Effects of B chromosomes on the activity of nucleolar organizer regions in the grasshopper *Eyprepocnemis plorans*: activation of a latent nucleolar organizer region on a B chromosome fused to an autosome

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Four nucleolar organizer regions (NORs) are active in standard males of the grasshopper *Eyprepocnemis plorans*. They are located near the centromeric regions of the S_9, S_{10}, S_{11}, and X chromosomes. Changes in the pattern of NOR activity have been observed in the presence of a B_2 type supernumerary chromosome. Males with one B show a higher mean number of active NORs per cell than do zero B males owing to significant increases in the activity of the NORs on the S_{11} and the X. Zero B and one B embryos, however, showed similar patterns of activity. In a male carrying a centric fusion between a B and one of the L_1 chromosomes, the activation of a latent NOR, present at the telomere of the long arm of the B, parallelled a significant decrease of NOR activity on the S_9 and S_{10} bivalents stemming from a competition between different NORs in the presence of the B. Thus, while in zero B males the activity of the S_{10} NOR influences that of the NORs on the X and S_9 in a negative way, in one B males it does not do so, although such an influence is observed in the B–L_1 fusion male where the activity of the S_{10} NOR again decreases significantly. On the other hand, the activities of the NORs on the S_9 and S_{11} show a significant positive interdependence in both zero B and one B males where S_{11} NOR activity is increased but do not do so in the B–L_1 fusion male, which shows a significant decrease in the S_9 NOR activity.

Key words: *Eyprepocnemis plorans*, B chromosome, nucleolus.

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Quatre régions organisatrices nucléolaires (NORs) sont actives chez les mâles standard des sauterelles *Eyprepocnemis plorans*. Elles sont localisées près des régions centromériques des chromosomes S_9, S_{10}, S_{11} et X. Des changements dans les modes d'activité NOR ont été observé en présence d'un chromosome surnuméraire de type B. Les mâles possédant le type B_1 présentent un nombre plus élevé de NORs actives par cellules que les mâles 0B, en raison d'augmentations significatives de l'activité des NORs de S_{11} et de X. Toutefois, les embryons 0B et 1B présentent des modes d'activité similaires. Chez un mâle porteur d'une fusion centrique entre un chromosome 1B et l'un des chromosomes L_1, l'activation d'une NOR latente présente sur le télomère du bras long du B a égalé une réduction de l'activité NOR sur les bivalents S_9 et S_{10}, en raison d'une compétition entre les différentes NORs en présence du B. Ainsi, tandis que chez les mâles 0B l'activité de la NOR du S_{10} influence celle des NORs de X et de S_9 de façon négative, chez les mâles 1B cette influence n'existe pas, bien qu'une telle influence soit observée chez les mâles ayant une fusion B–L_1 où l'activité de la NOR S_{10} diminue ici encore de façon significative. D'autre part, les activités des NORs de S_9 et de S_{11} présentent une interdépendance positive significative, tant chez les mâles 0B que 1B, où l'activité des NORs de S_{11} est augmentée; toutefois, cette situation n'existe pas chez les mâles ayant une fusion B–L_1, lesquels présentent une diminution significative de l'activité NOR de S_9.

Mots clés : *Eyprepocnemis plorans*, chromosomes B, nucleole.

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Introduction

Supernumerary or B chromosomes have been reported in many plant and animal species (Jones and Rees 1982). On the basis of their harmful effects on carrier individuals most of them are regarded as "parasitic," maintaining themselves in natural populations by accumulation mechanisms (Nur 1977). The heterochromatic nature of some B chromosomes argues for the genetic inactivity of the DNA contained in them. However, exceptions are known with specific B chrmosomes showing particular genic activities with a great variety of effects (see Jones and Rees 1982), including effects at the isozyme level (Ruiz Rejón et al. 1980; Oliver et al. 1982). On the other hand, B chromosomes may also contain structural genes, predominantly the ribosomal RNA genes contained in active NORs present in several plant species (see Guillén and Ruiz Rejón 1984). Translocations between A and B chromosomes have been induced experimentally in several plant species (Roman 1947; Rakha and Robertson 1970; Evans and Macefield 1977; Beckett 1978; Pushpa 1980; Maguire 1984). Spontaneous A–B translocations, however, have only been detected in the grasshopper *Eyprepocnemis plorans*, where they involve either a B_1 type supernumerary and a medium autosome or else a B_1 and the X chromosome (Henriques-Gil et al. 1983).

The present report demonstrates changes in the activity pattern of the standard nucleolar organizing regions (NORs) of the grasshopper *Eyprepocnemis plorans* in the presence of a B_2 type supernumerary. Furthermore, a spontaneous centric fusion between the B_2 and the longest autosome (L_1) has been found to activate a latent NOR distally located on the B_2, which is normally inactive.

Materials and methods

Fifty-seven adult males were collected at Salobreña (Granada, Spain) during the autumn of 1984, one of which carried a centric fusion

between a B_2 and the L_1. Testes were fixed in acetic acid – ethanol (1:3) and were subsequently studied cytologically by the C-banding technique described by Camacho et al. (1984) and the silver impregnation technique of Rufas et al. (1982). To locate kinetochores, a brief pretreatment with 2 × SSC at 60°C was applied prior to the silver impregnation technique.

Results

Standard individuals of the grasshopper *Eyprepocnemis plorans* possess $2n = 22 + X0♂/XX♀$ telocentric chromosomes but the species is also characterized by a complex polymorphism for B chromosomes (Camacho et al. 1980; Henriques-Gil et al. 1984). One of the 57 males analysed in the present study carried a heterozygous centric fusion between a B_2 chromosome and one of the longest (L_1) autosomes (Figs. 1*a* and 1*b*). Given that the kinetochore of the B–L_1 chromosome was double the size of those in the B and L_1 chromosomes, it may be inferred that the former has arisen by a strict centric fusion between both kinetochores, with the loss of the short arm of the B but with little loss of L_1 material. The acentric fragment resulting from the centric fusion was presumably lost in the first cell division after the origin of the chromosomal rearrangement. Consequently, we have not detected it in the mutant male.

First meiocytes of standard males of *E. plorans* show four active NORs located subproximally on the S_9–S_{11} bivalents and the X univalent (Fig. 1*c*). While the B chromosomes of this species have never been seen to organize nucleoli (Fig. 1*d* and see Henriques-Gil 1984), when fused to the L_1, the B_2 was frequently associated with one or two nucleoli (Figs. 1*e* and 1*f*). This demonstrates that the B_2 chromosome must possess a distal NOR in its long arm that is normally inactive but which, as a result of the fusion with the L_1, is activated in many cells. To confirm this we scored NOR activity in zero B, one B, and B–L_1 fusion males using the methodology described in Cabrero et al. (1986). Table 1 shows total NOR activity per cell in the three types of males. Zero B males showed a mean number of NOR-active bivalents per cell significantly lower than those of both one B males ($t = 6.29$, $P < 0.001$) and the B–L_1 fusion male ($t = 4.55$, $P < 0.001$). The one B and B–L_1 fusion males, however, did not differ significantly one another ($t = 0.55$, $P = 0.5$–0.9). When NOR activity was compared in individual S bivalents and the X univalent (Table 2), the S_9 bivalent showed significantly less NOR activity in the B–L_1 fusion male than in zero ($t = 4.20$, $P < 0.001$) and one B ($t = 5.10$, $P < 0.001$) males, while the two latter types did not differ significantly from each other ($t = 1.46$, $P = 0.1$–0.2). Likewise, the NOR on the S_{10} bivalent is significantly less active in the B–L_1 fusion male than in zero B ($t = 2.35$, $P = 0.01$–0.02) and one B ($t = 2.30$, $P = 0.02$–0.05) males, the two latter showing a similar level of activity ($t = 0.10$, $P > 0.90$). In contrast, the NOR on the S_{11} bivalent is significantly less active in zero B males than in both one B ($t = 1.99$, $P = 0.02$–0.05) and the B–L_1 fusion male ($t = 3.52$, $P < 0.001$), while the two latter types of male do not differ significantly ($t = 1.73$, $P = 0.05$–0.10). NOR activity in the X chromosome shows a pattern of variation similar to that of the S_{11} bivalent, being significantly lower in zero B males than in both one B males ($t = 10.14$, $P < 0.001$) and the B–L_1 fusion male ($t = 6.28$, $P < 0.001$), but equally active in the two latter male types ($t = 1.07$, $P = 0.2$–0.5). Finally, while the B chromosome did not show NOR activity in any of the 103 cells analysed from one B males, it was associated by the telomere of its long arm to one or two nucleoli in 40.7% of the 54 silver-stained diplotene cells analysed from the B–L_1 fusion male (Figs. 1*e* and 1*f*).

TABLE 1. Total cell NOR activity ($\bar{X}$) in zero B, one B, and B–L_1 fusion males

Male type	No. of diplotene cells with 1 to 5 bivalents showing NOR activity					Total cells	$\bar{X}_c$	SE
	1	2	3	4	5			
Zero B	71	55	37	3	0	166	1.831	0.065
One B	18	30	31	24	0	103	2.592	0.102
B–L_1 fusion	7	22	18	5	2	54	2.500	0.132

Eyprepocnemis plorans has, at least, four (five considering that on the B) NORs, which implies that a complex mechanism of gene regulation must exist for the cellular expression of NOR activity. In an attempt to clarify this situation we have analysed activity dependence between the different NORs in two ways. First, we studied the relationship between the activity of each NOR and those of the remainder taken as a whole. For this purpose we selected a specific NOR and then compared the mean activity of the remaining NORs between cells in which the selected NOR was active and cells in which it was inactive (Table 3). Thus, the activity of the S_{10} and X NORs in zero B males seems to influence the activity of the remaining NORs negatively since in both cases the mean cell NOR activity ($\bar{X}_c$) in the other NORs was significantly higher in cells with the selected NOR (S_{10} or X) inactive. In one B males no such influence was observed. Here, on the contrary, there was a positive influence of the S_{11} NOR activity on that of the remainder since $\bar{X}_c$ is significantly higher in cells where the S_{11} NOR is active. In the B–L_1 fusion male, however, no interaction between the activity of NORs was observed.

The second approach to the analysis of interdependence in NOR activity was to test its extent between all possible pairs of NORs using a contingency χ^2 test (Table 4). In zero B males, NOR activity on the S_{10} was found to influence the activity negatively on the S_9 and the X. On the other hand, NORs on the S_9 and the S_{11} show a positive interdependence in activity patterns. In one B males, while the positive dependence between S_9 and S_{11} NORs was maintained, the negative influence of the S_{10} NOR on the S_9 and X NORs was no longer evident. In the B–L_1 fusion male the positive relation between S_9 and S_{11} NORs was not observed but here NOR activity on the S_{10} and the X was negatively related.

To test whether the NOR on the B chromosome, which is usually inactive during spermatogenesis, might be active during other developmental stages, we scored the number of nucleoli in silver-stained interphase cells from zero B and one B embryos (Table 5). Both types of embryo showed a similar mean number of nucleoli ($t = 0.57$, $P = 0.5$–0.9).

Discussion

We have observed remarkable changes in NOR activity patterns in the presence of the B chromosome as well as to the activation of a latent NOR located distally on the B itself in the B–L_1 fusion. The former provides evidence for a regulatory effect of the B on NOR activity in the standard chromosomes while the latter results from the derepression of the structural genes (rRNA) contained in the B itself.

The regulatory role of the B chromosome on NOR activity in *E. plorans* is shown by the existence of a significantly higher mean cell NOR activity in one B males compared with zero B males (see Table 1) resulting from increases in NOR activity on the S_{11} and the X (see Table 2). To determine if an equivalent

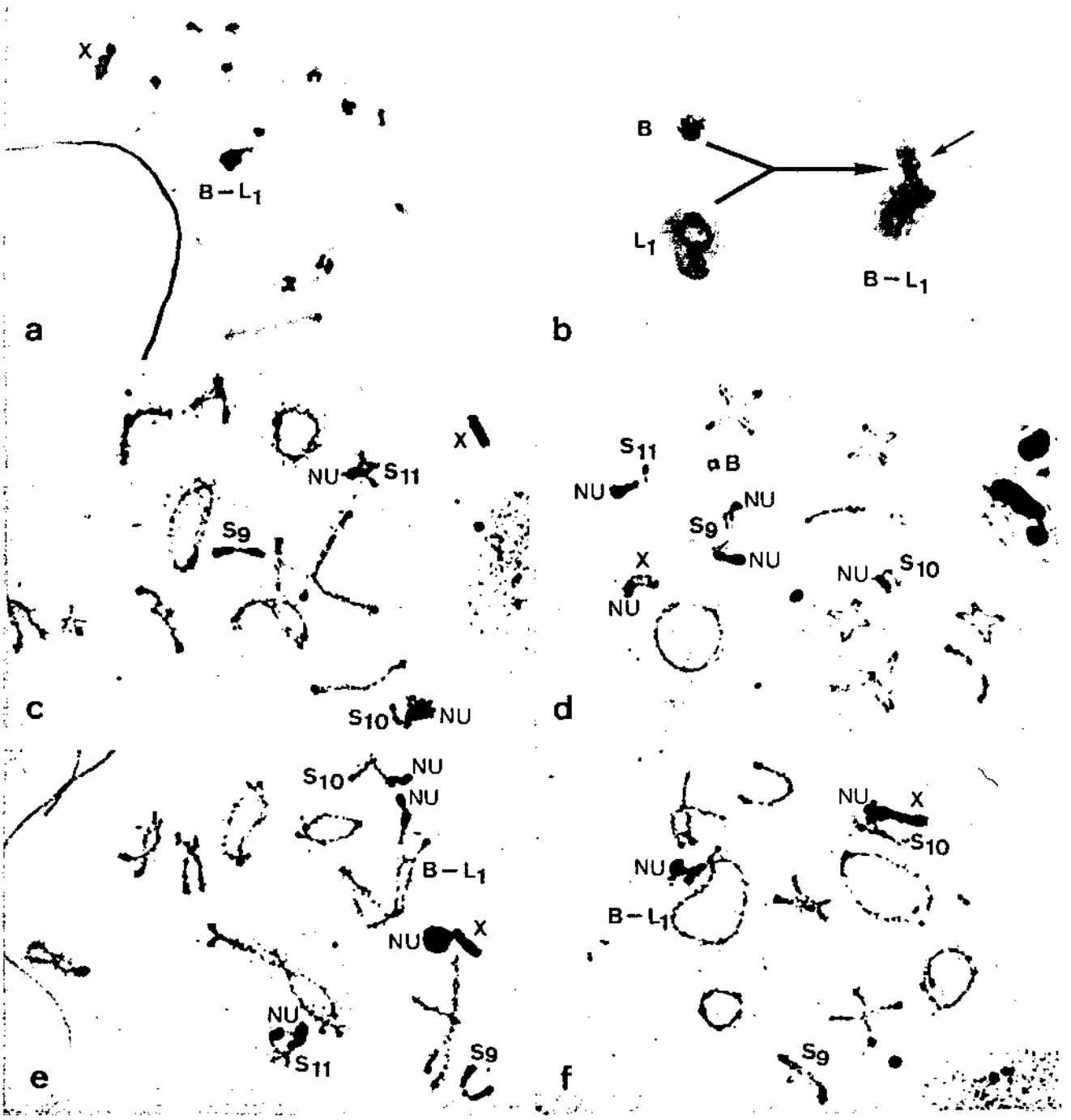

FIG. 1. B–L_1 centric fusion (*a*, *b*) and nucleolar activity in zero B (*c*), one B (*d*), and B–L_1 fusion (*e*, *f*) males of the grasshopper *Eyprepocnemis plorans*. (*a*) C-banded diplotene cell showing the B–L_1 fusion. (*b*) Selected L_1 bivalent, B univalent, and B–L_1 bivalent from metaphase I cells treated with 2 × SSC and silver impregnation. Note the presence of axial cores and the double-sized kinetochore of the B–L_1 chromosome (arrow) compared with those of B and L_1 chromosomes. (*c*) Silver-stained diplotene cell from a zero B male showing NOR activity in S_{10} and S_{11} bivalents. (*d*) Silver-stained diplotene cell from a one B male showing NOR activity in the X, S_9, S_{10}, and S_{11}. (*e*) Silver-stained diplotene cell from the B–L_1 fusion male showing NOR activity in the X, S_{10}, S_{11}, and B–L_1. (*f*) Silver-stained diplotene cell from the B–L_1 fusion male showing NOR activity in the X, S_{10}, and B–L_1. Note that in *f* the S_{11} is missing from this cell.

regulatory effect is present in other types of cells than spermatocytes, we scored the number of nucleoli in silver-stained interphase cells from zero B and one B embryos. Since each pair of homologues are separated at mitotic interphase they may yield two independent nucleoli. Our scores at diplotene from adult males, on the other hand, were made on bivalents showing NOR activity regardless of whether one or both homologues took part in the nucleolus formation. Thus the number of nucleoli at interphase can be assumed to be double that of bivalents showing NOR activity at diplotene. With this point in mind, the mean number of nucleoli per interphase cell in zero B embryos (3.665) is very similar to twice the mean number of bivalents showing NOR activity per diplotene cell in zero B males (1.831 × 2 = 3.662). On the other hand, the mean frequency of nucleoli in one B embryos (3.718) is significantly lower than double the mean number of bivalents showing NOR activity in one B males (2.592 × 2 = 5.184) and very similar to that of zero B embryos. These results indicate that the regulatory

TABLE 2. Mean NOR activity per bivalent or chromosome (± SE) in zero B, one B, and B–L_1 fusion males

Male type	Total cells	S_9	S_{10}	S_{11}	X	B–L_1
Zero B	166	0.512±0.039	0.801±0.031	0.380±0.038	0.139±0.027	—
One B	103	0.602±0.048	0.806±0.039	0.505±0.050	0.680±0.046	—
B–L_1 fusion	54	0.222±0.057	0.630±0.066	0.648±0.066	0.593±0.067	0.407±0.067

TABLE 3. Activity dependence between NORs

Male type and selected NOR	Total cells	Remainder NORs: Selected NOR active $\bar{X}_c$	SE	N	Selected NOR inactive $\bar{X}_c$	SE	N	t	P
Zero B	166								
S_9		1.329	0.086	85	1.309	0.055	81	0.20	0.5–0.9
S_{10}		0.947	0.075	133	1.364	0.085	33	3.68	<0.001
S_{11}		1.571	0.090	63	1.379	0.052	103	1.85	0.05–0.1
X		1.304	0.171	23	1.755	0.068	143	2.45	0.01–0.02
One B	103								
S_9		2.097	0.105	62	1.829	0.130	41	1.60	0.1–0.2
S_{10}		1.771	0.115	83	1.850	0.109	20	0.50	0.5–0.9
S_{11}		2.346	0.099	52	1.824	0.096	51	3.79	<0.001
X		1.929	0.110	70	1.879	0.155	33	0.26	0.5–0.9
B–L_1 fusion	54								
S_9		2.167	0.345	12	2.310	0.125	42	0.39	0.5–0.9
S_{10}		1.794	0.168	34	2.000	0.162	20	0.88	0.2–0.5
S_{11}		1.714	0.167	35	2.105	0.186	19	1.56	0.1–0.2
X		1.813	0.171	32	2.045	0.167	22	0.97	0.2–0.5
B–L_1		2.136	0.190	22	2.063	0.134	32	0.31	0.5–0.9

NOTE: $\bar{X}_c$, mean cell NOR activity; SE, standard error; N, number of cells.

TABLE 4. NOR activity dependence between pairs of NORs

Male type	NORs selected 1	2	No. of cells with: both NORs inactive	NOR 1 active	NOR 2 active	both NORs active	$\chi^2_{(1)}$	P	Type of dependence
Zero B	S_9	S_{10}	8	25	73	60	8.75	0.001–0.01	–
	S_9	S_{11}	58	45	23	40	5.37	0.02–0.05	+
	S_9	X	71	72	10	13	0.11	0.7–0.9	
	S_{10}	S_{11}	24	79	9	54	1.47	0.2–0.3	
	S_{10}	X	22	121	11	12	11.13	<0.001	–
	S_{11}	X	85	58	18	5	2.23	0.1–0.2	
One B	S_9	S_{10}	6	14	35	48	0.55	0.3–0.5	
	S_9	S_{11}	27	24	14	38	6.23	0.01–0.02	+
	S_9	X	15	18	26	44	0.35	0.5–0.7	
	S_{10}	S_{11}	14	37	6	46	3.21	0.05–0.1	
	S_{10}	X	3	30	17	53	2.41	0.1–0.2	
	S_{11}	X	19	14	32	38	0.83	0.3–0.5	
B–L_1 fusion	S_9	S_{10}	16	4	26	8	0	1	
	S_9	S_{11}	14	5	28	7	0.04	0.7–0.9	
	S_9	X	17	5	25	7	0	1	
	S_9	B–L_1	24	8	18	4	0.07	0.7–0.9	
	S_{10}	S_{11}	7	12	13	22	0	1	
	S_{10}	X	4	18	16	16	4.38	0.02–0.05	–
	S_{10}	B–L_1	13	19	7	15	0.14	0.7–0.8	
	S_{11}	X	6	16	13	19	0.52	0.3–0.5	
	S_{11}	B–L_1	9	23	10	12	1.04	0.3–0.5	
	X	B–L_1	16	16	6	16	1.93	0.1–0.2	

TABLE 5. Number of nucleoli in silver-stained interphase cells of zero B and one B embryos

Embryo type	No. of cells with 1 to 6 nucleoli						Total cells	Mean no. of nucleoli	SE
	1	2	3	4	5	6			
Zero B	—	14	85	87	31	4	221	3.665	0.058
One B	4	22	53	89	32	9	209	3.718	0.072

role of the B chromosomes on NOR activity is not evident in embryonic cells.

In the B–L_1 fusion male an increase was also observed in the activity of the NORs on the S_{11} and the X with respect to that observed in zero B males (see Table 2) but here changes also affect other NORs. Thus the activity levels of S_9 and S_{10} NORs decreased significantly in the B–L_1 fusion male compared with those observed in zero B and one B males. An extra active NOR on the B–L_1 chromosome appeared in 40.7% of the analysed cells (Table 2) so that total cell NOR activity of the B–L_1 fusion male was similar to that of one B males but significantly higher than that of zero B males (see Table 1). Consequently, the regulatory effect of the B chromosome, in increasing the NOR activity in S_{11} and X, is maintained in the B–L_1 fusion male, but it is partly modified by the rearrangement resulting in the appearance of an extra active NOR on the B–L_1 chromosome. This activity, however, is accompanied by a decrease in the activity levels of both the S_9 and the S_{10} NORs. These changes demonstrate that a very complex mechanism must be involved in the regulation of NOR expression. It is possible to distinguish two types of changes in this regulatory mechanism. On the one hand the increase in NOR activity on S_{11} and X chromosomes is not affected by the B–L_1 fusion, which indicates that the repositioning of the B does not modify its influence on these two NORs. Even so, the reactivation of the latent NOR on the B–L_1 and the decrease of NOR activity in the S_9 and S_{10} NORs are directly influenced by the fusion event. This could result either from contact of the B with the L_1 chromosome or else from the loss of the short arms of both chromosomes. In the first case, the functioning of presumed regulatory genes for NOR expression within the L_1 could be modified by means of a position effect. Such an effect has been recently described in the grasshopper *Chorthippus binotatus* where the presence of distal supernumerary chromosome segments on the M_5 is associated with significant increases of activity in paracentromeric secondary NORs on the M_4 and M_5 (Cabrero et al. 1986). In this case it is necessary to assume that genes that suppress the activity of these secondary NORs are located distally on the M_5 and that their normal functioning is affected by the presence of the extra heterochromatin. Under the hypothesis that the observed changes in B effects stem from the loss of chromosome material during the B–L_1 fusion, it could be argued that the short arm of the B carries suppressor genes for the activity of the rRNA genes contained in the B chromosome NOR and that their loss would lead to its expression. However, while all cells of the B–L_1 fusion male lacked the short arm of the B, the NOR on the B–L_1 chromosome was active only in 40.7% of cells, which indicates that its activity does not depend exclusively on the presence or absence of the B short arm.

An alternative approach to the regulation of expression of the different rRNA gene clusters in *E. plorans* was to study competition phenomena between NORs. We have analysed this by means of two types of statistical tests that examined,on the one hand, the interdependence of activity between each individual NOR and the remainder and,on the other hand, the interdependence of activity between pairs of NORs. Both analyses involved zero B, one B, and B–L_1 males to examine B effects on NOR activity.

In zero B males NOR activity in the S_{10} and the X is correlated with reduced activity in the remaining NORs (see Table 3), which identifies a competitive interaction between the S_{10} NOR and those on the X and the S_9 (see Table 4). On the other hand, NOR activity in the S_9 and the S_{11} are positively correlated (see Table 4). In one B males no competition was observed between the S_{10} NOR and those on X and S_9, but the positive influence exerted by the activity of the S_{11} NOR on those of the remainder (see Table 3), and especially on the S_9 NOR (see Table 4), was maintained. In the B–L_1 fusion male there was only a minor competitive interaction between the S_{10} and X NORs (see Tables 3 and 4). These results indicate that the observed competition between NORs in *E. plorans* is modified by both the presence of the B chromosome and the centric fusion of the B with the L_1, both modifications being consistent with the B effects on NOR activity. Thus in zero B males, the S_{10} NOR is the most active and influences negatively the NOR activity on the X and the S_9. In one B males, however, both the X and the S_9 NORs gain activity. In the B–L_1 fusion male, S_{10} NOR activity is very much reduced as a result of heightened X chromosome NOR activity. On the other hand, the activities of the S_9 and the S_{11} chromosome NORs show positive interdependence in both zero B and one B males, where there is a significant increase of the S_{11} NOR activity. This is not observed in the B–L_1 fusion male where S_9 chromosome NOR activity decreases significantly. These results confirm the existence of a complex mechanism for the regulation of gene expression in the different rRNA clusters of *E. plorans*.

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