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Population differences in the expression of nucleolus organizer regions in the grasshopper *Eyprepocnemis plorans*

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Summary. Fluorescence in situ hybridization revealed the presence of ribosomal RNA genes in paracentromeric regions of all A chromosomes and in the distal half of B chromosomes in embryonic cells from Moroccan specimens of the grasshopper *Eyprepocnemis plorans*. The expression of these genes was monitored by the presence of nucleoli attached to each chromosome bivalent in diplotene cells from males collected from two different Moroccan populations and was compared to previous data of Spanish populations. Whereas only the nucleolus organizer regions (NORs) on S_9–S_{11} and X chromosomes were active in the Spanish specimens, Moroccan individuals showed NOR activity in all chromosomes. The rRNA genes on the B chromosome were inactive in both populations. The S_9 and S_{10} NORs were less active in Moroccan specimens than in Spanish specimen, which might be partly explained by the negative interdependence for expression of the S_{10} NOR with respect to those on L_2 and X chromosomes. On the other hand, the X NOR was more active in Moroccan specimens than in Spanish specimens, and this might be partly due to the positive effect that the presence of B chromosomes has on the expression of this NOR. The implications of these observations on current models of NOR activity regulation are discussed.

Keywords: rDNA; Nucleolus organizer region; B chromosome; Grasshopper; *Eyprepocnemis plorans*.

Abbreviation: NOR nucleolus organizer region.

Introduction

In species with nucleolus organizer regions (NORs) in several chromosomes a regulatory mechanism is expected to exist in order that each cell gets an appropriate amount of rRNA. The elucidation of such a mechanism is still far from being completely understood, but recent research has provided some interesting insights. Schubert and Künzel (1990) have suggested that the number of rDNA cistrons is correlated to the expression level of a NOR. Forty years earlier, the size of the nucleolus was related to the amount of rRNA synthesized (Caspersson 1950). Recently, Zurita et al. (1998) have suggested that, because of their high competitiveness for transcriptional factors, NORs with the greatest amount of rDNA are preferentially activated.

Interindividual differences in the amount of rDNA have been reported in *Bufo marinus* (Miller and Brown 1969), *Homo sapiens* (Evans et al. 1974), *Ambystoma mexicanus* (Sinclair et al. 1974), *Salmo irideus* (Schmidtke 1976), and *Rattus norvegicus* (Sasaki et al. 1986), and the variation observed by fluorescence in situ hybridization (FISH) reveals differences in the number of cistrons (Wachtler et al. 1986, Suzuki et al. 1990, King et al. 1990, Leitch and Heslop-Harrison 1992).

López-León et al. (1994) reported the presence of rDNA in all standard chromosomes from Spanish specimens of the grasshopper *Eyprepocnemis plorans* and distinguished between large (located on the chromosomes S_9, S_{10}, S_{11}, and X) and small (located on the chromosomes L_1 to M_8) rDNA clusters. For simplicity, we will name primary NORs the large rDNA clusters and secondary NORs the small ones. With the exception of a single male found in Salobreña (Granada, Spain) by J. S. Rufas (Universidad Autónoma de Madrid, Spain, pers. commun.), only primary NORs are active in Spanish specimens of *E. plorans* (Cabrero et al. 1987; López-León et al. 1991, 1995).

In addition to the standard (A) chromosomes, almost all populations of *E. plorans* harbor a poly-

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morphism for supernumerary (B) chromosomes (for a review, see Camacho et al. 2000). These B chromosomes contain the greatest amount of rDNA in the genome (López-León et al. 1994, Cabrero et al. 1999), but with the exception of a single male carrying a centric fusion between the B and the longest autosome (Cabrero et al. 1987), the rDNA contained in the B chromosome has never been found to be active.

In this paper we analyze the localization and expression of the NORs in Moroccan specimens of *E. plorans*. The frequency of NOR expression was analyzed and compared with data reported by López-León et al. (1995) for Spanish specimens. Finally, the interdependence for expression between the different NORs, including the possible effects of B chromosome was also analyzed.

This work was orally presented at the VIII Congreso de la Sociedad Española de Biología Celular held in Granada (December 17–21, 1999).

Material and methods

Adult males and females of the grasshopper *Eyprepocnemis plorans* were sampled from the Moroccan populations of SO. DE. A. and Frain (for geographical locations see the map in Bakkali et al. 1999). Testes were immediately fixed in 3 : 1 ethanol-acetic acid, in order to analyze NOR expression by means of the silver nitrate impregnation technique (Rufas et al. 1982). A total of 11 males from SO. DE. A. and 9 from Frain were used for this purpose, from which 128 first spermatocytes at diplotene were analyzed, a meiotic stage where previous activity of each NOR may be evaluated by the presence of a nucleolus attached to it.

Females were kept alive in the laboratory by placing them in a cage with grass and humid vermiculite to facilitate egg laying. Egg pods were incubated at 28 °C for ten days and each egg was dissected in insect saline solution. The embryos were then fixed for cytological analysis as described in Camacho et al. (1991). Chromosome localization of rDNA was performed on embryo cells by the FISH technique described in López-León et al. (1994) and Cabrero et al. (1999).

To compare the expression frequency of the different NORs between Moroccan and Spanish specimens, we used data from Jete (Granada, Spain) reported by López-León et al. (1995). The frequency of activity per cell for each NOR was compared between specimens of the two geographical zones by means of the Mann–Whitney test. To test the interdependence for activity between the different NORs in the same chromosome complement, a series of logistic regression tests were done with each NOR as the dependent variable and the remaining NORs as independent ones. This analysis is very appropriate for binary variables such as NOR expression, where two states where recorded for each one, i.e., active or inactive. In addition, we calculated the odds ratio, a useful index for the interpretation of the results of logistic regression. It is computed from a 2×2 classification table, which displays the predicted and observed classification of cases for a binary dependent variable. An odds ratio lower than 1, with 95% confidence limits not including the value 1, indicates negative association between the two variables involved. If the odds ratio is higher than 1 and the 95% confidence limits exclude the value 1, the association is positive. Finally, the Mann–Whitney test was used to investigate possible effects of B chromosomes on the expression of the NORs on the A chromosomes. When multiple univariant tests were performed, we applied the sequential Bonferroni test to avoid type I errors (Rice 1989).

Results

The karyotype of *E. plorans* consists of 22 plus X0 (♂♂) or XX (♀♀) telocentric chromosomes which are conventionally classified into three size groups: three long (L_1–L_3), five medium (M_4–M_8), and three short (S_9–S_{11}) chromosomes; the size of the X chromosome being intermediate between those of L_2 and L_3 (John and Lewis 1965). In addition, specimens of this species can carry B chromosomes, which are found in almost all natural populations (Camacho et al. 2000).

In Moroccan specimens, the FISH technique showed the presence of rDNA in the paracentromeric region of all A chromosomes and the distal half of B chromosomes (Fig. 1). The largest cluster of rDNA was found on the B chromosome, followed by those on the X, S_9, S_{10}, and S_{11} chromosomes, i.e., those A chromosomes harboring the primary NORs. The remaining autosomes (L_1 to M_8) showed minute amounts of rDNA (see Fig. 1 b). Similar results have been previously reported in specimens from Spanish populations of this grasshopper (López-León et al. 1994).

A score of 128 diplotene cells from males collected at both Moroccan populations showed nonsignificant differences in the activity per cell of any of the NORs between SO. DE. A (61 cells) and Frain (67 cells) (results not shown). This permits using the whole collection of cells (128) as a single sample for comparisons with previous data obtained in Jete (Spain) by López-León et al. (1995). On average, 5.54 NORs out of the 12 existing on the A genome were active in Moroccan specimens (Fig. 1 c, d), which was significantly higher than the 2.25 observed in Spanish specimens (Mann–Whitney: $U = 4443$, $P < 0.001$). This higher number of active NORs per cell was due to the activity on the L_1–M_8 chromosomes, which never show NOR activity in Spanish populations (Cabrero et al. 1987, López-León et al. 1995) (Fig. 2). The rRNA genes located on B chromosomes in Morrocan specimens were always inactive.

Whereas primary NORs usually yielded large nucleoli, secondary NORs in Moroccan specimens always yielded small nucleoli (see Fig. 1 c, d).

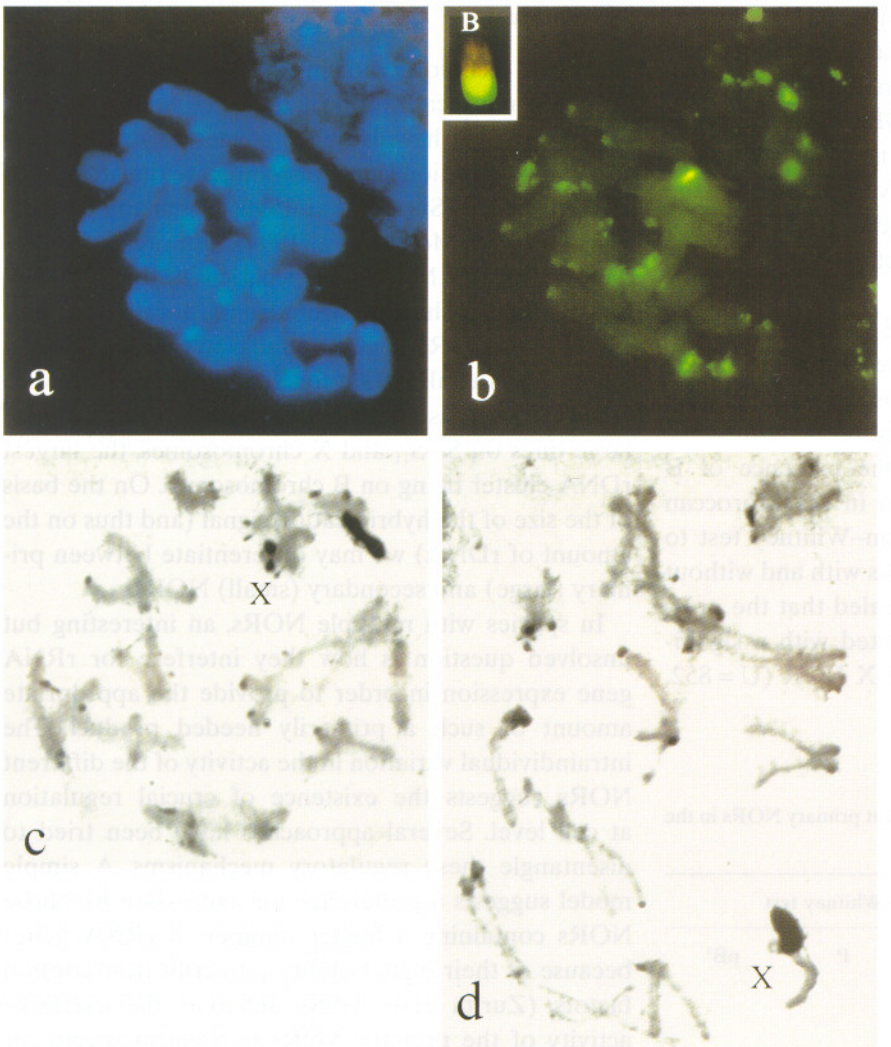

Fig. 1a–d. Localization and expression of rRNA genes in the grasshopper *E. plorans*. **a** and **b** Embryo cell submitted to staining with 4′,6-diamidino-2-phenylindole (**a**) and FISH with an rDNA probe (**b**). **b** Note the presence of large and small rDNA clusters, the largest one being distally located on the B chromosome (**inset**). **c** and **d** First spermatocytes at diplotene after silver staining, showing large and small nucleoli

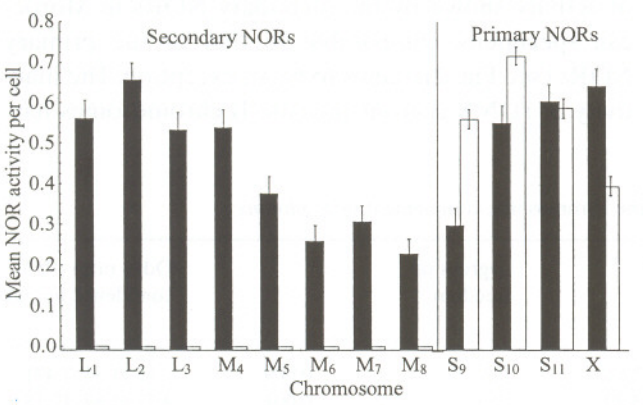

Fig. 2. Mean NOR activity per cell in Moroccan (black bars) and Spanish (white bars) specimens of the grasshopper *E. plorans*

A comparison of the mean activity per cell shown by the primary NORs indicated significant differences between the cells of Moroccan and Spanish specimens (Table 1), the S_9 and S_{10} NORs being less active and the X NOR being more active in Moroccan specimens, the S_{11} not showing significant differences (see also Fig. 2). As a whole, the primary NORs were active in less cells in Moroccan specimens (46.2%) than in the Spanish ones (56.3%) (Table 1). This suggests that the activity of secondary NORs in Moroccan specimens negatively influences the activity of S_9 and S_{10} NORs but positively influences that of the X chromosome. To test this possibility, an analysis of NOR activity interdependence was performed by means of logistic regression. It revealed a positive interdependence for activity among some secondary NORs (M_4 with L_1 and L_2, and L_3 with M_5) and a negative one between S_{10} and

L_2 and between S_{10} and X activities (Table 2). A similar analysis of the Spanish data revealed a significantly negative interdependence between the X NOR and the three remaining primary NORs (Table 2). It is therefore reasonable to conclude that the activity of secondary NORs (e.g., L_2) negatively influences the activity of some primary NORs (e.g., S_{10}) but is not responsible for the higher activity of the X NOR in the cells of the Moroccan specimens.

Logistic regression applied to the Spanish data from Jete (López-León et al. 1995) showed a negative interdependence between the X chromosome and the three remaining primary NORs (Table 2).

To detect possible effects of the presence of B chromosomes on NOR expression in the Moroccan specimens, we performed the Mann–Whitney test to compare NOR activity between cells with and without B chromosomes. This analysis revealed that the presence of B chromosomes is associated with a significant increase in the activity of the X NOR ($U = 852$, $P = 0.017$).

Table 1. Mean activity per cell of the different primary NORs in the genome of *E. plorans* males

Chromosome NOR	Mean NOR activity per cell of population:		Mann–Whitney test		
			U	P	pB[a]
	Morocco (n = 128)	Spain (n = 417)			
S_9	0.297	0.559	19699.0	7.3×10^{-6}	<0.001
S_{10}	0.547	0.715	22211.0	4.1×10^{-3}	0.0082
S_{11}	0.602	0.585	26249.5	7.8×10^{-1}	0.7784
X	0.641	0.393	20087.0	2.3×10^{-5}	<0.001
Mean	0.462	0.563	19581.0	5.1×10^{-6}	<0.001

[a] pB, probability corrected by the sequential Bonferroni method

Discussion

The FISH technique with an rDNA probe provides the chromosome localization of both active and inactive rRNA genes. It has also been suggested that the size of the hybridization signal may indicate the amount of rDNA present (Suzuki et al. 1990, Leitch and Heslop-Harrison 1992, Mellink et al. 1994). Our present results have shown that Moroccan specimens of the grasshopper *E. plorans* show a chromosomal localization and distribution of rRNA genes similar to that reported by López-León et al. (1994) for Spanish specimens, with small rDNA clusters on L_1 to M_8 chromosomes and large ones on S_9–S_{11} and X chromosomes, the largest rDNA cluster being on B chromosomes. On the basis of the size of the hybridization signal (and thus on the amount of rDNA) we may differentiate between primary (large) and secondary (small) NORs.

In species with multiple NORs, an interesting but unsolved question is how they interfere for rRNA gene expression in order to provide the appropriate amount of such a primarily needed product. The intraindividual variation in the activity of the different NORs suggests the existence of crucial regulation at cell level. Several approaches have been tried to disentangle these regulatory mechanisms. A simple model suggests a preference for expression for those NORs containing a higher number of rRNA genes because of their higher ability to recruit transcription factors (Zurita et al. 1998). Whereas the exclusive activity of the primary NORs in Spanish specimens (Cabrero et al. 1987; López-León et al. 1991, 1995) is in agreement with this hypothesis, the high frequency of activity shown by the secondary NORs in Moroccan specimens, comparable to that of the primary NORs (see Fig. 2), seems to be an exception. The inactivity of rDNA cistrons in some B chromosomes har-

Table 2. Interdependences of the activities of the different NORs in the chromosome complement of *E. plorans*

Population	NORs with significant interdependence	Type of association	t	degrees of freedom	P	Odds ratio (95% confidence limits)
Morocco	L_1–M_4	+	2.64	116	0.009	3.15 (1.33–7.47)
	L_2–M_4	+	3.30	116	0.001	4.77 (1.87–12.18)
	L_3–M_5	+	3.12	116	0.002	4.36 (1.71–11.12)
	L_2–S_{10}	–	–2.51	116	0.014	0.31 (0.13–0.78)
	X–S_{10}	–	–2.12	116	0.036	0.40 (0.17–0.94)
Spain	X–S_9	–	–3.49	413	<0.001	0.48 (0.31–0.72)
	X–S_{10}	–	–4.46	413	<0.001	0.36 (0.23–0.56)
	X–S_{11}	–	–2.56	413	0.011	0.58 (0.38–0.88)

boring the largest rDNA cluster in the genome, e.g., B chromosomes in *E. plorans* (Cabrero et al. 1987, López-León et al. 1995, this paper) and *Rattus rattus* (Stitou et al. 2000), is another exception, although in this case the B NOR inactivity might be a consequence of their general heterochromatinization and genetic inactivation caused by host genome mechanisms for defence against intragenomic parasites (Yoder et al. 1997). In such a case, the B NOR might be completely unavailable to regulatory mechanisms for A chromosomes. On the other hand, the activation of secondary NORs (small rDNA clusters) in Moroccan *E. plorans* cells where some of the primary NORs (large rDNA clusters) were inactive suggests that more complex models are necessary to explain this case.

Although secondary NORs in Moroccan specimens were comparatively as active as primary ones, the size of the nucleoli yielded was rather different, the primary NORs yielding large nucleoli and the secondary NORs very small ones. This is consistent with the existence of a relationship between the number of rRNA genes in a NOR and the amount of rRNA synthesized and, consequently, the nucleolus size (De Capoa et al. 1988, Shubert and Künzel 1990, Zatsepina et al. 1996).

The activation of the secondary NORs in Moroccan specimens might be due to some genetical and/or environmental differences determining regulatory changes in these individuals with respect to those from Spain. Since NORs are usually highly polymorphic (Henderson et al. 1976, Schmidtke 1976, Galleti et al. 1985, Sasaki et al. 1986, Sánchez et al. 1989), the possibility remains that primary NORs in Moroccan specimens could harbor less rDNA than Spanish ones, so that the activity of secondary ones is needed. Quantitative FISH of primary NORs in both Moroccan and Spanish specimens is needed to test this hypothesis. In addition, a detailed comparison of the nucleolus size between Moroccan and Spanish cells would help to ascertain whether rRNA requirements differ between specimens from the two geographical origins.

The analysis of NOR expression interdependence indicates a negative association between the S_9–S_{11} NORs and the X one, in Spanish specimens. In Moroccan specimens, the activity of the secondary NORs was paralleled by a decrease in the activity of the S_9 and S_{10} NORs and an increase in the activity of the X NOR. But only the S_{10} decrease was directly dependent on the expression of the secondary NORs since it was negatively associated to the activity of the NOR on the L_2 chromosome. It is also remarkable that the only negative interdependence between primary NORs in Moroccan specimens was found between the S_{10} and the X, those involving the S_9 and S_{11} NORs in Spanish specimens not appearing in Morocco. Since S_{10} harbors the only primary NOR in Moroccan specimens which is not accompanied by the 180 pb DNA tandem repeat reported by López-León et al. (1994) (J. Cabrero et al. unpubl.), the possibility that this repeat might play a role in NOR activity regulation, as was suggested by López-León et al. (1995), is not unlikely. The repeat could act in some unknown way protecting the adjacent NOR from inactivation or interchromosomal dependence. The S_{10} NOR, by being devoid of this repeat, would be prone to suffer dependencies, whereas the remaining NORs would be more or less blind to some interchromosomal effects, perhaps depending on their amount of DNA repeat.

The NOR on the X chromosome was significantly more active in Moroccan specimens than in Spanish ones, and this was not due to the expression of the secondary NORs since no activity interdependence was observed between the X and the L_1–M_8 chromosomes (see Table 2). More likely is the possibility that this higher activity is due to the presence of B chromosomes since the X was significantly more active in cells harboring B chromosomes. However, this cannot be the whole explanation since the X NOR was significantly more active in 0B cells from Morocco than in 0B cells from Spain ($U = 8678$, $P = 0.0002$). Increases in the activity of the X NOR associated to the presence of B chromosomes were reported by Cabrero et al. (1987) from a Spanish population of *E. plorans* collected at Salobreña. In the Jete population analyzed by López-León et al. (1995), the X NOR also tended to be more active in the presence of B chromosomes, but the difference did not reach significance ($U = 19543$, $P = 0.079$). Changes in NOR activity patterns associated to the presence of B chromosomes have also been reported in the migratory locust (Salcedo et al. 1988). Whether the higher frequency of X-NOR expression in B-carrying cells has a relationship with the presumed origin of the B chromosome from the X chromosome (López-León et al. 1994) is an interesting subject for future studies.

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