

Ribosomal Gene Reiteration in a Marsupial Species with an X-linked Nucleolar Organizer

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Abstract

The number of ribosomal cistrons in somatic cells of males and females of *Macropus eugenii* have been estimated using RNA/DNA hybridization in 70% (v/v) formamide. The male has one X-linked active nucleolar organizer and the female two and while the number of ribosomal cistrons is variable between the four males examined (31–65 copies), it is substantially less than the number present in the three females (94–103 copies). There is, therefore, no evidence of dosage compensation by amplification.

Introduction

Extensive cytogenetic studies with a range of marsupial species have indicated considerable diversity in the number and location of nucleolar organizer regions on marsupial chromosomes (Hayman and Rofe 1977). Of particular interest are those species in which the nucleolar organizer is found only on the X chromosome, since these species will have twice as many organizers in the female as in the male. Hayman and Rofe (1977) showed that in such females there are two nucleoli formed in the interphase nuclei, and that both the X chromosomes in dividing cells of the female respond to silver staining (Bloom and Goodpasture 1976)—see Fig. 1. Both of these observations strongly suggest that both X-chromosomal loci are active in the female. Consequently there is an absence of the usual mammalian X inactivation for this region of the chromosome and the female has, in theory, twice as many ribosomal cistrons as the male. The possibility exists that this substantial difference between the sexes could be overcome in somatic cells by differential amplification of the ribosomal cistrons in the single X of the male. This paper describes experiments to test this possibility using *Macropus eugenii*, the Kangaroo Island wallaby. The silver staining pattern in the male and female are shown in Figs 1a and 1b respectively.

Gene reiteration frequencies have often been estimated by C_0t analysis (Britten and Kohne 1968). While this method is appropriate for obtaining approximate reiteration values, it lacks precision. A second method employed to quantify gene reiteration frequency involves saturation hybridization of a specific sequence to filter-bound DNA. In practice this method suffers from several disadvantages. These include variation in binding efficiency of DNA to different batches of nitrocellulose and incomplete hybridization of complementary sequences as a result of either steric hindrance of filter-bound DNA or an inability to fully saturate the bound DNA with the complementary probe (Grunstein and Hogness 1975).

A reproducible and sensitive method to quantify ribosomal RNA gene numbers accurately was required. Vogelstein and Gillespie (1977) have described conditions in which hybridization of RNA to denatured DNA will occur without reannealing of DNA. We have used these conditions with ^{125}I -labelled rRNA to investigate the number of ribosomal genes in DNA isolated from several animals of *M. eugenii*.

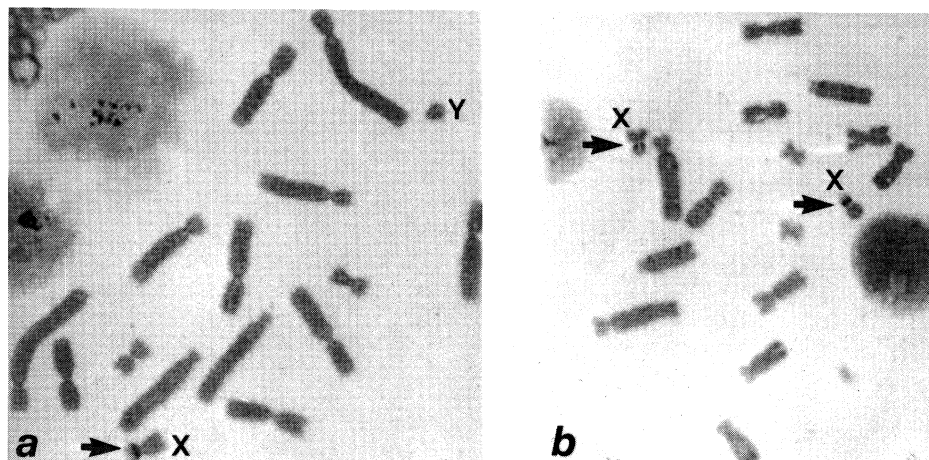


Fig. 1. Chromosome preparations from lymphocytes of (a) the male, and (b) the female of *M. eugenii* stained with silver to show active nucleolar organizer regions. The arrows indicate the silver-staining X chromosomes.

Materials and Methods

Preparation of DNA

Livers removed from freshly killed animals were immediately placed on ice and snap frozen. The tissue was homogenized in the presence of sodium dodecyl sulfate, crude nuclei prepared and nucleic acids isolated by phenol extraction. Purified, high molecular weight DNA was obtained after chloroform-octanol extractions, treatments with ribonuclease and proteinase K and a final precipitation with isopropanol.

Isolation of rRNA

Polysomes were prepared from red blood cells of either chickens or possums (*Trichosurus vulpecula*) by standard procedures. The animals were first made anaemic by five (chickens) or three (possums) subcutaneous injections of phenylhydrazine. Polysomes were either extracted with phenol or dissociated with sodium dodecyl sulfate and 18 S and 28 S rRNA purified by two rounds of sucrose-gradient centrifugation. The purity of 18 S rRNA was checked by gel electrophoresis and by C_{60} analysis. The latter analysis showed that the 18 S rRNA contained less than 5% contamination by 28 S rRNA.

Iodination of RNA

After purification, rRNA was extracted with phenol and twice precipitated with ethanol. The final precipitate was washed extensively with cold ethanol and dried. The RNA was resuspended in water to a concentration of 1 mg/ml and iodinated by the method of Commerford as described by Prenskey (1976). After incubation at 60°C for 10 min, the reaction mix was placed on ice and one-tenth volume of β -mercaptoethanol was added. The reaction volume was then increased to 0.1 ml with water and fractionated on a G50 Sephadex column (0.5 by 10 cm). Peak fractions containing radiolabelled RNA were pooled and frozen. Specific activities were in the range of 5×10^7 – 2×10^8 dpm/ μg RNA.

DNA-RNA Hybridizations in Formamide

DNA samples were subjected to ultrasonic disruption (2×30 s at setting 6 with a Branson sonicator), precipitated with ethanol, and resuspended in formamide to a concentration of 2.86 mg/ml.

Seven parts of the DNA solution were added to three parts of ^{125}I -labelled 18 S rRNA in potassium phosphate buffer, pH 6.8 (final concentration of phosphate in reaction mix is 0.36 M). The reaction mix was boiled for 1 min and then incubated at 45°C .

Samples were taken at various times and stored at -20°C .

Assay for RNA Hybridization

RNA hybridization was assayed essentially by the method of Ross (1976). Samples of hybridization mixes were diluted with 0.5 ml of 0.2 M NaCl, 4 mM EDTA, 10 mM Tris-Cl, pH 7.4. Further additions of *E. coli* tRNA (30 μg), ribonuclease (4 μg) and T1 ribonuclease (100 units) were made and the mixture incubated at 37°C for 40 min. After incubation, 50 μg of bovine serum albumin and 1 ml of 20% (w/v) trichloroacetic acid were added, the mix left on ice for 15 min and precipitated material collected on GF/A filters, washed with 5% (w/v) sodium pyrophosphate, dried and counted in a scintillation spectrometer.

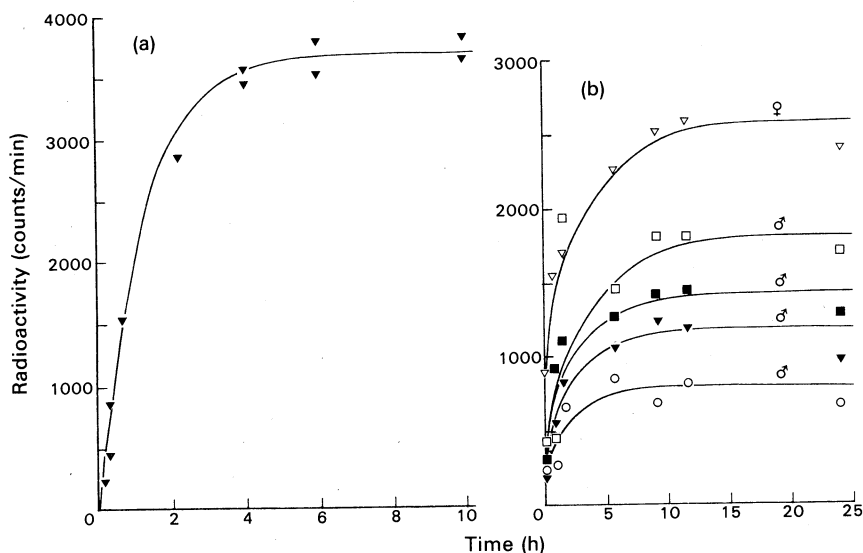


Fig. 2. Gene reiteration frequency of chicken 18 S rRNA genes (a) and of ribosomal cistrons in *M. eugenii* (b). Using 'R-looping' conditions ^{125}I -labelled 18 S rRNA was hybridized to either 20 μg of chicken genomic DNA (a) or 20 μg of liver genomic DNA from five different individuals (b). Each point represents the amount of hybridization for the times indicated. The plateau of hybridization was used to determine the number of copies of 18 S rRNA genes.

Results*Calculation of Ribosomal Gene Reiteration Frequency**Validity of the new method*

We have previously used C_0t analysis to estimate that the ribosomal genes are reiterated 100-fold in chicken DNA (A. J. Robins *et al.*, unpublished data). Before investigating the marsupial samples, the validity of saturation hybridization of RNA under 'R-loop' conditions as an independent method of estimating gene reiteration was tested (Fig. 2a). Taking the values of 1.2×10^{12} daltons and 0.7×10^6 daltons as the molecular mass of the chicken genome and of 18 S rRNA respectively, the plateau value for hybridization can be used to calculate a figure of 90 for the reiteration of ribosomal genes. This is in good agreement with the value of 100 previously determined by C_0t analysis. We conclude

therefore that RNA/DNA hybridization in 70% (v/v) formamide is a valid method for the determination of gene reiteration frequency. The method is particularly suitable to quantify gene numbers which may vary by twofold or less.

Application of the technique to quantify reiteration frequency of ribosomal genes in M. eugenii

The results of saturation hybridization of ^{125}I -labelled marsupial 18 S rRNA to five separate *M. eugenii* liver DNA samples are shown in Fig. 2b. With the four male samples, the gene numbers vary over a twofold range (31–65 copies). For the female DNA sample the reiteration frequency estimate from the data is 97 and in a separate experiment with two other samples of female DNA, and using RNA of different specific activity (data not shown), the reiteration frequency was 94 and 103 copies respectively. Experiments with DNA from the same animal were repeated several times with different batches of ^{125}I -labelled rRNA of different specific activities. Calculations from this data gave rRNA gene reiteration frequencies which varied by less than 10%, showing that the results are highly repeatable.

Discussion

The method described here should be applicable to any system in which a pure RNA probe is available. It has the advantage that saturation hybridization is possible under 'ideal' conditions, i.e. in solution but effectively without competition from a second DNA strand. In addition, high sensitivity is possible (since RNA can be iodinated *in vitro* to high specific activities) and the method is reproducible.

Nevertheless one caution is necessary. In developing the method for ribosomal gene titration, we initially used iodinated 18 S and 28 S rRNA as probes. However, with ^{125}I -labelled 28 S rRNA we continually encountered high background values, for example, in zero time samples about 30% of the radioactivity was not digested by ribonuclease. This is probably due to a high proportion of stable secondary structure in 28 S rRNA. Fortunately, 18 S rRNA did not present the same problem.

The data for *M. eugenii* illustrate that variation in ribosomal gene number can occur between different individuals. This suggests caution in assigning an absolute number for gene reiteration. Despite this caution and the limited number of individual samples investigated, it seems likely that for this species females on average possess twice as many ribosomal cistrons as males.

Consequently there is no evidence of a phenomenon like 'amplification' in somatic cells of the male to accommodate for the difference in the number of active nucleolar organizer regions between the sexes. Considerable variation in number of ribosomal cistrons exists between individual male animals without any obvious phenotypic effect. The variation in the number of ribosomal cistrons between the male and female animals is presumably accommodated in the same manner as is the variation which occurs between males.

The nucleolar organizer region therefore differs from other sex-linked loci in marsupials and eutheria. These loci achieve dosage compensation between the sexes by the X-inactivation mechanism whereby there is only one X chromosome transcriptionally active in the diploid cells of normal males and females. A possible, admittedly speculative, explanation for this difference is suggested by the observation that the ribosomal cistrons are not usually located on the X chromosome. In marsupials they are only found on the X or Y chromosome of species in certain taxonomic groups (Hayman and Rofe 1977; Hayman and Sharp 1982). They are more frequently found on the autosomes. The X-inactivation mechanism presumably arose in an X chromosome which lacked the nucleolar organizer region. When such regions are subsequently transposed to the X chromosome as an evolutionary novelty the X-inactivation mechanism has not been able to include

them. DNA measurements which would support this hypothesis have been reported (Hayman *et al.* 1982).

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