Acta Genet Med Gamellol 30:39-49 (1981)
The Mendel Institute/Alan R. Liss, Inc.

Received 22 August 1980
Final 2 October 1980

Quinacrine Mustard and Nucleolar Organizer Region Heteromorphisms in Twins

C.C. Morton, L.A. Corey, W.E. Nance, J.A. Brown

Department of Human Genetics, Medical College of Virginia, Richmond

Patterns of NOR activity in 640 metaphase spreads from twelve monozygotic (MZ) and eight dizygotic (DZ) twin pairs were studied to evaluate the heritability of this chromosomal heteromorphism. NORs were stained by a modification of the Ag-AS technique and counterstained with quinacrine mustard dihydrochloride to facilitate chromosome identification and assess their value in zygosity determination. In this study, all karyotypes were read blind with respect to zygosity and pair membership.

A discriminant function analysis of pair score differences in MZ and DZ twins revealed that, in our sample, the probability of accurately determining zygosity with NOR scores was 0.93 and with QFQ scores was 0.99. We conclude that NOR and QFQ scores are highly heritable and of great value in zygosity determination.

Data were collected from 687 metaphase spreads on the frequency with which an acrocentric chromosome was found in a satellite association. A significant correlation was found between this frequency and the degree of Ag-AS stain of the NOR. This study, therefore, confirms previous results showing that a high degree of NOR activity is found in those chromosomes most often involved in satellite associations.

Key words: Nucleolar organizer regions (NOR), Chromosome heteromorphism, Q-banding, Satellite association, Zygosity analyses

INTRODUCTION

The usefulness of human chromosome heteromorphisms in twin zygosity determination has been demonstrated previously for those regions visible by Q (quinacrine mustard dihydrochloride)- and C (centromeric heterochromatin)-banding [12, 23]. The development of the ammoniacal-silver (Ag-AS) stain has made possible the observation of yet another group of heteromorphic regions, the loci for rRNA, known as the nucleolar organizer regions (NOR).

Previous studies [3, 14] employing mouse-human somatic cell hybrids have shown that the staining of the NOR depends not only on the presence of rDNA but also on the cellular production of 18S and 28S rRNA. This physiologic activity has been implicated as a contributory factor in the associative tendency of the human acrocentric chromosomes

0001-5660/81/3001-0039$03.50

observed at metaphase. Data presented by Miller et al [15] and subsequent investigators [4, 7, 21] have shown that chromosomes that had even a small amount of Ag-AS stain were more frequently in association than chromosomes that lacked Ag-AS staining. Furthermore, Warburton et al [25] reported a positive correlation between the frequency of participation of a given chromosome in satellite association and its rDNA content. Evans et al [5], however, reported that the frequency with which a chromosome was involved in an association was not a simple function of its rDNA content and that other factors should be considered.

Subsequent research in this area has been directed toward an understanding of the heritability and regulation of the NOR. In a study of six Down syndrome children and their parents, Markovic et al [11] noted a constant number and distribution of NORs on specific chromosomes within an individual, but no report of the applicability of NOR activity patterns to twin zygosity determination has been made. If the number and distribution of NORs is an inherited property, a high correlation among monozygotic twin pairs for NOR scores should be observed.

This study investigates the extent to which differences in the heteromorphic NOR are heritable and useful in zygosity analyses; reassesses previously published findings employing QFQ heteromorphisms for zygosity determination; and further evaluates the relationship between Ag-AS staining of the NOR and satellite association.

METHODS

Forty peripheral blood samples were obtained from 12 monozygotic (MZ) and eight dizygotic (DZ) twin pairs seen in the Medical College of Virginia twin clinic. The population ranged in age from three to 76 years. There were 21 females and 19 males in a total sample of three black and 17 white twin pairs. Blood samples were coded by a nonparticipant and cultured by a modification of the procedure of Moorehead et al [17]. Slides were prepared and analyzed with the observer blinded to both zygosity and pair membership.

Metaphase chromosomes were stained by a modification of the ammoniacal-silver technique of Bloom and Goodpasture [1] and counterstained with quinacrine mustard dihydrochloride for unequivocal identification. The slides were examined using a Leitz Ortholox II fluorescence microscope with a combination of incident ultraviolet and transmitted visible light.

The NOR score for each acrocentric chromosome was based on visual estimation of the size of the stained region, following the system of Markovic et al [11]. Each chromosome was thus assigned a value of zero (no NOR stain visible) to four. Approximately 15 cells were scored for each member of a twin pair. Many of the same cells in which the size of the NOR was determined were also evaluated for satellite association. A chromosome was considered to be in association if Ag-AS stain was present and contiguous with the secondary constriction of another acrocentric chromosome. The total number of chromosomes participating in associations in each individual was divided by the number of cells scored to obtain a mean number of associations per cell.

QFQ heteromorphisms were scored for 14 variable regions (the centromeric regions of chromosomes 3, 4, 13, and 22 and the short arm and satellite regions of all ten acrocentric chromosomes) in approximately seven cells per twin pair member. Each chromosome was assigned a QFQ score ranging from one (negative for fluorescence) to four (brilliant) (Fig. 1).

After all analyses were completed, the code was broken and the twin zygosity determined based upon genotyping results for the loci of blood groups ABO, Rh, Sec, MNSs, Fy, P, K, and Jk^a; the enzymes *Hp*, *PGM*, *AP*, *G6PD*, *Cat*, *LDH*, and *6PGD*; and *Hb*.

NOR and QFQ scores for the cells examined were averaged individually, providing a mean score for a homologous chromosome pair in an individual. It was necessary to calculate the mean score for a homologous pair because it was not always possible to identify, unequivocally, individual homologs.

An adjustment was made for the nonindependence of the karyotypes by using Cotterman's weighting system for the estimation of frequencies of genes without dominance from family data [2]. This adjustment involved multiplying each twin score by the reciprocal of $(1 + r)$, where r is equal to the coefficient of relationship for the twin pair. Thus, each MZ score was halved and each DZ score multiplied by 2/3. These adjusted data were employed in further analyses.

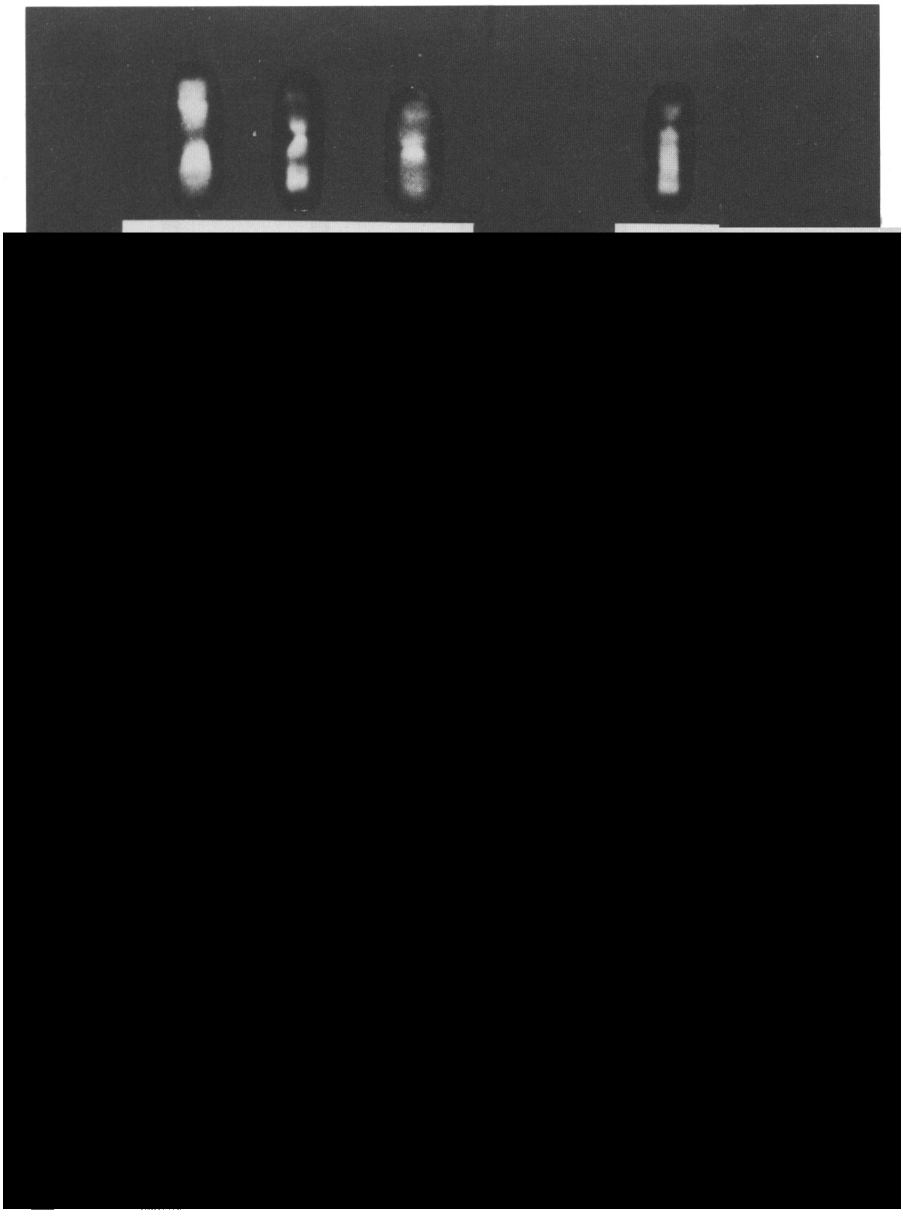

Fig. 1. Chromosome heteromorphisms as seen after staining with quinacrine mustard dihydrochloride. Key to scores: 1, negative; 2, pale; 3, bright; 4, brilliant.

RESULTS

Zygosity Determination Using NOR Heteromorphisms

The overall distribution of NOR scores for each homologous chromosome pair in the total population is presented in Figure 2. The most frequently observed NOR score was 2, accounting for approximately 29% of all scores, and the least frequently observed NOR score was 4, representing approximately 14% of all scores.

https://doi.org/10.1017/S0001566000006607 Published online by Cambridge University Press

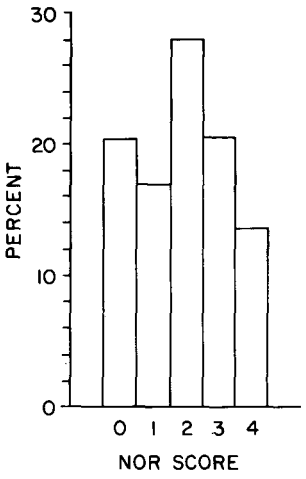

Fig. 2. Distribution of NOR scores in all acrocentric chromosomes.

A chi-square analysis of the frequency of NOR scores was performed on the data adjusted for nonindependence of the karyotypes, and revealed a highly significant difference (χ^2 = 105.4, 16 df) among chromosome pairs in the distribution of scores (Fig. 3). Chromosome 22 was noted to have the largest percentage of NORs with a score of zero, an observation in agreement with that reported by Mikelsaar et al [13]. This result was reflected in an analysis of the 6,336 acrocentric chromosomes examined in which the smallest mean score was found for chromosome 22, 1.88 ± 0.05 (Table 1). In this population, chromosome 13 had the largest mean score, 2.48 ± 0.05.

The data were plotted to compare for MZ and DZ twins the relationship between twins in their overall mean NOR scores (Fig. 4). A double entry point plot was generated in order to avoid bias in the selection of twin vs co-twin. The scatter of points around the line of identity for MZ twins is markedly different from the pattern seen in the DZ pairs. The intraclass correlation for the overall mean NOR score in MZ twins was 0.86 ± 0.03, and that of the DZ twins was 0.19 ± 0.16. The intraclass correlation for individual chromosome pair scores ranged from 0.80 in MZ twins for chromosome 13 to 0.92 for chromosomes 14 and 22 (Table 2). All correlations of homologous chromosome pairs in MZ twins were significantly different from the corresponding correlations in DZ twins.

A discriminant function analysis of mean pair score differences in MZ and DZ twins resulted in the misclassification of only one DZ pair with regard to zygosity by NOR scoring alone. In this sample, the probability of accurately determining zygosity with NOR scores was 0.93.

Zygosity Determination Using QFQ Heteromorphisms

The overall distribution of QFQ scores in this population is shown in Figure 5. Distributions for several heteromorphisms were quite heterogeneous, whereas others demonstrated the rarity, as well as the difficulty in precise discrimination, of certain variants, notably those of the short arm and satellite regions. A significant difference between blacks and whites was found in the distribution of the following heteromorphic regions: 3c, 13p, 13s, 15p, 15s, 21s, and 22s (Fig. 6). Similar significant differences have been reported previously [10, 18, 19], and deviations in our study sample may be attributed to sample size.

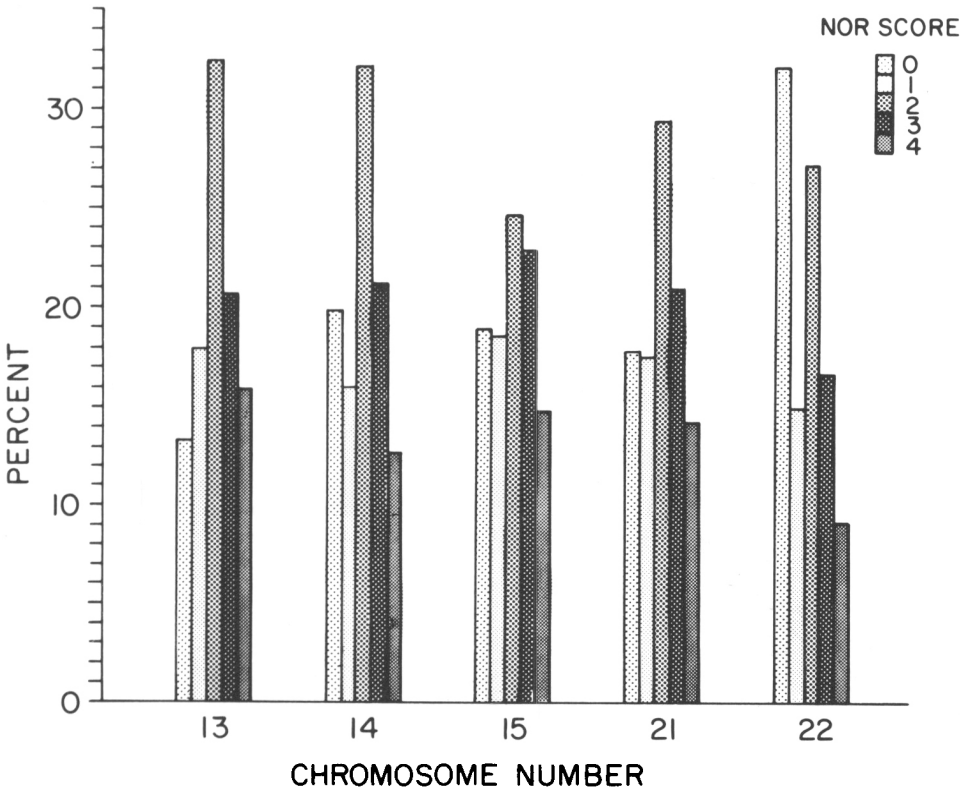

Fig. 3. Distribution of NOR scores in each acrocentric chromosome.

TABLE 1. Mean NOR Score

Chromosome number	n	Mean ± SE
13	1,280	2.48 ± 0.05
14	1,248	2.24 ± 0.05
15	1,280	2.33 ± 0.04
21	1,248	2.30 ± 0.05
22	1,280	1.88 ± 0.05

TABLE 2. Intraclass Correlations for Homologous Acrocentric Pairs

	Twin type	
Chromosome number	Monozygotic	Dizygotic
13	0.80 ± 0.11	0.15 ± 0.37
14	0.92 ± 0.05	0.47 ± 0.30
15	0.90 ± 0.06	0.08 ± 0.38
21	0.83 ± 0.09	−0.29 ± 0.35
22	0.92 ± 0.05	0.31 ± 0.34

https://doi.org/10.1017/S0001566000006607 Published online by Cambridge University Press

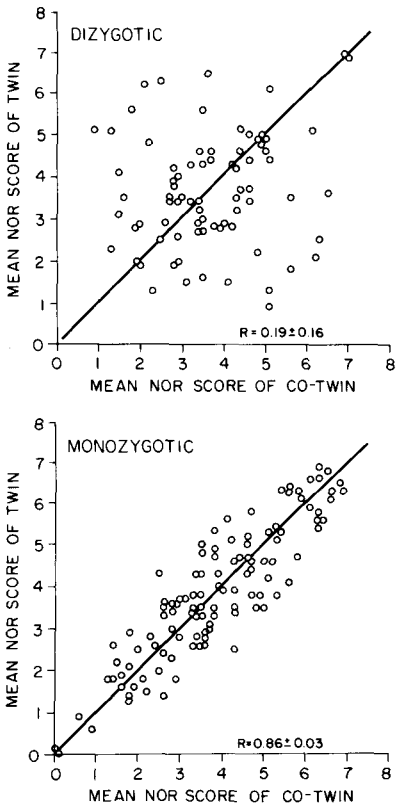

Fig. 4. Double entry point plot demonstrating the relationship between the mean NOR scores of all dizygotic (upper) and monozygotic (lower) twins with their co-twins.

To compare these data to several other published studies, the negative and dull scores were combined into a negative (–) classification, and the bright and brilliant scores were combined for a positive (+) classification. The estimates obtained in this study for the centromeric fluorescent variants were somewhat lower than other studies, with the exception of 3c in the black Virginia population. It was not possible to make direct comparisons of the frequencies of short arm and satellite variants from the present study, as these regions were combined in reports published previously. However, overall the estimates appear to agree quite well.

An analysis of the range of differences in QFQ scores in MZ and DZ twins revealed a larger spread in the scores in DZ twins for 11 of the 14 heteromorphic regions. A total of 83.5% of MZ twins had differences less than one, whereas 60.6% of DZ twins fell in this range. The intraclass correlation for the pooled chromosomes of the MZ twins was 0.67 ± 0.04, and that for the DZ twins was 0.36 ± 0.09. Intraclass correlations for individual chromosome pair scores ranged from 0.0 ± 0.30 for 22c to 0.96 ± 0.03 for 3c in MZ twins. Corresponding correlations in DZ twins were 0.0 ± 0.38 and 0.25 ± 0.36, respectively.

A discriminant function analysis of pair score differences resulted in the correct classification of zygosity by QFQ scoring for all 20 twin pairs. Thus, the probability of accurately determining zygosity with QFQ scores alone in this sample was 0.99.

https://doi.org/10.1017/S0001566000006607 Published online by Cambridge University Press

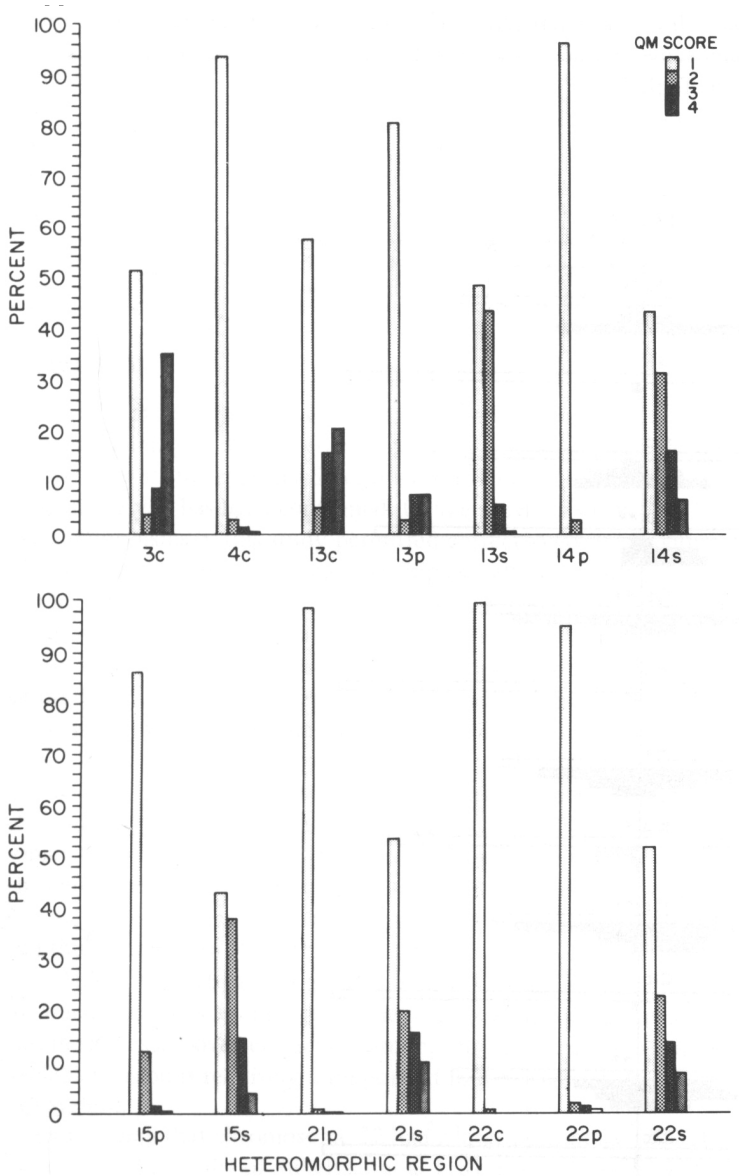

Fig. 5. Distribution of QFQ scores for heteromorphic regions in total twin pair sample.

NOR Activity and Satellite Association

The mean number of satellite associations per cell was 1.5 ± 0.13, with a range of 0.0–3.75 and a median value of 1.33. The frequency with which a particular acrocentric chromosome was found to be involved in satellite associations ranged from 16.6% for chromosome 22 to 21.9% for chromosome 14. A test for the differences between two proportions [24] showed that chromosome 22 was involved in associations significantly less often than were chromosomes 14, 15, and 21. Analyses of the relationship between the mean number of

https://doi.org/10.1017/S0001566000006607 Published online by Cambridge University Press

satellite associations per cell for each acrocentric chromosome and the mean NOR score for the respective chromosome revealed a significant positive correlation for all five acrocentric pairs, as shown in Table 3.

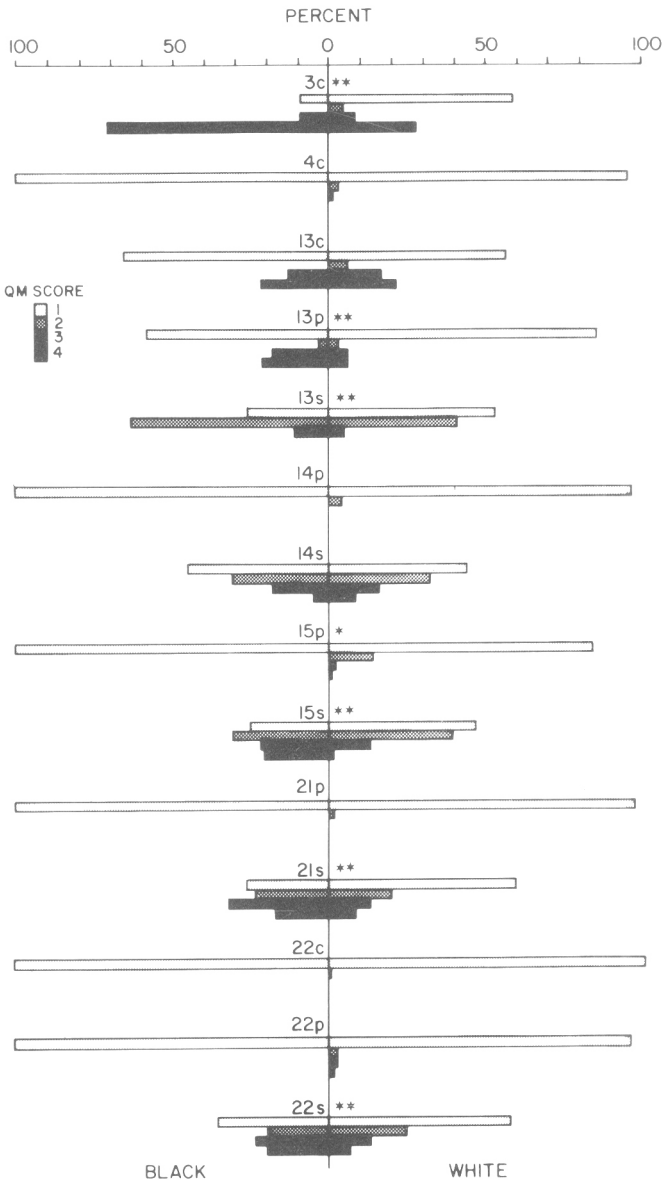

*Fig. 6. Distribution of QFQ scores for heteromorphic regions in black (n = 6) and white (n = 34) twins in twin pair sample. *, **Indicate distributions significantly different at $p < 0.05$ and $p < 0.01$, respectively.*

https://doi.org/10.1017/S0001566000006607 Published online by Cambridge University Press

TABLE 3. Pearson Correlations for Mean Number of Satellite Associations and Mean NOR Score

Chromosome number	R
13	0.353*
14	0.550**
15	0.369*
21	0.542**
22	0.393*

*, ** Indicates correlation significantly different from zero at $p < 0.05$ and $p < 0.01$, respectively.

DISCUSSION

The results of this study have shown that there is a high degree of heritability in the differential staining of the nucleolar organizing region, and have established the value of these genetic variants for zygosity determination. The usefulness of QFQ heteromorphisms in such an analysis has been confirmed. Unlike previous work [12, 23], the use of QFQ heteromorphisms in this study permitted correct zygosity assignments even though the observer was blinded to pair membership as well as zygosity.

Our results show that the frequency with which a particular acrocentric chromosome was found in satellite association was positively correlated with the size of the Ag-staining region at the NOR. These results are in agreement with those reported by several investigators [4, 7, 15, 22] and suggest that NOR activity may in some way influence the frequency with which an acrocentric chromosome participates in the organization of a nucleolus.

The high correlation for size of Ag-staining region and satellite association in both chromosome 14 and chromosome 21 may be related to the frequency of the 14/21 Robertsonian translocation in man [8]. Fusion of nucleolar organizing regions brings certain chromosomes into proximity, and Ohno et al [20] suggested that this process might predispose chromosomes with NORs to centric fusion, producing Robertsonian translocations, and thus account for the rather common occurrence of these translocations in man. However, Miller et al [16] investigated this theory in the mouse and proposed that, although the nucleolus plays an important role in Robertsonian translocations, because NOR-bearing chromosomes are overrepresented among chromosomes involved in these translocations, nucleolar fusion is relatively unimportant in the origin of Robertsonian translocations in this system.

Our finding that chromosome 22 had a large percentage of NORs with a score of zero agrees with the results of Mikelsaar et al [13] in a study of 51 karyotypically normal Caucasian individuals from Vienna and Ulm. The small size of the NOR on this chromosome is consistent with the possibility that there are few ribosomal cistrons on chromosome 22 [9, 13]. The fact that this chromosome is infrequently involved in satellite associations is also of interest in view of the infrequent occurrence of trisomy 22 compared to trisomy 21. Satellite associations involving chromosome 21 have been shown to occur with increased frequency in the mothers of patients with Down syndrome [6], and it is possible that the tendency for nondisjunction by acrocentric chromosomes is correlated in some way with NOR staining intensity, possibly mediated by their predisposition to form satellite associations.

Because of the heterogeneity of certain heteromorphisms in the population, some may prove to be singularly valuable in making zygosity determinations. From this sample, a survey of the intraclass correlations of NOR and QFQ mean scores in MZ and DZ twins indicated that the heteromorphisms of chromosomes 21 and 3c, respectively, are very useful in making these determinations.

Furthermore, NOR heteromorphisms may be used in conjunction with other chromosomal heteromorphisms in the identification of the origin of chromosomal aneuploidy, the prenatal determination of twin zygosity, paternity testing, and the exclusion of maternal contamination in antenatal studies.

REFERENCES

1. Bloom SE, Goodpasture C (1976): An improved technique for selective silver staining of nucleolar organizer regions in human chromosomes. Hum Genet 34:199–206.
2. Cotterman CW (1947): A weighting system for the estimation of gene frequencies from family records. Contrib Lab Vert Biol, University of Michigan, 33:1–21.
3. Croce CM, Talavera A, Basilico C, Miller OJ (1977): Suppression of the production of mouse 28S ribosomal RNA in mouse-human hybrids segregating mouse chromosomes. Proc Natl Acad Sci USA 74:694–697.
4. de Capoa A, Ferraro M, Menendez F, Mostacci C, Pelliccia F, Rocchi A (1978): Ag staining of the nucleolus organizer (NO) and its relationship to satellite association. Hum Genet 44:71–77.
5. Evans HJ, Buckland RA, Pardue ML (1974): Location of the genes coding for 18S and 28S ribosomal RNA in the human genome. Chromosoma (Berl) 48:405–426.
6. Hansson A (1979): Satellite association in human metaphases. A comparative study of normal individuals, patients with Down syndrome and their parents. Hereditas 90:59–83.
7. Hayata I, Oshimura M, Sandberg AA (1977): N-band polymorphism of human acrocentric chromosomes and its relevance to satellite association. Hum Genet 36:55–61.
8. Hecht F, Kimberling WJ (1971): Patterns of D chromosome involvement in human (DqDq) and (DqGq) Robertsonian rearrangements. Am J Hum Genet 23:361–367.
9. Kohno S, Abe S, Matsui S, Sandberg AA (1979): Chromosomes and causation of human cancer and leukemia, XXXVII. Nucleolus organizers on the Ph′ chromosome in chronic myelocytic leukemia. Cancer Genet Cytogenet 1:15–30.
10. Lubs HA, Patil SA, Kimberling WJ, Brown J, Cohen M, Gerald P, Hecht F, Myrianthopoulos N, Summitt RL (1976): Q and C banding polymorphisms in 7 and 8 year old children: Racial differences and clinical significance. In Hook EB, Porter IH (eds): "Population Cytogenetics." New York: Academic Press, pp 133–159.
11. Markovic VD, Worton RG, Berg JM (1978): Evidence for the inheritance of silver-stained nucleolus organizer regions. Hum Genet 41:181–187.
12. McCracken AA, Daly PA, Zolnick MR, Clark AM (1978): Twins and Q-banded chromosome polymorphisms. Hum Genet 45:253–258.
13. Mikelsaar A-V, Schmid M, Krone W, Schwarzacher HG, Schnedl W (1977): Frequency of Ag-stained nucleolus organizer regions in the acrocentric chromosomes of man. Hum Genet 37:73–77.
14. Miller DA, Dev VG, Tantravahi R, Miller OJ (1976): Suppression of human nucleolus organizer activity in mouse-human somatic hybrid cells. Exp Cell Res 101:235–243.
15. Miller DA, Tantravahi R, Dev VG, Miller OJ (1977): Frequency of satellite association of human chromosomes is correlated with amount of Ag-staining of the nucleolus organizer region. J Hum Genet 29:490–502.
16. Miller OJ, Miller DA, Tantravahi R, Dev VG (1978): Nucleolus organizer activity and the origin of Robertsonian translocations. Cytogenet Cell Genet 20:40–50.
17. Moorehead PS, Nowell PC, Mellman WJ, Battips DM, Hungerford DA (1960): Chromosome preparation of leukocytes cultured from human peripheral blood. Exp Cell Res 20:613–616.
18. Muller H, Klinger HP (1975): Chromosome polymorphism in a human newborn population. In Pearson PL, Lewis KR (eds): "Chromosomes Today." Vol 5. Jerusalem: John Wiley, pp 249–260.
19. Muller H, Klinger HP, Glasser M (1975): Chromosome polymorphism in a human newborn population. II. Potentials of polymorphic chromosome variants for characterizing the idiogram of an individual. Cytogenet Cell Genet 15:239–255.

20. Ohno S, Trujillo JM, Kaplan WD, Kinosita R (1961): Nucleolus-organizers in the causation of chromosomal anomalies in man. Lancet 2:123–126.
21. Schmid M, Krone W (1974): On the relationship between the frequency of association and the nucleolar constriction of individual acrocentric chromosomes. Humangenetik 23:267–277.
22. Sigmund J, Schwarzacher HG, Mikelsaar A-V (1979): Satellite association frequency and number of nucleoli depend on cell cycle duration and NOR-activity. Hum Genet 50:81–91.
23. Van Dyke DL, Palmer CG, Nance WE, Yu P-L (1977): Chromosome polymorphism and twin zygosity. Am J Hum Genet 29:431–447.
24. Walpole RE, Myers RH (1972): Testing the difference between two proportions. In "Probability and Statistics for Engineers and Scientists." New York: The Macmillan Company, pp 261–263.
25. Warburton D, Atwood KC, Henderson AS (1976): Variation in the number of genes for rRNA among human acrocentric chromosomes: Correlation with frequency of satellite association. Cytogenet Cell Genet 17:221–230.

Correspondence: Dr. J.A. Brown, Department of Human Genetics, MCV Station, Box 33, Richmond, VA 23298, USA.