Acta Genet Med Gemellol 34:141-151 (1985)

Received 17 May 1984
Final 29 April 1985

NOR Variability in Twins

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Abstract. The number of AgNOR (NOR^+) and the amount of AgNOR ($NORM^+$) were analysed by means of two multilevel analyses of variance in a total of 12 twin pairs: 3 female and 4 male MZ and 5 male DZ pairs. In the first analysis, only zygosity was controlled; in the second, chromosome types D and G were controlled as well as the interaction between chromosome type and zygosity. For NOR^+ and $NORM^+$, when chromosome types D and G are not distinguished, the within-pair variance is greater, though not significantly, in DZ than in MZ pairs; but it is highly significantly greater when chromosome type (D or G type) is under control. This confirms an important genetic determination of NOR^+ and $NORM^+$ when in the ANOVA model the D and G types are controlled. However, nongenetic factors also influence the Ag-NOR patterns, but not enough to conceal the genetically defined rDNA pattern. Indeed, about 50% of the cells transcribe their rDNA in a way not closely dependent on the rDNA background and significant intrapair differences of NOR^+ pattern exist in MZ twins.

Key words: Twins, NOR staining, Analysis of variance, D and G group chromosomes

INTRODUCTION

In man, the major genes responsible for ribosomal RNA synthesis (NORs) and therefore for the organisation of nucleolar components [for a review, see 24] are localized on the stalks of the short arms of the ten acrocentric chromosomes [4,7,9,10]. The number of ribosomal DNA (rDNA) genes per human NOR is highly variable [28] and the total number of ribosomal gene copies varies from individual to individual [5].

Under given controlled conditions, NORs are specifically stained by silver [8], which binds to a chromosome-associated protein [24] identified by Hubbell et al [13]. In man, however, the 10 NORs do not always react positively. The number of silverstained NORs

(Ag-NORs) has been found to be characteristic, though variable, within an individual [1,11,15]. It has also been shown that only those NORs, which were functionally active in rRNA synthesis during the preceding interphase [17,18], are stainable with silver. Whether the observed variability of Ag-NORs reflects individual difference in the amount of rDNA or the influence of other factors on rRNA activity is not clear.

In human cells, good correlations have been obtained between amount of rDNA and participation in satellite associations [4,28], between NOR size and participation in satellite associations [19], between NOR staining and intercentromeric distances [11], and finally between amount of rDNA and NOR staining in 6 out of 8 individuals [29]. However, there still appear exceptions to these relationships, since NOR negative chromosomes are sometimes also involved in associations [11,19], since some acrocentrics with large amounts of rDNA do not show higher frequencies of associations [7,28], and finally since no correlation between amount of rDNA and NOR staining was found in 2 out of 8 individuals [29].

In somatic cell hybrids, NOR activation of human chromosomes progessively occurs in mouse-human hybrids [3,18] destined to lose human chromosomes, but in the early stages of hybrid growth the time of disappearance of human NORs is not closely correlated with loss of human chromosomes. There is no evidence of inactivation in rDNA genes in mouse-Chinese hamster hybrids [30] nor in mouse-Syrian hamster hybrids [6, 21,26] even after loss of chromosomes of either species.

These data suggest that the individual NOR pattern is influenced by both hereditary components and environmental factors.

To compare the influence of genetic and regulatory factors, we performed a study on cultured peripheral lymphocytes from twins. We analysed the variation with respect to Ag stainability of NORs within and between individuals, including a comparison between MZ and DZ same sexed twin pairs.

MATERIAL AND METHODS

1. Description of samples

A total of 12 twin pairs was studied: 3 female and 4 male MZ and 5 male DZ pairs. All were healthy Caucasoids, with ages varying from 18 to 25 years; their socio-economic level could be described as medium or high. They were collected through inquiries at university level and all were living in Belgium.

The twin were tested for 8 blood groups (ABO, Rhesus, MNSs, P, Kell, Lewis, Duffy and Kidd), 7 serum groups (Hp, Gc, Gm, Km, Bf, C_3 and Tf), 8 enzymatic groups (A_cp_1, PGM_1, AK, ADA, GPT, PGD, EsD, GLO) and the HLA types. The 12 pairs were divided into two groups: 7 pairs concordant for all the tested parameters and thus probably MZ, and 5 pairs discordant for at least one parameter and therefore DZ. The probability of dizygosity for blood-concordant pairs has an expected value of 0.00022, the extreme values being 0.00003 and 0.00091. The probabilities were calculated by the method of Race and Sanger [23].

No twin included in this study had any acute of chronic hematological disease or suffered from acute or chronic virus disease (except for one case of mononucleosis). None had received blood transfusions nor had been treated with radiotherapy.

2. Cytogenetic analysis

Blood from all subjects was cultured for 48 hr following standard methods. Chromosome preparations were always spread after fixation for 40 min (1:3 acetic and methanol treatment) and Ag-stained according to Howell [12] and Schwarzacher et al [24] one or two weeks later.

About 50 metaphase cells per individual were photographed and analysed. Only euploid metaphases in which at least one positive NOR was observed were selected on a negative projection table. The number of positive D or G type chromosomes and the size of the silver precipitate (0 = absence, 1 = small amount, 2 = moderate amount, 3 = large amount) were registered by the same person according to Miller et al [19].

The results of the cytogenetic analysis are given in Table 3 together with relevant mean values. For NOR^+, the mean number of silver stained D or G type chromosomes per metaphase was calculated for each individual.

For $NORM^+$, mean NORM values were calculated for D and G type chromosomes as shown in Table 3. The relative amount of $NORM^+$ on D-group chromosomes is obtained for each individual by the following formula:

$$(\text{mean NORM}^+ \text{ on D}) / (\text{mean NORM}^+ \text{ on D} + \text{G}) \times 100.$$

3. Analysis of variance

Variability between MZ and DZ twins for both the number of NOR^+s and the amount of NOR^+ ($NORM^+$) was analysed by means of two multilevel analyses of variance. In the first, only zygosity was controlled; in the second, both chromosome types D and G were controlled as well as the interaction between chromosome type and zygosity.

The following variables were used:

1) Number of NOR^+

1st analysis (D and G type chromosomes not distinguished): the variable is, per cell, the mean number of acrocentric chromosomes with a NOR^+; the variable ranges from almost 0 to 1.

2nd analysis (chromosome of the D and G types are distinguished in each cell): there are two variables per cell, the mean number of D chromosomes with a NOR^+ and the mean number of G chromosomes with a NOR^+. Both variables range from almost 0 to 1.

2) Amount of NOR^+ ($NORM^+$)

1st analysis (D and G type chromosomes not distinguished): the variable is, per cell, the mean amount of NOR^+ on the acrocentric chromosomes; the variable ranges from almost 0 to 3.

2nd analysis (chromosomes of the D and G types are distinguished in each cell): there are two variables per cell, the mean amount of NOR^+ on the D chromosome and the mean amount on the G chromosome. Both variables range from almost 0 to 3.

Note: To characterize the amount of NOR^+ in a cell, as stated before, an arbitrary value (0, 1, 2, 3) is given to each NOR^+, f_0, f_1, f_2, f_3 denoting the frequencies of the chromosomes with respective values 0, 1, 2, 3; the variable measuring the mean amount of NOR^+ in a cell is

$$(0 \times f_0 + 1 \times f_1 + 2 \times f_2 + 3 \times f_3) / (f_0 + f_1 + f_2 + f_3).$$

https://doi.org/10.1017/S0001566000004669 Published online by Cambridge University Press

The denominator is respectively 10, 6 or 4, according to wether all the acrocentrics of the cell are considered or only the chromosomes of D type or those of the G type.

In the first analysis, we applied an ANOVA model with one fixed effect and three random effects as shown in Table 1.

Table 1.

Source of variation	Sum of squares	Degrees of freedom
Between zygosities (fixed)	Q_4	1
Among pairs within zygosities (random)	Q_3	10
Between pairs within MZ	Q'_3	6
Between pairs within DZ	Q''_3	4
Between individuals within pairs (random)	Q_2	12
Between individuals within MZ pairs	Q'_2	7
Between individuals within DZ pairs	Q''_2	5
Among cells within individuals (random)	Q_1	1,176
Total	Q	1,199

In the second analysis, the variability of the mean NOR^+ and the mean $NORM^+$ per D and per G type chromosome is also studied by an ANOVA mixed model but with two fixed effects and three random effects (Table 2). Actually the two analyses together constitute one analysis of variance of a complex repeated measures type [31].

Table 2.

Source of variation	Sum of squares	Degrees of freedom
Between chromosome types (D vs G, fixed)	Q_5	1
Type X Zygosity (fixed)	Q_4	1
Type X Pairs within zygosities (random)	Q_3	10
Type X Pairs among MZ	Q'_3	6
Type X Pairs among DZ	Q''_3	4
Type X Individual within pairs (random)	Q_2	12
Type X Individual within MZ pairs	Q'_2	7
Type X Individual within DZ pairs	Q''_2	5
Type X Cells within individuals (random)	Q_1	1,176
Total	Q	1,200

F and F' tests were performed at each line of the two ANOVA's. We chose $\alpha = 5\%$ as level of significance.

RESULTS

1. Cytogenetic data

Table 3 summarizes the number of metaphases analysed for the different MZ and DZ twin pairs, the frequencies of cells (%) with different NOR^+ values, the relative $NORM^+$

https://doi.org/10.1017/S0001566000004669 Published online by Cambridge University Press

Table 3. Data from the Cytogenetic Analysis

Twin	Sex	n	NOR+ % cells with NOR+ per metaphase									Mean D-NOR+ per metaphase	Mean G-NOR+ per metaphase	NORM+ Relative NORM+ on D-chrom.	*Mean D-NORM+ per metaphase	*Mean G-NORM+ per metaphase
			2	3	4	5	6	7	8	9	10					
MZ 011	F	50				4	6	12	18	**40**	20	5.06	3.36	60.0	6.28	4.14
012	F	50			2	4	10	16	**28**	**28**	12	4.64	3.32	58.3	6.02	4.26
MZ 021	M	50				8	10	22	36	18	8	4.14	3.52	54.0	4.68	4.08
022	M	50				6	18	**30**	18	26	2	4.10	3.36	54.9	4.52	3.62
MZ 041	M	50		2	6	16	28	**32**	10	2	4	3.82	2.58	59.7	4.22	3.12
042	M	50		2		4	**32**	24	28	10		4.10	2.92	58.4	4.32	3.28
MZ 051	F	50	2	2	6	18	10	**26**	14	18	4	4.14	2.86	59.1	4.62	3.20
052	F	50		6	2	8	12	24	**36**	10	2	4.10	2.70	60.3	5.16	3.72
MZ 061	M	50		2	6	8	**32**	24	16	6	4	4.34	2.24	66.0	4.52	2.44
062	M	50				12	14	**30**	**30**	12	2	4.90	2.32	67.9	5.40	2.72
MZ 111	F	50			4	6	**28**	24	20	14	4	3.74	2.34	52.8	4.12	2.56
112	F	50			6	10	20	**32**	22	8	2	3.46	2.40	59.0	4.06	2.86
MZ 121	M	50			4	6	24	**28**	22	14	2	3.88	2.94	56.9	4.06	3.18
122	M	50			4	12	**26**	**26**	22	8	2	4.04	3.04	57.1	4.36	3.40
DZ 031	M	50			8	10	**32**	22	22	6		4.10	2.46	62.5	6.72	3.34
032	M	45			2	11	20	**27**	20	20		3.87	3.24	54.4	5.4	4.31
DZ 071	M	50			4	14	**34**	30	16	2		3.64	2.84	56.2	3.84	3.32
072	M	50			10	10	24	18	**28**	14	4	4.32	2.80	60.7	5.44	3.76
DZ 081	M	50		2		18	**28**	16	**28**	8	0	3.52	3.20	52.4	4.06	3.80
082	M	50		2	2	16	**26**	18	20	14	2	3.50	3.30	51.5	3.70	3.58
DZ 091	M	50		2	10	24	24	**26**	14			4.08	1.98	67.3	4.24	2.26
092	M	50		2	24	16	**32**	14	4	4	4	3.04	2.76	52.4	3.80	2.98
DZ 101	M	50		2	6	2	22	18	**24**	20	6	4.68	2.62	64.1	5.32	3.02
102	M	50			6	14	**28**	22	16	12	2	4.18	2.56	62.0	4.54	2.74

Table 4. NOR Amount (NOR^+) in Human Lymphocytes

Author	No. of Subjects		No. of metaphases	Intraindividual variation estimated through: Range of NOR^+ in the metaphases of one individual	Intraindividual variation estimated through: % of metaphases with the modal NOR^+ number		Interindividual variation estimated through: $\%D^+ / (\%D^+ + \%G^+)$		Interindividual variation estimated through: Modal NOR^+ per individual	Average NOR^+/cel	
	F	M			mean	range	mean	range	range	mean	range
Goodpasture et al 1976	2		25	5 → 8	48	44 → 52			7 → 8	6.9	6.5 → 7.3
		2	25	8 → 10	54.5	46 → 48			9 → 10	9.3	9.2 → 9.3
Bloom et al 1976		16			± 61				8 → 9 6 → 7		
Varley 1977	11			(3 → 7)(6 → 8)			57.2	21.7 → 78.0	4 → 7		
		9		(3 → 7)(4 → 9)			65.9	52.0 → 73.7	3.5 → 7		
Mikelsaar et al 1977	20		3 → 14						7 → 10		
		31	3 → 14						6 → 10		
Lau et al 1978	2		8 → 13				63.5	56 → 71		7.2	7.1 → 7.4
		3	8 → 14				60	54 → 64		7.6	6.9 → 8.6
Mikelsaar and Schwarzacher 1978	1		26		34.6	34.6			8	8.2	8.2
		2	82 → 99		46	33 → 58.7			8 → 10	8.7	8.2 → 9.3
Ray and Pearson 1979	12		2 → 14	3 → 10			62.3		5 → 10	7.7	
		16	2 → 14	3 → 10							
Hens et al 1980	1		72	6 → 10	55.5						
Zakharov et al 1982	20		50								
		20	50							8.2	
This work	6		50	(5 → 10)(2 → 10)	31.7	26 → 40	58.3	52.8 → 60.3	5 → 9	7.0	5.9 → 8.4
		18	50	(5 → 10)(3 → 8)	29.6	24 → 36	58.8	51.5 → 67.9	5 → 8	6.8	5.8 → 7.7

Table 5. Analysis of Variance for NOR$^+$ and NORM$^+$

Source of variation	NOR$^+$ Sum of squares		d f	F and F' (*) values		d f	P	NORM$^+$ Sum of squares		d f	F and F' (*) values		d f	P
Analysis 1														
Between zygosities (fixed)	0.36		1	1.16(*)		(1,10)	ns	0.01		1	0.01(*)		(1,9)	ns
Among pairs within zygosities (random)	3.63		10	7.35(*)		(10,13)	$P<0.001$	14.02		10	7.59(*)		(10,10)	$0.001<P<0.005$
Between pairs within MZ		2.89	6		11.34	(6,7)	$0.001<P<0.005$		8.00	6		11.45	(6,7)	$0.001<P<0.005$
Between pairs within DZ		0.73	4		3.04	(4,5)	ns		6.02	4		5.26	(4,5)	$0.025<P<0.05$
Between individuals within pairs (random)	0.60		12	2.45		(12,1176)	$0.001<P<0.005$	2.25		12	3.35		(12,1176)	$P<0.001$
Between individuals within MZ pairs		0.30	7		2.10	(7,1176)	$0.025<P<0.05$		0.82	7		2.09	(7,1176)	$0.025<P<0.05$
Between individuals within DZ pairs		0.30	5		2.94	(5,1176)	$0.01<P<0.025$		1.43	5		5.11	(5,1176)	$P<0.001$
Among cells within individuals (random)	23.93		1176					65.80		1176				
Total	28.51		1199					82.07		1199				
Analysis 2														
Between chromosome types (D vs G, fixed)	0.51		1	0.77(*)		(1,10)	ns	1.29		1	1.50(*)		(1,9)	ns
Type X Zygosity (fixed)	0.13		1	0.20(*)		(1,9)	ns	0.02		1	0.02(*)		(1,8)	ns
Type X Pairs within zygosities (random)	6.57		10	2.80(*)		(10,6)	ns	8.50		10	1.99(*)		(10,6)	ns
Type X Pairs among MZ		3.87	6		21.50	(6,7)	$P<0.001$		4.23	6		14.10	(6,7)	$0.001<P<0.005$
Type X Pairs among DZ		2.70	4		1.24	(4,5)	ns		4.28	4		1.08	(4,5)	ns
Type X Individual within pairs (random)	2.92		12	7.06		(12,1176)	$P<0.001$	5.31		12	7.72		(12,1176)	$P<0.001$
Type X Ind. within MZ pairs		0.21	7		0.88	(7,1176)	ns		0.36	7		0.88	(7,1176)	ns
Type X Ind. within DZ pairs		2.72	5		16.00	(5,1136)	$P<0.001$		4.96	5		17.37	(5,1176)	$P<0.001$
Type X Cells within individuals (random)	39.76		1176					66.97		1176				
Total	49.89		1200					82.09		1200				

on the D-type chromosomes ($NORM^+$ on D) / ($NORM^+$ on D + G); the mean D-NOR^+ and G-NOR^+ per metaphase and the mean D-$NORM^+$ and G-$NORM^+$ per metaphase were added to make our data comparable with results from the literature (see Table 4).

A clearcut intraindividual variation of NOR^+ is found since within an individual the metaphases with the modal NOR^+ value account for only 24 to 40% of the total number of metaphases. It means that at least 60% of the cells present a NOR^+ pattern different from the dominating NOR^+ pattern of this individual.

Interindividual variation is observed for both the mean NOR^+ value on D + G type chromosomes and the mean $NORM^+$ value on D + G type chromosomes per metaphase; these values range from 5.80 to 8.42 and from 6.50 to 10.42 for NOR^+ and $NORM^+$, respectively.

2. Analysis of variance

The results of the statistical analysis of variance are collected in Table 5. This table gives, from the left to right, the considered source of variation, the sum of squares with the degrees of freedom and the level of significance of the F and F' values.

In analysis 1, the results for both NOR^+ and $NORM^+$ are nearly similar. In both cases, significance is found for variation of individuals within twin pairs, but more for DZ than for MZ pairs. When variation between pairs within zygosities is considered, high significance is found among MZ pairs but none or a low one among DZ pairs. No significant effect is found between zygosities.

Analysis 2, instead of considering the mean NOR^+ or the mean $NORM^+$ per acrocentric chromosome, uses the mean NOR^+ or the mean $NORM^+$ per D and G type chromosome, respectively. In this way, D and G type chromosomes are controlled. Since the levels of significance of the F and F' values are nearly identical for NOR^+ and $NORM^+$, we will consider that the results for NOR^+ and $NORM^+$ are similar:

1) a significant interaction is found between chromosome type × individuals within pairs, an interaction which appears to occur primarily in DZ ($P < 0.001$) but not in MZ pairs;
2) although there is no significant overall interaction for chromosome type × pairs within zygosities, a significant interaction is found for chromosome type × MZ pairs ($0.001 < P < 0.005$) but not for chromosome type × DZ pairs;
3) no significant interaction is observed between chromosome type × zygosity;
4) no statistically significant difference is found between NOR^+ pattern on D or G type chromosomes.

DISCUSSION

Although intraindividual and interindividual variability of Ag-stainable NOR patterns has been observed by different authors, few data are available on the mathematical estimation of this variability.

Estimations of the genetic and environmental component were obtained in two analyses of NOR activity in human lymphocytes from twins. One study performed in 19 MZ and 21 DZ twin pairs [2] indicated a low heritability of $NORM^+$ but a higher genetic influence on NOR^+. A more recent work on 20 MZ and 20 DZ pairs showed by an analysis of intrapair concordance as well as intrapair variance [32] that NOR^+ and $NORM^+$ are

highly heritable traits; the degree of genetic determination proved to be 0.98 when the NOR^+ was studied and 0.94 if $NORM^+$ was analysed. Moreover, the intrapair differences for any particular acrocentric chromosome proved to be significantly greater in DZ than in MZ twins.

We performed two multilevel analyses of the variance; in both analyses the results for NOR^+ and $NORM^+$ are quite similar.

In the first analysis, where only zygosity is controlled, the within MZ pairs variance is less significant than the within DZ pairs variance, as expected. The variation between pairs is highly significant for MZ pairs but not significant for DZ pairs; this may be due just to chance. But no difference is observed between the zygosities.

In second analysis, bot chromosome types D and G are controlled as well as zygosity; there is no significant interaction between chromosome type and zygosity, and no significant differences are observed when the mean NOR^+ (or $NORM^+$) are compared on D and G chromosome types.

The interaction between chromosome type X pairs is statistically significant among MZ pairs but not among DZ pairs; in contrast, the interaction between chromosome type X individuals within pairs is statistically significant within DZ pairs but not within MZ pairs. This is because MZ cotwins are genetically identical but the variation from one pair to another is just like the variation from one independent individual to another. In contrast, for DZ twins the genetic variation is partitioned both among and within twin pairs.

Both analyses thus confirm that NOR^+ and $NORM^+$ are more similar in two individuals of a MZ twin pair than in two individuals of a DZ twin pair; however, the interpair variability is so important that a comparison between zygosities shows no statistically significant differences.

Since the ratio, variation within DZ pairs on variation within MZ pairs, is essential for the genetic determination of NOR behavior, we performed a complementary test comparing the within pairs variation in DZ and MZ pairs with F equal to

$$F_{5,7} = \frac{\text{mean squares within DZ}}{\text{mean squares within MZ}}$$

The results of this analysis are given in Table 6.

Table 6. F Values Obtained by Comparing the Within Pairs Variation in DZ and MZ Pairs when D and G Type Chromosomes are or are not Under Control

	NOR^+	$NORM^+$
Analysis 1	$F_{5,7} = 1.399$	$F_{5,7} = 2.444$
(D and G type not distinguished)	ns	ns
Analysis 2	$F_{5,7} = 18.13$	$F_{5,7} = 19.80$
(D and G type controlled)	$P < 0.001$	$P < 0.001$

$$F = \frac{\text{mean squares within DZ}}{\text{mean squares within MZ}}$$

https://doi.org/10.1017/S0001566000004669 Published online by Cambridge University Press

For NOR^+ and $NORM^+$, when chromosome types D and G are not distinguished, the DZ within pair variance is greater, though not significantly, than the MZ within pair variance. But for both NOR^+ and $NORM^+$, the within pair variance is highly significantly greater in DZ than in MZ pairs when chromosome type (D or G type) is controlled.

Therefore, even with fewer twin pairs than others [2,32], we have also found results pointing to an important genetic determination of NOR^+ and $NORM^+$ when in the ANOVA the D and G types are under control.

CONCLUSION

From experiments correlating rDNA gene content of individual chromosomes with NOR stainability of these acrocentrics [29], it is considered that in a large majority of individuals (6 out of the 8 examined), the NOR pattern reflects the relative amount of rDNA present in these chromosomes. Numerous other data from the literature [1,11,15] indicate the presence of a genetic component in NOR^+ patterns.

Our results confirm the existence of an important genetic influence on the AgNOR pattern. However, one might wonder which factors are able to modify the expression of the genetically determined rDNA pattern. One may distinguish between external factors, such as living conditions, viral infection, culture conditions, and internal factors (which might be genetically determined) such as regulatory mechanisms, existence of different lymphocytic subpopulations with different responses to PHA stimulation, role of non-rDNA chromosomal material (spacer DNA) responsible for nucleolar association and therefore for activation of rDNA transcription [29].

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