The comparative analysis of NOR polymorphism detected by FISH and Ag-staining on horse chromosomes

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SUMMARY - The nucleolus organizer regions were investigated by FISH with biotinylated rDNA probe and silver staining on chromosomes of the domestic horse (*Equus caballus*). Ribosomal RNA loci were mapped at the secondary constrictions of the short arm of chromosome 1 and at the pericentromeric regions of chromosomes 27, 28 and 31. A new nucleolus-organizing chromosome 27 was identified. The interindividual, and interchromosomal polymorphism of NORs was described in 26 horses from 5 breeds. The relative rRNA gene activity was evaluated by the number of silver-stained chromosomes, and the size of silver deposits. The relative amount of rDNA in NORs was estimated by intensity of fluorescent hybridization signals. The size of silver deposits and intensity of fluorescence after FISH were determined for each NOR using arbitrary scales of 0-3 and 0-5, respectively. The statistical analysis of these data revealed a tendency for differentiation of NOR-bearing chromosome pairs by the rRNA gene number of copies, and the activity. A close correlation between activity of the rRNA genes and the relative number of rRNA gene copies was not found for NORs of homologous chromosomes.

Key words: FISH, Ag-staining, NORs, polymorphism, horse.

INTRODUCTION

Nucleolus organizer regions (NORs) are ones of the most intensively investigated chromosome sites. They are a suitable model to study structural-and-functional organization of chromosome. These chromosomal domains contain the major ribosomal RNA genes (18S, 28S and 5.8S). In situ hybridization techniques allow to visualize rRNA gene location. Silver staining creates the opportunities to evaluate the activity of the ribosomal genes directly on cytological preparations. Results of in situ hybridization reveal that observed number of copies of the rRNA genes varies within species (WACHTLER *et al.* 1986; MELLINK *et al.* 1991; SUZUKI *et al.* 1992). The irregularity comprises species, breed, individual, and intercellular variations both in the number and size of silver deposits (WINKING *et al.* 1980; ZAKHAROV *et al.* 1982; SOZANSKY *et*

al. 1985; MELLINK *et al.* 1991, 1992; SUZUKI *et al.* 1992). The relationship between Ag-staining properties and the amount of rRNA gene copies is not exactly known until now. There are few investigations and they dealt with human chromosomes mainly (MILLER *et al.* 1978; WARBURTON and HENDERSON 1979; BERNSTEIN *et al.* 1981; WACHTLER *et al.* 1986; DE CAPOA *et al.* 1988; MKHITAROVA *et al.* 1988).

In the karyotype of the domestic horse *(Equus caballus)* NORs have been detected by silver staining (RICHER *et al.* 1991) and FISH (MILLON *et al.* 1993). SWITONSKI *et al.* (1994) observed polymorphism of Ag-stained NORs on horse chromosomes. Previous studies do not collect the NOR polymorphism with the exact location on horse chromosomes. We present in this paper a new NOR-bearing chromosome and detail description of NOR polymorphism by semiquantitative analysis of FISH, and Ag-NOR patterns on identified R-banded horse chromosomes.

MATERIALS AND METHODS

Animals. - The study was carried out on 26 horses from the following breeds: Arabian (2 females, 4 males), Thoroughbred (1 female, 4 males), Polish Primitive horse (3 females, 3 males), Hucul (5 females, 3 males), and Cold-blood (1 male).

Chromosome preparations. - Chromosome spreads were prepared from blood lymphocyte cultures. Lymphocytes were cultured in medium RPMI-1640 with addition of autologous serum and pokeweed mitogen. To obtain R-banding BrdU (50 ìg/ml) was introduced into the culture 7h prior to harvesting.

Fluorescence in situ hybridization. - The probe used was a plasmid pUCI9, containing a 7.3 kb fragment of cluster of the human 18S-5.8S-28S rRNA genes (SKRYABIN *et a!.* 1989). The whole plasmid was biotinylated by nick translation with Biotin-16-dUTP (Boehringer, Mannheim) .

In situ hybridization was performed using standard procedure (LICHTER and CREMER 1992). Hybridization signals were visualized by fluorescein-conjugated avidin DCS (Vector Laboratories) and the amplification of signal was done using biotinylated anti-avidin D antibodies (Vector Laboratories)

Direct banding of BrdU-substituted chromosomes was obtained by modification of the technique of RONNE (1992) that omits Giemsa staining. After the first layer of avidin-FITC the slides were placed in Hoechst 33258 solution (1 ~g/ml, 2xSSC) and illuminated with UV-light for 1 h. Then the slides were incubated in fresh 2xSSC for 1 h at 65°C and second round of signal amplification was carried out for the detection of the hybridized probe on the banded chromosomes. The slides were mounted in antifade solution containing 1 ìg/ml propidium iodide.

Silver staining. - Well-spread metaphases were subjected to silver staining using the method of HOWELL and BLACK (1980). Slides with Ag-NORs were stained by means of FPG-technique (RONNE 1992) for the simultaneous visualization of silver deposits and banded chromosomes.

Analysis. - R-banded horse chromosomes were identified according to the standard karyotype of the domestic horse (RICHER *et al.* 1990). Chromosome prepara-

tions were examined using Vanox- T (Olympus) microscope equipped with FITC and propidium iodide filters.
The intensity of fluorescent hybridization signals was visually valued for each chromosome using six-mark grading system: 0 - absence of fluorescence, 1 - very small double-spot fluorescent signal, which is hardly visible on the maximum resolution of light microscope, 2 - distinct small double-spot fluorescent signal, the diameter of fluorescent spot is smaller than a chromatid width, 3 - medium-size fluorescent signal the diameter of fluorescent spot is equal or slightly over chromatid width, signals on two chromatids can be fused, 4 - large fluorescent signal, large integrated conglomerate of fluorescent grains well-defined even on the small microscope magnification, 5 very large fluorescent signal, fluorescence is observed in the region which is at least equal to one sixth of the short arm of chromosome 1.
To describe Ag-NOR patterns we counted the number of Ag-stained chromosomes and estimated the size of silver deposits on each NOR using four-mark grading system (0 - absence of silver staining, up to 3 - large size of silver deposit) which is similar to one of MKHITAROVA *et a!.* (1988).

RESULTS

Polymo1phism of FISH patterns.

At least 30 cells per individual animal were analysed after FISH. Ribosomal RNA genes in the domestic horse have been found on four pairs of autosomes: 1,28,31 and 27. The data of chromosomal location of the rRNA genes in 26 horses from 5 breeds are exhibited in T able 1. Figure 1 shows metaphase spread with 7 hybridization signals. Fluorescent signals are observed on the secondary constrictions of the short arm of chromosome 1 and near the centromeric regions of chromosomes 28, 31 and 27.
FISH cannot serve as a method for accounting the number of rRNA gene copies. The fluorescence intensity depends on several factors and is difficult to measure. We analysed metaphase spreads with similar hybridization efficiency and equal degree of chromosome condensation to approximate to the objectivity of estimation. The six-mark grading system we used does not allow to determine the exact number of rRNA gene copies in NORs but reveals an opportunity to range NOR-bearing chromosomes on the relative amount of the rDNA.
We noted the interindividual and interchromosomal polymorphism of intensity of fluorescence but not cell-to-cell. There was only one exception. The Thoroughbred male had cells (15.6%) with unusual large fluorescent signal on chromosome 28 which differed from other cells of the same individual.
The relative size of fluorescent signals varied both between pairs of NOR-bearing chromosomes and between two homologues of a particular pair. In almost all cases the largest signal was on chromosome 1 and the smallest one on chromosomes 28 and 27 (Fig. 2). Although in few animals fluorescent signals

on chromosomes 1,31 and 28 were roughly equal, the Student's t-test showed a statistically significant difference between the size of fluorescent signals on the particular NOR-bearing chromosome pairs: tl-31 = 9.611, tl-28 = 10.596, tl-27=22.988, t31-28=4.908, t31-27=17.882, t28-27=9.810; P<0.001. It is worth noting that there were five animals with small signal on one of the homologues of chromosome 1 (Fig. 1) or 31 but there were no animals with such one on both homologues of the same chromosomes .

TABLE 1- Location of rRNA loci an Ag-NORs on horse chromosomes.

Case number & sex	Breed	Number of chromosomes with fluorescent signals (per cell)				Modal number of chromosomes with Ag-NOR (per cell)			Ag-NORs on chromosome 27
		chr1	chr28	chr31	chr27	chr1	chr28	chr31	
1♀		2*	2	2	1	2*	2	2	+
2♀	Polish	2	2*	2*	2*	2	0	2*	–
3♂		2	2*	2*	2	2*	2	2	+
4♀	Primitive	2*	2*	2*	0	2	2*	2*	–
5♂	horse	2	2	2*	0	2	1	2*	–
6♂		2*	2*	2	0	2*	1	2	–
7♂		2	1	2	0	2	0	2	–
8♂		2	2*	2*	1	2*	0	1	–
9♂		2	2*	2*	0	2	0	2*	–
10♀	Hucul	2	2*	2*	0	2	0	1	–
11♀		2	2*	2	0	2	1	2*	–
12♀		2	2	2*	0	2*	1	1	–
13♀		2	2	2*	0	2	0	2*	–
14♀		2	2	2	1	2	0	2*	–
15♂		2*	2*	2*	1	2	1	2*	–
16♂	Thorou-	2	2*	2*	0	2	1	2*	–
17♂	ghbred	2*	2	2*	0	2*	0	2	–
18♀		2	2*	2	0	2	1	2	–
19♂		2*	2*	2	2*	2*	2*	2*	–
20♂		2	2*	2	0	2*	1	2	–
21♂		2*	1	2	0	2	0	2	–
22♂	Arabian	2	2	2	0	2	2	2*	
23♀		2*	2	2*	2*	2*	0	2*	–
24♂		2	2*	2	0	2	0	2*	–
25♀[xo]		2*	2*	2*	2*	2	0	2*	–
26♂	Cold-blood	2	2*	2	2	2	1	2	+

* difference of the fluorescent signal sizes or of the silver deposits between two homologues; + presence of silver deposits, - absence of silver deposits; !/XQ abnormal female with 2*n* = 63, XO karyotype.

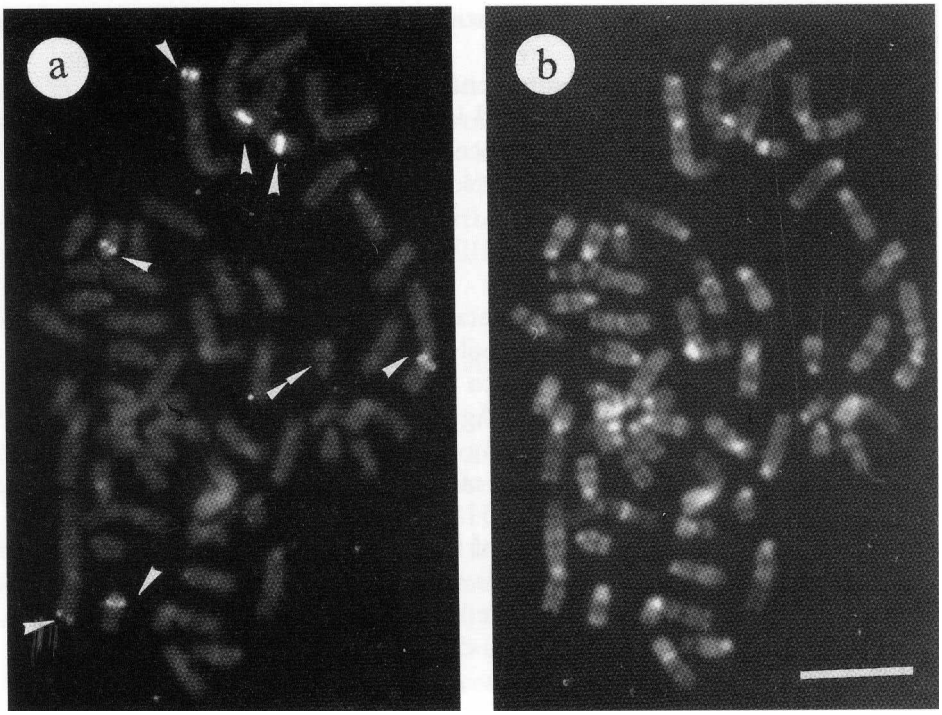

Fig. 1. - Fluorescence in situ hybridization of biotinylated rDNA probe to horse chromosomes. The fluorescent signals (a) are indicated by arrows. Double arrow shows chromosome 27 without hybridization signal. R-banded pattern is demonstrated on b. Bar equals 10 ~m.

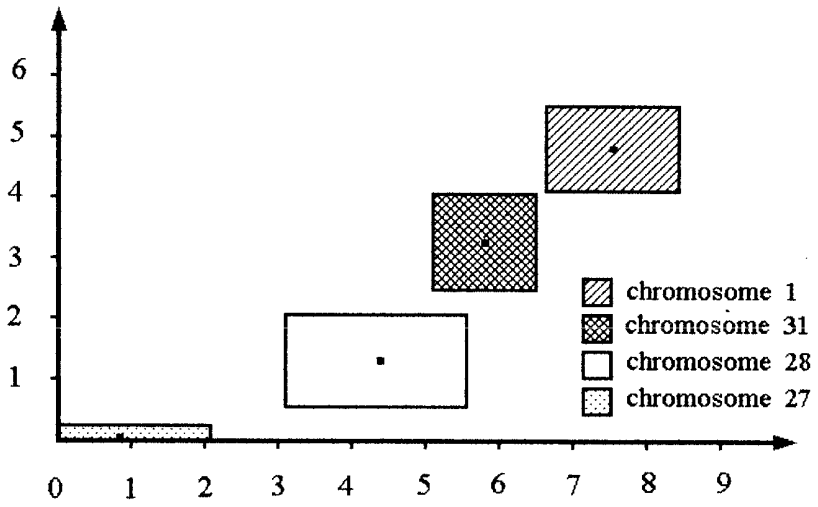

Fig. 2. - Graphical representation of the results of semiquantitative analysis of FISH with rDNA probe, and Ag-staining patterns in cells of 26 horses. The abscissa shows mean sizes of fluorescent signals and their standard deviations, the ordinate shows mean sizes of Ag-NORs and their standard deviations for chromosomes 1,27,28 and 31

Identical and different fluorescent signals on two homologues of the particular chromosomes were observed in animals from various breeds (Table 1), but we have not found any differences in fluorescence between homologues of chromosome pair 1 in the Hucul horses.

Polymorphism of Ag-NOR patterns.

At least 50 best silver-stained metaphase spreads from each animal were analysed for evaluating the Ag-NOR polymorphism.
Investigated animals had from 3 to 8 Ag-NORs per cell and the interindividual and intercellular variations of Ag-NOR patterns were observed. Fig. 3 shows the metaphase spread with 8 Ag-NORs. The number of silver stained NOR-bearing chromosomes per cell in modal class are presented for each horse in Table 1.
Statistical analysis of the results of four-marked estimation of intensity of Ag-staining revealed that in all horses (the data for each individual are not presented) Ag-NORs on chromosome 1 were largest, an intensity of Ag-

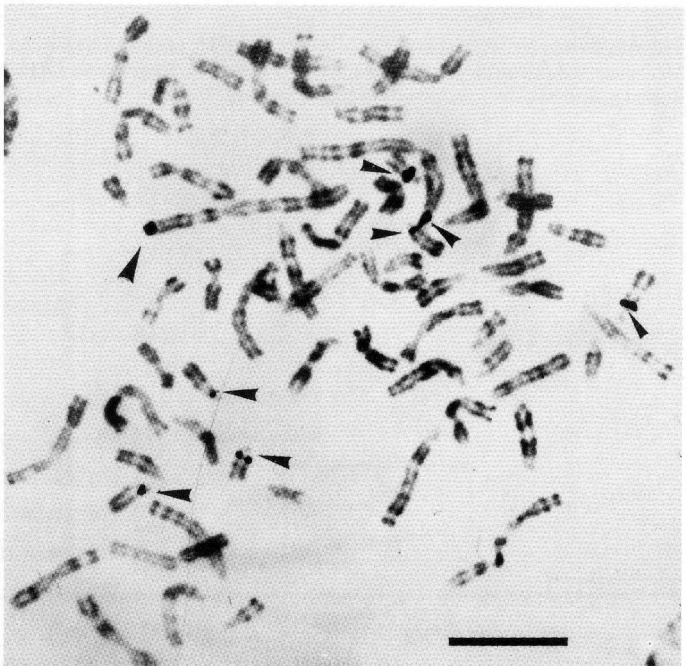

Fig. 3. - Silver staining of RBG banded horse chromosomes. Eight NOR-bearing chromosomes have silver deposits (arrowheads). Bar equals 10 ~m.

staining of chromosome 28 was smaller than chromosome 31 and the smallest intensity of Ag-staining was on chromosome 27. Thus the sizes of silver deposits on chromosome pairs 1, 31, 28 and 27 (Fig. 2) differ significantly: tl-31=7.313, tl-28=17.409, tl-27=33.862, t31-28=9.057, t31-27=19.874, t28-27 = 8.399; p< 0.001. Together with differences of silver deposits between NOR-bearing chromosome pairs the differences between homologues were also observed (Table 1; Fig. 4).

Relationship between polymorphism of Ag-NORs and FISH patterns.

The summarized data on the dependence of Ag-staining of NOR-bearing chromosomes on relative sizes of fluorescent signals are shown on Fig. 2. A positive correlation was found between the size of silver deposits and the amount of rDNA estimated by FISH on NOR-bearing chromosome pairs (r = 0.861, p < 0.001). However, the frequency of variants of Ag-staining of homologous chromosomes either depends very weakly (chromosomes 1 and 31, r=0.25, p<0.001 and r=0.38, p<0.001, respectively) or does not depend (chromosomes 28 and 27, r=0.07, p>0.05 and r=0,00, p>0.05, respectively) on the ratio of intensity of fluorescent signals (Fig. 4).

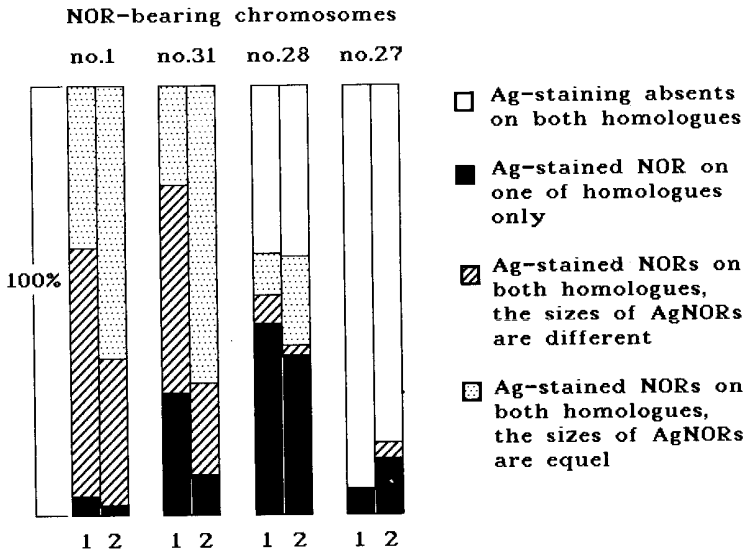

Fig. 4. - Frequency of four variants of Ag-NORs in analysed horse cells with different (1) and identical (2) fluorescent signals on the homologues of NOR-bearing chromosomes

DISCUSSION

In the normal karyotype the number of NOR-bearing chromosomes is as a rule species-specific and constant. Though the variable number of NORs at the intraspecific level has been noted for animals such as mouse (SUZUKI *et al.* 1992), fish (GARRIDO-RAMOS *et al.* 1995), amphibian species (SCHMID *et al.* 1995) and plants (GARRIDO *et al.* 1994, FUKUI *et al.* 1994). We present here the similar phenomenon of a variation in the rRNA gene location in the domestic horse (see Table 1). For the first time NOR was found on horse chromosome 27. The fact that rRNA genes have not been mapped yet on chromosome 27 (MILLON *et al.* 1993) can be explained by the fact that not all animals have them or they do not have the sufficient number of copies of the rRNA genes to be easily detected by FISH. Moreover rRNA genes on this chromosome are usually inactive. Ag-staining is out (RICHER *et al.* 1990). We observed silver deposits on chromosome 27 only in few cells from 3 of 26 analysed animals. The NORs of the Polish Primitive horses as compared to others are apparently more active. This breed (for history of creating these tarpan-like horses see the monograph by PRUSKI, 1959) is said to be phylogenetically close to the wild horse *Equus gmelini* which inhabited once Eastern Europe. It could suggest that wild members of the genus *Equus* as well as E. *przewalskii* (GAD! and RyDER 1983) possess higher number of Ag-NORs than domestic horse breeds. Similar data were obtained in other families. In wild Suidae the number of Ag-NORs is higher than in domestic breeds (MELLINK *et al.* 1992). Mice *(Mus musculus)* from different natural populations have additional AgNORs in comparison to laboratory strains (WINKING *et al.* 1980). Anyway this judgment is not always valid. Chinese Meishan breed of domestic pig is in AgNOR pattern very similar to the wild Asian suids (MELLINK *et al.* 1992). In our study the Cold-blood stallion has Ag-NOR on chromosome 27 which is characteristic for primitive horses.

Semi-quantitative analysis of the FISH patterns and Ag-staining on horse chromosomes confirmed once again wide polymorphism of NORs. The case of cell-to-cell polymorphism of rDNA distribution observed here is hardly likely a result of the culturing. Rather, chromosome rearrangement involving NORs has occurred in vivo and the population of lymphocytes was heterogeneous before culturing. This finding demonstrates NOR instability. The only case of rDNA amount variability within individual does not explain a wide intercellular polymorphism of rRNA gene activity as detected by Ag-staining.

An interesting phenomenon which emerged from our study is the statistically significant difference of activity, and rDNA amount at the different NOR-bearing chromosome pairs. In addition, an intensity of Ag-staining differed even in the cases when NOR-bearing chromosome pairs had identical fluorescent signals. Hence a divergence of functional activity likely precedes

distinction of NOR-bearing chromosome pairs on rDNA content. It seems there is a process of inactivation of NORs, the final stage of which is their disappearance in certain chromosomes (chromosomes 27 and 28). In support of this hypothesis species-specific regulation of rRNA synthesis in somatic cell hybrids occurs through inactivation of NORs with following chromosome elimination (MILLER *et al. 1976a, b).* Also the inactivation of loci is ensuing of the first steps in the process of diploidization in tetraploids (GARRIDO *et al.* 1994). However the two processes appear to be in the nature of equine NORs functionality. One of them leads through the most economical usage of genetic material to the elimination of the surplus of a genetic material. Another goes through maintaining the high degree of diversity by saving the reserve copies of rRNA genes in certain NOR-bearing chromosomes. As a result there are the numerous NOR-bearing chromosomes including those with inactivated NORs containing the relatively small number of rRNA gene copies.

Chromosomes 1 and 31 may have small NOR on one of the homologues, but we have not observed small NORs on both homologues of chromosomes 1 or 31. There is the critical level of the rRNA gene copies needed for normal cell vitality. Individuals which have less amount of rDNA in the NORs may not be viable. For instance, in chicken very small size NOR variants in a homozygous status were associated with embryonic mortality (DELANY and BLOOM 1993). Although in horse karyotype there are four pairs of NOR-bearing chromosomes, NORs on chromosomes 28 and 27 cannot apparently compensate for the lack of the rRNA gene copies on chromosomes 1 and 31. On the other hand a deficiency of the rRNA genes in chromosomes 27 and 28 does not affect viability. This finding corroborates once again the NOR functional difference between NOR-bearing chromosome pairs.

Analysing the data described in the literature we can suppose that tendency to NOR differentiation by the rDNA amount and their functional activity is general. The same has been noted for rabbit (MARTIN-DELEON *et al.* 1978; MARTIN-DELEON 1980), swine (MELLINK *et al.* 1991) and river buffalo (IANNUZZI *et al.* 1996) NOR-bearing chromosomes. This observation seems to be true also for plants (GARRIDO *et al.* 1994). However it is not always that simple. NOR of human chromosome 15 can be similar to horse NORs of chromosomes 27 and 28 as it has tendency to eliminate functional activity and rDNA (ZAKHAROV *et al.* 1982; SOZANSKY *et al.* 1985; NAZARENKO and KARTASHEVA 1991; LIAPUNOVA *et al.* 1994) but other investigations do not confirm that (DE CAPOA *et al.* 1988; MKHITAROVA *et al.* 1988).

The fact that there is a positive correlation ($r = 0.861$) between functional activity and relative amount of rDNA on the NOR-bearing chromosome pairs shows only that a process of NOR differentiation by functional activity is closely connected to a process of NOR differentiation on rDNA amount. So the data of a weak correlation between Ag-staining of the homologues and FISH patterns only for chromosomes 1 and 31 do not allow to suggest that the

amount of rDNA per NOR plays the principal role in the regulation of rRNA

gene transcription which involves many various factors.

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