Comparative DNA analysis of three South American marsupials

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Received 17 June 1982; Accepted 20 August 1982

ABSTRACT.
Published information on marsupials DNA is limited to a group of species belonging to only one genus. No previous reports have been written on South American species. In this paper we characterize the DNA of three out of the four marsupials found in Uruguay. Analytical and preparative ultracentrifugations in neutral CsCl gradients, including four intercalating agents and in Cs2SO4 gradients in presence of increasing amounts of Hg2+ ion did not allow us to separate any satellite fraction. The buoyant density of the unique peak measured in CsCl gradients was in every case 1.697 g/cc with a G-C content of 37.7%. Digestion of total DNA with 11 restriction endonucleases produced a different pattern of bands for the three species, although some possible homologies could be established. Hybridization with 32P-rRNA of Southern blots of the gels containing digested DNAs demonstrated that the repeated sequences evidenced do not correspond to the ribosomal cistrons.

INTRODUCTION.
Most, if not all, eukaryotic genomes are constituted by short and simple DNA sequences repeated many times, contained into a larger proportion of unique sequences (1). Repeated DNA can be shown as a satellite DNA in CsCl salts gradient centrifugation, provided its base composition is different from the bulk DNA, or if it represents a significant proportion of total DNA (2).

The structure, organization and nucleotide sequence of the repeated DNAs of a few species is well known (3). In situ hybridization indicates that in these species, repeated fractions are usually located in pericentromeric heterochromatin (4,5). Despite the great number of papers published on this subject, no conclusive results on the function of highly repeated DNAs have been obtained up to now (6).

It seems that repeated DNAs might be quite unrelated in a species or between different species, according to the variability shown in the amount, buoyant density and pattern of bands with restriction endonucleases of this class of DNA. Several studies on satellite DNA have been accomplished to the
moment, and all of them have demonstrated that these repeated DNAs present a high proportion of a rather short, basic repeated sequence, together with a variable amount of other sequences. However, this proportion seems to vary widely in the species studied so far. For instance, Drosophila highly repeated DNA has a sequence homogeneity of 90% in each satellite, while rodents apparently show a higher degree of heterogeneity in the base composition of their satellite DNAs (7).

Comparative analysis on the DNA of related species can throw light on the evolution of this type of DNA. Such type of studies have been performed in a relative large number of rodents and in several species of the genus Drosophila, but there is little information about other groups. The validity of the use of unique or repeated sequences in DNA comparative analyses with phylogenetic aim has been discussed (8).

Among mammals, the order Marsupialia is one of the greatest evolutive interest. They diverged very early from the general line of mammalian evolution and are considered by some authors as a different alternative of viviparity (9). Marsupials have had an explosive adaptative radiation in Australia and South America. In the later, almost 60 species are found. From the cytogenetical point of view, this order is one of the best known taxa (10). Such type of information is available in 40% of the total number of marsupial species.

However, little is known about marsupial DNA, being the information restricted to eleven australian kangaroo species, all of them belonging to the Macropodidae family (11-14).

In this paper we present a comparative study on the DNA from three out of the four opossums found in Uruguay: Monodelphis dimidiata, Lutreolina crassicaudata and Didelphis albiventris. Their DNAs have been characterized by analytical ultracentrifugation in CsCl and Cs$_2$SO$_4$ gradients, DNA-RNA hybridization, digestion of total DNA with restriction enzymes (RE) and hybridization of electrophoresed RE digestion fragments with $^{32}$P-rRNA after Southern blotting.

Our results show the presence of repeated fractions evidenced by RE digestion. These fractions could not be isolated by ultracentrifugation in Cs salts gradients. DNA-RNA hybridization demonstrated that these repeated DNAs are not related to the 18 and 28 S ribosomal DNA.

**MATERIALS AND METHODS.**

**Animals.**

All the specimens were caught in the wild. *Didelphis albiventris* was
Monodelphis dimidiata was caught in Colonia and Canelones, Lutreolina crassicaudata was caught in Río Negro. DNA extraction.

DNA was extracted from frozen livers according to a modification of the Marmur technique (15). First deproteinizations were carried out with the chloroform-isoamyl alcohol mixture (25:1) and the second ones with water saturated phenol. DNA was precipitated with ethanol and then dialized against Tris 10 mM or Na₂SO₄ 10 mM.

Analytical ultracentrifugation.

Analytical ultracentrifugations were carried out in neutral CsCl gradients and in Cs₂SO₄ gradients. In CsCl gradients Micrococcus luteus DNA was used as a buoyant density marker. Hg⁺⁺ was added to Cs₂SO₄ gradients to obtain Hg⁺⁺/DNA ratios of 0.05, 0.10, 0.15 and 0.20. The runs were performed in a Beckman ANG rotor and a Beckman Model E ultracentrifuge, at 44000 rpm during 20 hours.

Preparative ultracentrifugation.

Four intercalating agents were added to DNA (40 μg): Actinomycin D, Dystamycin A, Hoechst 33258 and ethidium bromide, in a DNA/intercalant ratio of 0.5, with Sarkosyl and Tris 0.1 M. CsCl was added to an appropriate density. The runs were carried out in a Beckman A1 50 rotor and a Sorvall OT D2 ultracentrifuge, at 44000 rpm and 18°C for 24 hours. 0.15 ml fractions were collected from the bottom of the tubes. The optical density was measured in an ISCO UA5 absorbance/fluorescence monitor, at 254 nm. DNA/³H-rRNA hybridization.

Molecular hybridizations were carried out according to Gillespie and Spiegelman (16). DNA from fractions collected after preparative ultracentrifugations was denatured with 0.1 M NaOH and neutralized with 0.05M HCl. DNA was loaded onto nitrocellulose filters (Millipore HAWP, 0.45 μm) and then hybridized with 18 and 28 S ³H-rRNA from Xenopus laevis. The radioactivity of the filters was measured in a Beckman LS 100C liquid scintillation counter in Fluoralloy cocktail. The measures were done in ³H channel during 10 minutes.

Restriction endonuclease digestion.

Restriction enzymes were a gift from Drs. Joseph Gall and Peter Rae. Digestions were carried out at 37°C for 1 hour (with the exception of Taq I) in the conditions recommended by the suppliers. Lambda DNA digested with Hind III was used as a molecular weight marker. Combined digestions were carried in some cases, in which Taq I digested pBR 322 DNA was included as a calibration for molecular weight.
Agarose gel electrophoresis.

1% or 1.4% agarose gels (Sea Kem and Sigma) were made in 1x electrophoresis buffer pH 8.0 (0.04 M Tris acetate, 0.002 M Na₂EDTA and 0.005 M Na acetate) and run in a vertical slab at approximately 65 mA and 200 V during 1 hour and a half at 4 °C. Gels were stained with ethidium bromide (0.5 μg/ml) for 20 minutes. Gels were screened in an UV transilluminator (366 nm) and photographed on Polaroid 105 sheet film.

Southern blots and 32P-rRNA hybridization.

Restriction enzyme digests of total DNA were transferred from the gels to nitrocellulose filters by the Southern technique (17). Filters were dried and hybridized with end-labeled 32P-rRNA (18) from CHO cells, for 18 h at 65°C. The filters were dried and autoradiographed on Kodak XO mat film for 24 and 72 hours at -70 °C.

RESULTS.

Analytical ultracentrifugations of DNA of the three studied species, in neutral CsCl equilibrium gradients, demonstrated a single symmetrical peak in each of them. The buoyant density of the three main peaks is 1.697 g/cc, with a G-C value of 37.7% (Fig. 1).

Analytical ultracentrifugations in Cs₂SO₄ gradients with increasing
Hg\(^{++}/\)DNA ratios (0.05, 0.10, 0.15 and 0.20) did not permit the separation of any satellite fractions. This result was similar for the DNA of the three species (Fig. 2).

Moreover, the use of four intercalating agents (Actinomycin D, Dystamycin A, Hoechst 33258 and ethidium bromide) in preparative ultracentrifugations of DNA of the three species in neutral CsCl gradients also failed to demonstrate satellite peaks. Three of these experiments are illustrated in Fig. 3.

Samples of the fractions collected from neutral CsCl gradients with intercalating agents were loaded onto nitrocellulose filters, which were hybridized with *Xenopus laevis* \(^3\)H-rRNA, to depict the location of the ribosomal genes within the gradients. The fractions that hybridized to
Figure 3. Preparative ultracentrifugations in CsCl gradients with intercalating agents: a) *M. dimidiata* DNA-Hoechst 33258, b) *L. crassicaudata* DNA-Actinomycin D, c) *D. albiventris* DNA-Hoechst 33258. Dotted line: hybridization of fractions with 3H-rRNA from *Xenopus laevis*.

$^3$H-rRNA were those corresponding exactly to the unique peak. Due to the similitude of the results obtained for all the species, we illustrate here only one of the experiments (Fig. 3c).

The studies carried out with eleven restriction endonucleases (Alu I, Bam HI, Bgl II, Eco RI, Hae III, Hha I, Hind III, Kpn I, Sac I, Sal I and Taq I) showed a different pattern of bands for each species.

Three out of the eleven restriction endonucleases (RE) employed produced the following bands in the species *M. dimidiata*: Eco RI, 2.20 kb and 1.50 kb; Hae III, 1.30 kb and 0.74 kb; Taq I, 1.77 kb, 1.35 kb, 0.78 kb and 0.52 kb (Fig. 4 and 9).

*L. crassicaudata* DNA showed bands with four enzymes: Bgl II, 2.10 kb, 1.92 kb, 1.12 kb and 0.72 kb; Hae III, 0.74 kb; Sal I, 1.10 kb; Sac I, 2.65 kb, 1.97 kb and 1.50 kb (Figs. 5 and 9).

In the species *D. albiventris* some bands were evidenced with three RE: Bgl II, 1.14 kb and 0.73 kb; Sac I, 1.72 kb and 1.41 kb; Taq I, 2.70 kb and 0.56 kb (Fig. 6 and 9).

Consecutive digestion of DNA of *L. crassicaudata* and *M. dimidiata* combining the RE that released bands in each species DNA did not permit to fractionate the original bands, (Fig. 7 and 8).

Autoradiographs following hybridization of $^{32}$P-rRNA from CHO cells to Southern transfers of restricted DNA of each species evidenced a low number of bands that do not correspond to the fluorescent bands visible in the gels. Autoradiographs bands and the size of the fragments expressed in
DISCUSSION.

Two different basic approaches have been employed in the comparative study of related species DNA with phylogenetic aim. A considerable number of studies have been centered in the analysis of satellite DNAs or highly and moderately repeated fractions (19,20), while a few papers were based on single copy DNA of related species (8,21). Analytical ultracentrifugation in cesium salts, ressociation kinetics and restriction enzyme digestions are the basic tools in the first mentioned approach. In the other type of experiments, ressociation kinetics of heterologous DNAs allowed to establish the single copy divergence or phylogenetic distance between the species compared.

Although both approaches seem to be very useful in evolutionary studies, the number of species analyzed up to date is still small to conclude which
one is more adequate for comparative purposes. We have selected the methodology used for detection of repeated sequences since most eutherian and all marsupial species have been studied mainly from this point of view.

The presence of conspicuous satellite DNAs or at least, cryptic repeated sequences has been sustained to be a constant feature of eukaryotic genomes (2). Satellite DNA of several rodents, Drosophila species and human have been extensively analyzed, many of them by cloning and sequencing techniques.

In 1970, Pardue and Gall (4) demonstrated that mouse satellite DNA was preferentially located on pericentromeric heterochromatin. Similar facts were posteriorly reported for other species (22). A group of related hypotheses on the possible function of satellite DNA or constitutive heterochromatin have been developed, suggesting a role in chromosomal speciation mechanisms (19,23,24). This point has been extensively discussed recently, and considerable experimental data that questioned the basis of the mentioned group of hypotheses were provided (6,7).

As we have already mentioned (see Introduction), cytogenetical information is available about a great number of marsupial species. These mammals have some peculiar chromosomal features: a) their chromosomes are larger than those of eutherians, though the amount of DNA is very similar in both groups, b) they present a great morphological homogeneity in size,
Figure 7. 1.4% agarose electrophoresis of total DNA of *Monodelphis dimidiata* digested with four restriction endonucleases alone (at left) and combined in pairs (at right). Lambda and pBR 322 were used as molecular weight markers.

Figure 8. 1.4% agarose electrophoresis of total DNA of *Lutreolina crassicaudata* digested with three restriction endonucleases alone (at left) and combined in pairs (at right). Lambda and pBR 322 were used as molecular weight markers.

centromere position and other parameters and c) the diploid number is usually low, varying from 2n=10 to 2n=32 (9). Marsupials chromosome number has a bimodal distribution, with one mode in 2n=14 and the other in 2n=22, the former is considered as the ancestral chromosome number by some authors (10,25). Two out of the three species studied in this paper belong to the 2n=22 mode: *Lutreolina crassicaudata* and *Didelphis albiventris*. *Monodelphis dimidiata* can be instead considered as belonging to a third intermediate mode of chromosome number distribution: 2n=18 (25).

Contrasting with the great amount of cytogenetic information on this taxon, published data on marsupial DNA are surprisingly restricted to only one australian genus: *Macropus*. Two species have been carefully analyzed including isolation of their satellite DNAs and cytological localization by in situ hybridization (11-13). A comparative study of the highly repeated
sequences of eleven species from this genus has been carried out (14) in order to establish their phylogenetic relationships. Three labeled probes hybridized on restriction enzyme digested total DNA of the other species have been used as the main tool for this analysis, that supports the monophyletic origin of this group.

The application of the usual Cs salts gradient ultracentrifugation, including several intercalating agents and four progressive concentrations of Hg$^{++}$ in Cs$_2$SO$_4$ gradients, did not allow us to separate any satellite DNA. These negative results might be due either to the lack of difference in the G-C content of the hypothetically cryptic satellite DNAs, or to the small proportion of these fractions when compared to the total amount of DNA. The last assumption might agree with the fact that these species have a relatively small quantity of heterochromatin (Seluja, personal communication).

The presence of several bands obtained by restriction enzyme digestion of DNA of each species demonstrated the existence of repeated sequences. Five out of the six RE that produced bands are unfrequent cutters (Bgl II, Eco RI, Sac I, Sal I and Taq I). The diversity in the recognition sites of the enzymes that released bands evidenced the absence of special affinity
for G-C or A-T rich zones.

The analysis of the sizes of the fragments produced by the four enzymes that released bands in M. dimidiata showed two similar bands with Hae III (0.74 kb) and Taq I (0.78 kb). The coincidence in the sizes of these fragments suggests that these bands might correspond to the same repeat, that has cleavage sites for both enzymes. The bands released by Taq I might correspond to a ladder-like repeat, with a supposed monomer of 200 base pairs. This possible monomer could not be visualized in the gels, may be because of its small size, and the bands are not disposed in a typical ladder-like distribution (Fig. 7 and 9).

In L. crassicaudata we have found two fragments of a very similar size produced by Bgl II (0.72 kb) and Hae III (0.70 kb). This species also shows two similar bands, one of them released by Bgl II (1.92 kb) and the other by Sac I (1.97 kb). Another possible ladder-like repeated DNA could be visualized in this species by Bgl II digestion. The bands obtained might correspond to a dimer, a trimer, a pentamer and a sixamer of a hypothetical 360 base pairs monomer, that is not visible in the gels (Fig. 8 and 9).

A comparison of the pattern of RE bands of the DNA of the three species allowed us to establish a few homologies that suggest the existence of common repeated fractions, although none of the enzymes used produced bands in all these species. The possible homologies found are three: a) Hae III releases a band of 0.74 kb in M. dimidiata and a band of 0.70 kb in L. crassicaudata; b) Bgl II- 0.72 kb in L. crassicaudata and 0.73 kb in D. albiventris; c) Taq I- 0.52 kb in M. dimidiata and 0.56 kb in D. albiventris. The fact that these possible homologies are scarce agrees with the fact that these species are not very closely related, since they belong to the same subfamily (Didelphinae) but to different genus.

The repeated DNAs evidenced by RE digestion do not contain the ribosomal cistrons, since the fluorescent bands on the electrophoresis gels do not correspond to those appeared in the $^{32}$P-rRNA hybridization on the nitrocellulose filters were the bands from the gels were transferred to, according to Southern (17) (Fig. 9 and 10).

ACKNOWLEDGEMENTS.

The authors are indebted to Prof. Joseph Gall from Yale University for the use of the facilities of his laboratory during some of the experiments, and also to Dr. Manuel O. Díaz for his generous help. We wish to thank Dr. Horacio Cardoso for his critical reading of the manuscript.
REFERENCES.