Journal of Cell Science 111, 1433-1439 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
JCS9698

Sequential silver staining and in situ hybridization reveal a direct association between rDNA levels and the expression of homologous nucleolar organizing regions: a hypothesis for NOR structure and function

F. Zurita, R. Jiménez, M. Burgos and R. Díaz de la Guardia*

Departamento de Genética, Facultad de Ciencias, Universidad de Granada, 18071 Granada, Spain
*Author for correspondence (e-mail: mburgos@goliat.ugr.es)

Accepted 18 February; published on WWW 29 April 1998

SUMMARY

We have developed a procedure for sequential silver staining and in situ hybridization to analyze the relationship between the amount of rDNA present in nucleolar organizer regions, as estimated by in situ hybridization, and their level of expression, as estimated by the silver signal. For simplicity we used cells from the insectivorous mole *Talpa occidentalis*, which have a single pair of nucleolar organizer regions in chromosome pair 3. The relative content of ribosomal cistrons was also related to the hierarchy of activation of the nucleolar organizer regions present in this chromosomal pair. Statistical analyses demonstrated that both the relative level of expression and the activation hierarchy depended mainly on the number of ribosomal cistrons in nucleolar organizer regions. We propose a functional two-step hypothesis, which is consistent with most known data concerning interchromosomal, intercellular and interindividual variation in a number of plant and animal species, including *Talpa occidentalis*. In step one, the first available transcription factors bind randomly to the ribosomal promoters, such that larger nucleolar organizer regions are more likely to recruit them. In the second step the remaining transcription factors are recruited in a cooperative way, thus completing activation of one nucleolar organizer region, before the next one becomes active.

Key words: Nuclear organizing region (NOR), *Talpa occidentalis*, Ribosomal cistron, Transcription activation

INTRODUCTION

Silver staining is the cytogenetic method most commonly used to detect the position of nuclear organizing regions (NORs) on chromosome preparations (Goodpasture and Bloom, 1975; Howell and Black, 1980; Rufas et al., 1982). However, the biological significance of this technique is not completely understood, and it has been the cause of much debate for several years. Some authors have suggested an association between silver staining and the transcriptional activity of ribosomal genes during the preceding interphase (see Hubbel, 1985). This is supported by the fact that several proteins (mainly C-23; Jordan, 1987), located exclusively in the fibrillar structure of the nucleolus, are selectively stained by silver (Goessens, 1984; Hernández-Verdún, 1986). However, biochemical studies of rRNA-synthesis inhibition led others to contradict this hypothesis (Clavaguera et al., 1983; Medina et al., 1983; Sánchez-Pina et al., 1984). In general, these latter authors assume that for silver staining to occur, only a decondensed state of the NOR chromatin (nucleolar constriction) is required. Jiménez et al. (1988) demonstrated that decondensation of NORs is neccesary but not sufficient for silver staining, and suggested that previous transcriptional activity may also be needed. Silver staining has also been demonstrated in chromosomal structures other than NORs, such as heterocromatic blocks of the Algerian hedgehog *Erinaceus algirus* (Sánchez et al., 1995) and *Gerbillus* (our unpublished data).

In situ hybridization (ISH) permits the unequivocal detection of both active and inactive NORs (see Hsu et al., 1975). Although this is a qualitative rather than a quantitative technique, an association between the size of the hybridization signal observed in a given NOR and the number of ribosomal cistrons it contains has been proposed (Suzuki et al., 1990; Leitch and Heslop-Harrison, 1992; Mellink et al., 1994). The size of the ISH signal was also proposed to be associated with the size of the silver signal (De Capoa et al., 1988; Shubert and Künzel, 1990).

The variability in both the size (Bickham and Rogers, 1985; Sánchez et al., 1989) and the number (Galleti et al., 1985; Jiménez et al., 1988) of stained NORs in many species may be a consequence of differences in the number of ribosomal cistrons in homologous and nonhomologous NORs. This would explain, at least in part, the differences in the size of the

silver signal displayed by the expressed NORs, and in their preference of activation, as demonstrated in the grasshopper *Locusta migratoria* (Díez and Puertas, 1986) and in the rodent *Eliomys quercinus* (Sánchez et al., 1989), among others.

We recently investigated this subject in the insectivorous mole *Talpa occidentalis* (Zurita et al., 1997), a species with a single pair of NOR-carrying chromosomes (Jiménez et al., 1984). The sizes of the ISH and silver signals were used to estimate the relative amount of ribosomal cistrons and the transcriptional activity of NORs, respectively. Both ISH and silver staining detected interchromosomal, intercellular and interindividual variability of NORs, suggesting that transcriptional activity of NORs does not depend exclusively on the number of ribosomal cistrons. Hence additional factors, probably responsible for transcription activation, may also be involved in this variation.

In this paper we present a new approach in which a single sample of cells was investigated sequentially to determine both the level and hierarchy of activation and the amount of rDNA. The first two were estimated from the size and the presence or absence of the silver signal, and the latter from the size of the ISH signals.

MATERIALS AND METHODS

Animals

Five individuals of the insectivorous mole species *Talpa occidentalis* (Insectivora, Mammalia) were trapped live in Vega de Granada (southern Spain) with home-made mole traps (our unpublished technique).

Cytogenetic procedures

Chromosome preparations were made from bone marrow cells in accordance with our standard air-drying procedure (Burgos et al., 1986). Silver staining was done according to the method of Rufas et al. (1982). Well-stained cells were photographed and then silver was removed with 7.5% potassium hexacianoferrate III for 4 minutes, followed by 20% sodium thiosulfate for 5 minutes according to Warburton and Henderson (1979). Preparations were checked under the light microscope to ensure that all silver had been removed, then the cells were photographed again and the slides were processed for in situ hybridization. We used a Syrian hamster rDNA probe consisting of sequences corresponding to the 3′ end of the 18S gene, the internal transcribed spacer, and almost the entire 28S gene (see Wahl et al., 1983). Nick-translation labelling with digoxygenin and hybridization were done according to the protocol recommended by the supplier (Boehringer-Mannheim). Considering the whole sequential staining procedure, modifications of the original method by Warburton and Henderson (1979) mainly concerned the silver staining and ISH procedures, whereas the intermediate silver-removing protocol was performed without any change.

Procedures for statistical analyses

Procedure 1

To investigate the possible influence of previous silver staining on the distribution of cell types that was subsequently obtained with the ISH technique during the sequential Ag-ISH procedure, a preliminary analysis was done in individuals T-426 and T-427. One sample of cells from each individual was sequentially treated for silver staining and in situ hybridization. The distribution of ISH cell types in these two samples was then compared by contingency table analysis with those of two other samples of cells (one from each individual) that were processed for the ISH technique only.

Procedure 2

Contingency table analysis was also used to study the possible relationship between the presence or absence of a decondensed secondary constriction and the presence or absence of an ISH signal in a total of 90 chromosomes of pair 3 from individual T-427.

Procedure 3

To study interindividual, intercellular and interchromosomal variation of NORs, 339 cells in total from five individuals were analyzed sequentially. Nine cell classes could be established in each individual, on the basis of the number and relative sizes of the silver and ISH signals. The resulting multiway table with three variables (individual, Ag cell type and ISH cell type) was statistically analyzed by using a log-linear procedure to detect possible associations between variables.

Procedure 4

The 678 potentially NOR-bearing chromosomes contained in the 339 sequentially treated cells were analyzed to establish the possible relationships between the amount of rDNA and both the level of transcriptional activity and the hierarchy of activation of NORs. We counted the number of cases in which the relative size of an ISH signal with respect to that of its homolog (larger, similar or smaller) was concordant with that of the silver signal in the same chromosome. Concordance was considered to occur when one chromosome showed either the larger or the smaller signal with both the silver staining and the ISH techniques, but not when the larger silver signal and smaller ISH signal, or vice versa, coincided in the same chromosome. For this purpose those chromosomes lacking any signal in cells with only one signal were considered to have the smaller signal. In addition, we counted two cases of concordance in cells with two similar signals with both the silver staining and the ISH techniques, but only one in cells with two equal signals for one technique and two different signals (or only one signal) for the other technique.

For statistical analyses we used the Statgraphics software package (Version 5, Statistical Graphics Corporation).

RESULTS

The method we used for sequential silver staining and ISH proved to be highly reproducible and very effective in detecting interchromosomal, intercellular and interindividual variations in the structure and function of NORs. Fig. 1 shows a representative sample of mole cells treated with the sequential procedure. Chi-squared values obtained by contingency table analysis with the data summarized in Table 1 demonstrated the absence of significant differences between two samples of in situ-hybridized cells that were previously silver-stained, and two other samples of in situ-hybridized cells without previous treatment (Procedure 1). These data clearly show that previous silver staining did not interfere with the subsequent ISH technique in the sequential procedure.

In contrast, contingency table analysis of the data summarized in Table 2 demonstrated a significant association between the presence or absence of an ISH signal and a decondensed secondary constriction (Procedure 2). We also observed that the secondary constriction was always located on the chromosome showing the larger ISH signal in cells with a single secondary constriction.

Log-linear statistical analysis with the data summarized in Table 3 detected significant interindividual differences with respect to the distribution of the nine cell types established in each individual, depending on the number and relative size of both the silver and the ISH signals (likelihood ratio χ^2=173.471, P=0.0000; Pearson's χ^2=169.235, P=0.0000; calculated for the

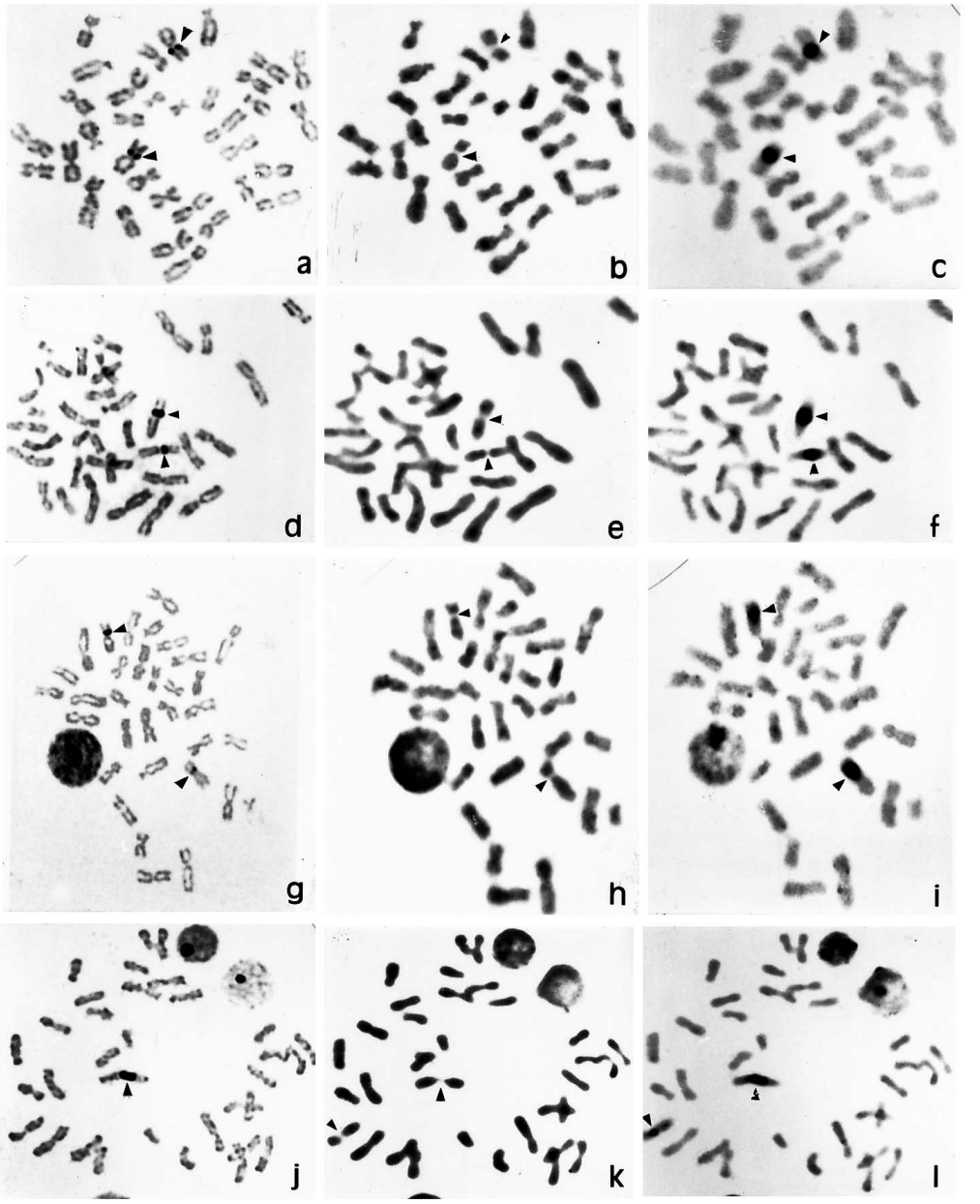

Fig. 1. Representative cells from *Talpa occidentalis* showing sequential Silver staining and in situ hybridization. Cells were silver-stained (a,d,g,j) then destained to reveal secondary constrictions (b,e,h,k), and then subjected to in situ hybridization with an rDNA probe (c,f,i,l). The cell shown in a, b and c produced two identical signals with both techniques. The relative size of the two different signals found in the cell in d, e, and f was similar with both techniques (concordance). In contrast, the relative size of the two silver signals in the cell shown in g, h and i was reversed after in situ hybridization (nonconcordance). The cell shown in j, k and l had two different hybridization signals but only one silver signal, corresponding to the larger nucleolar organizer regions.

model of independence between the 'individual' variable and the other two variables, i.e. the Ag-cell type and the ISH-cell type; Procedure 3). These data were further analyzed by applying the same log-linear model to several subsamples obtained by excluding specific individuals from the whole sample of five individuals (not shown). When we excluded data from individuals T-427 or T-425, which had the highest and lowest percentage of cells with heteromorphic NORs, respectively (cells with either one or two different ISH or silver signals; see Table 3), the log-linear model detected no significant difference between the remaining individuals. This is evidence for continuous variation of this character between individuals.

Table 1. Contingency table showing chi-squared values and significance levels calculated to test for possible interference of silver staining (Ag) on in situ hybridization (ISH) in sequentially treated cells from two individuals of *Talpa occidentalis*

		Number of cells with			
Individual	Technique	1 ISH signal	2 identical ISH signals	2 different ISH signals	Statistics
T-426	ISH alone	4	36	18	χ^2=0.528
	Sequential Ag-ISH	2	34	17	*P*=0.7679
T-427	ISH alone	6	8	56	χ^2=0.373
	Sequential Ag-ISH	9	14	74	*P*=0.8298

Table 2. Contingency table showing chi-squared values and significance levels calculated to test the relationship between the presence (+) or absence (–) of an in situ hybridization signal and a decondensed secondary constriction in chromosomes from one individual of *Talpa occidentalis*

Secondary constriction	In situ hybridization signal		Total
	+	–	
+	45	0	45
–	34	11	45
Total	79	11	90

χ^2=10.3567 *P*=0.0013.

Table 4 summarizes the number of cases of concordance and nonconcordance counted in the five individuals analyzed, and those expected from a random distribution of the relative size of both the silver and the ISH signals observed in each individual chromosome, with respect to those observed in its homologous chromosome (Procedure 4). Simple chi-squared analysis demonstrated that the number of concordances was significantly higher than nonconcordances in all five individuals. These results are direct evidence of a strong association between the size of the silver and the ISH signals. Individual T-427 showed the highest level of association between the size of both the silver and the ISH signals, and also had the highest percentage of cells with heteromorphic NORs (Table 3). This raised the question as to whether the percentage of concordances was correlated with the percentage of cells with heteromorphic NORs. Regression analysis demonstrated that these two variables fit both a linear model (correlation coefficient=0.8887, *P*=0.0438) and an exponential model (correlation coefficient=0.9014, *P*=0.0366; Fig. 2). Interestingly, individual T-427 also showed the most evident differences between the size of the signals (silver or ISH) of the two homologous chromosomes of pair 3 (Fig. 1j-l).

The relationship between the amount of rDNA in NORs and their hierarchy (preference) of activation was studied in contingency tables containing chromosomal data from cells showing two different ISH signals and only one silver signal (Table 5). Statistical analyses demonstrated a clearly significant association in three individuals (T-427, T-428 and T-429), no significant association in T-426, and an association approaching significance in T-425. Note that these later two individuals had the lowest numbers of cells with heteromorphic NORs (Table 3).

DISCUSSION

In situ hybridization directly detects ribosomal DNA, so that variations observed with this method are evidence of polymorphism for the number of ribosomal cistrons present in NORs. Silver staining has consistently been related to NOR activity, and is thus suitable for investigating NOR functionality. The use of these two methods thus helps to establish the possible relationship between the structure and function of NORs. However, the lack of a reproducible method for sequential silver staining followed by ISH made it

Table 3. Numbers of cells scored in five individuals of the species *Talpa occidentalis*, classified according to the number and relative size of the silver (Ag) and ISH signals, and the percentage of cells with heteromorphic NORs

Individual	Ag-NOR	ISH			Total	Percentage of cells with heteromorphic NORs	
		1 single signal	2 identical signals	2 different signals(*)		Ag signal	ISH signal
T-425	1 single signal	6	22	11(8+3)	39	69.44	34.72
	2 identical signals	0	17	5	22		
	2 different signals	0	8	3(2+1)	11		
	Total	6	47	19	72		
T-426	1 single signal	3	11	11(7+4)	25	61.19	40.29
	2 identical signals	0	18	8	26		
	2 different signals	0	11	5(4+1)	16		
	Total	3	40	24	67		
T-427	1 single signal	9	10	73(70+3)	92	96.91	85.57
	2 identical signals	0	3	0	3		
	2 different signals	0	1	1(1+0)	2		
	Total	9	14	74	97		
T-428	1 single signal	4	4	20(18+2)	28	79.25	83.02
	2 identical signals	0	3	8	11		
	2 different signals	0	2	12(10+2)	14		
	Total	4	9	40	53		
T-429	1 single signal	4	17	8(8+0)	29	92.00	48.00
	2 identical signals	0	3	1	4		
	2 different signals	0	6	11(8+3)	17		
	Total	4	26	20	50		

*Values in parentheses indicate the number of cells showing concordance (left) and nonconcordance (right) between the relative size of the Ag signal and the ISH signal of the two homologous NOR-bearing chromosomes in the group of cells with two different ISH signals.

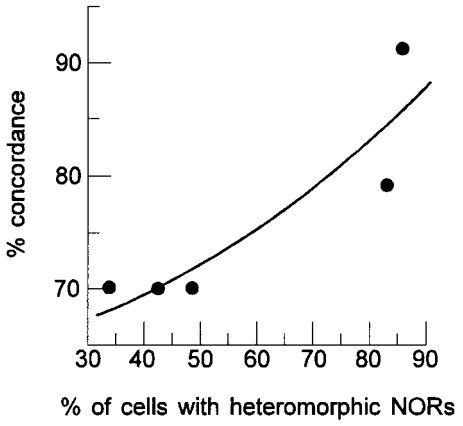

Fig. 2. Significant exponential correlation between the percentage of chromosomes with concordant silver and in situ hybridization signals and the percentage of cells with heteromorphic nucleolar organizer regions, in five individuals of *Talpa occidentalis*.

necessary to perform the two techniques in separate cell samples, so that no direct evidence could be obtained concerning the relationship between the structural and functional variability of NORs (see Zurita et al., 1997). Warburton and Henderson (1979) described a procedure for sequential silver staining-ISH, although no graphic evidence of their results was presented. Using their protocol as a starting point, we developed a modified procedure which is highly reproducible and provides good quality, sequentially stained metaphase plates in several mammalian species, including the insectivorous mole *Talpa occidentalis* (this paper) and the rodent *Eliomys quercinus* (our unpublished data). We have statistically demonstrated that silver staining does not interfere with subsequent ISH in the sequential procedure, a fact that validates the results obtained with this technique.

Regarding the significance silver staining in mitotic NORs, we previously showed that a decondensed state of NOR chromatin is neccesary, but not sufficient, to ensure silver staining, and suggested that additional factors, probably involved in transcription activation, may be needed (Jiménez et al., 1988). Our present results demonstrate that the presence of a visible (decondensed) secondary constriction is significantly associated with an ISH signal (Table 2). Furthermore, the observations that (1) chromosomes showing no ISH signal never had a secondary constriction, and (2) chromosomes with an ISH signal do not necessarily have a secondary constriction, are clear evidence that the existence of rDNA is also necessary but not sufficient to induce decondensation of the NOR chromatin. We also observed that the larger the ISH signal is, the larger the resulting secondary constriction appears (Fig. 1). In the light of these results we suggest that the funtionality of NORs is subject to the following chronological order of events: (1) presence in the NOR of a minimum amount of ribosomal cistrons (detectable by the ISH procedure), (2) decondensation of the NOR chromatin and formation of a secondary constriction, and (3) transcription of rDNA (detectable with silver staining). Although the steps should take place in this order, the existence of large amounts of rDNA in a given NOR does not ensure that a secondary constriction will be seen, nor does the observation of a secondary constriction ensure that silver staining will be positive (see Jiménez et al., 1988).

Our results clearly demonstrate a direct association between the amount of rDNA (ISH signal) and the level of expression of NORs (Ag signal), and are evidence that the level of transcriptional activity of NORs is directly related to the number of ribosomal cistrons. This is in clear agreement with recent molecular cytogenetic analyses by Zatsepina et al. (1996), who demonstrated that the degree of association to mitotic NORs of the transcription complexes, including the RNA polymerase I transcription factor UBF, is determined by the number of rDNA repeats rather than by any differential affinity of NORs to bind UBF. We also found that NORs

Table 4. Observed and expected numbers of NOR-bearing chromosomes showing concordance and nonconcordance between the relative size of the Ag and the ISH signals obtained with a sequential procedure, scored in five individuals of *Talpa occidentalis

Individual		Number of chromosomes with concordance	Number of chromosomes without concordance	Statistics
T-425	Observed	101	43	χ^2=23.36
	Expected	72	72	*P*=0.0000
T-426	Observed	94	40	χ^2=21.76
	Expected	67	67	*P*=0.0000
T-427	Observed	177	17	χ^2=131.96
	Expected	97	97	*P*=0.0000
T-428	Observed	84	22	χ^2=36.26
	Expected	53	53	*P*=0.0000
T-429	Observed	70	30	χ^2=16
	Expected	50	50	*P*=0.0000

*The numbers of concordances and nonconcordances were calculated from data in Table 3. For example, the number of chromosomes showing concordance in individual T-425 was calculated as follows: (6×2)+22+(8×2)+(17×2)+5+8+(2×2)=101. Similarly, the number of chromosomes showing nonconcordance was: 22+(3×2)+5+8+(1×2)=43

Table 5. Contingency table showing data for five different individuals of *Talpa occidentalis* with chi-squared values, significance levels and contingency coefficients (cc) calculated to test the possible association between the relative size of the ISH signal of NOR-bearing chromosomes and their order of activation

Individual	Ag signal	Relative size of the ISH signal		Statistics
		Larger	Smaller	
T-425	+	8	3	χ^2=2.9090
				P=0.0881
				cc=0.4138
	–	3	8	
T-426	+	7	4	χ^2=0.7273
				P=0.3938
				cc=0.2631
	–	4	7	
T-427	+	70	3	χ^2=119.342
				P=0.0000
				cc=0.6761
	–	3	70	
T-428	+	18	2	χ^2=22.5000
				P=0.0000
				cc=0.6247
	–	2	18	
T-429	+	8	0	χ^2=12.2500
				P=0.0005
				cc=0.7071
	–	0	8	

Data were obtained from cells with two different ISH signals but only one Ag signal. +, present; –, absent.

showing the largest ISH signals were preferentially activated, a fact indicating that the hierarchy of NOR activation is also conditioned by the number of ribosomal cistrons. However, a small number of cells in all individuals did not display this tendency, showing that the presence of abundant ribosomal genes in a given NOR makes it more likely, but does not ensure, that this NOR will be activated preferentially or will have a level of expression higher than its homologous.

Transcriptional activation of ribosomal cistrons, and hence NOR expression, requires the binding of transcription factors to promoters. Therefore, any model to explain the hierarchy and the relative level of expression of homologous and nonhomologous NORs should take into account how, when and at which level binding takes place. On the basis of these considerations we propose the following hypothesis, which is consistent with current knowledge on the structure and function of NORs.

Step 1. At the moment when the pattern of expression of NORs in a given cell is determined, transcription factors bind randomly to ribosomal cistrons so that any cistron is equally likely to recruit the required transcription factors. As a result, if the NORs of that cell have different numbers of ribosomal cistrons, those with more cistrons would be more likely to bind the first factors, and thus become activated preferentially. This explains the hierarchy of activation we found in *Talpa occidentalis*, where the larger NOR frequently showed the greatest activity in cells with heteromorphic NORs. Nevertheless, in a minority of these cells, the smallest NOR might by chance bind the first available transcription factors, an eventuality that would explain the appearance of cells in which the smallest NOR is activated preferentially.

Step 2. Once the first available transcription factors are bound, the rest would bind in a cooperative manner, so that they would have a higher affinity for the promoter of cistrons adjacent to those that have already recruited transcription factors. This may be the result of local alterations in NOR chromatin or rDNA structure, or a consequence of the action of transcription enhancers (see Sollner-Webb and Tower, 1986). As a consequence of cooperative binding there would be a strong tendency for the first NOR to be completely activated before the homologues begin to bind any transcription factors, a phenomenon that would take place only if the first NOR were unable to satisfy the ribosomal requirements of the cell. Cooperative binding is consistent with most of our observations in *Talpa occidentalis*. In individuals such a T-427, showing a high proportion of cells with heteromorphic NORs, most of these cells activate only the largest NOR (see Table 3). Furthermore, cells with two NORs identical in size also very frequently express only one of them (Table 3), a fact that would be difficult to explain if cooperative binding did not occur. Similarly, the hypothesis is also consistent with cells showing two different silver signals, i.e. cells with two differentially expressed NORs, most of which showed the highest activity in the largest NOR. Cells showing the opposite situation were very scarce.

Our hypothesis is also consistent with the results of a number of molecular studies on the transcription of eukaryotic ribosomal genes. In vitro template commitment assays have shown that when one rDNA template is expossed to the transcription machinary in a cell extract prior to the addition of a second rDNA template, transcription occurs only from the first template (Wandelt and Grummt, 1983; Cizewski and Sollner-Webb, 1983). This indicates that stable rDNA transcription complexes are formed with the first template, which sequesters all the available components (see Sollner-Webb and Tower, 1986, for a review). A similar phenomenon may involve the relationship between the two homologous NORs in *Talpa occidentalis* in vivo, according to our hypothesis. Furthermore, it has been suggested that transcription enhancers, located in the spacers between ribosomal cistrons (Moss, 1983), function by favoring either sliding or cooperative interaction. This would account for the rapid formation of stable transcriptional complexes (Sollner-Webb and Tower, 1986).

It is known that the binding of the RNA polymerase I complex to the active NORs in interphase persists during most of mitosis (from prophase to the anaphase-telophase boundary), when NORs are inactive (Roussel et al., 1996). These findings suggest that assembly of this transcription machinary prior to mitosis might determine the pattern of NOR expression at the beginning of the next cell cycle, and hence that this pattern may be inherited from cell to cell. However, the existence of interindividual, intercellular and interchromosomal differences in a variety of taxa suggests that the expression pattern of NORs is redefined not only in each individual generation, but probably in each cell cycle. The question as to when the expression pattern of NORs is redefined remains unanswered, but could occur during

replication, when transcriptional factors must be released from DNA to permit the replisome to advance along it.

Methylation of the dinucleotide cytosine-guanine (CpG) is generally, but not always, occurs in nontranscribed regions of most vertebrate genomes, including rDNA. Brock and Bird (1997) suggested that inactivity of a ribosomal gene might lead to methylation, as a result of which the gene could no longer be transcribed again. Methylation is known to represses nontranscribed genes stably in differentiated somatic cells, but a mechanism is presumed to exist which permits demethylation of genes that must pass from an inactive to an active status (see Kass et al., 1997, for a review). The demonstrated versatility of NOR expression clearly suggests that such a mechanism may be involved in the control of NOR activity. It is therefore possible that the pattern of methylated regions in rDNA is also redefined subsequently to that of NOR expression. In this context it is interesting to note that both NOR activity and methylation-mediated repression are closely related to chromatin structure.

We would like to thank K. Shashok for revising the English style of the manuscript. This work was supported by the Spanish DGICYT through project no. PB92-0951, and by the Junta de Andalucía through group no. CVI0109.

REFERENCES

Bickham, J. W. and Rogers, D. S. (1985). Structure and variation of the nucleolus organizer regions in turtles. *Genetica* **67**, 171-184.

Brock, G. J. R. and Bird, A. (1997). Mosaic methylation of the repeat unit of the human ribosomal RNA genes. *Hum. Mol. Genet.* **6**, 451-456.

Burgos, M., Jiménez, R. and Díaz de la Guardia, R. (1986). A rapid, simple and reliable combined method for G-banding mammalian and human chromosomes. *Stain Technol.* **61**, 257-260.

Cizewski, V. and Sollner-Webb, B. (1983). A stable transcription complex directs mouse ribosomal RNA synthesis by RNA polymerase I. *Nucleic Acids Res.* **11**, 7043-7056.

Clavaguera, A., Querol, E., Dolors, C., Genesca, J. and Egozcue, J. (1983). Cytochemical studies on the nature of NOR (nucleolus organizer region) siver stainability. *Cell. Mol. Biol.* **29**, 255-259.

De Capoa, A., Felli, M. P., Baldini, A., Rocchi, M., Archidiacono, N., Aleixandre C. and Miller, O. J. (1988). Relationship between the number and function of ribosomal genes. *Human Genet.* **79**, 301-304.

Díez, M. and Puertas, M. J. (1986). Quantitative analysis of the formation of nucleoli in *Locusta migratoria*. *Can. J. Genet. Cytol.* **28**, 207-218.

Galleti, P. M. Jr., Barbosa da Silva, E. and Germinaro, R. J. (1985). A multiple NOR system in the fish *Serrasalmus spilopleura* (Serrasalminae, Characidae). *Rev. Brasil Genet.* **8**, 479-484.

Goessens, G. (1984). Nucleolar structure. *Int. Rev. Cytol.* **84**, 107-158.

Goodpasture, C. and Bloom S. E. (1975). Visualization of nucleolar organizer regions in mammalian chromosomes using silver staining. *Chromosoma* **53**, 37-50.

Hernández-Verdún, D. (1986). Structural organization of the nucleolus in mammalian cells. *Meth. Archiv. Exp. Pathol.* **12**, 26-62.

Howell, W. M. and Black, D. A. (1980). Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a one step method. *Experientia* **36**, 1014-1015.

Hsu, T. C., Sperito, S. E., Pardue, M. L. (1975). Distribution of 18-28S ribosomal genes in mammalian genomes. *Chromosoma* **53**, 25-36.

Hubbel, H. R. (1985). Silver staining as an indicator of active ribosomal genes. *Stain Technol.* **60**, 285-294.

Jiménez, R., Burgos, M. and Diaz de la Guardia, R. (1984). Karyotype and chromosome banding in the mole (*Talpa occidentalis*) from the south-east of the Iberian peninsula. Implications on its taxonomic position. *Caryologia* **37**, 253-258.

Jiménez, R., Burgos, M. and Diaz de la Guardia, R. (1988). A study of the Silver staining significance in mitotic NORs. *Heredity* **60**, 125-127.

Jordan, G. (1987). At the heart of the nucleolus. *Nature* **329**, 489-490.

Kass, S. U., Pruss, D. and Wolffe, A. P. (1997). How does DNA methylation repress transcription? *Trends Genet.* **13**, 444-449.

Leitch, I. J. and Heslop-Harrison, J. S. (1992). Physical mapping of the 18S-5.8S-26S rRNA genes in barley by in situ hybridization. *Genome* **35**, 1013-1018.

Medina, F. J., Risueño, M. C., Sánchez-Pina, M. A. and Fernandez-Gomez, M. E. (1983). A study on nucleolar silver staining in plant cells. The role of argyrophylic proteins in nucleolar physiology. *Chromosoma* **88**, 149-155.

Mellink, C. H. M., Bosma, A. A. and De Haan, N. A. (1994). Variation in size of Ag-NORs and fluorescent rDNA in situ hybridization signals in six breeds of domestic pig. *Hereditas* **120**, 141-149.

Moss, T. (1983). A transcriptional function for the repetitive ribosomal spacer in *Xenopus laevis*. *Nature* **302**, 223-228.

Roussel, P., Andre, P., Comai, L. and Hernández-Verdún, D. (1996). The rDNA transcription machinery is assembled during mitosis in active NORs and absent in inactive NORs. *J. Cell Biol.* **133**, 235-246.

Rufas, J. S., Iturra, P., De Souza, W. and Esponda, P. (1982). Simple silver staining procedure for the localization of nucleolus and nucleolar organizers under light and electron microscopy. *Arch. Biol.* **93**, 267-276.

Sánchez, A., Burgos, M., Jimenez, R. and Díaz de la Guardia, R. (1989). Quantitative analysis of silver staining of the nucleolar organizing region in *Eliomys quercinus*. *Genome* **32,** 978-982.

Sánchez, A., Stitou, S., Zurita, F., Jiménez, R., Burgos, M. and Diaz de la Guardia, R. (1995). Cytogenetic peculiarites in the Algerian Hedgehog: silver stains not only NORs but also heterocromatics blocks. *Heredity* **75**, 10-16.

Sánchez-Pina, M. A., Medina, F. J., Fernández-Gómez, M. E. and Risueño, M. C. (1984). Ag-NOR proteins are present when transcription is impaired. *Biol. Cell.* **5**, 199-202.

Shubert, I. and Künzel, G. (1990). Position dependent NOR activity in barley. *Chromosoma* **99**, 352-359.

Sollner-Webb, B. and Tower, J. (1986). Transcription of cloned eukaryotic ribosomal RNA genes. *Ann. Rev. Biochem.* **55**, 801-30.

Suzuki, H., Kusihara, Y., Kaneisa, T. and Moriwaki, K. (1990). Variation in the distribution of the siver staining nucleolar organizer regions on the chromosomes of the wild mouse, *Mus musculus*. *Mol. Biol. Evol.* **7**, 271-282.

Wahl, G. M., Vitto, L. and Rubnitz, J. (1983). Co-amplification of rRNA genes with CAD genes in N-(phosphonacetyl)-L-aspartate-resistant Syrian hamster cells. *Mol. Cell Biol.* **3**, 2066-2075.

Wandelt, C. and Grummt, I. (1983). Formation of stable preinitiation complexes is a prerequisite for ribosomal DNA transcription in vitro. *Nucleic Acid Res.* **11**, 3795-3809.

Warburton, D. and Henderson, A. S. (1979). Sequential silver staining and hybridization in situ on nucleolus organizing regions in human cells. *Cytogenet. Cell Genet.* **24**, 168-175.

Zatsepina, O. V., Schofer, C., Weipoltshammer, K., Mosgoeller, W., Almeder, M., Stefanova, V. N., Jordan, E. G. and Wachtler, F. (1996). The RNA-polymerase-I transcription factor UBF and rDNA are located at the same major sites in both interphase and mitotic pig embryonic kidney. *Cytogenet. Cell Genet.* **73**, 274-278

Zurita, F., Sánchez, A., Burgos, M., Jiménez, R. and Díaz de la Guardia, R. (1997). Interchromosomal, intercellular and interindividual variability of NORs studied with silver staining and in situ hybridization. *Heredity* **78**, 229-234.