two species, a character considered to be diagnostic in most *Scuiridae*.

Mating behavior, gestation period and development of the two species are reported to be similar (Muul 1969). *G. sabrinus* breeds once a year in the late winter or early spring; *G. volans* often breeds twice a year, first in late winter and again in late spring (Hall and Kelson 1959). Whether a strict reproductive isolation between the two species is maintained in areas where they are sympatric is not known. Hall and Kelson (1959) state, however, that in some areas (e.g. New York and Virginia) where northern and southern flying squirrels occur together, certain specimens are identifiable as to species only with difficulty. Nevertheless, it is not at present known whether or not interspecific hybridization does in fact occur.

Our findings do not preclude the possibility of hybrid fertilization; however, it is possible that anatomical, behavioral and other subtle differences serve to maintain a reproductive isolation between the species.

Autoradiography

Table 1 gives the results of our autoradiographic studies and the types of tissues used. Large blocs of late replicating heterochromatin were found in all 5 animals

Table 1. Autoradiographic studies on various tissues from animals of both species *Glaucomys volans* and *sabrinus*

Animal	Sex	Tissue	Number of cells analyzed	Late replicating chromosomes
<i>Gl. volans</i> No. 2	♀	Kidney	17	Chrom. No. 1+2
" <i>volans</i> No. 3	♀	Skin	21	Acrocentric chrom.
" <i>volans</i> No. 6	♂	Kidney	21	" "
" <i>volans</i> No. 9	♂	Kidney	6	" "
" <i>sabrinus</i> No. 2	♂	Diaphragm	21	" "

studied and in all three types of cells used. The asynchronously replicating chromosome segments were very easily identified: they were heavily labelled in all otherwise lightly labelled cells (51% of all cells analyzed); they showed a particularly dense grain pattern in cells with a medium to heavy amount of label (33%); and they were the only chromosomes labelled in 17% of cells. The first animal studied, with its particularly fuzzy chromosomes No. 1 and 2 (Fig. 1), showed late labelling over these segments (Fig. 3). The four animals studied subsequently, showed consistently late labelling over one of the large pairs of acrocentric chromosomes (Fig. 4). The two patterns appeared mutually exclusive, since no cell was found in which the large submetacentric and the acrocentric chromosomes were late replicating. As Table 1 demonstrates, these 2 patterns do not express a species difference, the same pattern as depicted in Fig. 4 in a *Glaucomys volans* having been found in

Fig. 3. Karyotype of a female *Glaucomys volans*, with H^3 -thymidine labelling. Chromosomes No. 1 and 2 are late replicating. Kidney epithelial cell culture (same animal as in Fig. 1).

Fig. 4. Karyotype of a male *Glaucomys volans*, with H^3 -thymidine labelling. The large acrocentric pair is late replicating. Kidney epithelial cell culture.

a *Glaucomys sabrinus*, thus suggesting an identical labelling pattern in both species. Tissue specificity is ruled out by the fact that both types of patterns were detected among the kidney cell cultures. Since only one animal, out of five studies, showed labelling of the 2 large submetacentric chromosomes, one might assume an individual aberration in this particular animal. That this is probably not so is suggested by the kidney cell cultures of another *Glaucomys volans*, a male, in which chromosomes No. 1 and 2 showed the same striking fuzziness as in the female *Glaucomys volans* No. 2. We assume, therefore, that this male would have shown the same replication pattern, although this has not been proven by autoradiography. A reciprocal translocation is an alternative (though rather unconvincing) hypothesis: the long arms of No. 1 plus the terminal segment of No. 2 seem to involve much longer chromosome segments than the late replicating chromosome arms of the acrocentric pair.

Unfortunately, only one tissue per animal has been studied, so that nothing is known about a possible variability of the labelling pattern within one animal.

If heterochromatin is considered genetically inactive, then its variable localization would imply the presence of large segments of duplicate and unnecessary genomes, since no concurrent phenotypic changes were observed. On the other hand, the possibility of incomplete genetic inactivation of heterochromatin has to be considered. Furthermore, the constant presence of large blocs of heterochromatin suggests some role other than inactivation of genomes, particularly since the "inactivated" genomes can vary in their location.

Chromocenters

The two types of cultures used did not show the same results. When cells had been subcultured and harvested as a cell suspension, with trypsinization prior to harvesting, all 3 types of cells (kidney epithelial cells and fibroblasts from diaphragm and skin) showed chromocenters. When the cells had been allowed to grow on coverslips and were harvested *in situ*, only kidney epithelial cells showed chromocenters. This particular cell type specificity, with regard to the presence of chromocenters, had already been shown by Schmid (1965) in *Microtus agrestis*, *Mesocricetus auratus*, and in *Cavia cobaya*. The different and probably more traumatic treatment of the cells by trypsinization abolishes this specificity. Nothing is known about the reasons for and the mechanism of visualization of chromocenters. Schmid (1965) interpreted the cell specific presence or absence of these bodies as an expression of different functions of the respective cells. We can only add that a different treatment of cells can possibly influence their formation or visualization (if not influence the biologic behavior of these cells).

The percentage of positive nuclei was also different in the two types of cultures. In trypsinized and subcultured cells, it varied from 15 to 30%; in kidney epithelial cells, grown on coverslips, practically all nuclei showed chromocenters. This difference might, in part, be attributable to the hypotonic treatment of the trypsinized cells, which tends to blow up the nuclei and to disperse the chromatin very finely.

The number of chromocenters per nucleus varied from 1 to 6, with 40% of cells

showing two large chromocenters (Fig. 5a, b, c), frequently with a third small one interpreted as nucleolus. About 25% of the cells had only one chromocenter and the remainder of the nuclei three or more. This pattern did not change in the various cultures analyzed. No difference of the size and the number of chromocenters was detected between animals exhibiting different labelling patterns.

The high frequency of two chromocenters is interpreted as interphase expression of the two late replicating autosomes. Cells with one chromocenter might be interpreted as due to fusion of the two chromocenters; they frequently were very large indeed. Pera (1969) has shown that in female *Microtus agrestis* one third of brain cell nuclei had one large chromocenter, having arisen by fusion of two. Whether this is really an expression of somatic pairing, as he suggests, remains to be seen. The relatively high percentage of cells with three or more chromocenters is not well explained by our autoradiographic findings. In some cells, however, with medium to light label, a medium sized submetacentric and a small metacentric

Fig. 5. Interphase nuclei with varying numbers of chromocenters. Carbofuchsin stain on subcultured cells: a) and c) skin fibroblasts of ♀ *Gl. volans*, b) kidney epithelial cells of ♂ *Gl. sabrinus*.

pair (Fig. 3) showed relatively late labelling, as compared to the remaining, euchromatic, autosomes. Either of these two pairs might form the additional chromocenters.

No difference was found between male and female cells. The chromocenters were generally too large to represent sex-chromatin, particularly since the X-chromosome in these two species is of the "standard" type, i.e. about 5% of the complement. Barr bodies were looked for in buccal smears and in histologic sections of epithelia and smooth muscle of the genito-urinary tract of several males and females of the species *Glaucomys volans*. Sex-chromatin-like bodies were found with equal frequency in both sexes in all tissues studied. The absence of sexual dimorphism in interphase nuclei of the flying squirrel and of several other rodents has already been demonstrated by Moore (1965) and appears to be due to their coarse chromatin distribution in interphase nuclei. This is true in tissue sections, but not in

buccal smears, where the scarcity of Barr bodies in females (1–2%) remains unexplained.

Meiosis

Analysis of first prophase stages showed the consistent presence of a condensed pair of chromosomes (Fig. 6a). From diplotene on, this pair became sufficiently individualized to show, in some well spread figures, a clear separation of

Fig. 6. Meiotic preparations. Testis of *Glaucomys volans*. One autosomal pair is strikingly heteropycnotic at pachytene (a), shows partial absence of pairing at diplotene (b) and at diakinesis (c), but does exhibit crossing-over at one end of the chromosome pair (d).

the two condensed arms (Fig. 6b). In over 50 diakinetic figures examined, this same pair could be identified because of its V-shape configuration (Fig. 6c), being paired at one end, with a definite cross-over figure in some cells (Fig. 6d). The remainder of the condensed arms never showed any sign of pairing and of crossing-over. The pronounced contraction of this pair, visible from zygotene on, was also evident at diakinesis, but subsided at metaphase, by gradual contraction of the

remaining pairs. No differential contraction could be found at second metaphase.

Comparable figures were demonstrated by Gropp (1969) in the European hedgehog, another species which possesses large blocs of autosomal heterochromatin and which exhibits similar meiotic configurations, suggesting absence of pairing, although early terminalization cannot be ruled out.

All tests analysed stemmed from animals with the same type of mitotic labelling pattern, i.e. with the late replicating acrocentric pair and so nothing is known about the meiotic behavior in animals possessing the late labelling chromosomes No. 1 and 2.

One cell type, in our testicular preparations, exhibited heteropycnosis of several chromosomes (Fig. 7). This cell was a prophase nucleus, interpreted as a spermatocyte I. The contracted chromosomes were clearly individualized against a background of thin threads of chromosomes corresponding with the leptotene stage. At zygotene, this type of heteropycnosis subsided, only one single pair showing the above mentioned differential contraction. The number of heteropycnotic chromosomes in this cell type varied, however, and a correlation between these chromosomes and the late replicating ones at mitosis is difficult. The significance of this transitory differential contraction is unclear. A correlation between the RNA-synthesis during zygotene and pachytene (Utakoji 1966) is possible but probably indirect, since heteropycnosis appears before the actual RNA-synthesis takes place.

Fig. 7. Spermatocyte nucleus of *Glaucomys volans* at leptotene. Several chromosomes show transitory heteropycnosis.

Summary

The chromosome complement of both species of New World flying squirrels, *Glaucomys volans* and *Glaucomys sabrinus*, comprise 48 chromosomes, with identical karyotypes. The northern flying squirrel (*Gl. sabrinus*) distinguishes itself from its southern counterpart (*Gl. volans*) mainly by different geographical distribution and by minor skeletal and coat differences. The discrete morphological differences and the identical karyotypes suggest that the two animals are taxonomically closely related.

Autoradiographic studies of somatic chromosomes have revealed the presence of large blocs of autosomal heterochromatin in both species. This heterochromatin was expressed at mitosis by a particular fuzziness of the respective chromosome

segments, at interphase as large chromocenters, and at meiosis by heteropycnosis during the first prophase and by diakinetik figures suggesting absence of pairing. The meiotic findings are interpreted as indirect evidence of genetic inactivity of heterochromatin.

Autosomal heterochromatin was present in all animals studied (5) and in both species. Two different locations of heterochromatin were found in different animals of the same species (*Gl. volans*). The two late-labelling patterns appeared mutually exclusive and did not express tissue specificity. This variability of the location of heterochromatin remains unexplained.

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