

## ALU I AND HAE III RESTRICTION ENDONUCLEASES, HUMAN CHROMOSOMES AND INFERTILITY

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### ABSTRACT

The Alu I and Hae III bands of twenty-five couples plus two isolated members of two other couples with reproductive problems, as well as the same number of appropriate controls were studied. Both qualitative and quantitative (densitometric) approaches were employed. Chromosome 1 was the most heteromorphic for the two enzymes, but others also showed heteromorphism frequencies equal or higher than 10% (Alu I: chromosomes 3, 4, 9, 15, 16, 19 and 22; Hae III: 16). The Alu I bands of chromosomes 9, 15, 19 and 22 were measured densitometrically. Those of chromosome 9 were larger than those observed in the other chromosomes, and those of chromosome 19 larger than those of chromosome 22. The heteromorphism indices proved to be strikingly similar. Agreement between qualitative and quantitative approaches was tested and found to be satisfactory. No significant differences were observed between the infertile and fertile individuals.

### INTRODUCTION

Restriction endonucleases are useful for the banding of human chromosomes (Miller *et al.*, 1983; Mezzanotte *et al.*, 1983a,b, 1985; Babu and Verma, 1985, 1986a, 1987; Bianchi *et al.*, 1985; Ferrucci *et al.*, 1987; Babu *et al.*, 1988; Zhang and Dong, 1989; review in Babu, 1988). Therefore, it is surprising that only two reports present results involving the frequencies of heteromorphisms induced by such enzymes, and these involved Alu I and chromosomes 16 and 18 only (Babu *et al.*, 1987; Stuppia *et al.*, 1989). We report here a detailed investigation considering all human chromosomes, which have been subjected to the action of Alu I and Hae III. For the first enzyme, besides

a qualitative evaluation, densitometric, quantitative measurements of the indicated pericentromeric bands were also performed. The samples included members of couples selected due to infertility problems, as well as of normal, fertile controls.

## MATERIAL AND METHODS

The criteria used for the establishment of a diagnosis of infertility problems due to genetic causes were: (a) Previous occurrence of at least two spontaneous abortions; (b) History of a spontaneous abortion and of a malformed child; (c) Absence of a clinically established gestation with at least two years of marital relations without the use of contraceptives; and (d) Lack of a clear non-genetic etiology for these problems.

The couples diagnosed in this way were ascertained through the following institutions or individuals: (a) Genetic Counseling Unit of our Department (9); (b) Fundação Universitária de Endocrinologia e Fertilidade (7); (c) Medical Genetics Unit of the Hospital de Clínicas de Porto Alegre (6); (d) Practicing physicians (5). Only 25 of these couples were completely studied. For two of them (one from b and the other from c) only the males could be cytogenetically examined.

Seven relatives of two patients who were carriers of a Robertsonian translocation were also studied, but with G-banding only, to ascertain if they were also carriers.

For each couple with infertility problems a control couple was investigated, which had the following requisites: (a) Same ethnic group of the index cases; (b) At least one healthy child; (c) Without previous history of spontaneous abortions, stillbirths or malformed children. Only one of them was composed by Black subjects; all the others were Caucasoid.

The methods for chromosome preparation and G-banding were the usual ones (Erdtmann and Mattevi, 1971; Seabright, 1971). Alu I and Hae III banding was induced as described by Babu and Verma (1985) with the following modifications: (a) 100  $\mu$ l instead of 40  $\mu$ l per slide; (b) enzyme treatment between the second and third days after fixation (around 60 hours); and (c) in the second lot of the enzymes (two lots were used), a longer incubation time (six to seven hours, instead of three to four). The enzymes were manufactured by Pharmacia LKB.

For each patient, as well as his(her) relatives, 11 metaphases with conventional (Giemsa) staining were analyzed under the microscope. The detection of aneuploid cells would lead to the increase of this number to 30. Photos of five G-banded cells were then obtained, three of which were karyotyped. Alu I and Hae III banding was qualitatively investigated under the microscope, considering five cells from each individual. In cases of doubt at least three cells were photographed and analyzed. The quantitative, densitometric Alu I band measurements were performed, adapting the procedures described by Erdtmann *et al.* (1982) for C-bands. Appropriate corrections were therefore made for

the chromosomes' levels of contraction. The measurement error was estimated by the examination of 1560 determinations (780 paired comparisons; 78 for the two homologues of pair 9 and 104 each for those of pairs 15, 19 and 22). The average error was determined as 2%, with minimal variation among chromosomes. The average of the two measurements was considered as the final band length.

The quantitative data were analysed using the SPSS program (Norusis, 1986) or other statistical methods, as described in Ayres and Ayres (1987).

## RESULTS

Tables I and II present a comparison between members of infertile and fertile couples in relation to Alu I and Hae III heteromorphisms, respectively, as assessed in a qualitative way. No significant differences were observed in relation to the two groups, so that they were considered together in the description of the findings. Considering first the Alu I results (Table I), the percentages of individuals with banded cells varied from 10% (chromosome 5) to 100% (chromosomes 1, 15, 16, 19, 22 and Y). Chromosome 1 was the most heteromorphic (21%), but several others (chromosomes 3, 4, 9, 15, 16, 19 and 22) showed heteromorphism frequencies equal to or greater than 10%. Therefore, the enzyme induced heteromorphisms not only in the large pericentromeric heterochromatic regions (those of chromosomes 1, 9 and 16), but in others as well. Representative examples of observations made in the eight chromosomes in which frequencies equal or greater than 10% were obtained are shown in Figure 1.

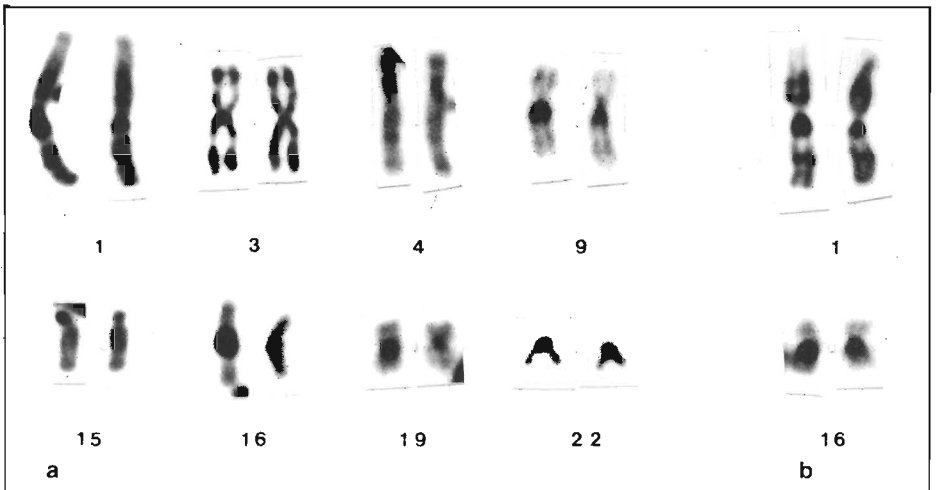


Figure 1 - Representative examples of cells in which heteromorphisms with frequencies of 10% or more were observed. (a) Alu I, chromosomes 1, 3, 4, 9, 15, 16, 19 and 22; (b) Hae III, chromosomes 1 and 16.

Table I - Comparison between members of infertile and fertile couples in relation to the prevalences (in percentages) of Alu I heteromorphisms.

Chromosome	Infertile couples (Males, N = 27; females, N = 25)				Fertile couples (Males, N = 27; females, N = 25)			
	% indiv. with banded cells <sup>a</sup>	Heteromorphism <sup>b</sup>			% indiv. with banded cells <sup>a</sup>	Heteromorphism <sup>b</sup>		
		Absent	Partial	Total		Absent	Partial	Total
1	100	79	21	-	100	79	21	-
3	44	34	4	6	35	23	8	4
4	54	42	8	4	60	52	6	2
5	10	10	-	-	10	10	-	-
7	75	71	2	2	77	73	4	-
9	73	60	13	-	75	67	8	-
10	13	13	-	-	10	10	-	-
13	60	56	4	-	58	56	2	-
14	19	19	-	-	12	12	-	-
15	100	90	10	-	100	85	15	-
16	100	90	10	-	100	83	17	-
18	19	13	6	-	17	15	2	-
19	100	88	12	-	100	85	15	-
20	13	11	2	-	13	13	-	-
21	10	10	-	-	13	13	-	-
22	100	88	12	-	100	88	10	2
Y	100	100	-	-	100	100	-	-

None of the differences, considering each chromosome of infertile and fertile individuals separately, was statistically significant (corrected chi-squares, alpha of 5%).

<sup>a</sup> All five cells of a given individual were always either banded or unbanded.

<sup>b</sup> *Total*: band present in one homologue but absent in the other; *partial*: bands present in both homologues, differing in size only.

Table II - Comparison between members of infertile and fertile couples in relation to the prevalences (in percentages) of Hae III heteromorphisms.

Chromosome	Infertile couples (Males, N = 25; females, N = 25)			Fertile couples (Males, N = 25; females, N = 25)		
	% indiv. with banded cells <sup>a</sup>	Heteromorphism <sup>b</sup>		% indiv. with banded cells <sup>a</sup>	Heteromorphism <sup>b</sup>	
		Absent	Partial		Absent	Partial
1	100	82	18	100	80	20
9	46	42	4	38	36	2
15	72	68	4	82	72	10
16	90	82	8	100	88	12
19	62	62	-	80	76	4
22	78	72	6	84	78	6
Y	100	100	-	100	100	-

None of the differences, considering each chromosome of infertile and fertile individuals separately, was statistically significant (corrected chi-squares, alpha of 5%).

<sup>a</sup> All five cells of a given individual were always either banded or unbanded.

<sup>b</sup> *Partial*: bands of different size present in both homologues.

The results concerning Hae III are shown in Table II. The number of banded chromosomes was smaller; and in those in which banding occurred the percentages of individuals with banded chromosomes was equal or somewhat lower than those observed for Alu I (ranging from 42% for chromosome 9 to 100% for chromosomes 1 and Y). Again, chromosome 1 was the most heteromorphic (19%), the frequency for chromosome 16 reaching also the two-digit level (10%; see Figure 1). After Hae III treatment as was observed by previous authors (Babu and Verma, 1985, 1986a; Bianchi *et al.*, 1985; Babu, 1988; Zhang and Dong, 1989), we obtained residual G-banding.

Quantitative data are given in Tables III and IV; averages from the actual measurements (corrected for differing levels of contraction) are given in Table III, and those for the heteromorphism indices in Table IV. Of the 16 comparisons made between members of infertile and fertile couples, only one reached statistical significance (a

somewhat higher level of heteromorphism in the controls, 0.896, as opposed to that of the patients, 0.846). The Alu I bands induced in chromosome 9 were larger than those observed in chromosomes 15, 19 and 22. Those of chromosome 19 were also, on average, larger than those of chromosome 22. A striking degree of similarity was obtained for the heteromorphism indices; without considering the two extreme values cited above, the averages varied from 0.87 to 0.89 only. Differences among chromosomes were statistically non-significant.

Table III - Comparison between patients and controls in relation to the size (in micra) of the Alu I bands of chromosomes 9, 15, 19 and 22.

Alu I band <sup>a</sup>	No. indiv. studied	Patients $\bar{X} \pm DP$	Controls $\bar{X} \pm DP$	t test	Significance
9 M	78	0.873 ± 0.238	0.811 ± 0.257	1.09	NS
9 m	78	0.731 ± 0.195	0.722 ± 0.211	0.20	NS
9 M + 9 m	78	1.604 ± 0.423	1.532 ± 0.464	0.70	NS
15 M	104	0.631 ± 0.167	0.640 ± 0.127	0.32	NS
15 m	104	0.557 ± 0.143	0.560 ± 0.117	0.13	NS
15 M + 15 m	104	1.188 ± 0.305	1.200 ± 0.235	0.22	NS
19 M	104	0.693 ± 0.156	0.689 ± 0.127	0.16	NS
19 m	104	0.604 ± 0.127	0.596 ± 0.131	0.30	NS
19 M + 19 m	104	1.297 ± 0.275	1.285 ± 0.248	0.24	NS
22 M	104	0.611 ± 0.147	0.593 ± 0.146	0.63	NS
22 m	104	0.541 ± 0.131	0.523 ± 0.135	0.71	NS
22 M + 22 m	104	1.152 ± 0.273	1.115 ± 0.275	0.68	NS

<sup>a</sup> M - Larger band; m - smaller band.

NS - Non-significant.

Table IV - Comparison between patients and controls in relation to the indices for heteromorphism of Alu I bands.

Chromosome	No. indiv. studied	Indices of heteromorphism <sup>a</sup>		t test	Significance
		Patients $\bar{X} \pm DP$	Controls $\bar{X} \pm DP$		
9	78	0.846 ± 0.096	0.896 ± 0.072	2.63	2.0%
15	104	0.888 ± 0.078	0.878 ± 0.094	0.59	NS
19	104	0.877 ± 0.085	0.866 ± 0.097	0.63	NS
22	104	0.887 ± 0.073	0.884 ± 0.087	0.21	NS

<sup>a</sup> Size of the smaller band divided by the size of the larger band.

NS - Non-significant.

The average number of heteromorphisms per individual (as assessed qualitatively for all chromosomes) was  $1.13 \pm 1.06$  for Alu I and  $0.47 \pm 0.64$  for Hae III.

Simultaneous heteromorphisms for Alu I and Hae III occurred most frequently for chromosomes 1 (present in 18 subjects, against four in which isolated Alu I and one in which isolated Hae III heteromorphisms were found) and 16 (nine, five and one individual, respectively). For the remaining autosomes in which both enzymes induced heteromorphisms (9, 15, 19 and 22) the higher amount of independence was observed for chromosome 22 (simultaneous heteromorphisms in two, Alu I only in 10, Hae III only in four).

To verify if there was agreement between the qualitative and quantitative approaches, the measurements on the individuals classified as heteromorphic on a subjective basis were separated from those considered as homomorphic. The heteromorphic indices obtained densitometrically were then averaged in the two sets. The averages of those previously classified as heteromorphic were 2.1x-4.2x higher than those of the homomorphic (all the four differences, concerning chromosomes 9, 15, 19, and 22 were highly significant, at the 0.1% level).

Two unrelated male subjects in the infertility group presented presumably the same 13;14 Robertsonian translocation, involving in one chromosome the distal and in the other the proximal pericentromeric region [45, XY, t(13;14) (13qter → cen → 14qter)]. The frequency of this major rearrangement in this group is, therefore,  $2 \times 100/52 = 3.8\%$ . In one family the mother and two sisters of the propositus (JCB) also carried the

translocation; one brother was normal. Five abortions occurred in the obstetrical history of JCB's mother, and three plus a stillborn in that of one of her carrier daughters. The other had three children and no abortions. JCB's wife had two abortions. He showed moderate oligospermia ( $31 \times 10^6$  spermatozoa per ml), and low-viability (35%-60%) spermatozoa.

As for the other family, only the parents of the propositus (LCR) could be tested. His mother was a carrier with no history of reproductive loss (having delivered LCR and two daughters). But the propositus' grandmother had five abortions in 19 pregnancies. LCR presented severe oligospermia ( $1-10 \times 10^6$  spermatozoa per ml), and low-viability (20%) spermatozoa. He was married for four years, with no children, despite the absence of contraceptive practices.

The chromosomes of the two propositi were examined after Alu I treatment. In both there was banding in the isolated chromosome 13 as well as in the 13/14 derivative, with no obvious differences between them.

## DISCUSSION

The first question asked in this investigation, whether significant differences in the Alu I and Hae III banding patterns could be verified between infertile and fertile individuals can now be answered in the negative, at least with the sample sizes investigated.

The representativeness of our patients is indicated by the presence of the two 13;14 Robertsonian translocation carriers, the frequency of major chromosome rearrangements found (3.8% per individual; 8% per couple) being of the same order of magnitude as those observed elsewhere, using the same procedures (2.9% per individual, review in Tharapel *et al.*, 1985; 2.3%-6.0% per couple - Fortuny *et al.*, 1988; Castle and Bernstein, 1988; Sider *et al.*, 1988; De Braekeleer and Dao, 1990).

The two previous reports describing frequencies of heteromorphisms for Alu I bands are those of Babu *et al.* (1987) and Stuppia *et al.* (1989). The first included the investigation of 50 normal Caucasians for chromosome 18. It cannot be strictly compared with the present study because they could obtain a much better resolution of the Alu I bands of this chromosome. They subdivided the bands in five sizes and four types, the latter depending upon the position relative to the centromere. Using this detailed subdivision they arrived at a very high frequency of heteromorphisms: 96%. In addition, they obtained banding in 100% of the treated cells, while we could get only 18%. These differences may be due at least in part to dissimilarities in the technique used in the two surveys. While Babu *et al.* (1987) used an enzyme concentration of 100  $\mu$ /ml and an incubation time of four-five hours, we employed 200  $\mu$ /ml and six-seven hours.

Stuppia *et al.* (1989) studied 20 normal subjects for the Alu I bands of chromosome 16, observing a heteromorphism frequency of 10%, very similar to that obtained here (13%).

The present investigation agrees with the findings of Mezzanotte *et al.* (1983a), and Babu and Verma (1985) in the presence or absence of Alu I bands in the chromosomes in general. Bianchi *et al.* (1985), however, had not been able to induce this type of bands in chromosomes 19 and 22. The occurrence of Hae III bands in chromosomes 1, 9, 15, 16 and Y had already been mentioned by Mezzanotte *et al.* (1983a), Babu and Verma (1985) and Bianchi *et al.* (1985). But their presence in high frequencies (above 70%) in chromosomes 19 and 22 had not been registered up to now. It is possible that this difference may be related to the greater incubation time employed in the present study.

There is much variation in the C band heteromorphism frequencies observed by different authors using subjective evaluations. Therefore, it is difficult to compare them with those obtained here after Alu I and Hae III treatments, but they show levels of the same order of magnitude in relation to chromosomes 1, 9 and 16. In addition, treatment with Alu I may induce heteromorphic bands in frequencies equal or greater than 10% in chromosomes 3, 4, 15, 19 and 22. The possibility of revealing heteromorphisms in other chromosomes besides those of no. 1, 9 and 16 had been already emphasized by Bianchi *et al.* (1985); Agarwal *et al.* (1986); Babu and Verma (1986b,c); and Babu *et al.* (1987, 1988).

Of the four chromosomes quantitatively measured in the present investigation only one (chromosome 9) had been previously studied densitometrically in relation to C bands (the reason for not including chromosomes 1 and 16 in our evaluations was that they had already been reasonably well studied in relation to the traditional C bands. Chromosome 9 was investigated because Flores (1984) had observed differences in the C bands of this chromosome among fertile and infertile individuals). The size of the Alu I band obtained (0.72-0.87  $\mu$ ) was always smaller than those observed for the C band of this chromosome in different studies which used similar methodologies, and persons sampled from the same ethnic group (0.89-1.11  $\mu$ ; Erdtmann *et al.*, 1981; Pedrosa *et al.* 1983; Cavalli *et al.*, 1984; Zanenga *et al.*, 1984; Flores, 1984). As was suggested by Bianchi (1986), this derives from the fact that part of the pericentromeric heterochromatin is digested by Alu I.

The method of inducing bands by restriction endonucleases is simple, quick, and preserves the morphology of the chromosomes, that can be generally identified by the residual G bands. On the other hand, there is variability in the degree of activity presented by different lots of the enzymes, and some points of the technique are critical, like the buffer's pH and the temperature of incubation. Future studies should expand the number of enzymes employed, which would allow the establishment of a chromosome map indicating, for each chromosome, the restriction enzyme capable of inducing the

highest degree of heteromorphism. This would be of obvious importance for both academic and applied investigations.

### ACKNOWLEDGMENTS

Thanks are due to Margarete S. Mattevi and Bernardo Erdtmann for useful suggestions; to Roberto Giugliani for the use, during part of the study, of the laboratories of the Medical Genetics Unit, Porto Alegre's Clinical Hospital; to the several physicians who referred patients for study, and to Sidia M. Callegari-Jacques, for precious collaboration in the analysis of the data. This study was financed by the Organização dos Estados Americanos (OEA), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Financiadora de Estudos e Projetos (FINEP), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

### RESUMO

Vinte e cinco casais, mais dois membros isolados de dois outros casais, todos com problemas reprodutivos, bem como número idêntico de controles apropriados, foram estudados com relação às bandas induzidas por Alu I e Hae III. Foram empregados enfoques tanto qualitativos como quantitativos (densitométricos). O cromossomo 1 foi o mais heteromórfico usando-se as duas enzimas, mas outros também mostraram freqüências de heteromorfismos iguais ou superiores a 10% (Alu I: cromossomos 3, 4, 9, 15, 16, 19 e 22; Hae III: 16). As bandas Alu I dos cromossomos 9, 15, 19 e 22 foram medidas densitometricamente. As do cromossomo 9 são maiores do que as observadas nos outros cromossomos, e as do cromossomo 19 maiores do que as do cromossomo 22. Os índices de heteromorfismo mostraram-se marcadamente similares. Foi testada a concordância entre os enfoques qualitativo e quantitativo, que resultou ser satisfatória. Não foram encontradas diferenças significativas entre os indivíduos inférteis e férteis.

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(Received March 21, 1991)