11. V. I. Kukharenko, K. N. Grinberg, and A. M. Kuliev, Hum. Genet., 42, 157 (1978).
12. E. L. Schneider and B. J. Powlkes, Exp. Cell Res., 98, 298 (1976).
13. J. E. Sisken and S. V. Bonner, Mech. Ageing Dev., 11, 191 (1979).
14. J. R. Smith, O. M. Pereira-Smith, and E. L. Schneider, Proc. Natl. Acad. Sci. USA, 75, 1353 (1978).
15. A. Vorbrodt, R. Charpentier, and V. J. Cristofalo, Mech. Ageing Dev., 11, 113 (1979).

CLONAL ANALYSIS OF INTERCELLULAR VARIABILITY OF NUCLEOLAR ORGANIZER ACTIVITY IN HUMAN CHROMOSOMES

O. A. Sozanskii and S. M. Terekhov

UDC 612.014.24:576.316.23

KEY WORDS: nucleolar organizer; chromosome; clone.

By the selective staining of nucleolar organizing regions (NOR) with silver (Ag staining) it has become possible to study the functioning of ribosomal genes by determining the product of their transcription activity directly in metaphase chromosomes. It has recently been shown that the character of Ag staining, which includes the number of NOR stained with silver, the intensity of their staining, and distribution with respect to 20 acrocentric chromosomes, is a stable, specific, and inheritable individual characteristic [12, 14]. Meanwhile most investigators have found intra-individual intercellular variability for the number of active NOR and link this phenomenon with technical errors of the Ag staining method [10]. In investigations on blood lymphocytes specially devoted to this problem, intercellular variability was shown to be a truly existing phenomenon [4, 15]. Essentially it consists of the presence of several subpopulations of cells differing in their number of active NOR. These differences arise on account of a change in the ability of one or two NOR of acrocentric chromosomes specific and characteristic for each individual, to stain with silver. It has also been found that intercellular variability does not depend on the order of cell division after the cells had emerged from the G_0 phase [3]. The observations described above can be explained in two ways: 1) In the early stages of ontogeny a definite character of activity

TABLE 1. Characteristics of Individual Chromosomes Varying in Ag-Staining of Parental Cultures and of Clonal Lines Isolated from Them

Individual	Culture	Varying chromosome	Relative percentages of two cell subpopulations
1	Parental	13	31:69
	Clone I	13	33:67
	» II	13	35:65
	» III	13	30:70
	» IV	13	36:64
2	Parental	14	37:63
	Clone I	14	50:50
	» II	14	37:63
	» III	14	39:61
3	Parental	15	52:48
	Clone I	15	54:46
	» II	15	48:52

Note. Number of metaphase plates studied for each case is shown in Fig. 1.

Institute of Medical Genetics, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR N. P. Bochkov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 97, No. 5, pp. 592-595, May, 1984. Original article submitted June 27, 1983.

 0007-4888/84/9705-0638$08.50

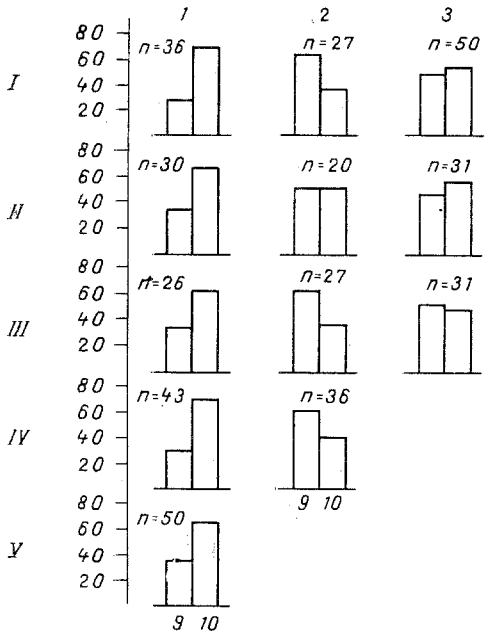

Fig. 1. Distribution of cells by total number of NOR stained with silver in parental cultures and clonal lines: 1, 2, 3) individuals: I) parental cultures; II, III, IV, V) clonal lines; n) number of cells studied; abscissa, total number of Ag^+-NOR in metaphase plate; ordinate, number of cells (in %);

of the NOR is fixed for different cells and is transmitted as a stable trait to all the progeny of the given cell; in this case the cell population would consist of several clonal lines with a stable character of Ag staining, inheritable in cell generations; 2) the phenomenon of intercellular variability arises *de novo* every time. This might mean that every cell, in its subsequent generations, can reproduce the heterogeneity of the cell population from which it was obtained.

The aim of this investigation was the experimental testing of these two alternative hypotheses by clonal analysis of the character of Ag staining of NOR of human metaphase chromosomes.

EXPERIMENTAL METHOD

As parental lines for the investigation cultures of skin fibroblasts were obtained from two human embryos aged 8-9 weeks, obtained during medical abortions, and conventionally described as 1 and 3, and from an individual aged 23 years, conventionally described as 2.

To isolate the cell lines a unicellular suspension was poured into a 96-well plate at the rate of one cell per well. Wells containing one cell were marked under an inverted microscope 6-8 h after adhesion of the cells. After 18 days of culture clonal lines of unicellular origin were isolated. Four clonal lines were isolated from parental culture 1, three from culture 2, and two from culture 3. Subsequent culture of the clonal lines was indistinguishable from the conditions of culture of the parental strains, which were described previously [5].

At the time of investigation the parental cultures were at the 10th-12th passage and the clonal lines at the 3rd-4th passage from the time of their isolation. During the 44 h before fixation, the cells were subcultured on slides. Preparations of metaphase chromosomes were obtained from cultures grown on slides without separating the cells from the glass.

Staining with silver was carried out by the use of the writers' own modification of the Ag-1 method [4]. Combined Ag and G staining was used for simultaneous assessment of the state of NOR and identification of acrocentric chromosomes carrying them. For this purpose preparations stained with silver were immersed in 0.2 N CsCl solution at 60°C for 6-7 min, after which they were stained with 2% phosphate-buffer solution of Giemsa stain (pH 6.8) for 6-7 min. To exclude artifacts, only metaphase plates with optimal silver saturation of NOR of

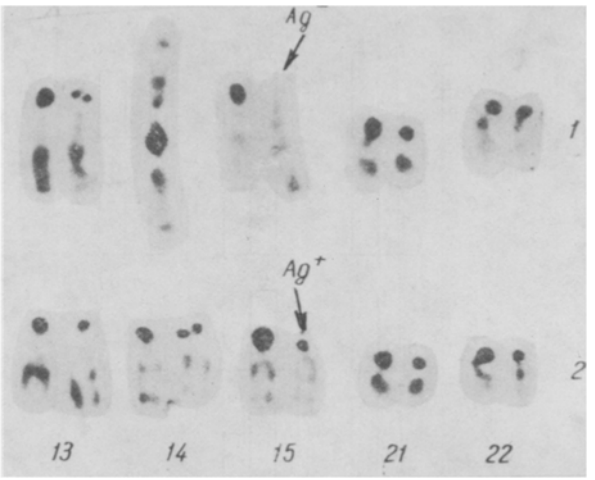

Fig. 2. Intercellular variability of NOR weakly stained with silver: 13-22) acrocentric chromosomes in metaphase plate; 1, 2) two different cells from clonal line II of individual 3. Arrow indicates NOR of one of the chromosomes of pair 15, Objective 100, ocular 10.

acrocentric chromosomes were analyzed, using the morphologic criterion of completion of Ag staining described previously [4].

EXPERIMENTAL RESULTS

It will be clear from the data in Fig. 1 that heterogeneity of the cell populations for the number of active NOR per metaphase plate was observed in all parental cultures and clonal lines isolated from them. Each culture consisted of two groups of cells, differing in the number of NOR stained with silver. The quantitative ratio between these groups of cells in the clonal lines remained the same as in parental cultures from which they were obtained. The results given in Table 1 are evidence that intercellular variability observed in the parental cultures was due to a change in the ability of NOR of one chromosome to stain with silver. For cultures 1 and 3, one of the chromosomes 13 and 15 respectively was varying. As an example of such variability, see Fig. 2.

For the culture from individual 2 this was one homolog of chromosome 14. As Table 1 shows, variability for silver uptake in the clonal lines was exhibited by the same chromosomes as in the parental cultures. The quantitative ratio between cells containing one or other type of variable chromosome also was the same in both clones and parental lines. Consequently, both quantitatively and qualitatively the phenomenon of variability in clonal lines was found to be the same as in the original parental cultures.

The intensity of Ag-staining of the varying chromosomes of cultures 1 and 3 was weak. Despite the use of a morphologic criterion for assessing completion of silver staining of NOR, in these cases it was impossible to rule out completely artifacts on account of incomplete Ag staining, which could make their contribution to the appearance of intercellular variability. Variability of staining of NOR of chromosome 14 in the culture from individual 2 was therefore particularly interesting. By routine staining it could be seen that this chromosome carried two satellites and two satellite threads both in the parental strain and in all clonal lines isolated from it, whereas in the remaining cells it was represented by a single-satellite variant. In clonal line I a two-satellite variant was observed in 40.6% of metaphases, in line II in 36%, and in line III in 38% of cells studied. On staining with silver the two-satellite chromosome always contained two active NOR, located in the satellite threads. In cells in which this chromosome carried one satellite thread, the presence of one active NOR was observed. Incidentally, one of the homologs of chromosome pair 22 never stained with silver in the karyotype of this individual. Consequently, the presence of intercellular variability for total number of active NOR per metaphase plate was due to a change in their number on chromosome 14 (as an example, see Fig. 3). It is extremely important to note that the presence of heterogeneity of the cell population for the number of

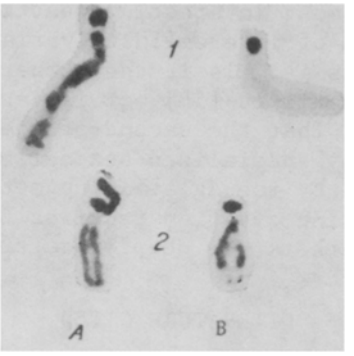

Fig. 3. Staining of NOR of two variants of chromosome 14 of individual 2 with silver with a greater (1) and lesser (2) degree of condensation. A) two blocks of silver, i.e., two active NORs; B) one block of silver, i.e., one active NOR. Objective 100, ocular 10.

active NOR, identified by Ag staining, was predetermined by the actual structure of this chromosome. On the basis of this fact we can completely exclude the influence of technical factors of silver staining on the appearance of the phenomenon of intercellular variability of NOR and it convincingly proves the existence of true heterogeneity of the cell population for activity of human ribosomal genes. The intensity of Ag staining of the remaining NOR and their distribution among individual chromosomes remained characteristic and stable for each parental culture and for the cell lines isolated from it.

It must therefore be emphasized that in none of the clonal lines tested was it possible to observe homogeneity of the cell population for the character of Ag staining of the NOR. Conversely, each clonal line was a heterogeneous cell population with respect to the feature studied. The qualitative and quantitative structure of this heterogeneity was the same as in the original parental culture from which the particular clonal line was obtained. Between the parental cultures and clonal lines, by the time of investigation of the latter there was a difference of 25-30 successions of cell generations. Taking this fact and all the observations described above into consideration, it must be concluded that the experiment fully confirmed the second of our hypotheses put forward above. Each cell which preserves its ability to proliferate is evidently capable of reproducing, in its subsequent generations, the heterogeneity of that cell population from which it was obtained, with respect to a complex feature such as the character of silver staining of NOR of human chromosomes. Preservation of the original character of Ag-staining, on the one hand, and recreation, both qualitatively and quantitatively, of the previous intercellular variability in a series of cell generations, on the other hand, make it possible to postulate that the potential ability of human ribosomal genes to change their function is itself genetically determined.

When the results of this investigation were described there was only one publication in the literature on clonal analysis of the character of Ag staining of NOR of human metaphase chromosomes [7]. The authors cited drew conclusions regarding clonal inheritance of the character of Ag staining of NOR of human chromosomes. Our own results do not agree with those described in this paper. Meanwhile a number of communications have confirmed our hypothesis of inheritance of a certain range of NOR variation in man at the organism level. A particularly noteworthy investigation is that of Kyaosaar [1], which preceded the introduction of Ag staining. In two generations of individuals (mother and son) this worker found a two-satellite variant of one chromosome of the D group in 40 and 35% of cells respectively, whereas in the remaining cells this chromosome carried one satellite thread and one satellite. This observation may be evidence of inheritance of a particular range of variation of structure of the region of the nucleolar organizer, not only in generations of somatic cells, but also in generations of individuals. Despite many investigations of "two-satellite" chromosomes, information on the presence of one- and two-satellite versions of marker chromosomes in one of eight individuals described by her, with a frequency characteristic for each individual, is given only in a publication by Tsvetkova [6]. The results of observations on the character of silver staining of NOR in familial [9] and twin [15] studies also confirm

the hypothesis submitted above. The most interesting question, namely the mechanisms of onset and maintenance of the phenomenon of intercellular variability in cell populations, remains unexplained. One of its aspects is: Is it the number of copies of ribosomal genes that changes in varying nucleolar organizers [13], or is variability based on a phenomenon of activation—inactivation of genes? That the second of these explanations is possible in principle has been described in highly specialized sex cells [8], tumor cells [2], and interspecific hybrid cells [11], tested by the Ag staining method. Both mechanisms are possible, but the study of this interesting question requires the use not only of Ag staining, but also of other techniques. The authors are deeply grateful to A. F. Zakharov for scientific direction of the research.

LITERATURE CITED

1. M. É. Kyaosaar, Genetika, No. 7, 114 (1971).
2. N. N. Mamaev, S. E. Mamaeva, D. Bandandoiya, et al., Tsitologiya, No. 2, 161 (1980).
3. O. A. Sozanskii, Byull. Éksp. Biol. Med., No. 1, 114 (1983).
4. O. A. Sozanskii, Byull. Éksp. Biol. Med., No. 4, 71 (1983).
5. S. M. Terekhov, Tsitologiya, No. 6, 717 (1981).
6. T. G. Tsvetkova, "Polymorphism of chromosomes in a contingent of married couples with abnormal obstetric history," Author's Abstract of Candidate's Dissertation, Moscow (1980).
7. M. Ferraro, P. Lavia, F. Pelliccia, et al., Chromosoma (Berlin), 84, 345 (1981).
8. M. Hartung, C. Mirre, and A. Stabl, Hum. Genet., 52, 295 (1979).
9. M. Jotterand-Bellomo and C. Van Mello, Hum. Genet., 59, 141 (1981).
10. A.-V Mikelsaar and H. G. Schwarsacher, Hum. Genet., 42, 291 (1978).
11. D. A. Miller, V. G. Dev, R. Tantravahi, et al., Exp. Cell Res., 101, 235 (1976).
12. E. F. Taylor and P. A. Martin De Leon, Am. J. Hum. Genet., 33, 67 (1981).
13. D. Warburton and A. S. Henderson, Cytogenet. Cell Genet., 24, 168 (1979).
14. A. F. Zakharov, A. Z. Davudov, V. A. Benjush, et al., Hum. Genet., 60, 24 (1982).
15. A. F. Zakharov, A. Z. Davudov, V. A. Benjush, et al., Hum. Genet., 60, 334 (1982).