Heredity **65** (1990) 7–10

Received 3 November 1990

Latent NORs in the species *Pycnogaster cucullata* (Orthoptera)

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The chromosomal location of the ribosomal cistrons 18+26S and 5S has been determined in spermatocytes of two cytological races of *Pycnogaster cucullata* by *in situ* hybridization using molecular probes from both types of rDNA. A comparative analysis with previous results suggested the existence of more 18+26S rDNA sites than active NORs as shown by silver impregnation, but 5S rDNA sites are outside the NORs. Thus, different categories of latent NORs have been shown in this specialised cell type which are discussed in relation to the evolutionary process of differentiation in this species.

INTRODUCTION

The species *Pycnogaster cucullata* has been studied in previous papers, and two cytological races were found differing by their sex chromosome system (XO and neo XY) (Fernández-Piqueras *et al.*, 1982, 1983; Sentís *et al.*, 1984). Silver impregnation in spermatocytes of this species has also served to demonstrate two different categories of Ag-NORs, named primary and secondary, which differ by the appearance and meiotic behaviour of their silver precipitates. Primary NORs are present in all primary spermatocytes from each individual whereas the other type (secondary NORs) seems to be dispensable and is only present in some individuals and even so, not in all primary spermatocytes of a given individual. In addition, other Ag-precipitates can be also observed at a very low frequency in the proximal region of the X chromosome (XO race), and five exceptional cells were found in a single male of the neo XY race which displayed a single active NOR at the distal secondary constriction of the X_L arms of the neo X (Sentís *et al.*, 1988).

We postulated that secondary NORs could be considered as a special category of *latent* NORs with variable transcriptional activity (Sentís *et al.*, 1988). To check this hypothesis, in situ hybridization using molecular probes from both types of ribosomal cistrons (18+26S and 5S) have been accomplished in the two cytological races of the species *P. cucullata.*

MATERIAL AND METHODS

Ten males from each cytological race of the species *Pycnogaster cucullata* (Orthoptera) have been collected from natural Spanish populations at Sierra de Gredos (Puerto del pico, XO race) and Sierra de Guadarrama (Puerto de Morcuera, neo XY race). Individuals used in this study were injected with 0·03 per cent colchicine in insect saline solution 6 h prior to standard fixation of gonadal tissues in ethanol: acetic acid (3 : 1) for 2 h. Meiotic chromosomes were obtained by the conventional squash method in a drop of 45 per cent acetic acid, and the coverslips were removed after immersing the slides in liquid nitrogen. The samples were then air dried for 15 min. One hundred meiotic cells were studied for each probe and individual.

The molecular probes used in this study have been obtained from *Artemia salina.* The 16·5 kb insert of clone pArtI contains the full 18+26S rRNA coding region and a considerable part of the non-transcribed spacer (NTS). The 700 bp insert of clone pVC9 contains the entire 5S rRNA coding region and a small part of adjacent regions. Clones were labelled by nick translation with (^{3}H) dTTP according to the method described by Rigby

et al. (1977). *In situ* hybridization of rDNA probes was carried out following the procedure described by Pardue (1986). Slides were immersed in Kodak NTB2 emulsion and stored 4-7 days at 4°C. Autoradiographs were developed with D-19 and stained with 2 per cent Giemsa.

RESULTS

The chromosomal sties for ribosomal DNA were observed in meiotic chromosomes (fig. 1). Those sites where labelling was found to occur symmetrically in both homologues and in more than 50 per cent of the examined cells (up to a maximum of 80 per cent) were considered as *major* sites. In addition, some *minor* sites were also observed in less than 50 per cent (from 10 to 50 per cent) of the examined cells; these did not occur symmetrically in both homologues. Interspersed silver grains have also been observed in other chromosomal sites in less than 10 per cent of the cells. They could be considered as non-specific labelling, in spite of the relative absence of background. The observed differences in the frequency of labelling at specific sites could be explained if we accept some variations in the amount of rDNA and/or a differential accessibility to molecular probes due to the chromatin conformation at these regions (fig. 2).

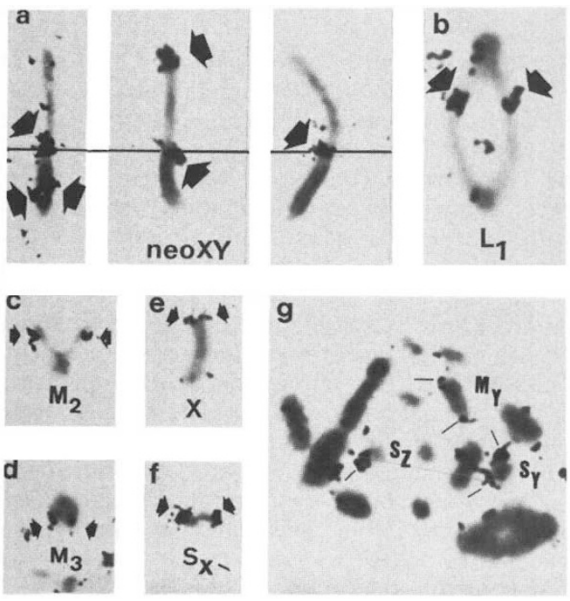

Figure 1 *Labelling of meiotic chromosomes.* (a-f) Chromosomal location of 18+26S rDNA (dark arrows). (g) Metaphase I cell showing 5S rDNA sites located at the M_Y bivalent and at the two short chromosome pairs (S_Y and S_Z) (lines).

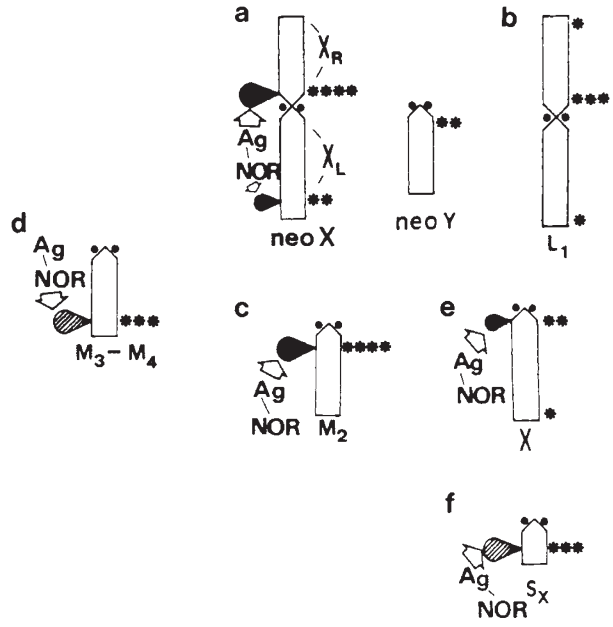

Figure 2 *Interpretative diagrams* showing the comparison of Ag-staining (Sentís *et al.*, 1988) and "*in situ*" hybridization of 18+26S results. Dark areas indicate active primary NORs, and striped areas denote active secondary NORs (Ag-NORs). Asterisks represent the chromosomal location and the cell frequency of labelling (*, less than 50 per cent of the analyzed cells. **, from 50-60 per cent. ***, from 60-70 per cent. ****, from 70 per cent to a maximum of 80 per cent).

Chromosomal location of 18+26S rDNA

XO race

Up to six *major* sites have been shown which are located at the proximal regions of chromosomes L_1, M_2 and X, at the distal region of two medium sized chromosomes (M_3 and M_4), and at an unidentified short chromosome (S_x) (figs. 1(b, c, d, e and f) and 2).

XY race

This race differs from the XO race because two *major* sites can be shown on chromosome neoX, one is close to the centromere and the other is at a distal position in the XL arm (which derives from a primitive X at XO state). Another proximal *major* site can be observed on the chromosome neo Y (fig. 1a and 2).

Minor sites for 18+26S rDNA have been located at the telomeric regions of L1 (XO and neo XY races) and X (XO race) chromosomes (fig. 1(b and e) and 2).

Chromosomal location of 5S rDNA

The chromosomal sites for these cistrons are not coincident with those of 18+26S rDNA, and have been located for both races at the paracentromeric regions of a medium sized bivalent M_Y (M_5 or M_6), and on two short bivalents apparently shorter than those carrying 18+26S sites (S_Y and S_Z). Nevertheless, the precise location of silver grains is difficult to ascertain in the short bivalents due to their extremely small size (fig. 1(g)). Chromosomes hybridized with the 5S probe usually show less labelling than that displayed with the 18+26S probe and silver grains are only observed in 40–60 per cent of the analysed cells.

DISCUSSION

Nucleolar organizer regions (NORs) have been described as those chromosomal sites which consist of the 18+26S rDNA sequences whereas 5S rDNA is usually outside the NORs in higher eukaryotes (Steffensen and Duffey, 1974; Vitelli *et al.*, 1982). Silver impregnation has been widely used as a fast and reliable method to identify active NORs during both mitotic and meiotic divisions (Goodpasture and Bloom, 1975; Hofgartner *et al.*, 1979), but the correspondence between Ag-NORs and the chromosomal location of the two ribosomal cistrons as shown by *in situ* hybridization is still controversial. Thus, Ag-NORs and 18+26S rDNA sites show an exact correlation in mammals (Tantravahi *et al.*, 1976), but in the parthenogenetic species *Warramaba virgo* only the chromosomal location of the 5S ribosomal cistrons can be seen on the mitotic metaphases by silver staining (White *et al.*, 1982). Furthermore, Schmid (1978) has described in *Rana catesbeiana* two different categories of NORs with Ag-staining named *standard* NOR and *small* NOR respectively. *In situ* hybridization of rDNA showed that *standard* NORs always hybridized with ribosomal 18+28S cistrons while at least one *small* NOR corresponded with the location of 5S rDNA sites (Vitelli *et al.*, 1982). All these data seem to indicate that silver impregnation is able to demonstrate either both types of active ribosomal cistrons or only one, depending on the analyzed species.

The chromosomal location of the 18+26S rDNA sites in the species *P. cucullata* reveals that all the previously described Ag-NORs (Sentís *et al.*, 1988), including primary and secondary Ag-NORs as well as those rarely observed in chromosomes X (proximal) and neoX (distal), consistent of 18+26S rDNA, whereas 5S ribosomal cistrons are located outside Ag-NORs (fig. 2).

These results confirm that secondary Ag-NORs in this species could be a special category of latent NORs (*facultative* latent NORs), which should be in turn under cellular controls related to the cellular requirements of ribosomal RNA synthesis in spermatocytes.

Nevertheless, there are two *major* chromosomal sites at the centromeric regions of L_1 and Y chromosomes which hybridize with the 18+26S probes but are not revealed by silver impregnation. In relation to the inactive ribosomal cistrons located on the L_1 chromosomes of *P. cucullata* it is worth noting that an active Ag-NOR had been located at the same position in the L_1 of the related species *P. finotii* (Sentís *et al.*, 1988). Thus, this NOR could be considered as a *strictly* latent NOR in *P. cucullata* similar to those described by King (1980) in the genus *Litoria*. It is conceivable that the inactivity or activity of this NOR is species specific. The existence of such latent NORs could be explained by the alteration of some factors which regulate the expression of these ribosomal cistrons, or by structural modifications of the rDNA itself during the evolutionary differentiation of the genus *Pycnogaster*. This may be reinforced by data reported in mammals, where species-specific transcription factors for rRNA genes have been described, and also by evidence for enhancer elements in rDNA of *Xenopus* species that occur in variable numbers in different ribosomal genes (see Reeder, 1985 for review).

With regard to *minor* labelled sites for 18+26S rDNA, they could also be considered as latent NORs. However, these sites coincide with some telomeric regions and it may be argued that the rDNA probe cross-hybridize with (G+C) sequences as those demonstrated in the telomeres of many species of eukaryotes (Allshire *et al.*, 1989).

In conclusion, we can distinguish two categories of latent NORs in *P, cucullata*. The *strictly* latent NORs consist of ribosomal cistrons (18+26S rDNA) which do not show nucleolar activity, at least in one very specialised cell type (spermatocytes). This could be the case of the ribosomal cistrons located at the L_1 chromosomes (fig. 1(b)). The second type, so called *facultative* latent NORs, include those 18+26S rDNA sites

which show a variable transcriptional activity within a single individual, thus suggesting the existence of a cellular control for the rRNA synthesis.

Further experiments should be performed on other cell types, in order to obtain a more general view of the types and behaviour of latent NORs in the species *Pycnogaster cucullata.*

Acknowledgements We wish to thank Dr Jaime Renar who kindly provided us rDNA clones that have been used in the present study. This work was supported by a grant from the Spanish CICYT PB 87/114.

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