

Satellite DNA Sequences in the Red Kangaroo (*Macropus rufus*)

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Abstract

There is a complex pattern of satellite DNA sequences in *M. rufus* which are revealed by addition of Ag⁺ or dye (Hoechst 33258) to the DNA in Cs₂SO₄ or CsCl equilibrium density gradients. Six satellite DNA fractions have been isolated; these have buoyant densities in neutral CsCl of 1.692, 1.704, 1.705, 1.707 (two), 1.710 and 1.712 g/ml compared with 1.696 g/ml for the main band DNA. Each satellite accounts for 1-3% of the DNA of the genome.

The satellites are located in the centromeric heterochromatin of the chromosomes, in the nucleolar organizer region and in interstitial bands on some of the autosomes, each satellite having a unique distribution. Nucleic acid hybridization showed that six of the satellite sequences are also present in the genomes of the wallaroo and the red-necked wallaby, with sequence divergences of only 1-2% relative to the sequences in the red kangaroo.

Extra keywords: *in situ* hybridization.

Introduction

The red kangaroo, *Macropus rufus*, is one of the 15 large marsupial species in the family Macropodidae. It has a diploid chromosome number of $2n = 20$, unlike most of the macropods which have $2n = 16$ (Hayman and Martin 1974). This difference in chromosome number led to the recognition of the red kangaroo as a monotypic genus *Megaleia*. As evidence accumulated, the relatedness of the red kangaroo to other *Macropus* species became clear and it is now recognized as a *Macropus* species (Kirsch and Calaby 1977).

Dunsmuir (1976) and Venolia and Peacock (1981), on the basis of the distribution of highly repeated DNA sequences isolated from the red-necked wallaby and the wallaroo, suggested that these two species are closely related to the red kangaroo. Peacock *et al.* (1981) isolated a highly repeated DNA sequence from the red kangaroo and used it as a hybridization probe which again suggested a close relationship between the red kangaroo, the wallaroo and the red-necked wallaby. We have isolated and characterized several satellite DNAs from the red kangaroo and shown that at least six of these sequences occur in the red-necked wallaby and the wallaroo genomes. Our study agrees with the previous results regarding relatedness of the three species and has indicated the complexity of the complement of highly repeated DNA in their genomes.

Materials and Methods

Characterization of Satellite DNA by Analytical Ultracentrifugation

Analytical ultracentrifugation was carried out in a Beckman Model E ultracentrifuge for 18 h at 44 000 rpm and 25°C. In neutral gradients a solution of 1–3 µg DNA in 0.01 M Tris-HCl, pH 8.4, 0.001 M EDTA (TE) was adjusted to a density of 1.71 g/ml with CsCl and 1 µg *M. luteus* DNA was added as a marker ($\rho = 1.731$ g/ml).

In Ag^+ - Cs_2SO_4 gradients, DNA in TE was complexed with Ag^+ by the addition of AgNO_3 solution. The amount of Ag^+ was adjusted until the buoyant density separation of satellites from the bulk of the DNA was maximized. Borate buffer (50 mM Na_2SO_4 , 25 mM $\text{Na}_2\text{B}_4\text{O}_7$, pH 9.1) was added to 23% (v/v) and Cs_2SO_4 was added to a density of 1.45–1.55 g/ml.

The dye Hoechst 33258 was added to DNA in TE at a ratio of about 1:1 (w/w, dye to DNA) and CsCl was added to a density of 1.68 g/ml.

In alkaline gradients DNA in TE was adjusted to 0.1 M NaOH and to a density of 1.78 g/ml with CsCl.

Isolation of Satellite DNA

DNA was isolated from red kangaroo liver tissue as described by Venolia and Peacock (1981). Satellite DNAs were isolated using preparative Ag^+ - Cs_2SO_4 and Hoechst 33258-CsCl gradients. Conditions for isolation were as established in analytical gradients. The solution was centrifuged at 44 000 rpm for at least 48 h in a Beckman Ti 50 rotor, and 0.25–0.5-ml fractions were collected from the top or bottom of the tube, according to satellite position relative to the main band of DNA. The pooled fractions were dialysed against 2 M NaCl to remove Ag^+ or extracted with isopropanol saturated with CsCl to remove Hoechst 33258. To remove Cs_2SO_4 or CsCl the solution was dialysed against TE. Single-stranded satellite DNA was isolated in alkaline CsCl gradients made up to conditions of the analytical gradients. Following centrifugation, peak fractions were combined and dialysed against TE to neutralize the DNA solution.

In situ and Filter Hybridization

Metaphase chromosomes were prepared as described by Venolia and Peacock (1981) except that colchicine was added to a final concentration of 1 µg/ml 1.5 h prior to harvest. Complementary RNA (cRNA) was synthesized and hybridized to metaphase chromosomes or nitrocellulose filters as described by Venolia and Peacock (1981). For determination of melting temperatures, filters were extensively washed after hybridization and radioactivity bound to the filter (from 8000 to 30 000 cpm) was eluted at successively higher temperatures with an initial temperature below the hybridization temperature.

Restriction Enzyme Digestion

Restriction enzymes were prepared by a modification of the method of Greene *et al.* (1978). 1–10 µg of DNA in 20–30 µl of appropriate buffer was digested for 1–2 h at 37°C, or 65°C for the enzyme TaqI. Assay conditions for restriction enzymes were as described in New England Biolabs catalogue (1978) (New England Biolabs Inc., 283 Cabot St, Beverly, Ma.). The quantity of enzyme used was the minimum amount which gave a pattern unaltered either by additional enzyme or by a prolonged incubation period (about 1–4 units per microgram of DNA). Reactions were stopped by heating the sample to 65°C for 5 min, with addition of 10 µl solution containing glycerol and bromophenol blue.

Gel Electrophoresis

Gels (6–12% w/v) contained acrylamide : bisacrylamide (50 : 1) in Leoning buffer (40 mM Tris, 20 mM NaCH_3COO , 2 mM EDTA, pH 7.5) and were subjected to electrophoresis vertically at about 15 mA at room temperature for 15 h. Gels were stained with ethidium bromide (EtBr) (1 µg/ml) for 10 min then illuminated with ultraviolet light and photographed with Polaroid film type 655 through a Kodak yellow filter (No. 15).

Results and Discussion

Characterization of Satellite DNA

In neutral CsCl gradients red kangaroo nuclear DNA forms a single asymmetrical band of density 1.696 g/ml with a slight shoulder on the heavy side (Fig. 1). The buoyant density of the main band corresponds to a GC content of 37% (Mandel *et al.* 1968). All other macropods examined have a similar density main band DNA (Peacock *et al.* 1981).

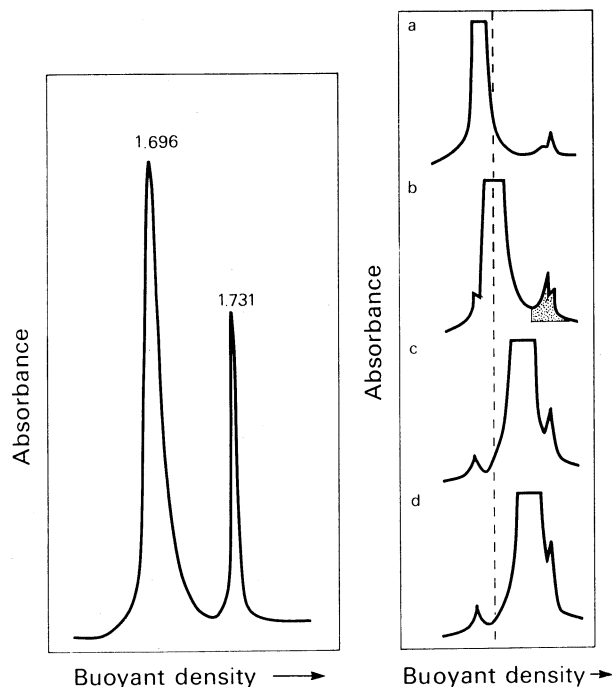


Fig. 1 (left). Analytical CsCl equilibrium gradient of 2 μ g red kangaroo DNA. The peak at 1.731 g/ml is a marker (0.5 μ g *Micrococcus luteus* DNA).

Fig. 2 (right). Analytical Ag⁺-Cs₂SO₄ density gradient profiles of red kangaroo DNA. Each gradient contained 34 μ g DNA complexed with Ag⁺ at the following R_F values (R_F = mole ratio of Ag⁺ to DNA phosphate): a, 0.03; b, 0.043; c, 0.055; d, 0.067. The dashed line represents a buoyant density of 1.500 g/ml. The hatched region in (b) was isolated and used as described in Fig. 3.

When red kangaroo DNA is complexed with Ag⁺, which binds preferentially to GC-rich sequences (Jensen and Davidson 1966), the DNA is resolved into main-band and satellite peaks (Figs 2a-2d). As increasing amounts of Ag⁺ are added to a constant amount of DNA, sequences binding the most Ag⁺ appear as a satellite on the heavy side of the gradient (Fig. 2a). With more Ag⁺ (Figs 2b-2d) additional DNA sequences resolve as satellites, indicating that there are a number of GC-rich sequence families, each with a different GC composition. With increasing Ag⁺ concentration the main-band sequences also bind more Ag⁺ and shift in the gradient disclosing, on the less-dense side of the gradient, satellite DNAs with lower Ag⁺-

binding capacity. At least four complex satellite peaks were revealed, each comprising 1–2% of the genome as estimated from areas underneath the satellite peaks in 265 nm absorbance profiles.

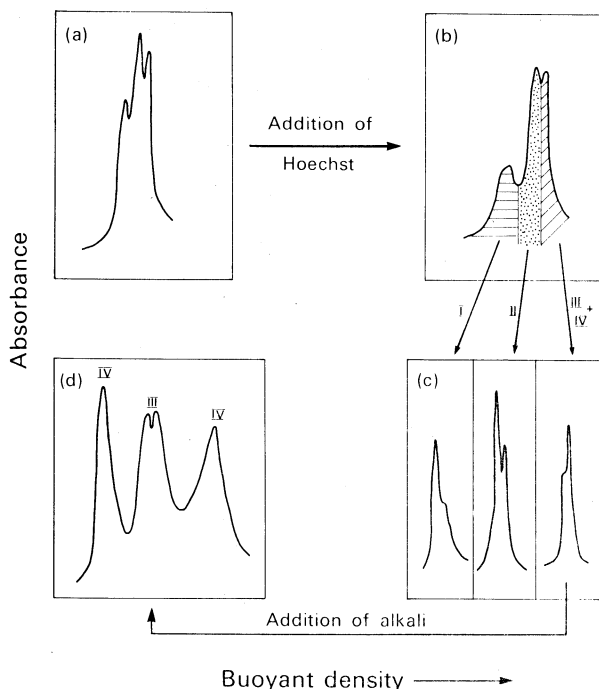


Fig. 3. Isolation of satellite DNA from the heavy side of a Ag^+ – Cs_2SO_4 gradient. (a) Analytical CsCl gradient of 3 μg of the satellite DNA hatched in Fig. 2b. (b) Analytical CsCl gradient of 3 μg of the satellite DNA in a complex with 2.5 μg Hoechst dye 33258. (c) Analytical CsCl gradients of 2–3 μg of each of the three fractions collected preparatively in (b). (d) Analytical alkaline CsCl gradient of fraction III + IV.

Four buoyant density species were isolated from the heavy side of the Ag^+ – Cs_2SO_4 gradient. Preparative Ag^+ conditions, as in Fig. 2b, were used to isolate a heterogeneous peak, which analytical buoyant density studies showed to be composed of a number of DNA species (Fig. 3a). Addition of Hoechst dye (which binds to AT-rich sequences) to this DNA in CsCl density gradients enabled further separation into three components (Fig. 3b). Fractions I and II have heterogeneous neutral and alkaline buoyant density profiles but were not further purified. The third component was shown by alkaline gradients to contain two DNA species (fractions III and IV) (Fig. 3d) which could be separated on a preparative scale. Satellite fractions V and VI were isolated from the light side of the gradient by successive Ag^+ – Cs_2SO_4 gradients (Fig. 4). An additional Ag^+ gradient resulted in the isolation of fraction VII (Fig. 4). The analytical buoyant density profiles suggest several of the fractions contain more than one buoyant density species (Table 1). It is likely that adjacent fractions in the gradient contain cross-contaminating sequences.

All the satellites were digested with a number of restriction enzymes (*EcoRI*, *HaeIII*, *TaqI*, *MboII*, *HindIII*, *BamHI*, *PstI*, *HhaI*) in an attempt to examine the

organization of their DNA sequences. Most enzymes left the satellite DNAs uncut, suggesting that all are composed of long stretches (more than 20 kb as indicated by gel electrophoresis) of tandemly arrayed sequences which do not contain recognition sites for the enzymes used. There were some exceptions. Satellite fraction I was

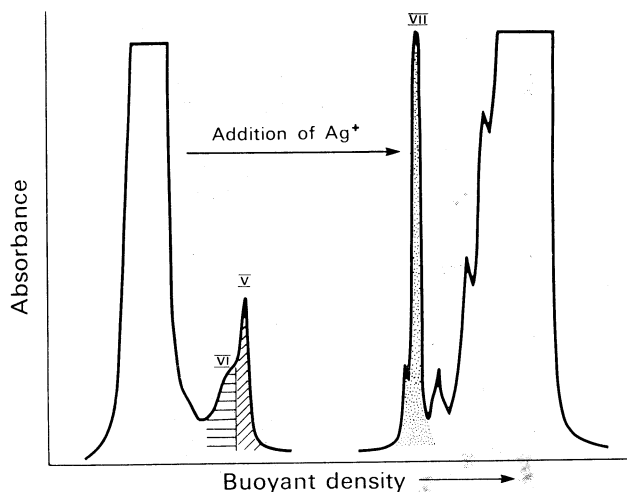


Fig. 4. Isolation of satellite DNA from the light side of the Ag^+ - Cs_2SO_4 gradient. (a) DNA from the least dense tenth of the gradient in Fig. 2c (including the satellite region on the left of the main band) was rerun in an analytical Ag^+ - Cs_2SO_4 gradient (Fig. 4a) with an R_F of 0.01. This resulted in the separation of two new satellite regions (hatched) which were isolated and correspond to fractions V and VI. (b) Analytical Ag^+ - Cs_2SO_4 gradient of 50 μg of the DNA remaining in Fig. 4a following removal of the fractions V and VI, and addition of Ag^+ ($R_F = 0.02$). The dotted region was isolated and corresponds to fraction VII.

digested by the enzyme *TaqI* to a series of fragments (Fig. 5a) and a remainder of uncut DNA at the top of the gel. The fragments form a complex pattern not corresponding to a simple monomer, dimer, trimer, etc. series. Although most of satellite fraction II is left undigested by *TaqI*, a small proportion of the DNA is digested into fragments having a pattern the same as that produced by satellite fraction I. This result is in agreement with the analytical buoyant density profiles of these satellites (Fig. 3) which indicated that the two fractions were cross contaminated.

Satellite fraction VII was digested by the enzyme *HaeIII* (Fig. 5b) leaving some DNA uncut in addition to producing bands with irregular periodicity. *HaeIII* and *TaqI* digest 20% of satellite fractions I and VII into discrete size fragments indicating they are composed of tandemly arrayed repeated sequences. The length and restriction map of these repeats could not be determined because of the complex band pattern and the lack of recognition sites for other enzymes, but the complexity in distribution of restriction sites and the varying intensities of bands indicate the presence of more than one repeating sequence in each fraction. Alternatively, the complexity could be due to subpopulations in the one satellite sequence similar to those found in a number of other satellite DNAs (Pech *et al.* 1979; Dennis *et al.* 1980).

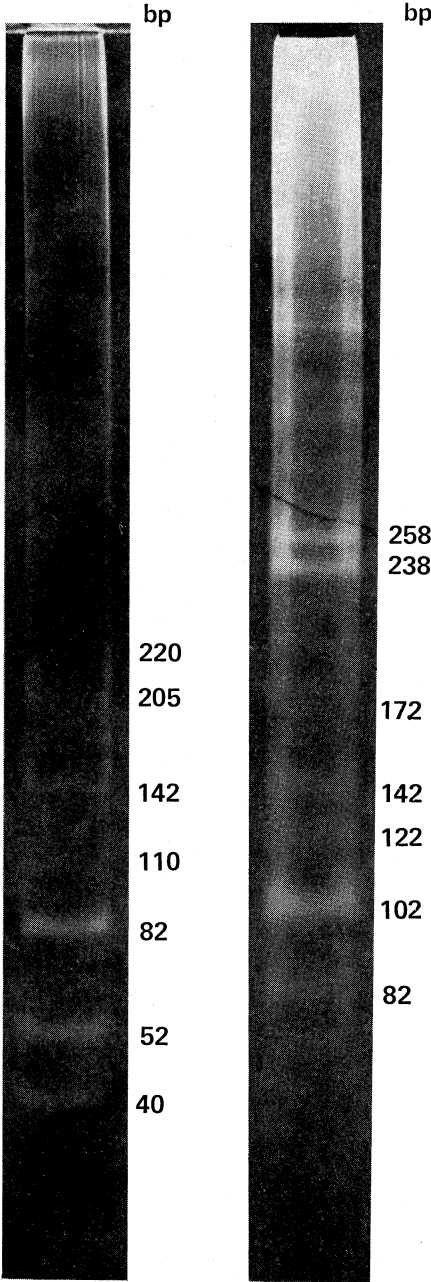


Fig. 5. Restriction enzyme analysis (10% w/v acrylamide gel). *Left:* 2 μ g of satellite fraction I digested with the restriction enzyme TaqI. *Right:* 2 μ g of satellite fraction VII digested with the restriction enzyme HaeIII.

Another way of characterizing and differentiating DNA species is to determine their melting profiles, the shape of the curve giving an indication of sequence homogeneity, and the melting temperature (T_m) reflecting base composition and sequence. [^3H]cRNA to each of the satellite fractions was hybridized to denatured red kangaroo DNA bound to nitrocellulose filters, and the T_m of each satellite was determined (Table 1).

Table 1. Characterization of satellite DNA fractions

Fraction	Centrifugation isolation procedures ^A	Buoyant density of major DNA species (g/ml)	T_m of cRNA/DNA hybrid (°C) ^B	Est. No. of DNA species in fraction
I	Ag ⁺ , Hoechst	1.692	45.0	> 1 ^C
II	Ag ⁺ , Hoechst	1.704	46.0	3
III	Ag ⁺ , Hoechst, alkaline	1.705	47.5	1
IV	Ag ⁺ , Hoechst, alkaline	1.710	47.5	1
V	Ag ⁺ , Ag ⁺	1.712	45.0	> 1 ^C
VI	Ag ⁺ , Ag ⁺	1.707	47.0	> 1 ^C
VII	Ag ⁺ , Ag ⁺ , Ag ⁺	1.707	52.5	> 1 ^C

^A Ag⁺, Ag⁺-Cs₂SO₄ gradient; Hoechst, Hoechst dye 33258-CsCl gradient.

^B All temperatures $\pm 0.5^\circ\text{C}$.

^C As indicated by analytical buoyant density gradients.

The melting profiles were monophasic and did not reflect the buoyant density complexity of some of the fractions. The different components in fractions I and II must have melting temperatures too similar to be distinguished. The melting profiles did differentiate satellite fractions VI and VII although their buoyant densities had suggested that their base compositions might be similar. Sequence organization of nucleotides within repeat units can affect both density and melting temperatures differentially as demonstrated by the 1.688 and 1.686 satellites from *Drosophila melanogaster*, two satellites which, although appearing as one peak in neutral CsCl gradients, have T_m 's differing by 8°C (Brutlag *et al.* 1977).

Chromosomal Location of Satellite DNA

The red kangaroo has a diploid chromosome number of 20 ($2n = 18 + \text{XY}$, $2n = 18 + \text{XX}$). All chromosomes are morphologically distinguishable except chromosomes 8 and 9, which can be differentiated by G-banding (Rofe 1979). In order to determine the chromosomal distribution of the various satellite fractions [^3H]cRNA copies of the two pure highly repeated DNA species (from fractions III and IV) and of four of the other satellite fractions I, II, V and VI were made. Each of these was hybridized to mitotic metaphase chromosomes prepared from peripheral leucocytes and autoradiographed. The major hybridization sites were determined (Figs 6a-6f) in five metaphase cells of each satellite.

Satellites III and IV, each composed of a single DNA species, had markedly different distributions on the autosomal chromosomes although their locations on the X-chromosome were similar. Satellite III is located in several large blocks on chromosomes 1, 3, 4 and X, and has minor centromeric locations on chromosomes 6 and 7. Not all sites on chromosomes 1, 3 and 4 were located around the centromeres; some

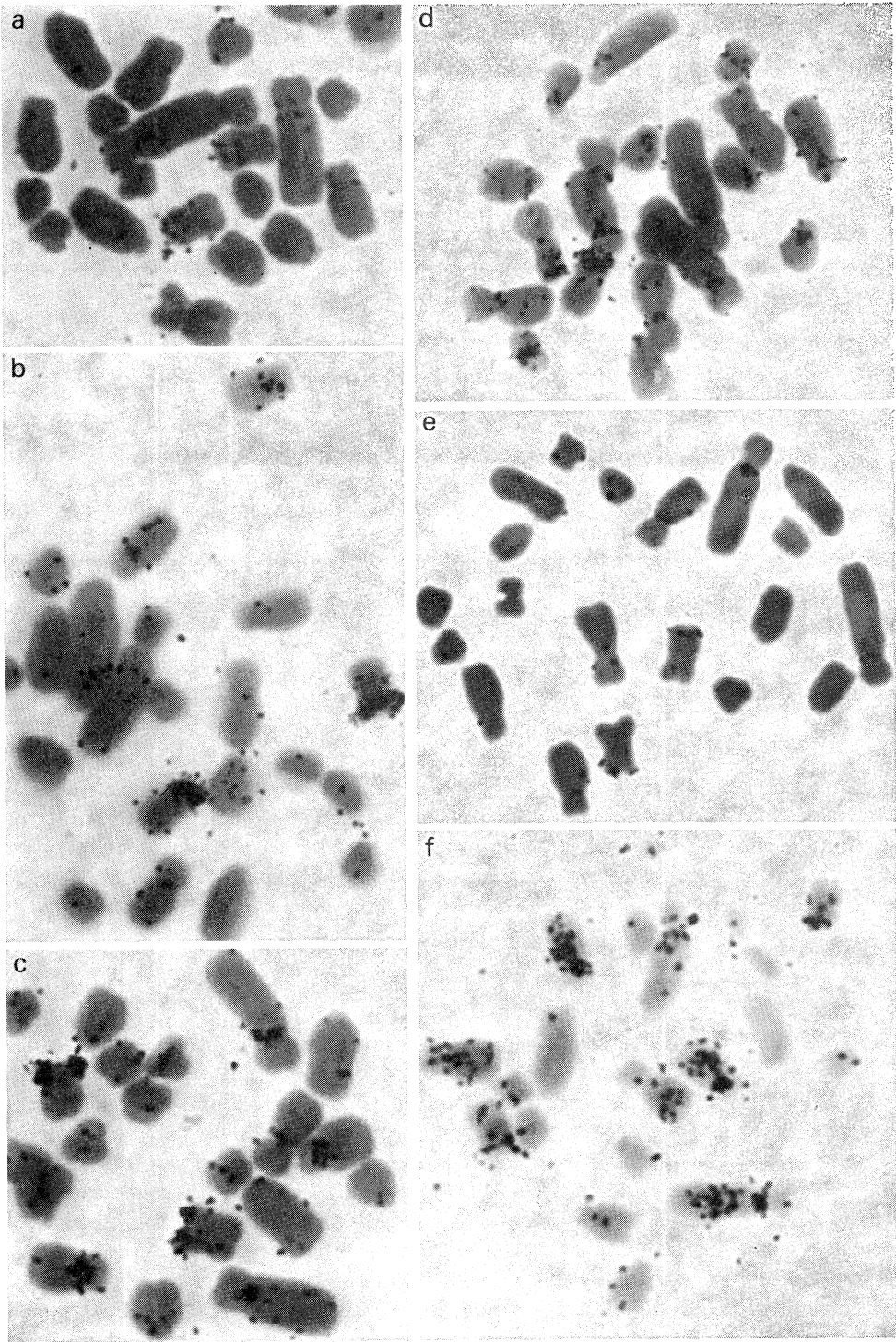


Fig. 6. *In situ* hybridization of [^3H]cRNA of individual satellite DNA fractions to red kangaroo metaphase chromosomes (*above*) and their corresponding diagrams (*opposite*): *a*, fraction II; *b*, fraction IV; *c*, fraction V; *d*, fraction I; *e*, fraction VI; *f*, fraction III.

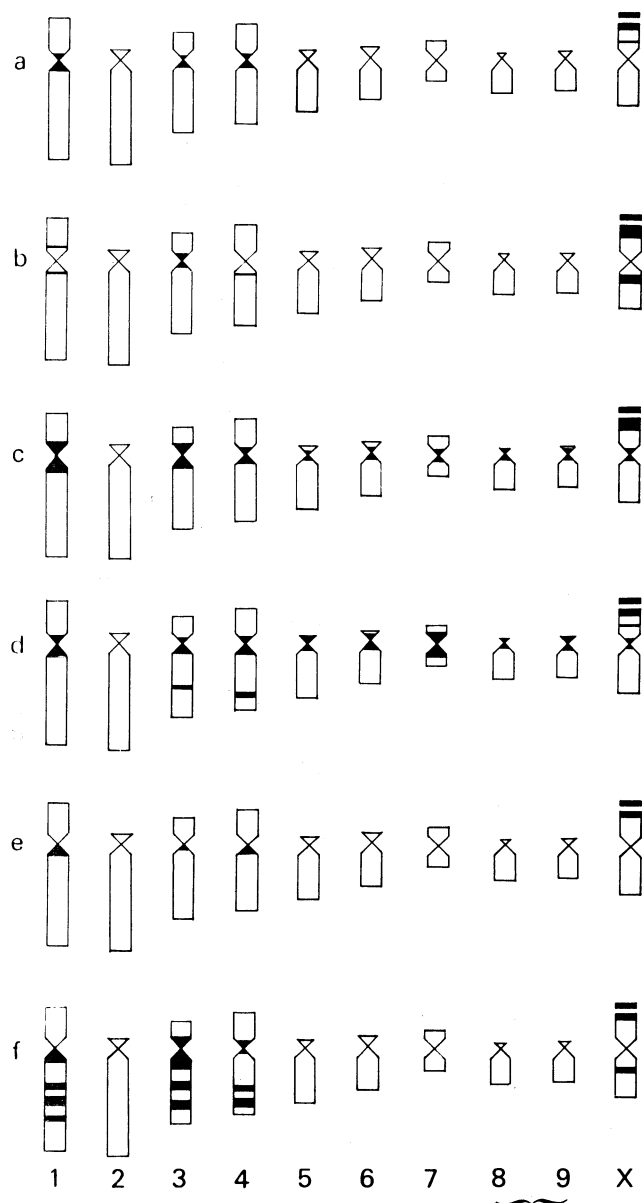


Fig. 6 (continued).

were distributed as interstitial bands on the long arms of the chromosomes. Satellite IV is mainly located on the X-chromosome with minor sites at the centromeric regions of chromosomes 1, 3, and 4, the site on chromosome 3 being most prominent.

Satellite fractions I and II, which were shown by the restriction enzyme analysis to share common sequences, also have some, but not all, common chromosomal sites. The common sites are the centromeric regions of chromosomes 1, 3 and 4, and the telomeric heterochromatin of the X-chromosome. In addition, satellite fraction 1 has sites on the long arm of chromosomes 3 and 4 and the centromeric

regions of chromosomes 5, 6, 7, 8, 9 and X. These additional locations must correspond to sequences unique to fraction I. Satellites V and VI were isolated as adjacent fractions in one gradient and both occur at the centromeres of chromosomes 1, 3 and 4, and at the telomeric segment of the heterochromatic arm of the X chromosome. Satellite fraction V has additional sites at the centromeres of all other chromosomes except chromosome 2, indicating the presence of a sequence component in fraction V which is not present in fraction VI.

Each fraction has a different chromosomal distribution (taking cross-contamination into account) and each centromeric block of heterochromatin differs in the amount and distribution of the different highly repeated DNA species present.

Table 2. Hybridization of [^3H]cRNA to red kangaroo, red-necked wallaby and wallaroo DNA

Values given are raw counts obtained in a single experiment. Each point is the average of two determinations

Satellite fraction	Labelled RNA (cpm) bound to 10 μg DNA		
	Red kangaroo	Red-necked wallaby	Wallaroo
I	18028	4452	5792
II	1659	1118	1268
III	21037	2901	5521
IV	8849	2667	4122
V	827	443	472
VII	5125	2052	4817

Not all hybridization sites are centromerically located. There are major sites on the long arms of chromosomes 1, 3 and 4, and in both arms of the X-chromosome. The distribution of the interstitial hybridization sites partially agrees with Rofe's (1979) C-banding data. There are interstitial C-bands on the long arms of chromosomes 1, 2, and 3, and on chromosomes 7 and X. The C-bands on chromosomes 1, 3 and X might correspond with some of the sites seen by *in situ* hybridization. The presence of *in situ* interstitial bands on chromosomes 1, 3 and 4 which do not have counterparts by C-banding could be a result of the higher sensitivity of the *in situ* hybridization technique. The lack of a C-band may be a consequence of the base composition of the highly repeated sequences which might affect staining properties.

Each of the red kangaroo satellites occurs near the nucleolar organizer region. The cytological association of highly repeated DNA sequences with the ribosomal gene region has also been observed in *Drosophila melanogaster* (Peacock *et al.* 1977) and previously in the macropods using the wallaroo satellite as a probe (Venolia and Peacock 1981). Although the cytological proximity does not necessarily indicate that the highly repeated sequences are directly adjacent to the ribosomal genes, they might be significant in providing the chromosomal environment needed for the transcription or amplification of the ribosomal genes.

Analysis of Homologous and Heterologous cRNA-DNA Hybrids

The presence of red kangaroo satellite sequences in the genome of the red-necked wallaby and the wallaroo was examined by filter hybridization using [^3H]cRNAs of

six of the satellite fractions. Sequences of all six satellites were present in the genomes of both species (Table 2). When the number of counts bound in each of the heterologous hybrids is compared with the homologous hybrids, a rough estimate of the relative amount of the different satellites in each genome is obtained. There is a marked difference in these relative values between satellites; for example, satellite fraction II showed a similar level of counts in the three species while satellite fraction III exhibited a great reduction in amount in the heterologous species compared with the homologous species. The different satellite sequences have changed reiteration frequencies independently during macropod speciation.

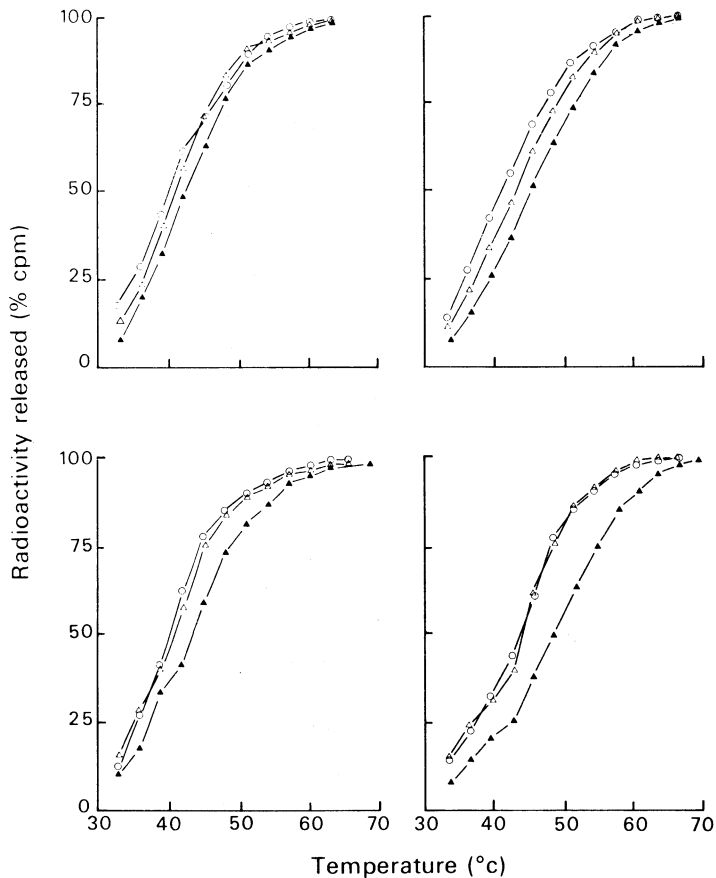


Fig. 7. Thermal denaturation profiles of hybrids formed between [^3H]cRNA of four satellite DNAs and DNA from three kangaroo species: *a*, fraction II; *b*, fraction III; *c*, fraction IV; *d*, fraction VII. $\blacktriangle$ Red kangaroo. $\circ$ Red-necked wallaby. $\triangle$ Wallaroo.

Difference in the melting temperature of homologous and heterologous hybrids is indicative of sequence divergence (Ullman and McCarthy 1973). Fig. 7 shows the melting profiles of four of the satellite fractions. Examining the change in melting temperature of the satellites in the heterologous species relative to the melting temperature of the satellites in the red kangaroo, the red-necked wallaby shows an equal

or greater sequence divergence than the wallaroo (Table 3). Satellite fraction III showed a great reduction in the amount of sequence present in the heterologous species relative to satellite fraction VII, but both fractions have a similar ΔT_m . This implies that nucleotide sequence and reiteration frequency vary independently.

Table 3. Melting temperatures of homologous and heterologous hybrids

All temperatures $\pm 0.5^\circ\text{C}$			
Satellite fraction	Melting temperatures ($^\circ\text{C}$)		
	Red kangaroo	Red-necked wallaby	Wallaroo
II	43.0	40.0	41.5
III	44.5	40.5	42.5
IV	43.5	40.5	40.5
VII	48.0	43.5	43.5

Conclusions

In $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ buoyant density gradients different Ag^+ concentrations have revealed a complex pattern of satellites in red kangaroo DNA. Seven different satellite fractions have been isolated, two being pure single highly repeated DNA species. The other five fractions contained a total of at least seven other highly repeated DNA species. Each highly repeated DNA has a unique chromosomal distribution, with locations in the centromeric heterochromatin, the nucleolar organizer region and some interstitial regions. It seems probable that even though the red kangaroo does not have a single major satellite like the red-necked wallaby (Dunsmuir 1976) and the wallaroo (Venolia and Peacock 1981), at least 15–20% of its genome is highly repeated DNA. The presence of six red kangaroo highly repeated sequences in the genomes of the red-necked wallaby and the wallaroo suggests that the underlying sequence constitution of highly repeated DNA in the red kangaroo is not so different from those of the red-necked wallaby and the wallaroo as was first indicated by the buoyant density analyses of the DNA of the three species. Other kangaroo species do not overlap in highly repeated DNA sequence composition to the same extent (Peacock *et al.* 1981). The finding that several highly repeated sequences isolated from the red kangaroo, red-necked wallaby and the wallaroo are common to the genomes of each of these species supports a close phyletic relationship.

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