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Evolution of Mammalian X-chromosome Inactivation: Sex Chromatin in Monotremes and Marsupials

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Abstract

The inactive mammalian X-chromosome is always late-replicating, and in eutherian mammals it is heterochromatic and hypermethylated. We propose that this multistep system has evolved from a more primitive system, remnants of which may be found in marsupials and monotremes.

The heterochromatic X (sex-chromatin body) is a distinctive feature of interphase cells of certain tissues in eutherian females but not males. Thus we have searched for a sex-specific chromatin body in these same tissues in marsupials (brush-tail possum, *Trichosurus vulpecula*) and monotremes (platypus, *Ornithorynchus anatinus*), using classical histological techniques. A female-specific chromatin body was observed at low frequency in nuclei of possum corneal epithelium, but not in any other tissues. No sex difference was observed in any monotreme tissue. These data suggest that stabilization of X-chromosome inactivation by heterochromatinization is tissue-specific in marsupials and absent in monotremes.

Extra keywords: Barr body.

Introduction

At an early stage in the development of female eutherian mammals, one X-chromosome becomes genetically inactive (Lyon 1961), transcriptionally silent (Graves and Gartler 1986), late-replicating (Morishima *et al.* 1962) and heterochromatic (Ohno and Hauschka 1960). The inactive X-chromosome, which may be either maternally or paternally derived, is detectable at interphase as the condensed sex-chromatin body (Barr and Bertram 1949; Morishima *et al.* 1962). Inactivation is random, occurs early in embryogenesis, and is stably maintained and clonally inherited *in vivo* and in culture (reviewed by Gartler and Riggs 1983; Graves 1983). Recent studies suggest that there are at least two mechanisms involved in human X-chromosome inactivation; DNA methylation and heterochromatinization, which may be uncoupled in cell hybrids and studied by induced reactivation (Gartler *et al.* 1985). These experiments suggest that heterochromatinization plays a stabilizing role in the maintenance of inactivity.

X-chromosome inactivation in marsupials (Infraclass Metatheria) is paternal rather than random, tissue-specific and incomplete (reviewed by Graves 1983; VandeBerg *et al.* 1986). The findings of paternal (Takagi and Sasaki 1975) and incomplete inactivation (Migeon *et al.* 1985) also in rodent and human extra-embryonic membranes suggests that a less stable paternal inactivation system was present in the common therian ancestor of eutherian and methatherian mammals. The observation of tissue-specific late replication, confined to the small region of the X not homologous to the Y in prototherian mammals (monotremes) (Wrigley and Graves 1988) suggests that at least a primitive form of X-chromosome inactivation existed in the common ancestor of the Theria and Prototheria (more than 150 million years ago).

It is of great interest therefore to consider how the stable X-chromosome inactivation exhibited by the human X could have evolved. To this end, it is instructive to compare the mechanisms of inactivation in eutherian mammals with the mechanisms in marsupials and monotremes, in an attempt to identify intermediates in a sequential evolution of a complex system. We

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have therefore examined the role played by heterochromatinization in marsupial and monotreme X-chromosome inactivation.

In somatic cells of female mammals, the inactive X may be observed as a condensed, heterochromatic body located at the periphery of the interphase nucleus (Ohno and Hauschka 1960; Morishima *et al.* 1962). Such a sex-chromatin body is apparent in many tissues of the human female; oral and vaginal mucosa as well as cultured skin fibroblasts have a high frequency (above 40%) of sex chromatin, while in the blood the inactive X is observed at a lower frequency (2–3%) as a 'drumstick'-shaped nuclear appendage in neutrophils (reviewed by Moore 1962).

There are a number of confusing reports of sex chromatin in marsupial cells. Studies involving *Didelphis virginiana* (Graham and Barr 1959; Ohno *et al.* 1959), *Philander opossum quica* (Perondini and Perondini 1966) and *Perameles nasuta* (Walton 1969) have all reported a conspicuous chromatin body in cells from both sexes. With the exception of Walton's study, all have reported this chromatin body to be larger in females than in males. It is therefore not clear how such chromatin bodies relate to the X and Y chromosomes, especially since recognition of sex-specific differences in nuclear morphology may have been confused by the presence of large blocks of constitutive heterochromatin in both sexes, which are evident using C-banding techniques developed since those early studies.

We have made a careful search for sex-specific differences in nuclear morphology in marsupial and monotreme cells. In this study sex chromatin is defined as a chromatin mass, of equal size in males and females, located either adjacent to the nucleolus or on the periphery of the nucleus in the corneal epithelium, buccal mucosa and fibroblasts. It is defined as a nucleolar satellite in the neuronal tissue (Barr and Bertram 1949) and as a drumstick (Davidson and Smith 1954; Levine 1971) in the blood smears. We have used the same tissues which in humans and other eutherian mammals possess readily identified sex chromatin. To avoid confusion by constitutive heterochromatin, we have chosen the brush-tail possum (*Trichosurus vulpecula*) for our study, since its karyotype contains little C-banded material. The platypus (*Ornithorhynchus anatinus*) was chosen to represent the monotremes, since this species (alone of the three extant monotreme species) possesses a simple XX/XY (rather than an $X_1X_1X_2X_2/X_1X_2Y$) sex determination system (Murtagh 1977; Wrigley and Graves 1988).

Materials and Methods

One mature male and female of both species were studied. Animals were anaesthetized with ether and a blood sample obtained, via heart puncture, using a heparinized syringe. A lethal injection of Nembutal was administered and the animals dissected immediately.

Neuronal Tissue

Sections 10 μm thick were cut from samples fixed immediately after death in 19% (v/v) formal saline for 20–24 h, dehydrated and embedded in paraffin. Slides were hydrolysed in 0.1 M HCl for 1 min, rinsed in distilled water, stained in Tetrachrome (Gurr) for 1 min (1 part Tetrachrome : 2 parts Gurr's buffer), rinsed again and air dryed.

Corneal Epithelium

Eyes removed from each specimen were fixed immediately in 95% (v/v) ethanol and slides prepared by a modification of Fredga's (1964) technique.

Buccal Mucosa

The buccal cavity was scraped using a blunt spatula. This first sample was discarded. The cavity was scraped again and the cells transferred to a clean slide, stained in lacto-acetic-orcein for 8 min, and gently squashed beneath a coverslip.

Tissue Culture

The derivation and culture of platypus cell lines has been described previously (Wrigley and Graves 1984). Possum cultures were initiated using the same method. Fibroblast lines were grown routinely in 25 cm^3 plastic tissue culture flasks in Dulbecco's modification of Eagle's medium supplemented with 10% (v/v) foetal calf serum (Flow), 60 μg/ml benzyl penicillin, 50 μg/ml dihydrosteptomycin and 100 μg/ml glutamine. Flasks were gassed with 10% CO_2 in air. Platypus fibroblasts were grown at 32°C and possum fibroblasts at 37°C. Cells were grown on coverslips until subconfluent, fixed *in situ* (3 parts methanol : 1 part acetic acid), hydrolysed for 1 min in 0.1 M HCl, stained with Tetrachrome for 1 min, rinsed and air dryed.

Other Preparations

Blood smears were stained in 10% (v/v) Giemsa for 5 min. C-banded slides were prepared by the procedure of Leversha *et al.* (1980). Late-replication banding was carried out by the method of Eichenbaum and Krumins (1982). All slides were examined using a Leitz Dialux microscope and photographed, using a Wild Photoautomat, on Kodak technical pan film.

Results

The chromosomes of the brush-tail possum were studied by C- , G- and late-replication banding. The $2n = 20$ karyotype, and specifically the X-chromosome, contains almost no C-banding material which could have confused our recognition of sex chromatin (Fig. 1*A*). The X-chromosome is the smallest in the female complement, accounting for 4% of the haploid chromosome length, and contains a nucleolus organizer region (Murray 1977). Late-replication banding studies demonstrate a late-replicating X at high frequencies in female-derived blood lymphocytes and fibroblasts (Fig. 1*B*).

The chromosomes of the platypus were studied using the same techniques. Several chromosomes do contain large heterochromatic regions, revealed by chromomycin A_3 staining and by C-banding (Fig. 1*C*). The X-chromosome, which is the seventh largest element in the $2n = 52$ karyotype, amounting to about 6% of the haploid length, has no such region, nor does it contain a nucleolus organizer region. Late-replication studies reveal tissue-specific asynchronous replication confined to the non-pairing region located on the short arm of the X-chromosomes (Wrigley and Graves 1988).

Interphase cells from a number of tissues were examined, in sectioned or whole-mount preparations, for consistent sex differences in nuclear morphology. Corneal epithelia, buccal mucosa and fibroblasts were scored for the presence of a chromatin mass on the periphery of the nucleus and adjacent to the nucleolus. Neuronal sections were scored for the presence of a nucleolar satellite as described by Barr and Bertram (1949) for cat. Blood smears were examined for the presence of polymorphonuclear neutrophilic drumsticks (Davidson and Smith 1954; Levine 1971). Large numbers of cells were scored in tissue from both sexes and the frequency of sex-chromatin positive nuclei compared between male and female derived cells using a χ^2 contingency test. The data are summarized in Table 1. No sex difference was detected in possum buccal epithelium, blood polymorphs, neurones or fibroblasts (Fig. 2*A*); however, a small proportion (about 6%) of corneal epithelial nuclei from female possum possessed a distinct peripheral chromatin body (Fig. 2*B*) which was never observed in nuclei of male cells. No sex difference in nuclear morphology was ever observed in platypus cells.

Discussion

Our study indicates that sex chromatin is confined to the corneal epithelia of the possum and is absent in the platypus. These results contradict previous studies in which sex chromatin was reported in both sexes in various tissues. However, these earlier studies suffered from difficulties in distinguishing between sex chromatin, which is sex specific, and constitutive heterochromatin, present in both sexes.

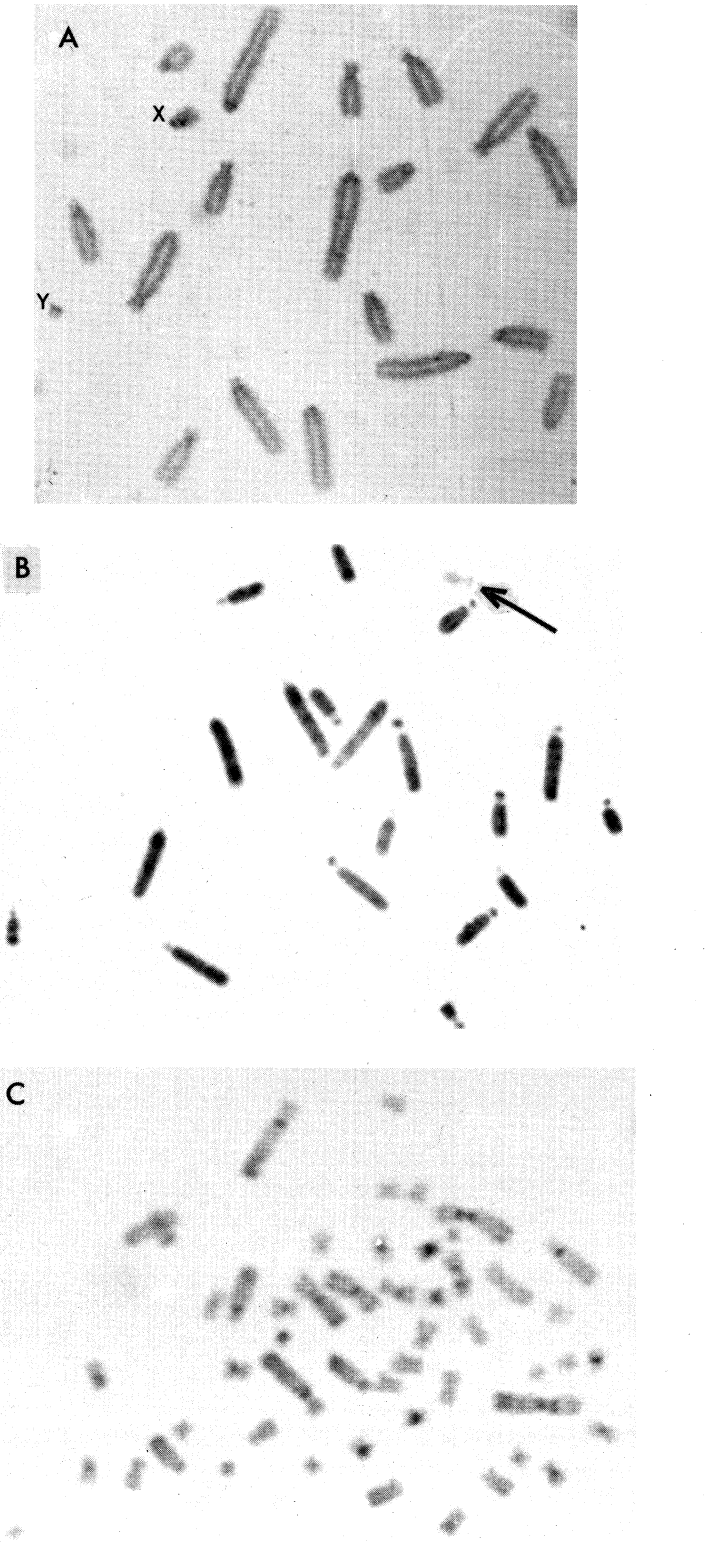

Fig. 1. C-banded chromosomes of brush-tail possum(*A*); late replication banded metaphase of brush-tail possum fibroblasts (*B*) — the arrow indicates the late-replicating X chromosome; C-banded chromosomes of the platypus (*C*).

Using the criteria for defining sex chromatin presented in the Introduction, we detected sex chromatin in only one tissue. Although small numbers of bodies were scored in both male and female tissues these were statistically insignificant and designated artifacts. Only in the corneal epithelium of the brush-tail possum was there a consistent sex difference in nuclear morphology present at equal frequency in both sexes. The presence of non-sex-specific dark-staining heterochromatic bodies was obviously a function of tissue, being particularly high in blood polymorphs and absent in the very large, pale nuclei of corneal epithelium.

The low incidence and restricted distribution of sex chromatin in the possum, and its absence in the platypus, may indicate differences in the role of heterochromatinization in the stability of X-chromosome inactivation in the three groups of mammals. In the Eutheria,

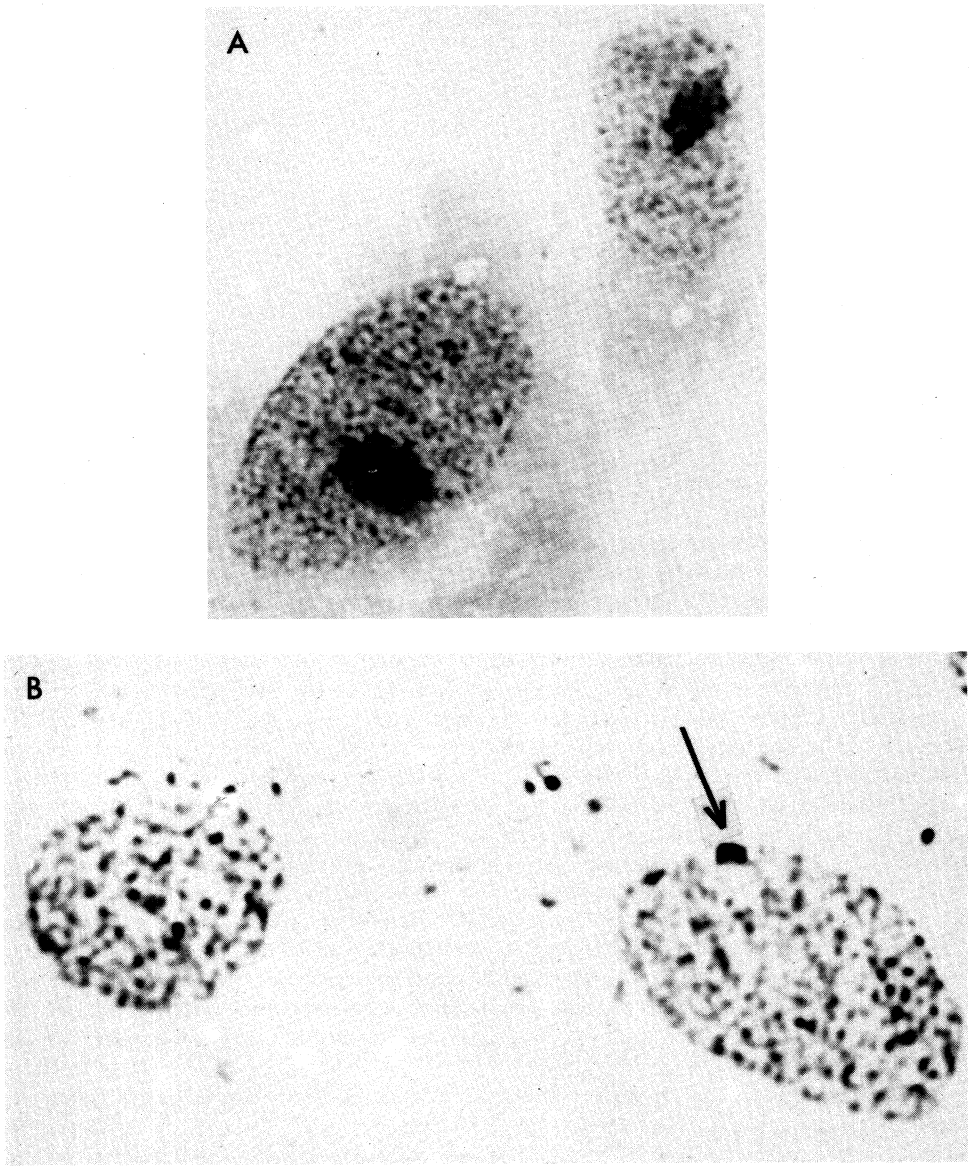

Fig. 2. Female brush-tail possum fibroblasts displaying no definitive Barr body (*A*); female brush-tail possum corneal epithelium — arrow indicates Barr body (*B*).

heterochromatinization and DNA methylation (and perhaps also late DNA replication) probably interact in maintaining the extremely stable, random, complete X-inactivation found in this mammalian infraclass. Gartler *et al.* (1985) have demonstrated that, in cell hybrids which retain a late-replicating, inactive, human X-chromosome but lack sex chromatin, inactivation is unstable as is indicated by their high spontaneous and 5-azacytidine-induced reactivation rate. Our interpretation of these results is that heterochromatinization, while not a necessary condition for X-chromosome inactivation, plays a major stabilizing role in the Eutheria.

Table 1. Presence or absence of sex chromatin in tissues of the platypus and brush-tail possum

Tissue	Male[A]		Female[A]		χ_1^2	P
	+	−	+	−		
			Platypus			
Buccal epithelium	3	197	4	196	0.145	$0.9>P>0.5$
Corneal epithelium	6	300	5	316	0.148	$0.9>P>0.5$
Blood polymorphs	14	527	17	547	0.184	$0.9>P>0.5$
Neurones	14	233	8	198	0.485	$0.5>P>0.1$
Fibroblasts	9	246	11	257	0.120	$0.9>P>0.5$
			Brush-tail possum			
Buccal epithelium	0	520	2	501	2.07	$0.9>P>0.5$
Corneal epithelium	0	700	30	480	42.38	$P<0.05$
Blood polymorphs	19	231	27	223	1.53	$0.9>P>0.5$
Neurones	5	250	6	244	0.114	$0.9>P>0.5$
Fibroblasts	6	263	5	250	0.044	$P>0.9$

[A] + = sex chromatin positive; − = sex chromatin negative.

The low frequency and restricted distribution of sex chromatin in marsupial cells, and its absence in monotremes, implies that heterochromatinization is not a necessary feature of inactivation in these mammalian groups but is present, and may play a minor role in, some tissues. The extent of X-chromosome inactivation in the Metatheria differs between tissues (summarized by Graves 1983; VanderBerg *et al.* 1986). In macropods the paternal X-chromosome is partially inactive in fibroblasts and fully inactive in lymphocytes. The pattern of inactivity of four loci, whose order is known, suggests that inactivation may be controlled by tissue-specific spreading from a single inactivation centre (Graves and Dawson 1987). The absence of sex chromatin in most, but not all, tissues of the possum may be a feature of a primitive, less stable, tissue-specific X-inactivation system found in the marsupials. The restriction of sex chromatin to the corneal epithelia could reflect a fully and stably inactive X-chromosome only in this tissue, while in other tissues inactivation is less rigidly controlled. Paternal phosphoglycerate kinase is fully inactive in corneal epithelium in the brush-tail possum (VandeBerg *et al.* 1979).

Our finding that heterochromatinization seems not to be involved at all in the monotremes and plays at most a minor role in marsupial X-chromosome inactivation, suggests that it became associated with X-chromosome inactivation after the divergence of the subclass Theria and Prototheria, more than 150 million years ago, but before the divergence of the infraclasses Eutheria and Metatheria 130 million years ago, and has assumed increasing importance during Eutherian evolution.

The paternal and/or incomplete X-chromosome inactivation of eutherian extra-embryonic tissues (Takagi and Sasaki 1975; Migeon *et al.* 1975) is phenotypically similar to the X-inactivation system in marsupials, and possibly these systems share a common mechanism, which evolved in an ancestral therian mammal. However, sex chromatin is present at high frequency in human extra-embryonic membranes (Klinger 1957), signifying that the minor role of heterochromatin is not a property of paternal or incomplete inactivation. Perhaps what these paternal systems

have in common (and what distinguishes paternal from random inactivation) is that paternal inactivation does not involve DNA methylation. Extra-embryonic tissues of eutherian mammals are significantly undermethylated (Sanford *et al.* 1985), and gene-transfer experiments (Kratzer *et al.* 1983) suggest that the hypoxanthine phosphoribosyl transferase (HPRT) allele on the inactive mouse X-chromosome is not methylated in extra-embryonic tissue. As yet, it is unclear whether DNA methylation is involved in marsupial X-chromosome inactivation; although there are significant sex differences in methylation patterns of internal sites in the kangaroo phosphoglycerate kinase and HPRT genes (Van Daal and Cooper, unpublished data), there are no consistent methylation differences, in the GC-rich regions 5′ to the glucose-6-phosphate dehydrogenase or HPRT genes (Kaslow *et al.* 1986). It is difficult to accept that paternal X-chromosome inactivation evolved independently in marsupials and eutherian mammals; if there is, indeed, a common mechanism, it is therefore more likely to involve late DNA replication, which is common to the inactive X-chromosome in *all* mammals.

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