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Familial Silver Staining Patterns of Human Nucleolus Organizer Regions (NORs)

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SUMMARY

The silver staining patterns of the nucleolus organizer regions (NORs), an indication of rDNA transcriptional activity, were studied in metaphases from lymphocyte cultures of 20 karyotypically normal members of three families selected for a large sibling number or a monozygotic twin pair. Quinacrine polymorphic markers and bands were used to identify the acrocentrics and to determine their parental origin. A comparison of the silver staining frequencies among siblings and between parent and child indicated no significant differences for any acrocentric in the twin pairs and significant differences ($P < .05$) for only one of the 20 acrocentrics segregating in each of two families. These two acrocentrics had short stalks with very small silver deposits (AgNORs). The mean size of the AgNOR, based on a relative score, was not significantly different ($P > .05$) for each homolog between the twin pair and in approximately 70% of the acrocentrics shared by members of the one family analyzed. The frequency with which a particular chromosome was silver stained demonstrated a significant correlation ($r^2 = .732$) with the size of AgNOR. There was a close correlation ($r^2 = .609$) between stalk length and the size of the AgNOR. We conclude that the frequency of silver staining and the mean size of the AgNOR are characteristics inherent in a particular chromosome carried from one generation to the next.

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INTRODUCTION

The nucleolus organizer regions (NORs) carry the structural genes for rRNA, and the silver stainability of these chromosomal regions is determined by their transcriptional activity [1, 2]. Each individual has a characteristic modal number and consistent pattern of distribution of silver-stained NORs [3–6]. These stained regions, located on the acrocentrics, demonstrate considerable polymorphism with respect to size [3, 7]. Analysis of the silver staining patterns of acrocentrics with polymorphic markers in Down syndrome children and in their parents [8, 9] has provided preliminary evidence that NOR silver stainability may be a heritable characteristic of a given chromosome.

To date, there have been no studies on the inheritance of NOR activity in families with karyotypically normal individuals or in twins. It is the purpose of this investigation to analyze the inheritance of silver stainability in 2-generation pedigrees with a large sibling number or a monozygotic twin pair in an attempt to understand the behavior of NORs. In a family study, a given chromosome tagged by its polymorphic markers can be observed in a variety of "chromosomal environments," which have been created by random assortment at gametogenesis. On the other hand, monozygotic twins act as a control since they provide a situation in which one can analyze identical chromosome complements in two different individuals.

MATERIALS AND METHODS

Subjects

Twenty individuals from three 2-generation pedigrees (tables 1–3) were studied. Twin zygosity was determined through *ABO, Rh, Kk, Mns*, and *Fy* genotyping in the immunohematology laboratory at the University of Delaware. Metaphases were prepared from routine short-term lymphocyte cultures after 1 hr fixation of cells. Slides from each individual were coded to preclude a bias in scoring. Repeat cultures from individuals in two families were prepared for analysis without knowledge of the original scores of the individuals.

Sequential Silver Staining and Quinacrine Banding

Silver staining was accomplished using the technique of Bloom and Goodpasture [10]. To identify the homologs and to observe the stalk and satellite polymorphisms of silver-stained chromosomes, slides were counterstained for 1 hr with quinacrine mustard (50 μg/ml) in Hank's buffer differentiating for 10 min in McIlvaine's phosphate-citric acid buffer, pH 5.5. Preparations mounted in this same buffer were analyzed with a Zeiss Photomicroscope II equipped with an HB050 mercury vapor lamp.

Chromosome Analysis

The parental origin of each acrocentric in the offspring was ascertained by polymorphic markers. In scoring the polymorphic short arm (p) regions of the acrocentrics, a reduction in the number of classes proposed by the Paris Conference Supplement [11] was employed to insure unambiguous assignment. These classifications were as follows: fluorescent intensity of the satellites (region p13) was scored as very bright (3), intermediate (2), or dull (1). Stalk length (region p12) was scored using the p arm of chromosome 18 as an internal standard for class 3, the very long stalks. Intermediate and short stalks were recorded as 2 and 1, respectively. Zero represented no stalks.

Once the polymorphic designations were made, the NOR-silver staining patterns could be analyzed. For each individual, 25 diploid metaphases (from three or more slides) were

TABLE 1

PARENTAL ACROCENTRIC POLYMORPHIC ASSIGNMENTS AND SILVER STAINING FREQUENCIES IN FAMILY A

	FATHER I-1										MOTHER I-2									
	13a	b*	14a	b	15a	b	21a	b	22a	b	13a	b	14a	b	15a	b	21a	b	22a	b
Polymorphisms:																				
Centromere	c+	...	...	...	...	...	...	...	...	...	c+	...	...	...	p+	...	...	...	...	...
Stalk	3	0	2	2	1	3	2	1	2	2	2	2	2	3	0	3	3	2	1	2
Satellite	3	1	2	1	3	1	3	1	3	1	1	2	3	2	3	2	3	2	1	2
No. AgNORs/ 25 cells	25	2	25	19	21	25	12	4	23	22	25	25	25	25	1	25	25	23	21	11
	No. AgNORs/25 cells																			
Siblings:																				
II-1	...	9	24	...	21	...	...	7	23	...	...	25	...	25	6	...	25	...	24	...
II-1 R†	...	13	25	...	24	...	...	9	22	...	...	25	...	25	4	...	25	...	25	...
II-2	25	...	23	...	16	...	...	7	...	25	...	25	...	25	...	25	25	...	...	16
II-3	...	1	19	...	21	...	...	7	...	23	...	25	...	25	...	25	25	...	...	17
II-4	25	...	...	19	19	...	11	...	...	24	...	25	25	...	...	25	25	...	...	11
II-5	25	...	...	19	16	...	...	3	...	22	25	...	...	25	4	...	25	...	...	14
II-6	...	19	...	23	...	23	19	...	...	25	25	...	...	25	11	...	...	25	...	22
II-7	...	7	...	21	...	21	12	...	19	...	...	22	...	24	7	...	...	23	24	...

*$\chi^2 = 26.99$, df = 5, $P < .001$.
†R = repeat culture.

TABLE 2

PARENTAL ACROCENTRIC POLYMORPHIC ASSIGNMENTS AND SILVER STAINING FREQUENCIES IN FAMILY B

	FATHER I-1										MOTHER I-2									
	13a	b	14a	b	15a	b	21a*	b	22a	b	13a	b	14a	b	15a	b	21a	b	22a	b
Polymorphisms:																				
Centromere	c+	c+	...	...	...	...	...	p+	...	...	c+	...	p+	...	...	p+	...	...	...	...
Stalk	3	2	3	1	3	1	1	0	3	0	3	0	2	1	1	0	3	2	1	1
Satellite	3	3	3	1	2	1	2	2	2	3	3	0	2	1	2	1	2	1	3	1
No. AgNORs/ 25 cells	25	25	25	14	25	21	7	1	25	0	25	0	25	11	25	1	25	21	25	24
	No. AgNORs/25 cells																			
Siblings:																				
II-1	...	25	...	15	25	...	20	...	25	...	...	2	24	...	...	6	25	...	...	22
II-1 R†	...	24	...	17	25	...	14	...	25	...	...	0	25	...	...	3	25	...	...	23
II-2‡	25	...	24	...	...	22	...	3	25	...	...	2	...	7	25	...	25	...	...	18
II-3‡	25	...	25	...	...	24	...	3	25	...	...	1	...	4	25	...	25	...	...	24
II-3 R†	25	...	25	...	...	22	...	5	25	...	...	1	...	14	25	...	25	...	...	14

*$\chi^2 = 6.20$, df = 2, $P < .05$.
†R = repeat culture.
‡Cotwin.

TABLE 3

PARENTAL ACROCENTRIC POLYMORPHIC ASSIGNMENTS AND SILVER STAINING FREQUENCIES IN FAMILY C

	FATHER I-1										MOTHER I-2									
	13a	b	14a	b	15a	b	21a	b	22a	b	13a	b	14a	b	15a	b	21a	b	22a	b
Polymorphisms:																				
Centromere	c+	c+	...	...	...	...	...	...	...	...	c+	...	...	...	...	...	...	p+	...	...
Stalk	1	1	2	1	0	3	2	1	2	3	2	1	2	1	1	1	2	1	3	2
Satellite	2	1	2	1	2	1	3	2	3s+	2	2	1	3	3	3	3	2	3	1	3
No. AgNORs/ 25 cells	25	23	24	20	1	24	25	21	25	25	25	24	25	19	...	...	25	23	24	2
	No. AgNORs/25 cells																			
Siblings:																				
II-1	24	...	...	16	2	...	...	23	25	...	...	22	24	...	...	23	25	...	25	...
II-2*	25	...	25	...	3	...	25	...	...	25	25	...	...	18	...	24	...	25	...	0
II-3*	25	...	25	...	1	...	25	...	...	25	25	...	...	21	...	23	...	25	...	1
II-4	25	...	...	20	2	...	25	...	...	25	...	25	25	...	...	24	...	25	25	...

NOTE: No significant differences were observed for any acrocentric.
*Cotwin.

microscopically scored for the presence of a silver deposit on the p12 region of each acrocentric. Identification of an acrocentric and its stainability were achieved simultaneously by varying the combination of uv and brightfield illumination. The cells of all members of family C (table 3) were analyzed for the relative size of AgNORs within each metaphase. In an arbitrary classification of size, a scale of 0–3 was used; 3 indicating the heaviest deposit, and 0, a complete absence of stain.

RESULTS

In the six genetically unrelated individuals studied (the parents of the three families), a wide range of polymorphisms could be detected in the short arm region of the 10 acrocentrics. Using only the three categories each for stalk length and satellite fluorescent intensity, one would expect to find homologs with identical stalk and satellite polymorphisms less than 2% of the time. This proportion decreases even further as additional criteria, such as centromeric band brightness (c+), enlarged short arms (p+), or large satellite size (s+), are considered. As a result, among the 20 individuals analyzed, polymorphic differences could be recognized for 98 of the 100 homologous pairs.

Identical polymorphic scores for homologs of each monozygotic twin pair, B II-2 and II-3 and C II-2 and II-3 (tables 2 and 3), were observed. Generally, the cells of each individual had acrocentrics with a consistent polymorphic assignment. Figure 1 illustrates assignments of a representative cell from C II-1 (table 3). Stability in assignment of polymorphic markers was also seen in their transmission from parent to offspring, where in all cases the origin of acrocentrics could be resolved unequivocally. In repeat cultures as well, only 8 one-step changes were recorded in 66 markers.

While the polymorphic markers of a given chromosome remained stable, its silver stainability varied. Tables 1–3 demonstrate the parental origin of each homolog and

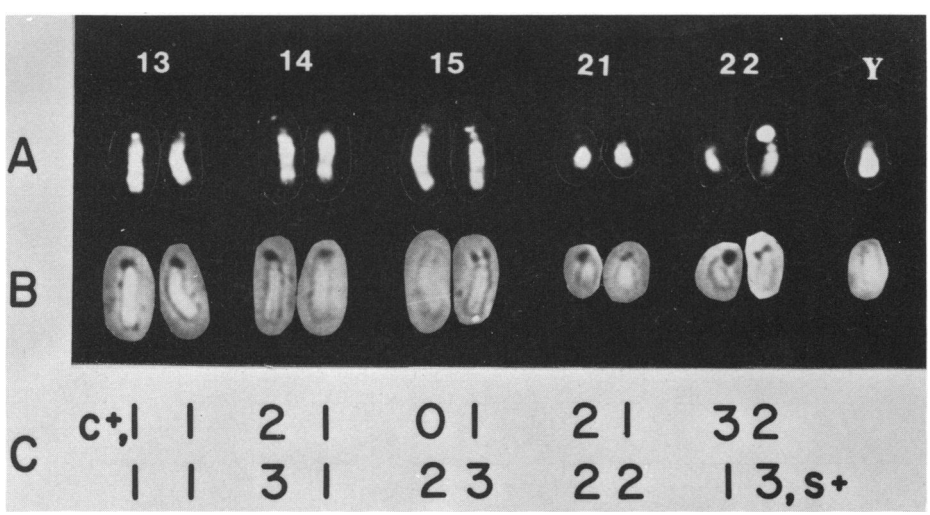

FIG. 1.—Partial karyotype of D- and G-group chromosomes of a representative cell from II-1 (table 3) illustrating: *A*, quinacrine banding and polymorphisms, and *B*, silver staining as observed simultaneously. Polymorphic assignments are shown in (*C*).

allow comparison of staining frequencies of NORs among the siblings, as well as between parent and child. Analysis of the staining frequencies showed no significant difference ($P > .05$) for any of the acrocentrics in members of family C, while only one acrocentric had significant differences in members of families A and B. The two chromosomes showing significant differences (A I-1 13b and B I-1 21a) both had extremely small secondary constrictions, and when Ag-positive, the staining was minimal. There were no significant differences between the original and repeat cultures in two of the three individuals (A II-1 and B II-1) subjected to a second analysis. In the third individual (B II-3), a significant difference in staining was detected for only the maternally derived chromosome 14 between the two cultures. However, the difference falls short of significance when the Ag-staining frequencies of this chromosome in the mother and the cotwin are considered. Again, this chromosome had a very short stalk and minimal staining.

The mean size of the AgNOR for each homolog in 25 cells is shown for family C in table 4. In 11 of the 16 (approximately 70%) acrocentrics inherited by one or more offspring, the mean size of the AgNOR was similar among the siblings and the contributing parent. For two of the homologs, only the two most distantly ranked means differed from each other. In three homologs, the mean size was the same for all except for one individual. No significant differences ($P > .05$) were detected in the size means of the individual homologs between the twins.

TABLE 4

MEAN AGNOR SIZE OF ACROCENTRICS IN FAMILY C

	PARENT	CHILDREN				
FATHER	I-1	II-1	II-2*	II-3*	II-4	$P < .05$
13a	2.16	2.24	2.40	2.23	2.32	...
13b	1.36	...	...	...	...	...
14a	2.18	...	1.68	1.52	...	†
14b	1.00	0.88	...	...	1.32	...
15a	0.04	0.08	0.12	0.04	0.08	...
15b	1.16	...	...	...	...	...
21a	2.60	...	2.44	2.68	2.76	‡
21b	1.24	1.24	...	...	...	...
22a	2.76	2.60	...	...	...	...
22b	2.92	...	2.88	2.88	2.60	†
MOTHER	I-2					
13a	2.60	...	2.56	2.84	...	...
13b	1.84	1.72	...	...	1.96	...
14a	1.72	1.92	...	...	2.32	†
14b	0.96	...	1.08	1.36	...	...
15a§ / 15b§	...	1.56	1.60	1.36	1.52	...
21a	2.32	1.92	1.92	2.12	...	...
21b	1.52	...	...	...	1.80	...
22a	2.60	3.00	...	...	2.80	‡
22b	0.16	...	0.00	0.04	...	...

*Cotwin.

Using Duncan's Multiple Range Test, significant differences were obtained for the following: †means are similar for all except one individual; and ‡only the most distantly ranked means differ from each other.

§ Homologs not differentiated.

Regression analysis of the relationship between the frequency of silver staining and size of the silver deposit suggested a highly significant correlation ($r^2 = .7318$). Generally, the size of the AgNOR was also closely correlated with the stalk length. Stalks that had been given a polymorphic assignment of 3 had the greatest amounts of stain and were most frequently silver stained, while chromosomes with minimal stalk length (1 or 0) were stained relatively less frequently and/or carried the least amount of staining. A comparison of the relative stalk length of the acrocentrics with the mean size of the deposit gave a correlation coefficient ($r^2 = .6047$) that indicated that 60% of the variation in silver stainability is related to stalk length. In one individual, there was an almost perfect correlation between the two factors.

DISCUSSION

Our data from the twin and repeat cultures markedly indicate the stability of the stalk and satellite region polymorphisms of an acrocentric. The data from parent and offspring set limits on the frequency of unequal crossing over. Other studies [12–15] have clearly documented the stability and heritability of human Q- and G-band polymorphisms. The reliability of the markers used in this study for the identification of each acrocentric in family members is, therefore, substantiated.

The frequency with which silver staining was observed on a particular homolog was, in almost all cases, similar among family members having that chromosome (tables 1–3). The consistent staining pattern of an NOR was most clearly demonstrated by the almost identical staining frequencies for each homolog from the members of each twin pair. Only 3% of the parental acrocentrics in all families showed significantly different staining frequencies among individuals sharing them. These two acrocentrics had small NOR sites, and technical factors, as suggested by Schwarzacher et al. [16], might be responsible for inconsistencies in their scores. Our results, therefore, not only support but also extend the findings of Mikelsaar et al. [8]. These workers, studying silver staining of only chromosome 21 in Down syndrome children and in their parents, suggested that silver stainability of an NOR might be a heritable characteristic of the chromosome. In the present study, the staining pattern of all the NORs were analyzed.

Similarity of the relative size of AgNORs on acrocentrics shared by different individuals was noted for the twin pair as well as for other family members. This finding supports that of Markovic et al. [9], who reported close correlation between the relative size of AgNORs in Down syndrome children and in their parents. It now seems very clear that the transcriptional activity of an NOR, as determined by qualitative and quantitative analysis of silver staining, is a fixed characteristic of each site and is not, in general, dependent on the other NORs in the complement. This fixed characteristic remains unchanged from one generation to the next.

In situ hybridization studies have shown that the number of rRNA genes correlates with stalk length: a smaller number of rRNA cistrons is present in the short stalks or small sites and vice versa [17]. Since larger silver deposits were generally found on longer stalks, it appears from our study that the amount of activity at an NOR may be largely explained by the number of rDNA copies in that NOR. Warburton and Henderson [18] have demonstrated a positive correlation between

the number of autoradiographic grains from in situ hybridization (an indication of gene numbers) and the size of the AgNOR in six of eight individuals. In the present study, although there was a general trend in which the stalk length closely paralleled the size of the AgNOR, the correlation was not perfect in all individuals. Warburton and Henderson [19] and Miller et al. [20] have reported that in certain chromosomes with high rDNA content, the amount of silver staining does not reflect the number of active genes, but may be influenced by other factors, such as the rate of transcription.

As far as the limited number of families in our study allows this conclusion, it seems that there are no preferences for acrocentrics with long stalks or for particular acrocentric combinations in the offspring (tables 1–4). Therefore, the adaptive significance of a multisite distribution of rDNA in the human is evident. Such a distribution leads to only a small proportion of individuals inheriting a combination of chromosomes in which each carries only a very small stalk with correspondingly low total number of rRNA genes and low activity.

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