

Original article

Interindividual and intercellular polymorphisms of Ag-NOR pattern in mink embryo siblings

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Summary – The variability of the silver-staining pattern of the nucleolus organizing regions (Ag-NOR pattern) was studied in hepatocytes from 9 mink embryo siblings, including a pair of mono chorionic (presumably monozygotic) co-twins. Both the number of Ag-NORs and the mean size of Ag-spots per cell were found to be identical in mono chorionic twins. All other sibs had patterns different from each other and from co-twins. Intercellular variation of both the number and size of Ag-stained regions, as measured by the coefficient of variation, was similar only in mono chorionic twins. The data indicate that both the interindividual and intercellular variations of the Ag-NOR pattern are highly heritable. The mechanisms underlying the Ag-NOR pattern polymorphisms are discussed. It is proposed that at least 2 independently inherited routes for the variable expression of the ribosomal gene system exist: 1) polymorphism for rDNA array; and 2) polymorphism for rRNA gene expression.

mink / embryo / chromosome / nucleolus organizing region / silver staining

Résumé – Polymorphisme interindividuel et intercellulaire de la coloration à l'argent des régions des organisateurs nucléolaires chez des embryons de vison. La variabilité de la coloration à l'argent des régions des organisateurs nucléolaires (Ag-NOR) a été étudiée sur des hépatocytes de 9 embryons de vison, germains de portée et incluant une paire de jumeaux mono chorioniques, présumés monozygotes. À la fois le nombre des Ag-NOR et la taille moyenne des taches d'argent par cellule se sont trouvés être identiques chez les jumeaux mono chorioniques. Les autres germains avaient tous des patrons différents entre eux et différents des jumeaux. La variation intercellulaire du nombre et de la taille des régions colorées à l'argent, mesurée par le coefficient de variation, n'était similaire que chez les jumeaux. Les données indiquent que les variations interindividuelles et intercellulaires du type d'Ag-NOR sont hautement hérissables. Les mécanismes génétiques sous-jacents sont discutés. Il est suggéré qu'au moins 2 voies génétiques indépendantes peuvent expliquer l'expression variable du système des gènes ribosomiques : i) polymorphisme des structures de l'ADN ribosomique; ii) polymorphisme de l'expression des gènes de l'ARN ribosomique.

vison / embryon / chromosome / organisateur nucléolaire / coloration à l'argent

INTRODUCTION

Nucleolus organizing regions (NORs) are some of the most intensely studied chromosome sites. The number of NORs in the normal karyotype is species-specific and constant. Goodpasture and Bloom (1975) developed the method to reveal NORs in metaphase cells with silver nitrate. Only those NORs that are transcriptionally active in the preceding interphase are stained (for a review, see Hubbell, 1985). Now we can visually distinguish active NORs from inactive ones. The Ag-NOR pattern, *ie* the number of Ag-stained NORs and the size of Ag-spots per cell, can be used to study the differential expression of rRNA gene clusters in ontogenesis and phylogenesis.

In all cytogenetic studies of different mammalian species including human, an interindividual variability of Ag-NOR pattern has been noted. The nature of the variability has been intensely studied. From studies of human families (Mikelsaar *et al*, 1977; Markovič *et al*, 1978), sheep (Henderson and Bruere, 1980), rabbits (Arruga and Monteagudo, 1989) and pigs (Vishnevskaya and Vsevolodov, 1986), it is apparent that the silver-staining property of each NOR-bearing chromosome is genetically determined and inherited in a simple Mendelian fashion. Further work has supported this conclusion. In studies of cell clones derived from a human fibroblast culture, the Ag-NOR pattern remained similar to that in the parental cell line (Ferraro *et al*, 1981). Taylor and Martin-DeLeon (1981) analyzed the karyotypes of the members of 2 monozygotic (MZ) twin pairs and found no significant differences for the number or size of Ag-NORs. Zakharov *et al* (1982) studied lymphocytes from 20 MZ and 20 dizygotic (DZ) twin pairs. Analysis of intrapair variance as well as intrapair concordance of the number of Ag-NORs and the size of Ag-deposits indicated that the Ag-NOR pattern is highly heritable.

Variation of the Ag-NOR pattern among cells from the same individual has, however, been noted in cultured lymphocytes from human subjects (Mikelsaar and Schwarzacher, 1978; Taylor and Martin-DeLeon, 1981; Zakharov *et al*, 1982; de Capoa *et al*, 1985; Sozansky *et al*, 1985; Liapunova *et al*, 1988), pigs (Stefanova, 1983; Troshina and Gustavsson, 1984; Vishnevskaya and Vsevolodov, 1986; Mellink *et al*, 1991), cattle (Di Berardino *et al*, 1981; Mayr *et al*, 1987) and also in fibroblast cultures from human subjects (Mikelsaar and Schwarzacher, 1978; de Capoa *et al*, 1985; Sozansky *et al*, 1985) and rabbits (Martin-DeLeon *et al*, 1978). Mikelsaar and Schwarzacher (1984) reported that variability can even occur within 1 cell clone. Zakharov *et al* (1982) and Sozansky *et al* (1985) suggested that the intercellular variability is genetically determined.

This conclusion can be supported by accumulating data from different animal species, tissues and cell types. Litters of multiparous animals containing MZ twins among sibs can be used as one of the most suitable models to reveal the possible genetic determination of the characters. In this paper, data on the Ag-NOR pattern variability in mink embryo siblings are presented. Two of the sibs were monochorionic (MCh) co-twins, which are considered to be MZ twins. A rather high (1–2%) incidence of MCh twin embryos is a characteristic feature of domestic mink (Hansson, 1947; Belyaev *et al*, 1983).

The location of NORs in the mink karyotype ($2n = 30$) corresponds to regions of secondary constrictions in chromosomes 2 and 8, which can be identified without

application of banding techniques (fig 1) (Isakova, 1989). The normal diploid mink karyotype contains 4 NORs.

MATERIALS AND METHODS

The embryos studied were from 2-year-old Standard Dark mink bred at the experimental farm (Novosibirsk). The dam was mated once to a Dark male on March 7 and autopsied on April 19 in 1991. Among 8 implantation sites, 7 contained single embryos, and in 1 fetal camera 2 embryos were found that shared a common chorion but had separate amnions and placentas. Single implanted embryos in multiparous animals should be considered as DZ twins; the MCh co-twins can only be derived from a single zygote, and are therefore MZ twins.

Embryos were removed, separated from their membranes and weighed. Table I contains the date of the development of the embryos. One had a normal body weight but was dead. Another embryo, which was alive and of normal body weight, was found to have an abundant microbial infection of unknown nature in preparations from its liver. Both MCh co-twins were alive but had a low body weight in comparison with other embryos. All sibs had a normal diploid karyotype. Both MCh co-twins had a male chromosome complement ($2n, XY$).

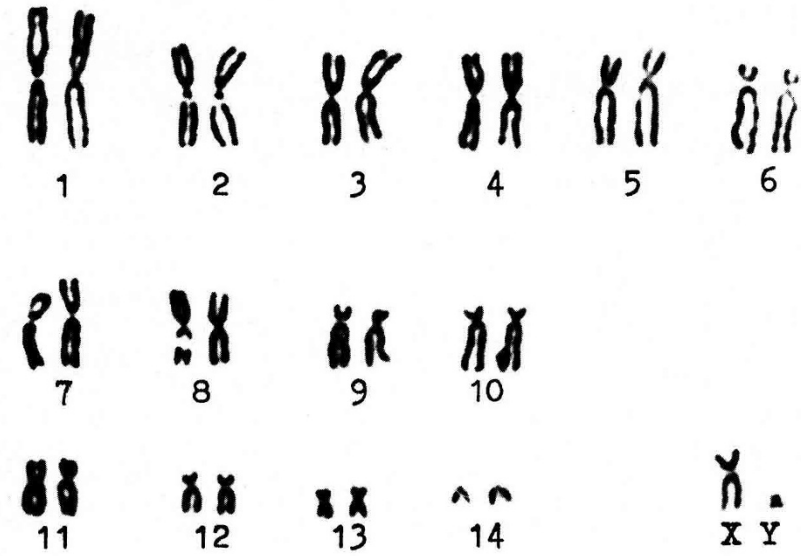
About half of the liver was taken from each embryo and placed in a glass tube containing 2 ml medium RPMI 1640, supplemented with 10% fetal calf serum and colchicine in the usual concentration. Cells were dispersed and suspended with a pipette, and incubated at 38°C for 1 h. Then, using the standard hypotonic and fixative solutions (Isakova, 1989), chromosome preparations were made. To reveal the NORs, the technique suggested by Howell and Black (1980) was used. From 15 to 25 metaphase spreads were analyzed from each embryo. The Ag-NOR pattern was characterized using 4 criteria for each NOR-bearing chromosome: 1) the number of Ag-stained NORs in the cell and their proportion relative to the maximum possible number of 4; 2) the size of Ag-NOR spots, estimated visually in arbitrary units on a scale from 0 (no staining) to 3 (maximum size); the score for each NOR-bearing chromosome was counted as the sum of both homologues; the mean values were calculated by dividing the sum obtained from all the cells analyzed by the number of cells; 3) intercellular variability of both the number and size of Ag-stained NORs, estimated by the coefficient of variation (C_v); and 4) the frequency of NOR-bearing chromosome associations.

RESULTS

Individual Ag-NOR patterns

The mean scores of the frequency with which the particular NORs were stained, and the size of Ag-spots per cell in each of 9 embryos are given in table II. All 4 (100%) NORs were stained in only 3 embryos. Chromosome 8, which possesses a longer secondary constriction than chromosome 2, had both homologous NORs stained in all embryos except the MCh co-twins and embryo 7 which had developmental deviations (table II). Particular NORs displayed different mean sizes of Ag-spots

A



B

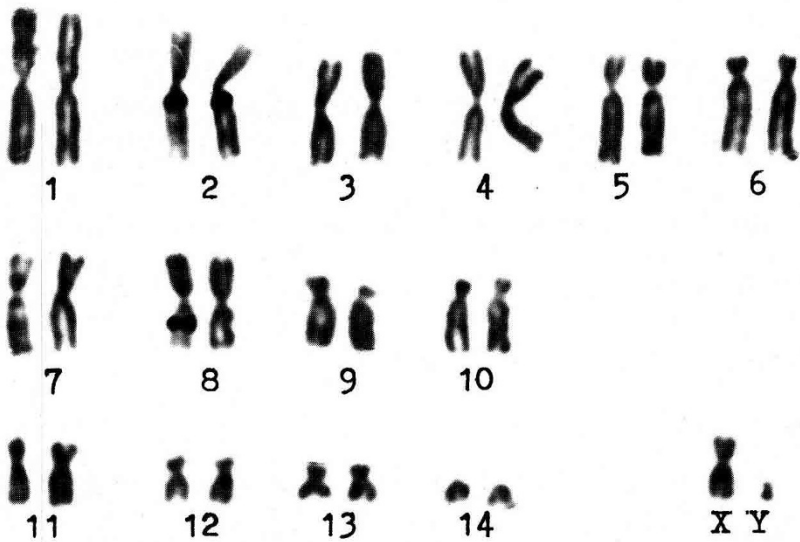


Fig 1. Karyotype of a mink male ($2n = 30$). A - Giemsa staining; and B - silver staining (location of the NORs).

Table I. Developmental characteristics of the embryos derived from 2-year-old mink 42 d after mating.

<i>Embryo</i>	<i>Implantation character</i>	<i>Body weight (g)</i>	<i>Developmental anomalies</i>	<i>No of cells analysed</i>	<i>Sex chromosomes complement</i>
1	Single embryo	1.15		25	XY
2	Single embryo	1.11	Resorbtion	12	XX
3	Single embryo	0.91		25	XX
4	Single embryo	1.14		25	XX
5	Single embryo	1.18		25	XY
6	Single embryo	1.34		25	XY
7	Single embryo	1.15	Microbial infection	12	XX
8*	Monochorionic co-twins	0.52	Resorbtion	25	XY
9*	Monochorionic co-twins	0.77	Resorbtion	12	XY

* Co-twins

Table II. Mean frequency of Ag-stained NORs and size of Ag-spots per cell in embryonic hepatocytes of mink siblings.

<i>Embryo</i>	<i>% Ag-NOR</i>			<i>Ag-spot size (arbitrary units)</i>		
	<i>Chromosome 2</i>	<i>Chromosome 8</i>	<i>Total</i>	<i>Chromosome 2</i>	<i>Chromosome 8</i>	<i>Total</i>
1	92	100	96	2.1	4.1	6.2
2	100	100	100	3.3	3.8	7.1
3	100	100	100	4.0	3.2	7.2
4	68	100	84	2.2	3.8	6.0
5	100	100	100	2.5	4.5	7.0
6	74	100	87	2.5	3.8	6.3
7	96	88	92	3.6	3.4	7.0
8*	100	92	96	3.9	3.3	7.2
9*	100	94	97	3.8	3.4	7.2

* Co-twins

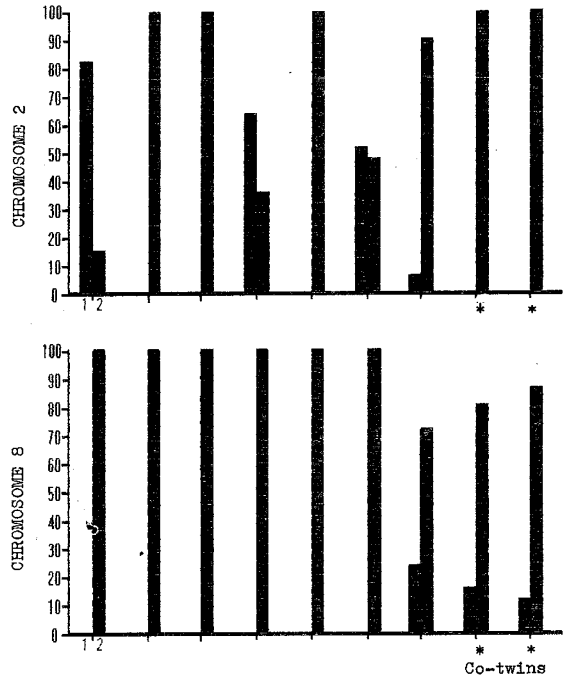
and, as a rule, differences between homologous NORs also existed. Each embryo differed from all others either for the frequency of staining of Ag-NORs, or for the Ag-spot sizes, or for both criteria. MCh co-twins however had nearly identical Ag-NOR pattern scores.

Intercellular Ag-NOR pattern variability

Diagrams showing the distribution of cells for the number of Ag-NORs are presented in figure 2. MCh twins had similar profiles for each NOR-bearing chromosome and for the total number per cell. Embryos 2, 3 and 5 revealed a constant maximal frequency of staining, and so their profiles appear identical in the image. All other embryos differed from each other and from the MCh twins. Both number and size of Ag-NORs on both chromosomes 2 and 8 were found to vary from cell to cell in all the embryos except for embryo 3, which only showed a variable expression of Ag-spot sizes for chromosome 8. MCh co-twins had similar coefficients of intercellular

variability except for a difference in the size of Ag-stained NORs on chromosome 2 (table III). All other sibs differed from each other and from the MCh twins. In embryos with both varying number and size of stained regions, the correlation coefficient between the 2 scores was highly significant (Table IV). Therefore, the mean number of Ag-NORs can be considered to be a reasonable indicator of NOR activity.

Fig 2. The distribution of embryonic hepatocytes for the number of Ag-stained NORs in the mink twins. Abscissa: number of Ag-stained NORs per cell; ordinate: percentage of cells. The 9 columns represent the 1st to 9th embryos assigned in table I.



Associations NOR-bearing chromosomes

Associations between NOR-bearing chromosomes, *ie* juxtapositions of 2 or more such chromosomes and the connection between them by an Ag-bridge, were rarely seen. In each embryo except numbers 4, 6 and 7, only one cell with an association was observed. Only associations between chromosomes 2 and 8 (2-8), and none between homologues occurred.

DISCUSSION

Genetic determination of interindividual Ag-NOR pattern polymorphism

If the Ag-staining property of each NOR-bearing chromosome is hereditary, each individual may inherit a set of NORs that are different for such characteristics, and may then be unique for its Ag-NOR pattern. Only genetically identical organisms

Table III. The coefficient of intercellular variation % for the number and size of Ag-NORs in mink siblings.

Embryo	No Ag-NORs/cell			Ag size/cell		
	Chromosome 2	Chromosome 8	Total	Chromosome 2	Chromosome 8	Total
1	32.3	0	11.8	20.7	4.9	7.7
2	0	0	0	14.8	12.1	9.4
3	0	0	0	0	12.8	5.7
4	36.0	0	14.6	26.7	13.2	13.9
5	0	0	0	23.6	14.6	14.3
6	34.4	0	14.7	20.6	19.7	9.8
7	15.1	25.8	13.4	18.7	26.4	12.2
8*	0	20.3	9.7	17.1	25.7	15.6
9*	0	18.2	8.8	10.6	23.9	14.6

* Co-twins

Table IV. Correlation coefficients between the number and size of Ag-NORs.

Embryo	Correlation coefficient		
	Chromosome 2	Chromosome 8	Total
1	0.89 ± 0.1***	—	0.79 ± 0.13***
2	—	—	—
3	—	—	—
4	0.83 ± 0.12***	—	0.67 ± 0.15***
5	—	—	—
6	0.87 ± 0.01***	—	0.66 ± 0.16***
7	0.75 ± 0.21**	0.95 ± 0.01***	0.87 ± 0.16***
8*	—	0.81 ± 0.12***	0.59 ± 0.17**
9*	—	0.67 ± 0.20**	0.44 ± 0.24†

* Co-twins; † $P < 0.1$; ** $P < 0.01$; *** $P < 0.001$.

would display identical Ag-NOR patterns. In the present study, each of 7 single implanted embryos (*ie* twins of different zygosity) was found to have a different Ag-NOR pattern, either for the number of Ag-NORs per cell or for Ag-spot sizes, or for both criteria. MCh co-twins (presumably MZ ones) were identical for all the scores. This signifies that the Ag-stainability of NORs in the mink embryonic hepatocytes is a strongly inherited property, and the Ag-NOR pattern can be used as a reliable genetic marker to diagnose twin zygosity. A similar conclusion was drawn by Zakharov *et al* (1982) from studies of human lymphocytes. It is probable that the Ag-NOR pattern is tissue-specific (Martin-DeLeon *et al*, 1978; Mikelsaar and Schwarzacher, 1978; de Capoa *et al*, 1985; Sozansky *et al*, 1985). Therefore, cells of same cell type should be used to test the Ag-NOR pattern identity. Moreover, the unique feature of Ag-staining of the NORs signifies a unique character of rRNA multigene families expression in each individual. Unique karyotypic features for

some chromosome regions revealed by the different banding techniques was noted in man (Van Dyke *et al*, 1977) and in pigs (Troshina and Gustavsson, 1984).

Genetic determination of intercellular Ag-NOR pattern polymorphism

Evidence in support of a hypothesis for genetic intercellular Ag-NOR variability was presented in 2 reports. Zakharov *et al* (1982) observed the distribution of both the number and size of Ag-NORs in lymphocytes from human MZ twins to be similar; Sozansky *et al* (1985) found a similar ratio of stained to unstained NORs in parental and clone human fibroblast cultures. In the present mink study, the coefficient of variation is similar in MCh co-twins, both for number and size of Ag-spots on each NOR-bearing chromosome, and differs from those characteristic of other sibs. The findings support the hypothesis for genetic control of Ag-staining intercellular variability.

The intercellular variability was noted to be different in lymphocytes and fibroblasts from the same individual, and the difference is probably due to different stainability of particular NORs (Mikelsaar and Schwarzacher, 1978; de Capoa *et al*, 1985).

Mechanisms underlying the Ag-NOR pattern polymorphisms

It is known that the underlying basis for the NOR staining with silver is the acidic nonhistone argentophilic proteins associated with transcriptions of rRNA genes (for reviews see: Schwarzacher and Wachtler, 1983; Hubbel, 1985; Dyban *et al*, 1990). There is not a strong correlation between the number of rRNA gene copies and staining intensity; NORs having a small number of the genes can express more intense staining than NORs containing many rRNA genes (de Capoa *et al*, 1988). Furthermore, the transcriptionally active NORs (Ag-NORs) were shown to be more sensitive to the DNase treatment and hypomethylated as compared to inactive ones (Ferraro and Prantera, 1988). These findings indicate that the role of Ag-proteins might be to maintain a different NOR chromatin conformation, which then facilitates different levels of rDNA transcription.

The origin of Ag-NOR proteins in ontogenesis is a question of special interest. Dyban *et al* (1990) detected argentophilic proteins in the pronucleoli of 1-cell mouse embryos, *ie* before transcription of rRNA genes started. It was suggested that the proteins (or their precursors) are inherited through the oocyte cytoplasm. The results of the present mink study indicate that the program of its expression during ontogenesis is also probably inherited.

The Ag-staining intensity of the NORs is considered to be an indicator of the level of nucleolus activity. The nucleolar size is also widely used to indicate its activity. Delany *et al* (1991), using their experimental model of chickens trisomic for the NOR-bearing chromosome, have shown that inherited polymorphisms for the number and size of nucleoli were caused by alterations of the rDNA array; it was also noted that the variability was not dependent on tissue type or developmental stage. In other work, however, the Ag-NOR pattern was shown to be independent of the number of rRNA genes (de Capoa *et al*, 1988), but dependent on tissue and cell type (Martin-DeLeon *et al*, 1978; Mikelsaar and Schwarzacher, 1978; de Capoa

et al., 1985), and stage of development (Martin-DeLeon *et al.*, 1978; de Capoa *et al.*, 1983; Patkin and Sorokin, 1983; King *et al.*, 1988). In plants, particular NORs ranked differently when studied by the 2 tests (for a review, see Mukai *et al.*, 1991). Therefore, at least 2 bases for the variable expression of the ribosomal gene system may be proposed. First, polymorphisms for rDNA array cause a diversity of the nucleolar morphology, and second polymorphisms for the Ag-NOR pattern, *ie* for rRNA gene expression may also cause variability of nucleolar morphology. Both routes are genetically determined, and each seems to be inherited independently.

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