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Autosomal assignment of OTC in marsupials and monotremes: implications for the evolution of sex chromosomes

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Summary

The OTC gene coding for ornithine transcarbamylase is sex linked and subject to *X* inactivation in humans and mice. We have used a rat cDNA probe to localize OTC by *in situ* hybridization in marsupials and monotremes. The gene maps to an autosomal site in two distantly related marsupial species and in one monotreme (the platypus); the first demonstration that a gene *X*-linked in one mammalian species may be autosomal in another. Since the conservation of the mammalian *X* is thought to be a consequence of its isolation by the inactivation mechanism, we propose that an autosomal or pseudoautosomal segment containing OTC has been recruited into the inactivated region of the *X* rather recently in eutherian evolution while it remained autosomal, or was translocated to an autosome, in metatherian and prototherian mammals.

1. Introduction

Marsupials (Infraclass: Metatheria) and placental mammals (Infraclass: Eutheria) diverged at least 130 m.y. ago (Air *et al.* 1971; Wainwright, 1984) while monotremes (Subclass: Prototheria) diverged even earlier, between 150–200 m.y. ago (Kemp, 1982). Hence, comparisons between eutherian, metatherian and prototherian gene maps may permit us to deduce ancient gene arrangements present in a common mammalian ancestor.

The eutherian *X* chromosome seems to have been entirely conserved in size (about 5% of the haploid chromosome length) and gene content (Lalley & McKusick, 1985). Ohno (1973) has suggested that this is a consequence of selection acting against disruption of the *X* chromosome, in order to preserve the *X* chromosome inactivation mechanism, and so maintain the correct dosage of gene products. The basic metatherian *X* is smaller (3% of the haploid length; Hayman *et al.* 1982) but contains the genes HPRT, PGK, G6PD, and GLA (Dawson & Graves, 1986; Dobrovic & Graves, 1986) which are present on the *X* of all eutherian species studied. The prototherian *X* is 5–6% of the haploid chromosome length, and is largely homologous to the *Y* chromosome (Wrigley & Graves, submitted).

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The exclusion of steroid sulphatase (STS) from the *X* in marsupials is an apparent exception to the conservation of the mammalian *X* chromosome. STS is *X*-linked in humans (Mohandas *et al.* 1979) and lies near the pseudoautosomal region of *X*–*Y* pairing (reviewed by Craig & Tolley, 1986), but is partially expressed when on the inactive *X* chromosome (Migeon *et al.* 1982; Lykkesfeldt *et al.* 1984). In the mouse, STS is also *X*-linked (Gartler & Rivest, 1983), and the presence of a functional *Y*-linked allele, which undergoes recombination with the *X* at meiosis (Keitges *et al.* 1985), means there are no observed sex differences in dosage (Crocker & Craig, 1983). Marsupial STS is not expressed by several cell hybrids retaining a marsupial *X* chromosome (Cooper *et al.* 1984; Dawson & Graves, 1986). These data would exclude the STS locus from the marsupial *X*; however, since no positive assignment could be made (few cell hybrids gratuitously retain marsupial autosomes) it is possible that the marsupial STS locus is present, but repressed, in cell hybrids.

Ornithine transcarbamylase (OTC), a trimeric enzyme synthesized in the liver, is imported into the mitochondria, where it catalyses a step in the urea cycle (Jones, 1961). OTC is *X*-linked in mouse (De Mars *et al.* 1976) and humans (Short *et al.* 1973; Ricciuti, 1976) and mosaicism in the livers of women heterozygous for OTC deficiency shows that it is subject to inactivation (Ricciuti, 1976). In humans, OTC is located distally on the short arm of Xp21·1

(Lingren *et al.* 1984), close to the STS locus and the pseudoautosomal region, which is exempt from inactivation.

We have used the rat OTC probe (McIntyre *et al.* 1984) for *in situ* hybridization to the chromosomes of two distantly related marsupial species; *Dasykaluta rosamondae* and *Macropus rufus*, as well as a monotreme species, the platypus, *Ornithorhynchus anatinus*.

2. Materials and Methods

The cells used were marsupial fibroblast lines established from ear punch, and platypus fibroblast lines derived from toe webbing. Cells were maintained in Dulbecco's modified Eagle medium (Gibco), supplemented with 10% foetal calf serum (Flow), 100 I.U./ml penicillin (Commonwealth Serum Laboratories, Melbourne), 50 mg/ml streptomycin sulphate (Glaxo) and 100 mg/ml glutamine (Merck) at 37 °C for marsupials and 32 °C for monotremes.

Chromosome spreads were prepared by arresting cells at metaphase with 0·005% colchicine (Commonwealth Serum Laboratories, Melbourne) for 1–2 h. Cells were harvested, treated with 0·075 M-KCl, fixed in 3:1 methanol:acetic acid, spread on slides and air dried.

The OTC gene probe we used contains a 1·3 kb rat cDNA OTC insert in a pUC9 vector (McIntyre *et al.* 1984). The probe was labelled by nick translation to a specific activity of $2{\cdot}1 \times 10^7$ cpm/μg with [^{3}H]dATP, [^{3}H]dCTP and [^{3}H]dGTP (Amersham).

A combination of the *in situ* hybridization methods of Harper & Saunders (1981), Trent *et al.* (1982), Donlon *et al.* (1983), Zabel *et al.* (1983) and Simmers *et al.* (1986) was used. Freshly prepared slides were treated with 200 μl of 100 μg/ml RNase A in 2 × SSC (pH 7) at 37 °C for 1 h, then washed in four changes of 2 × SSC (pH 7) for 2 min each, dehydrated through an ethanol series and air dried. Chromosomal DNA was denatured by placing slides in 70% formamide/2 × SSC, pH 7 at 70 °C for 2 min, then 70% ethanol at −20 °C for 1 min followed by 95% ethanol at −20 °C for 1 min and air drying. The labelled probe DNA was prepared in concentrations ranging from 0·05 to 0·4 μg/ml in a mixture containing 50% formamide, 10% dextran sulphate, 2 × SSCCP (pH 7) and a 200-fold excess of sheared salmon sperm DNA. The probe mixtures were denatured at 100 °C for 5 min and then stored on ice. Hybridization was carried out overnight at 37 °C in a 2 × SSC saturated environment with 20 μl of probe mixture, sealed under a siliconized coverslip with rubber cement. Coverslips were removed and the slides washed in three changes of 50% formamide in 2 × SSC (pH 7) at 40 °C for 10 min each, three changes in 2 × SSC (pH 7) at 40 °C and three changes in 2 × SSC (pH 7) at room temperature, dehydrated through an ethanol series and air dried.

The slides were coated with Ilford K2 nuclear track emulsion, diluted 1:2 with water and 2% glycerol, exposed at 4 °C with desiccant for 20–25 days, developed with Kodak D19 and stained with Giemsa.

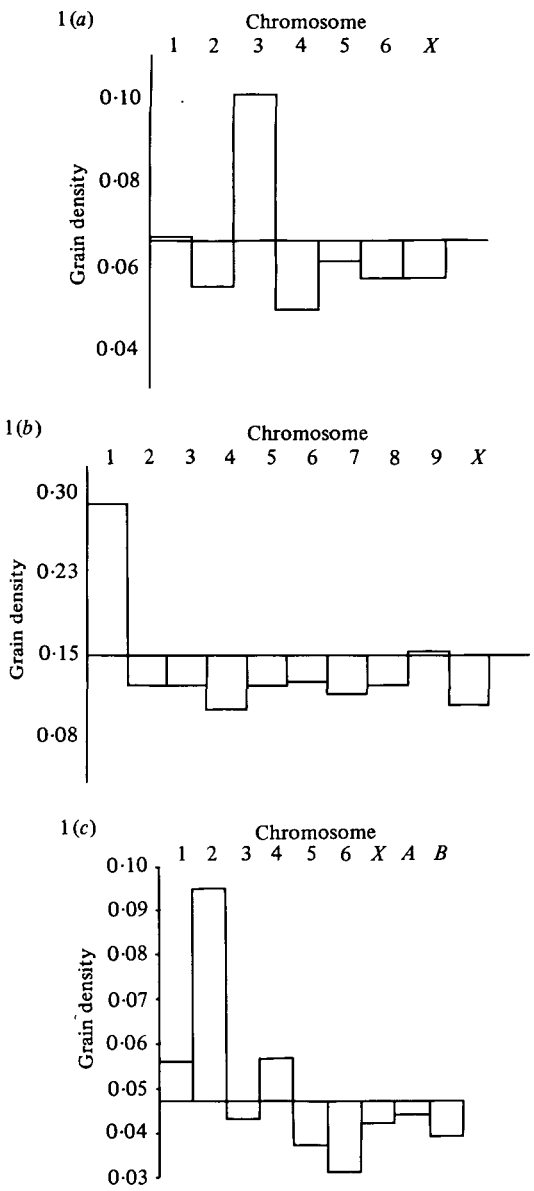

Fig. 1. Grain density (the number of silver grains per relative length of chromosome per metaphase) for each chromosome. (*a*) *D. rosamondae*, (*b*) *M. rufus*, (*c*) *O. anatinus*.

3. Results

The karyotypes of both marsupial species are ideal for *in situ* hybridization because of the low diploid number, and the large size and distinctive morphology of chromosomes, such that G-banding was not required to identify individual chromosomes.

D. rosamondae has a modal number of $2n = 14$, with each chromosome recognized by size and morphology. The cell line retained a nearly normal diploid karyotype. A total of 250 metaphases were scored at

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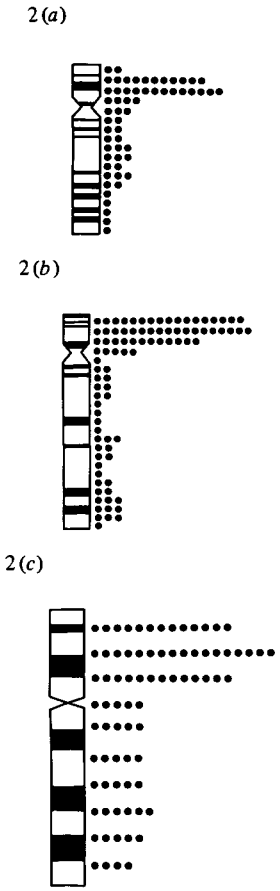

Fig. 2. Ideograms of (*a*) *D. rosamondae* chromosome 3, (*b*) *M. rufus* chromosome 1, (*c*) *O. anatinus* chromosome 2 showing labelled sites on 100 labelled chromosomes following hybridization with a probe for OTC.

the optimum probe concentration of 0·1 μg/ml. Only grains that were touching one or other chromatid were scored, otherwise they were regarded as background. Analysis showed 30% (125) of the grains were on chromosome 3 and of these, 54% (68) were on the short arm. (Fig. 1*a*, 2*a* and 3*a*.)

The *M. rufus* line had the normal $2n = 20$ complement, and again each chromosome was identifiable by size and centromere position. Analysis of 100 metaphases at probe concentrations of 0·1 μg/ml showed 38% (89) grains on chromosome 1, with 60% (53) of these on the short arm. (Fig. 1*b*, 2*b* and 3*b*.)

The platypus has $2n = 52$ and the fibroblast line studied remained diploid. Although the sex chromosome and the six largest autosomal pairs were readily identifiable by their size and centromere position, it was impossible to distinguish most of the smaller elements, even with G-banding. Thus, these smaller chromosomes were assigned to two groups, A (the larger elements, chromosomes 7–17*a*) and B (the smaller elements, chromosomes 18–23*b*, *c*, *d*). Grains over 100 metaphases were scored at a probe concentration of 0·1 μg/ml. Analysis showed 16% (77) grains on chromosome 2 and 56% (42) of these were

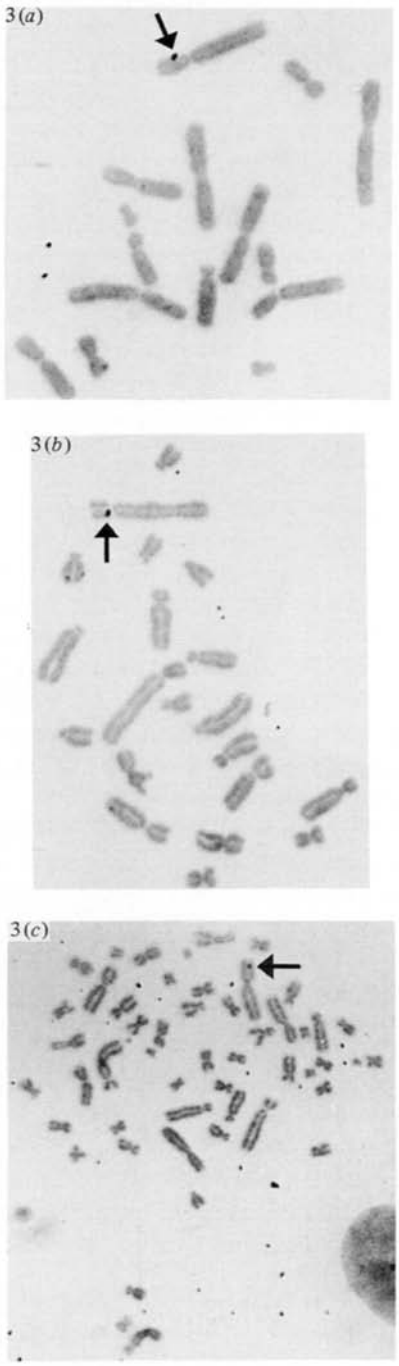

Fig. 3. Metaphases of (*a*) *D. rosamondae*, (*b*) *M. rufus* and (*c*) *O. anatinus* showing silver grains on chromosomes 3, 1 and 2 respectively.

on the short arm. (Fig. 1*c*, 2*c* and 3*c*.) See Appendix for analysis of *in situ* hybridization data.

In all these species, no other sites were observed which registered above background level.

4. Discussion

In situ hybridization of the OTC probe revealed a single site of hybridization in both marsupial species. This site was on chromosome 3p in *D. rosamondae* and chromosome 1p in *M. rufus*. Since these two

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species represent orders which diverged 45 m.y. ago (Archer, 1984), it is likely that the autosomal location of OTC is a characteristic of the whole infraclass Metatheria.

The OTC probe was localized to an autosome (2p) in the platypus. There was no other chromosome significantly labelled by the probe, and the only qualification to our assignment to 2p is our inability to identify the small chromosomes, grouped in A and B. It is possible that a second significantly labelled region on one of these small elements could have been undetected because of the necessity to pool grain counts for these groups. However, the significant hybridization to chromosome 2p, and the absence of significant label over group A or B makes it likely that the OTC gene is on 2p in the platypus.

OTC is located on the *X* chromosome in eutherian mammals. The autosomal location for OTC in marsupials and monotremes thus constitutes the first clear exception to the hypothesis that the mammalian *X* chromosome has been conserved *in toto*. The assignment of OTC makes it appear more likely that STS is also autosomal in marsupials, as the exclusion data from cell hybrids suggest (Cooper *et al.* 1984; Dawson & Graves, 1986). Thus the mammalian *X* has been rearranged during mammalian evolution. It is therefore of great interest to consider the implications of this rearrangement to our understanding of the evolution of mammalian sex chromosomes, and of *X* chromosome inactivation.

There are two alternative hypotheses for the rearrangement in the three mammalian groups. Since OTC is autosomal in monotremes and marsupials, but sex-linked in eutherians, the simplest hypothesis is that the region containing this locus (and STS) was originally autosomal in the common mammalian ancestor, and was translocated to the *X* only in the eutherian lineage. Once a part of the *X*, the OTC–STS region may have increasingly come under the influence of *X* chromosome inactivation during eutherian evolution.

The alternative hypothesis is that OTC was originally located on the *X*, within the pseudoautosomal region, and has been translocated from it independently in the monotreme and marsupial lineages. A comparison of the lengths and the extent of homology of the *X* and *Y* chromosomes of the three groups of mammals suggests that the *X* and *Y* differentiated gradually from an originally homomorphic sex pair (Wrigley & Graves, submitted). The monotremes retained a rather early stage of differentiation, in which the *X* (5% of the haploid length) and the *Y* are largely homologous, whereas the marsupials appear to have a smaller *X* (3% of the haploid length) and have lost the pairing segment altogether (Sharp, 1982). Eutherian sex chromosomes retain only a very short pairing segment. Since alleles on the pseudoautosomal region of the *X* would have had a homologue on the *Y*, dosage compensation would not be necessary; thus this region of the *X* would be free to undergo exchange with autosomal regions and would therefore not be expected to have been conserved.

If the OTC gene were originally on the large pseudoautosomal region of the *X* in a common ancestor, it could well have undergone exchange with an autosome in the prototherian line of descent. A region containing the OTC and STS genes could have been lost independently in the marsupial lineage by translocation of all or part of a pseudoautosomal region, at least 2% of the haploid length; this idea implies that the *X* and *Y* chromosome of a common therian ancestor shared a larger pseudoautosomal region than seen in extant eutherians, and, that, like monotremes, marsupials represent an earlier stage in *X*–*Y* chromosome differentiation (Wrigley & Graves, submitted).

Either hyopothesis requires that the OTC gene in the common therian ancestor and the common mammalian ancestor, was located on an autosomal or pseudoautosomal region, and was not subject to inactivation. We propose that in the eutherian lineage, as the *Y* became progressively reduced, the OTC gene became unpaired and was recruited into the segment of the *X* which was subject to inactivation. Its position on the conserved region of the *X* then became fixed, because of selection against the disruption of the dosage compensation mechanism.

Spreading from an inactivation centre is observed in *X*-autosome translocations in the mouse (Cattanach, 1975), and has been proposed to explain the tissue-specific patterns of *X* inactivation in marsupials (Graves & Dawson, in preparation). We therefore suggest that the recruitment of the OTC gene into the inactivated region of the *X* may have occurred during evolution by selection of variants in which this spreading of inactivation is more extensive. Perhaps this evolutionary spreading still continues; the partial inactivation of the human STS locus which is near, but not in, the pseudoautosomal region of the human *X*, suggests that inactivation is creeping up on it.

Appendix: analysis of *in situ* hybridization data

Assuming a random distribution of label, the background number of silver grains over a particular chromosome in a cell population will have a Poisson distribution with a mean (or expected value) proportional to the area of the chromosome. When a gene probe hybridizes to a particular chromosome, the grain number over that chromosome, while still having a Poisson distribution, will have a mean value which is disproportionately large compared to its area.

We modelled the number of silver grains over all the chromosomes excluding a particular one, as having a mean proportional to the chromosome area. If the

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chromosome omitted is the one to which the gene hybridized, the observed distribution should fit well with that predicted by the model. If the omitted chromosome is not the site of hybridization, then the observed distribution will differ significantly from that predicted by the model.

The program GLIM was used to fit the model and give a measure of adequacy of fit, termed deviance, when each chromosome in turn is omitted; the deviance may then be compared with the significance levels on a χ^2-distribution with degrees of freedom (n-2). When the model is fitted omitting one chromosome, then the model can be used to give an expected count for the chromosome omitted. If the chromosome omitted is the site of hybridization, the observed count will be much larger than this expected count.

Calculating the standardized residual, $r =$ (observed-expected)/(expected)$^{\frac{1}{2}}$, we have a quantity which would be an observation on a standard normal distribution if the gene does not hybridize to that chromosome. The hybridization of a gene probe to a chromosome will cause the standardized residual to be very large compared to the standard normal distribution.

A GLIM program was written to analyse *in situ* hybridization data and clearly indicate to which chromosome the gene has hybridized. An example of the output obtained in the present study is given below.

Analysis of OTC Data for the Platypus

Chromosome	Deviance	Standardized residual
B	37·63	−2·35
A	41·46	−1·22
X	41·76	−0·85
6	38·02	−2·00
5	41·09	−1·16
4	41·01	1·31
3	42·14	−0·60
2	10·8*	6·87*
1	40·89	1·37

The deviances must be compared with the χ^2 distribution with (n-2) = 7 degrees of freedom. Only for the model omitting chromosome 2 is the fit adequate. Also for that model the standardized residual is highly significant compared to the standard normal distribution. The results convincingly demonstrate that the OTC gene probe hybridized to chromosome 2 in the platypus. Similar analysis showed OTC hybridized to chromosome 1 in *M. Rufus* and chromosome 3 in *D. rosamondae*.

A more formal technical description of this procedure is being prepared by A. H. Sinclar and D. Scott for publication elsewhere.

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