A Highly Repeated DNA from the Genome of the Wallaroo (Macropus robustus robustus)

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Abstract

The major satellite of M. r. robustus DNA has been isolated in a Ag^+–Cs_2SO_4 gradient. It has a density of 1.710 g/cm^3 compared with 1.697 g/cm^3 for the bulk of the DNA, and accounts for about 10% of the total DNA. Sequence heterogeneity within the satellite was shown by an increase in density to 1.715 g/cm^3 and by a reduction of 12°C in the temperature of denaturation (T_m) after renaturation. The satellite was found to occur in the centromeric regions of all chromosomes. This pattern of distribution was essentially duplicated in the genomes of other members of the wallaroo group. A polymorphism for a major block of the sequences on chromosome 5 occurred in both M. r. erubescens and M. antilopinus.

Species in the wallaroo group all contained about the same amount of this sequence, while M. rufus contained 30% and M. rufogriseus 16% of the wallaroo content; all other macropods and marsupials tested had 5% or less of the wallaroo level. The thermal denaturation profiles of the hybrids formed between cRNA (to the satellite) and DNA of species in the wallaroo group were identical; hybrids formed with DNA of other kangaroo species had a ΔT_m of approximately 4°C relative to the wallaroo.

The chromosomal locations of the sequence in M. rufus and M. rufogriseus were in major blocks on the X chromosome, in association with the nucleolus organizer. The sequence also occurred at the centromere of the Y chromosome in M. rufogriseus, and in centromeric blocks on the four small acrocentric chromosomes of M. rufus which are believed to be related to metacentric chromosomes of 2n = 16 karyotypes by fusion or fission.

Introduction

On the basis of comparisons of buoyant densities and melting profiles, highly repetitive DNA sequences (satellite DNA sequences) have been considered to be labile components of eukaryote genomes (Hennig and Walker 1970; Walker 1971). However, current evidence from sequence determinations shows that highly repetitive sequences may be conserved in evolution relative to other types of DNA sequences (Fry and Salser 1977; Peacock et al. 1977). The functions of highly repetitive sequences are still unresolved, and their organization, chromosomal locations and contents are as yet well characterized in only a few organisms.

Because the Australian kangaroos and wallabies (macropods) form a well-defined evolutionary group, they present a favourable situation for examining highly repetitive DNA sequences. The basic outline of macropod phylogeny is established but there are uncertainties in some of their relationships, due largely to a lack of fossil evidence and the homogeneity of the group. DNA sequence comparisons used in conjunction with classical techniques could assist in a better understanding of the evolutionary history of the group.
The macropod group has the advantage of low chromosome numbers \((2n = 10-20)\) and large and often individually distinguishable chromosomes. The macropods of central interest in this paper are the wallaroos, which include *Macropus robustus* (wallaroo), *M. r. erubescens* (euro) and *M. antilopinus* (antilope). They present subspecific and specific variation, and in addition a number of geographically localized forms are known. Their closest relative is the red kangaroo (*M. rufus* or *Megaleia rufa*), and there is a group of less-related wallabies and kangaroos, which includes the grey kangaroos (*Macropus fuliginosus* and *M. giganteus*) and the wallabies *M. rufogriseus* (red-necked wallaby) and *M. eugenii* (tammar wallaby).

The major satellite sequence of *M. r. robustus* has been isolated, characterized and used as an *in situ* hybridization probe on the genomes of the above species. Where possible the resulting data have been interpreted in terms of macropod phylogeny and changes in chromosome morphology.

**Materials and Methods**

**Isolation of DNA**

Frozen liver tissue (20 g) was homogenized in buffer (10 mM MgCl₂; 0·25 mM sucrose; 10 mM Tris–HCL, pH 8·4) in a Waring blender at high speed for 1 min, then at low speed for 4 min. The homogenate was filtered through four layers of cheese cloth and two layers of Miracloth. The filtrate was centrifuged at 1500 g for 7 min and the nuclear pellet washed twice in homogenization buffer, then resuspended in 10 mM Tris, 1 mM EDTA, pH 8·4 (TE). The nuclei were lysed by adding sodium lauryl sarcosinate (Sarkosyl N98-Geigy) to a final concentration of 1% (w/v). Solid CsCl was added to a density of 1·70 g/cm³ and this solution was centrifuged at 44000 rpm for at least 48 h in a Beckman Ti-60 rotor. DNA-containing fractions, collected from the top of the tube, were pooled and dialysed against TE to remove CsCl.

**Analytical Ultracentrifugation**

Analytical ultracentrifugation was carried out in a Beckman Model E ultracentrifuge for 18 h at 44000 rpm and 25°C. In neutral gradients 1–3 µg DNA in TE was adjusted to a density of 1·71 g/cm³ with CsCl and 1 µg *Micrococcus luteus* DNA was added as a marker \((\rho = 1·731 \text{ g/cm}^3)\). In alkaline gradients 1–3 µg DNA in TE was adjusted to 0·1 M NaOH and to a density of 1·76 or 1·78 g/cm³ with CsCl. In Ag⁺–Cs₂SO₄ gradients 10 µg of DNA in 10 mM Tris–sulfate, 1 mM EDTA was complexed with the required amount of AgNO₃ solution. The amount of Ag⁺ was adjusted until the separation of the satellite from the bulk of the DNA was maximized. Borate buffer (50 mM Na₂SO₄, 25 mM Na₂B₄O₇, pH 9·1) was added to 23% (v/v) and Cs₂SO₄ was added to a density of 1·50 g/cm³.

**Optical Thermal Denaturation of DNA**

The melting behaviour of unfractionated DNA was examined in a Gilford Model 2000 spectrophotometer. The temperature of the DNA solution (10–15 µg DNA/ml TE) was raised by 1° per minute with both temperature and optical density being recorded every few seconds. \(T_m\) was defined as the temperature at the midpoint of the increase in optical density (Mandel and Marmur 1968), relative to an *E. coli* standard.

**Isolation of Satellite DNA**

Satellite DNA was isolated using preparative Ag⁺–Cs₂SO₄ gradients. The concentration of DNA relative to Ag⁺ was maintained at the level of a successful analytical gradient. The solution was centrifuged at 44000 rpm for at least 48 h in a Beckman Ti50 rotor. The gradient was fractionated from the bottom at about 0·3 ml per fraction. Peak fractions were combined and dialysed in TE.

**Thermal Denaturation and Renaturation of Satellite DNA**

Satellite DNA was subjected to ultrasonic disruption three times on ice for 30-s intervals at level 4 on a Branson sonifier B-12, yielding fragments of less than 700 base pairs in length. It was dialysed
in 0·12 M phosphate buffer and melted in a Gilford spectrophotometer as before. For renaturation, the same DNA was heated at 98°C to denature it [as measured by maximized optical density at 260 nm (OD_{260})] then placed in water at 65°C (T_m−25°C) until the OD_{260} had levelled. A sample was then examined in an analytical CsCl gradient and the remainder was remelted in the Gilford spectrophotometer as before.

Synthesis of Complementary RNA

cRNA was synthesized on a template of purified heavy strand (SS ³HcRNA) or native DNA (DS ³HcRNA) in an E. coli RNA nucleotidyl transferase reaction. The 0·1-ml reaction contained 10 µl enzyme, 2 µg DNA, 100 µCl ³H-labelled CTP or UTP, and 80–100 µM cold UTP or CTP, 80–100 µM cold GTP, and 100–1000 µM cold ATP. The buffer contained 0·04 M Tris–HCl, pH 7·9, 0·01 M MgCl₂, 0·16 M KCl and 0·0002 M dithiothreitol. Following DNAse (Worthington DPFF) treatment (100 µg/ml) and phenol extraction, the cRNA was isolated by passage through a Sephadex G-75 column.

Filter Hybridization

Nitrocellulose filters (Sartorius, pore size 45 µm) were washed twice in 4 × SSC (SSC is 0·15 M NaCl, 0·015 M sodium citrate, pH 7·2) for 15 min. The DNA solution (about 10 µg DNA/25-mm diam. filter) in 4 × SSC was denatured in 0·05–0·1 M KOH and placed on ice after 5 min. The solution was neutralized with HCl and loaded onto filters largely without vacuum. Duplicates were generally used. The filters were then washed twice with 3 × SSC and dried overnight at 60°C in a vacuum. They were soaked in the hybridization solution of 3 × SSC–50% (v/v) formamide [form-amide, Fluka purum; refractive index of 3 × SSC–50% (v/v) formamide (3 × SSC–F) at 25°C was 1·3655 for all hybridizations] for 2 min at the proposed hybridization temperature. The filters were transferred to vials containing 3 × SSC–F, 10 µg yeast RNA and ³HcRNA at 2 × 10^5–8 × 10^5 cpm/ml, and the vials were tightly capped. If preheating was desired, the vials were held in water hotter than 80°C for 1 min. They were then shaken at the hybridization temperature for 3 h, and washed once in 3 × SSC–F and three times in 3 × SSC for 5 min at the hybridization temperature. After RNAse treatment (T₁RNAse, Calbiochem, 20 units/ml; RNAse A, Sigma, 4 µg/ml, in 3 × SSC at about 30°C for 30 min), the filters were washed three times in 3 × SSC for 5 min. They were assayed for either the amount of cRNA hybridized to DNA, by drying and counting in a toluene-based scintillant, or the mean melting temperature of the RNA–DNA hybrids. Blank filters were treated as above, except that no DNA loading solution was used. For melting temperature analysis, filters were used at the 25-mm diameter size or cut to a 7-mm diameter. A filter was washed briefly in 3 × SSC–F, then placed in a vial containing 1 ml or 200 µl of 3 × SSC–F, depending on size. It was held at a particular temperature for 5 min, then serially transferred to a fresh vial as the temperature was raised by 4°C. Temperature points were generally started at or below the hybridization temperature. The solution remaining in each vial was assayed for ³H-cRNA in a scintillation counter by combining about 200 µl of the solution with 100 µl of water and 8 ml of a Triton X–toluene based scintillant. Drying and counting the filters established that all radioactivity had been removed. The average radioactivity (cpm) at each temperature point minus the radioactivity (cpm) of a blank filter at that temperature was plotted as cumulative radioactivity v. temperature.

Gradient Hybridization

Aliquots (20 µl) of each DNA-containing fraction from a Ag⁺–Cs₂SO₄ preparative gradient were loaded onto filters as discussed above. The peak satellite aliquot contained about 1·2 µg of DNA. Filters were preheated and hybridized with DS ³HcRNA at 45°C for 3 h, and, after RNAse treatment, were dried and counted.

Leucocyte Culture

Freshly collected blood (8 ml) was incubated at 37°C for 3 min–1 h with 2 ml dextran to precipitate the red cells. The leucocytes were collected and added at a concentration of 10% (v/v) to Hamm's F10 medium supplemented with 10% (v/v) foetal calf serum, 4% (v/v) phytohaemagglutinin, 60 µg/ml penicillin and 100 µg/ml streptomycin. After incubation for 60–69 h at 37°C, colchicine was added to a final concentration of 2·5 × 10⁻⁷ M for 3 h prior to harvest. Following hypotonic treatment in 0·075 M KCl (15 min, 37°C) the cells were fixed in 95% (v/v) ethanol: 45% (v/v) acetic acid (1 : 1).

* Four times the concentration of SSC as given.
Cytological Hybridization

In situ hybridization was by a modification of the method of Pardue and Gall (1970). Air-dried slides were dehydrated in 100% ethanol, incubated at 37°C for 10 min in 0.2 M HCl to denature the DNA, then dehydrated through an alcohol series and air dried. 5–10 µl of DS 3HcRNA in 3 × SSC–F (5 × 10⁵–10 × 10⁶ cpm) were placed on each slide which was then covered with a 22-mm² coverslip and sealed with rubber solution. The slides were held in water at 65°C for 5 min, then incubated at 35°C for 3 h. The coverslips were removed in two, 3 × SSC–F rinses at the incubation temperature, and the slides washed twice in 2 × SSC. Treatment with pancreatic and T1 RNAses in 2 × SSC at room temperature for 1 h was followed by extensive washing in 2 × SSC, dehydration in ethanol and coating with Ilford K2 emulsion (50% dilution) for exposure in plastic boxes at 4°C for at least 4 days.

![Buoyant density vs Temperature](image)

**Fig. 1.** (a) Neutral CsCl gradient of 2 µg of wallaroo DNA. The peak at 1.731 g/cm³ is a marker (1 µg of Micrococcus luteus DNA). (b) Thermal denaturation of wallaroo total DNA in TE (●). Optical density was measured at 260 nm. E. coli DNA was included as a control (○).

C-banding

C-banding was by a modification of the Sumner (1972) Ba(OH)₂ method. Slides were precleaned with chromic acid before cells were applied. Ba(OH)₂ was made fresh each time, as a hot, saturated solution. Slides were placed in it for less than 5 min, rinsed briefly in distilled water, and incubated in 2 × SSC at 65°C for 1 h. After being rinsed in distilled water they were stained horizontally for 10–60 min at room temperature in 10% Giemsa. They were rinsed quickly with buffer, then with water and dried.
Results

Characterization of Wallaroo DNA

In neutral CsCl gradients total nuclear DNA of M. r. robustus was resolved into two bands (Fig. 1a): a major peak (mainband DNA) at a density of 1·697 g/cm³ and a small peak (satellite DNA) amounting to about 8·5% of the total DNA (estimated from areas in analytical traces) at a density of 1·710 g/cm³. The buoyant density of the satellite corresponds to a guanine + cytosine content of 51% compared with guanine + cytosine content of 38% for the mainband DNA (Mandel et al. 1968). The melting profile of total wallaroo DNA (Fig. 1b) shows a broadening at higher temperatures, which may be due in part to the satellite DNA sequences.

![Optical density at 260 nm](image)

Fig. 2. (a) Preparative Ag⁺–Cs₂SO₄ gradient of wallaroo DNA. Moles of Ag⁺ per optical density unit of DNA = 5 x 10⁻⁴. MB, mainband DNA. (b) Neutral CsCl gradient of impure wallaroo satellit e DNA and M. luteus marker. Main-band DNA is evident. (c) Neutral CsCl gradient of wallaroo purified satellite DNA and M. luteus marker.

Isolation and Characterization of Satellite DNA

The satellite DNA fraction cannot be isolated in pure form from neutral CsCl gradients so Ag⁺–CsSO₄ gradients were used to achieve better separation of the satellite DNA from mainband DNA as shown in Figs 2a and 2b. Examination of DNA from pooled ‘satellite’ fractions in a neutral CsCl gradient results in satellite DNA free from any apparent main-band contamination (Fig. 2c). In alkaline gradients the complementary strands have densities of 1·746 and 1·816 g/cm³.

Thermal denaturations of native and renatured satellite DNA (in 0·12 M phosphate buffer, equimolar NaH₂PO₄ and Na₂HPO₄) are shown in Fig. 3. Tₘ of the native satellite is 90°C which indicates a guanine + cytosine content of 50·2% (Mandel and Marmur 1968), in good agreement with the estimate of 51% based on buoyant density. The renatured satellite shows a much broader melt with a Tₘ of 78°C. The ΔTₘ of 12°C between native and renatured satellite indicates 9% sequence mismatch in the renatured molecules (Blumenfeld 1973). The renatured satellite DNA bands at a density of 1·715 g/cm³ which is an increase of 0·005 g/cm³ from native satellite, and is consistent with sequence heterogeneity of the magnitude estimated from the ΔTₘ.
**Fig. 3.** Thermal denaturations in 0·18 M Na+ of native satellite DNA (●), renatured satellite DNA (▲), and E. coli standard (○). Optical density was measured at 260 nm.

**Fig. 4.** Thermal denaturation of wallaroo satellite filter cRNA/DNA hybrids (○) relative to D. melanogaster cRNA/DNA hybrids (●). Details are given in Materials and Methods.

**Fig. 5.** Gradient hybridization of M. r. robustus satellite 3HcRNA (●) to fractions of an Ag+–CS2SO4 gradient of total DNA (see Materials and Methods) indicated by optical density at 260 nm (■). Radioactivity in the peak satellite fraction was measured at 29000 cpm.
Characterizations using Satellite cRNA

Radioactive complementary RNA (\(3^3\text{HcRNA}\)) was synthesized from the pure double-stranded satellite DNA. To enable the use of this \(3^3\text{HcRNA}\) in filter hybridization studies, an accurate determination of the \(T_m\) of the wallaroo RNA–DNA hybrid was made by simultaneous comparison with a melt of hybrids of the 1.705 g/cm\(^3\) satellite probe of *Drosophila melanogaster* with total DNA of *D. melanogaster (\(T_m\)
determined as 61°C in 3×SSC-F, Brutlag et al. 1977). Duplicate filters gave a corrected value of 52.5°C for the \( T_m \) of wallaroo cRNA–DNA hybrids in 3×SSC-F (Fig. 4).

### Table 1. In situ hybridization of \(^{3}\)HcRNA to wallaroo chromosomes

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Total No. of grains stored</th>
<th>Mean No. of grains per chromosome</th>
<th>s.e.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1174</td>
<td>16.3</td>
<td>±0.52</td>
</tr>
<tr>
<td>2</td>
<td>740</td>
<td>10.3</td>
<td>±0.37</td>
</tr>
<tr>
<td>3</td>
<td>686</td>
<td>9.5</td>
<td>±0.39</td>
</tr>
<tr>
<td>4</td>
<td>1150</td>
<td>16.0</td>
<td>±0.46</td>
</tr>
<tr>
<td>5</td>
<td>1098</td>
<td>15.3</td>
<td>±0.58</td>
</tr>
<tr>
<td>6</td>
<td>681</td>
<td>9.5</td>
<td>±0.37</td>
</tr>
<tr>
<td>7</td>
<td>17</td>
<td>0.24</td>
<td>±0.058</td>
</tr>
<tr>
<td>X</td>
<td>305</td>
<td>8.5</td>
<td>±0.49</td>
</tr>
<tr>
<td>Y</td>
<td>39</td>
<td>1.6</td>
<td>±0.23</td>
</tr>
</tbody>
</table>

The \(^{3}\)HcRNA was hybridized to each fraction of a \( \text{Ag}^+ \text{Cs}_2\text{SO}_4 \) preparative gradient, and the results in Fig. 5 show that the satellite sequences are not extensively interspersed with other sequences of the genome. The satellite sequences are almost entirely contained in the fractions of the satellite DNA peak, suggesting that the satellite sequences occur principally in long blocks in the genome.

### Table 2. Hybridization of wallaroo satellite \(^{3}\)HcRNA to filter-bound DNA of macropod species

<table>
<thead>
<tr>
<th>Source of DNA bound to filter</th>
<th>Radioactivity per 10 ( \mu )g of bound DNA (cpm)</th>
<th>Radioactivity relative to that of ( M. \text{r. robustus} ) (% cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( M. \text{r. robustus} ) (wallaroo)</td>
<td>22000</td>
<td>100</td>
</tr>
<tr>
<td>( M. \text{r. erubescens} ) (euro)</td>
<td>28500; 27000(^a)</td>
<td>130; 126(^a)</td>
</tr>
<tr>
<td>( M. \text{antilopinus} ) (antilopine)</td>
<td>20300</td>
<td>93</td>
</tr>
<tr>
<td>( M. \text{rufus} ) (red kangaroo)</td>
<td>6600</td>
<td>30</td>
</tr>
<tr>
<td>( M. \text{rufogriseus} ) (red-necked wallaby)</td>
<td>3500</td>
<td>16</td>
</tr>
<tr>
<td>( M. \text{giganteus} ) (eastern grey kangaroo)</td>
<td>1000</td>
<td>5</td>
</tr>
<tr>
<td>( M. \text{fuliginosus} ) (western grey kangaroo)</td>
<td>470</td>
<td>2</td>
</tr>
<tr>
<td>( M. \text{eugenii} )(^b) (tammar)</td>
<td>380</td>
<td>2</td>
</tr>
<tr>
<td>( \text{Wallabia bicolor} )(^b) (swamp wallaby)</td>
<td>350</td>
<td>2</td>
</tr>
<tr>
<td>( \text{Petrogale xanthopus} ) (rock wallaby)</td>
<td>550</td>
<td>3</td>
</tr>
<tr>
<td>( \text{Petaurus breviceps} ) (feather glider)</td>
<td>360</td>
<td>2</td>
</tr>
</tbody>
</table>

\(^a\) Replicate experiments.

\(^b\) \( M. \text{eugenii} \) and \( W. \text{bicolor} \) data were obtained from thermal denaturation experiments.

**Chromosomal Location of Satellite DNA**

Analysis of the distribution of satellite sequences by in situ hybridization of cRNA to metaphase chromosomes from peripheral lymphocytes showed that the blocks of satellite sequences have a centromeric location and that there are different-sized blocks on the different chromosomes (Fig. 6). This pattern has been quantified by grain counts on 36 chromosome sets (Table 1). Chromosomes 1, 4 and 5 show the largest satellite blocks; 2, 3, 6 and X show an intermediate amount; and 7 and Y have very few sequences. The labelled blocks correspond to heterochromatic regions indicated by C-banding.
Presence of Wallaroo Satellite Sequences in Other Macropods

$^3$HcRNA of wallaroo satellite DNA was used as a probe for identification of related sequences in other species in the macropod group.

Analysis by filter hybridization

In other macropods, the presence of sequences similar to those of wallaroo satellite DNA was monitored by the hybridization of the satellite cRNA to filter-bound DNA of the other species. The extent of hybridization is shown in Table 2. Of the species tested, all are in the Macropodinae, except *P. breviceps* which is in the Petauridae; all are included in the Phalangeroidea. The wallaroo, euro, and antilopine (wallaroo group) show the greatest content of satellite sequences in their genomes, and they are also the most closely related of those species tested. An important distinguishing feature of the wallaroo and euro subspecies is that the euro has a larger heterochromatic X chromosome (Sharman 1974); this may partly account for the larger amount of satellite sequences present in the euro. The red kangaroo, which is considered to be closely related to the wallaroo group, shows an intermediate level of satellite sequences. *M. rufogriseus* has not been thought to be particularly related to the wallaroos, yet it has 16\% of the amount of satellite sequence occurring in the wallaroo. The data are not definitive in a quantitative sense as there was no measure of retention of unlabelled DNA on the filters, and it is not known if saturating conditions were used, but the relative contents are similar in replicate experiments.

![Fig. 7. Thermal denaturation of hybrids of satellite $^3$HcRNA of *M. r. robustus* and filter-bound DNA of several macropods. Melting profiles of *M. r. robustus*, *M. r. erubescens* and *M. antilopinus* (---) were superimposed with a $T_m$ of 52°C. Profiles for *M. fuliginosus* ($T_m = 48.2°C$), *M. rufogriseus* ($T_m = 48.4°C$), *M. eugenii* ($T_m = 47.3°C$), *M. rufus* ($T_m = 48.4°C$), *W. bicolor* ($T_m = 49.1°C$) and *M. giganteus* ($T_m = 49.1°C$) all fell into the hatched region in the figure.](image)

The fidelity of the hybrids formed in the experiments of Table 2 was examined in order to test the degree of conservation of sequence; any differences in $T_m$ between these hybrids and the homologous wallaroo–wallaroo hybrids would indicate sequence differences (Fig. 7). The wallaroo, antilopine and euro yield virtually identical melting plots. The other species show a $\Delta T_m$ of about 4°C, indicating about 3\%
Table 3. In situ hybridization to the species of the wallaroo group

Numbers in brackets are the percentage content of satellite sequences in each chromosome of the complement.

<table>
<thead>
<tr>
<th>Source of $^3$HcRNA/ source of chromosomes</th>
<th>No. of cells</th>
<th>Chromosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Wallaroo/wallaroo (W/W)$^b$</td>
<td>36</td>
<td>1174(19.9)</td>
</tr>
<tr>
<td>Wallaroo/euro (W/E)$^a$</td>
<td>33</td>
<td>686(19.3)</td>
</tr>
<tr>
<td>Wallaroo/western euro (W/C)$^A$</td>
<td>36</td>
<td>894(19.6)</td>
</tr>
<tr>
<td>Wallaroo/antilopine (W/A)</td>
<td>40</td>
<td>825(29.1)</td>
</tr>
<tr>
<td>Euro/wallaroo (E/W)</td>
<td>24</td>
<td>719(19.9)</td>
</tr>
<tr>
<td>Euro/western euro (E/C)$^A$</td>
<td>24</td>
<td>536(20.8)</td>
</tr>
</tbody>
</table>

$^a$ $\chi^2$ heterogeneity: W/C v. W/E, $\chi^2_{(3)} = 68.5, p \ll 0.001$; W/C v. E/C, $\chi^2_{(3)} = 37.3, p \ll 0.001$.

$^b$ $\chi^2$ homogeneity: two sets of data from W/W, $\chi^2_{(8)} = 6.05, 0.70 > p > 0.50$. 

\[ \begin{align*} \chi^2 \text{ heterogeneity: W/C v. W/E, } \chi^2_{(3)} &= 68.5, p \ll 0.001; \\ \chi^2 \text{ homogeneity: two sets of data from W/W, } \chi^2_{(8)} &= 6.05, 0.70 > p > 0.50. \end{align*} \]
sequence mismatch relative to the wallaroo homologous reaction. The differences between these and other species cannot be considered significant as the amount of $^3$HcRNA bound to many of the macropod DNAs was one to two orders of magnitude lower than the binding to wallaroo DNA (Table 2). Thus it appears that the sequences in the wallaroo satellite are conserved in the closely related wallaroo group, and have only slightly 'diverged' in the remainder of the macropods.

Fig. 8. Karyotypes from *in situ* hybridization of wallaroo satellite $^3$HcRNA to metaphase chromosomes of (a) wallaroo, (b) euro, (d) antilopine, (e) red-necked wallaby, (f) red kangaroo. Row (c) shows C-banded chromosomes of the euro. Chromosome numbering does not correspond exactly to the red kangaroo as it has a $2n=20$ complement and is a female. A chromosome of pair 1 was missing from the photograph of the karyotype of the red-necked wallaby, so the chromosome 1 which was present is shown twice.

*Analysis by in situ hybridization*

The question of the chromosomal distribution of the satellite sequences was examined by *in situ* hybridization. Two $^3$HcRNA probes were used, one from the wallaroo satellite DNA and the other from the euro satellite DNA, isolated by the
same method. Grain counts were taken on 24–40 sets of metaphase chromosomes in each case. The basic pattern of centromeric hybridization seen in the wallaroo is observed in the other members of the wallaroo group (Table 3). Again chromosomes 1, 4 and 5 show the largest satellite blocks, 2, 3, 6 and X have intermediate blocks, and 7 and Y show little satellite sequence. $\chi^2$ contingency tests comparing the wallaroo distribution with each other species do show significant differences between them, whereas a $\chi^2$ test on two sets of data from the homologous wallaroo hybridization shows homogeneity (Table 3). Since the homogeneous $\chi^2$ data are from one experiment it can be concluded that any errors in experimental scoring are not significant.

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>X</th>
<th>Y</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. robustus robustus</em> (wallaroo)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><em>M. rufus</em> (red kangaroo)</td>
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<td><em>M. rufogriseus</em> (red-necked wallaby)</td>
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**Fig. 9.** Chromosomal distribution of wallaroo satellite sequences (shown in black on the chromosomes) of the wallaroo, red kangaroo, and red-necked wallaby. Arrangement of the chromosomes of the red kangaroo is after Hayman and Martin (1974).

However, until further data are collected using more than one animal in both homologous and heterologous hybridizations, the observed inter- and subspecies differences cannot be considered biologically significant. This same qualification applies to differences which have been shown with probes of satellite $^3$HcRNA from the wallaroo and euro (Table 3). The heterogeneity observed in any particular comparison cannot be ascribed to specific chromosomes, but rather is due to quantitative differences in most of the chromosomes. An exception is in the striking lack of wallaroo satellite sequences on chromosome-pair 5 in the antilopine. This was correlated with a lack of heterochromatin by the C-banding technique. A second antilopine, paternally related to the first, showed one chromosome of pair 5 unlabelled, while the other bound a reduced amount of cRNA compared with the wallaroo pair-5 chromosome. The
first euro which was examined showed cRNA binding to only one chromosome 5, and a second euro showed the same heterozygosity; this was also confirmed by C-banding (Fig. 8). Both euros were collected in the wild, about 100 miles apart. An absence of C-banding on one chromosome of the fifth pair in a wallaroo has also been observed (G. B. Sharman, personal communication).

The differences in amount of sequences similar to those of wallaroo satellite DNA in other macropods may reflect proportional decreases of the sequence on all of the chromosomes, or could represent decreases on only certain chromosomes. The locations of the sequences present in the genomes of *M. rufus* and *M. rufogriseus* were examined by *in situ* hybridization (Fig. 8). Binding of the cRNA was mainly to the X chromosome in both cases, in large, non-centromeric blocks and in blocks in the region of the nucleolus organizer (Fig. 9). *M. rufogriseus* also showed significant binding to the centromere of the Y chromosome, but a male red kangaroo was not examined. The only major blocks on autosomes were those at the centromeres of the four subtelocentric chromosomes in the red kangaroo. These four chromosomes are thought to be related to chromosomes 5 and 6 in the wallaroo karyotype, either by centromeric fusion or fission (Hayman and Martin 1974). The presence of sequence blocks like those of wallaroo satellite DNA on only certain chromosomes of the red kangaroo and red-necked wallaby showed that, in this case, the ‘reduction’ in the amount of the sequences indicated by filter binding was not proportional on all the chromosomes.

**Discussion**

*Buoyant Density*

The data in this study describe the isolation and characterization of the major buoyant-density satellite of *M. r. robustus*, in terms of its structure and organization within the wallaroo genome, and its presence and conservation in the genomes of related macropods. The buoyant-density profile of total DNA of *M. r. robustus* can be compared with those of other macropods, with the qualification that similarities in buoyant density need not imply major sequence homology. *M. rufogriseus* shows two peaks in a neutral CsCl gradient: a major peak of density 1·698 g/cm³ and a satellite peak with a density of 1·708 g/cm³ (Dunsmuir 1976). There is also a major satellite which can be isolated in an actinomycin D–CsCl gradient, and has a density of 1·698 g/cm³ in neutral CsCl. The satellites account for about 20 and 5% of the total DNA, respectively. Other macropods showing a distinct satellite in neutral CsCl are *M. r. erubescens* and *M. antilopinus* (Peacock and Venolia, unpublished data). *M. r. erubescens* shows a main-band DNA at a density of 1·697 g/cm³ and about 10% of the DNA in a satellite at 1·709 g/cm³. *M. antilopinus* has a mainband density of 1·695 g/cm³ and about 10% of its DNA in a satellite with density 1·710 g/cm³. The close relationships between *M. r. robustus*, *M. antilopinus* and *M. r. erubescens* suggest that the satellites seen in the three taxa are composed of the same highly repeated DNA sequence and the hybridization data of the present study confirm that this is the case.

The other macropod DNA density profiles we have examined are of the red kangaroo, eastern and western grey kangaroos, and the swamp wallaby, *Wallabia bicolor*. All show a major DNA peak at a density of about 1·696 g/cm³, and all but *W. bicolor* have a heavy shoulder. The small amount of sequences like those
of wallaroo satellite DNA detected in these species by filter-binding experiments would not be expected to appear as a distinct satellite, but could be contained within the heavy shoulder.

Chromosomal Distribution

The wallaroo

The homologous in situ hybridization of the wallaroo satellite to wallaroo nuclei showed that the chromosomes have different sizes of satellite blocks at their centromeres. These blocks range in size from about $2.7 \times 10^7$ base pairs on chromosome 1 to $4 \times 10^3$ base pairs on chromosome 7. (These results are derived from DNA values cited by Rendel and Kellerman (1955) and Hayman and Martin (1974), and the relative satellite distribution has been determined by grain counts.) However, several chromosomes have similarly sized blocks, and thus size difference alone does not completely differentiate the centromere regions of all chromosomes. Data on sequence locations in other eukaryotes suggest that the heterochromatin of each chromosome has a specific array (location and amount) of sequences (Peacock et al. 1977). In M. rufogriseus there is some variation in the locations of the two analysed satellites, distinguishing a few chromosomes (Dunsmuir 1976). However, the major satellite shows extensive, non-random sequence changes (Dennis et al. 1980), and these tandemly arranged isomers may provide a level of chromosomal specificity equal to that seen in Drosophila melanogaster. The same possibility applies to the wallaroo since sequence heterogeneity within the wallaroo satellite was shown by its increased buoyant density and lowered $T_m$ following renaturation. The observed mismatching could result either from random mutation of base pairs or from improper pairing of isomers of a basic repeating unit (Brutlag and Peacock 1975). Nucleotide sequencing of the satellite will distinguish between these two possibilities.

The wallaroo group

Data from thermal denaturation studies (Fig. 7) showed no detectable differences between the DNA sequences of the wallaroo, euro and antilopine. The contingency tests on the in situ hybridization data (Table 3) showed heterogeneity for the different animals, but not enough animals have been tested to determine if these differences are biologically significant. With the exception of chromosome 5, the qualitative distribution of sequences similar to those of wallaroo satellite DNA is the same on the chromosomes of the wallaroo, euro, antilopine and M. r. cervinus. The data indicate that the antilopine may be quantitatively different from the wallaroo, and a confirmation of this difference would be consistent with a slightly greater phylogenetic distance from the wallaroo.

The close relationships in the wallaroo group are underscored by the presence of the previously noted 'deletion-polymorphism' on chromosomes of the fifth pair. Even though the 'deletion' is substantial, the occasional autoradiographic grain seen on these chromosomes indicates that as many as $5 \times 10^4$ base pairs could still be present. It is not possible to state when the 'deletion' occurred, but its presence in the three taxa suggests that it originated prior to speciation. Another explanation is that the polymorphism occurred in one of the species and has been transferred by introgression into the others. Richardson and Sharman (1976) show some evidence for introgression between antilopine and euro populations in the Northern Territory.
Other related species

While the presence of sequences similar to those of wallaroo satellite DNA has been shown in many related macropods (i.e. *M. rufus, M. rufogriseus, M. giganteus, M. fuliginosus, M. eugenii* and *W. bicolor*), the hybrid $\Delta T_m$ showed that the sequences are slightly different. An experiment using the euro satellite probe gave parallel results to those of the wallaroo satellite. The locations of the wallaroo satellite sequences on the chromosomes of these related species have been examined only in *M. rufus* and *M. rufogriseus*. It was shown that the sequence has not been proportionally reduced on all the chromosomes. In both species there are large blocks on the X chromosome, some being associated with the nucleolus organizer. In *M. rufogriseus* the sequences also occur on the Y chromosome, and in *M. rufus* centromeric segments are found on four acrocentric autosomes. The association of the wallaroo satellite sequence with the nucleolus organizer in the wallaroo group and in *M. rufus* and *M. rufogriseus* may indicate a function of this highly repetitive sequence. Furthermore, it is possible that the sequence occurring near the organizers is a particular sequence isomer of the wallaroo satellite sequences. The evidence of hybrid $\Delta T_m$ shows that the sequences on the chromosomes of *M. rufus* and *M. rufogriseus* are ‘diverged’ from the bulk of the wallaroo sequences by about 3%. The particular set of repeats of the sequence on the wallaroo X chromosome may also show this ‘divergence’ from those elsewhere in the complement. Speculations on the function of the sequence might include a role in ‘escorting’ the nucleolus organizer region in the nucleus, a role in guiding particular elements of ribosomal synthesis to their proper locations, or a role in the transcription or amplification of the region.

The presence of the wallaroo satellite sequence on the four acrocentric autosomes of *M. rufus* represents a highly non-random occurrence of these sequences in the genome, since these particular chromosomes are involved in chromosome alterations which differentiate macropod species groups. These four autosomes are considered to be related to pairs 5 and 6 of the more common 2n = 16 karyotypes, the differences in chromosome number being explained by fusion or fission of these chromosomes (Hayman and Martin 1974). The argument has been presented that the primitive macropod karyotype is 2n = 22 (cf. Hayman and Martin 1969; Sharman 1973), indicating that the four autosomes in question have remained unfused in the red kangaroo. In any case, it is clearly of interest to determine the other highly repeated sequences present on the acrocentric autosomes of *M. rufus* to provide more clues as to the direction and nature of karyotype change.

Phylogenetic Implications

The analysis of the wallaroo satellite sequences in the genomes and chromosome complements of the wallaroo group confirm the integrity of this grouping. As discussed above, if any one taxon of those studied is to be set apart from the others in satellite distribution it is the antilopine. *M. r. cervinus*, the western euro, has been considered a separate subspecies, mainly because of its different coat colour. Although the data presented here show small, statistically significant differences in the distribution of the wallaroo satellite on the euro and on *M. r. cervinus*, they do more to emphasize the relatedness of the two taxa than to show any subspecific differentiation.

The wallaroo satellite data indicate that both *M. rufus* and *M. rufogriseus* are related to the wallaroo group, and while this relationship has been previously shown
by other data for \textit{M. rufus}, this has not been the case with \textit{M. rufogriseus}. A closer relationship of \textit{M. rufogriseus} to \textit{M. rufus} than had previously been anticipated was also suggested by data of Dunsmuir (1975) in her analysis of both satellites of \textit{M. rufogriseus}. She showed that their presence in the genome of \textit{M. rufus} exceeded that in either grey kangaroo or in the swamp wallaby, and that the minor satellite sequence was unchanged in \textit{M. rufus}.

The suggestion of an evolutionary relationship between \textit{M. rufogriseus} and the wallaroo/\textit{M. rufus} group depends largely on the presence of sequences similar to those of wallaroo satellite DNA in significant amounts on the X and Y chromosomes. Hayman and Martin (1974), in discussing the implications of the size of the X chromosome in the Macropodidae which show a continuous distribution from small to large X chromosomes, suggested the changes are largely due to increased heterochromatin content. Hayman and Martin suggested that the continuous size distribution is consistent with local increases of relatively small units in the chromosomes. The 'wallaroo satellite' blocks on the large X chromosomes of \textit{M. rufogriseus} and \textit{M. rufus} may reflect such segmental amplifications of a particular repeating sequence. The blocks of the satellite on the X chromosomes of these two species are actually greater (two to five times) than that on the wallaroo X chromosome. It will be of interest to determine if similar amplifications have occurred in \textit{M. parryi} which has the largest X chromosome of the macropods.

As information on other highly repeated sequences in this group of species, becomes available, some light may be thrown on the question as to whether there is any significance to the 'choice' of the wallaroo satellite sequence for amplification

on the sex chromosomes, particularly around the nucleolus-organizer region.

References


Repeated DNA from the Wallaroo


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