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Flow cytometry estimation of nuclear size and ploidy level of habituated calli of sugar beet

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Abstract

A fully habituated (auxin- and cytokinin-independent) self-regenerating (organogenic) sugar beet cell line (HO) and a fully habituated non-organogenic one (HNO) derived from the former one, were analyzed as to their nuclear size and DNA content. Flow cytometry and image analysis were used and cells of certified diploid leaves of the same sugar beet strain served as controls. The HNO cells had been shown previously to have many characteristics of cancerous cells. The analyses made on leaves and HNO cells indicated the presence of only one population of cycling cells. In HO cells, two cycling populations were detected: the first one had the same DNA content as the leaves while the second one contained two fold more DNA than the first population. HNO cells showed the higher nuclear size and DNA content. HNO cells also showed evidence of aneuploidy. Thus, nuclear size, DNA content and ploidy level increase together with the neoplasic progression to culminate in HNO cells with the loss of organogenic totipotency.

Additional key words: aneuploidy, *Beta vulgaris*, habituation.

Introduction

Flow cytometric methods have several advantages over conventional microscopic procedures for determining the ploidy level of cells (Muirhead *et al.* 1985). In particular, they make it possible to estimate the intercellular variation of DNA content and to recognize cell subpopulations (Doležel 1991). Unfortunately, their

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application in plant biology has been delayed, largely due to the fact that flow cytometry requires single-cell suspensions (Alan 1985), or the isolation of protoplasts (Houssa *et al.* 1991) or cell nuclei (Hopping 1993, Doležel *et al.* 1994). In plants, the technique has mostly been applied to the measurement of nuclear-DNA content (Hopping 1993) in relation with cell cycle analyses (Nicoloso *et al.* 1994) and ploidy changes (Pijnacker *et al.* 1989) and for measuring the variation in nuclear-DNA content (Gielis *et al.* 1997).

In the present paper, the method was applied to determine the ploidy level of habituated calli of *Beta vulgaris*. Habituation is defined as the stable, heritable loss in the requirement of cultured plant cells for growth hormones (Meins 1989). It is known that genetic and epigenetic variation occurs in cultured cells, tissues and regenerated plants (Phillips *et al.* 1990, Geier *et al.* 1992, Jemmali *et al.* 1995). These variant cell types have been shown to arise during *in vitro* cultivation, especially under conditions favouring unorganized growth (Winfield *et al.* 1993), and calli are defined as resulting from anarchic prolifération of undifferentiated cells. The loss of growth control in plant or animal neoplasia must be due to the activation and expression of genes bypassing the requirement for specific growth factors (Bishop 1987). The process of habituation is considered to be part of a neoplastic progression leading to a complete loss of organogenic totipotency (Gaspar *et al.* 1991).

A fully habituated organogenic callus (HO) of sugar beet was obtained by De Greef and Jacobs (1979). From this organogenic callus, they isolated a fully habituated nonorganogenic callus (HNO). HNO cells divide actively, are of monoclonal origin (Kevers *et al.* 1981a,b), accumulate polyamines as a result of an alteration of nitrogen metabolism (Le Dily *et al.* 1993), present apoptotic bodies (Hagège *et al.* 1991) and exhibit reduced cell-to-cell adhesion due to highly acetylated pectins (Liners *et al.* 1994).

The present study compares nuclear size and ploidy level in the two types of calli with those from cells of diploid leaves of a plant of the same strain.

Materials and methods

Plants: Experimental conditions for obtaining fully habituated organogenic (HO) and nonorganogenic (HNO) calli of sugar beet (*Beta vulgaris* L. var. *altissima*), and for maintaining these tissues in solid stock cultures have been reported elsewhere (Kevers *et al.* 1981b). Such calli when subcultured every 2 weeks on solid medium did not show any visible change over a period of 17 years. Some morphological and biological characteristics of these lines have been described previously (Kevers *et al.* 1981a, Hagège *et al.* 1991, Le Dily *et al.* 1993). Samples of calli were taken after about 2 weeks of culture. Leaves of *in vitro* cultured, certified diploid plants of the same strain were provided by the *SES* company (Tienen, Belgium) and used as control.

Isolation of nuclei: Intact cell nuclei were isolated mechanically according to Galbraith *et al.* (1983) with some modifications. All steps were performed on ice.

Samples (10 g) were fixed by chopping HO callus or leaf tissues in 5 cm^3 of cold extraction buffer (EXB: 10 mM Tris, 10 mM Na_2EDTA, 100 mM NaCl, 0.2 % Triton X-100, pH 7.4) supplemented with formaldehyde (1.5 %) (Sgorbati *et al.* 1988). After 5 min, the samples were recovered by filtration (64 μm) and were washed twice for 15 min with EXB. The material was chopped with a razor blade in a glass Petri plate in 1 cm^3 of EXB and incubated 30 min. The extract was filtered through a 64 μm nylon mesh to remove large tissue debris. The filtrate was centrifuged for 10 min at 750 *g* and the pellet was resuspended in 0.5 cm^3 of cold EXB and passed several times in a syringe (needle 0.3 mm in diameter) to eliminate aggregates. HNO cells were treated in a similar manner except that instead of the second chopping stage it was necessary to squash them delicately for 1 min using a mortar and pestle.

Nuclear size: The nuclei were stained with toluidine blue (*Sigma*, St Louis, USA). Nuclear sizes of HO and HNO calli were measured with a computer analysis system (*CAS 200* image analysis system, *Becton Dickinson*, Leiden, The Netherlands). This system permitted morphological observations and analyses of nuclei with a microscope connected to a computer.

Flow cytometry: Suspension of isolated nuclei were kept cold on ice, and stained with an equivalent volume of 1 mg cm^{-3} propidium iodide (PI) (*Sigma*, St. Louis, USA). This dye was selected because it has an excitation spectrum (peak at 493 nm) compatible with the blue argon-laser present on most flow cytometers, and was found to be the best for determining DNA content in plant material (Michaelson *et al.* 1991). Stained nuclei were analyzed directly in the dye solution. Nuclei were run on a *FACStar*+ flow cytometer equipped with the *FACStation* computer management system (*Macintosh CellQuest* software; *Becton Dickinson*, Sunyvale, USA) and with an argon-ion laser emitting a 488-nm beam at 200 mW of power (*Spinnaker 1161* model; *Spectra-Physics*, Mountain View, USA). The nuclei were injected through a 70-μm nozzle tip at a low rate of analysis (< 200 events s^{-1}) to achieve optimal resolution of the measurements. Simultaneous recordings of forward angle light scatter, related to particle size, and the DNA-PI complex red fluorescence signal were made from 10 000 to 50 000 nuclei per sample. The fluorescence signal at 620 nm of the DNA-PI complex was processed for each nuclei as two distinct parameters related either to DNA content (pulse-area) or to the transit time (time of flight) of a nucleus through the laser beam (pulse-width; pulse duration). Two nuclei in G_1 phase of the cell cycle which are stuck together (doublets) will have the same DNA content as a single nucleus in G_2M phase. To avoid this artifact, DNA histograms (linear scale; 1.024 channels) were obtained after gating on pulse-area versus pulse-width red fluorescence bivariate dot plots to minimize clumps of nuclei and doublets contribution as described elsewhere (Ormerod 1994) (Fig. 2). In some experiments, the red fluorescence pulse-area was used as the threshold trigger for eliminating a major part of numerous debris with very low fluorescence or DNA content. The DNA index (DI) of the sample was calculated by dividing the mean fluorescence channel of the sample G_1 peak by the mean channel of the diploid G_1 peak of *Beta vulgaris* leaves nuclei used as an internal standard. The coefficient of variation (CV) for

G_1 peaks in histogram was calculated by the standard deviation × 100/mean ratio. This gave the resolution of the DNA measurements which is within the accepted value of CV% = 10 for plant material (Houssa *et al.* 1991). The fraction of nuclei in each G_1, S and G_2M phases of the cell cycle was calculated using the ModFit cell-cycle software (*Verity*) which applies Marquardt nonlinear least-squares curve-fitting optimization, plus operator verification, to produce the best modeling of an observed distribution of DNA content in nuclei after background residual debris subtraction.

Results

Nuclear size: Fifty nuclei from each type of callus (HNO and HO) were analyzed. The histogram (Fig. 1) indicates the distribution of nuclear size. Nuclei from HO callus were smaller (35 ± 11 μm^2) than those from HNO callus (138 ± 47 μm^2). The distribution of the nuclear size of the HNO cells was rather heterogeneous.

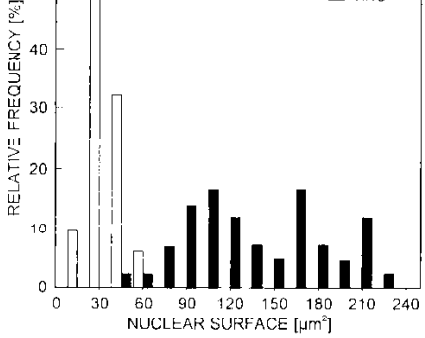

Fig. 1. Nuclear surface of HO and HNO callus cells.

Flow cytometric DNA distributions: The procedure used to analyze the DNA flow cytometry data for nuclei isolated from diploid cells of *Beta vulgaris* leaves is illustrated on Fig. 2. In each experiment, the C.V. value obtained was under 10 % (Table 1). On the DNA histogram (Fig. 3*A*), peaks corresponding to two different DNA contents (2C and 4C) could be distinguished. 66 and 25 % of nuclei were respectively in G_0G_1 and in G_2M phases respectively (Table 1). The same method was applied to *in vitro* callus cultures. The histograms of nuclear DNA content in HNO cells was similar to these obtained with leaf tissues: two clear peaks corresponded to two different DNA content levels (Fig. 3*C*) with a ratio of fluorescence intensities G_1/G_2 near 2 in one population of cells. In this case, 61 % of cells were in G_0G_1 and 25 % in G_2M phases (Table 1). In HO callus, the *Cell Cycle Analysis Software* identified two populations of cells: one population, representing 75 % of total nuclei, with 76 % in G_0G_1 and 12 % in G_2M phases, and a second

population (25 % of total nuclei) with 63 % in G_0G_1, 12 % in G_2M phases and a big S phase (25 %) (Fig. 3*B*, Table 1).

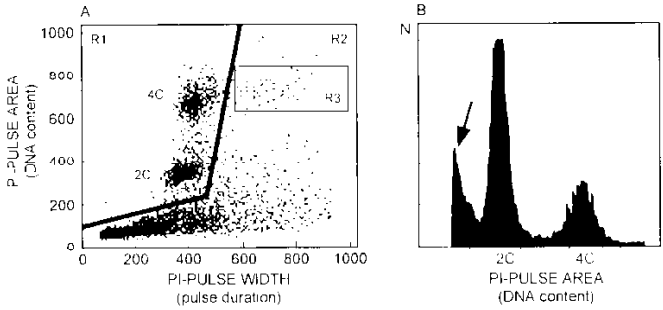

Fig. 2. DNA flow cytometry of nuclei isolated from diploid *Beta vulgaris* leaves cells (*A*) and DNA histogram obtained after selection of the intact single nuclei (*B*). The integrated area and the duration of the signal (pulse area and width, respectively) from the DNA-PI complex fluorescence of 20 000 particles are plotted as a bivariate dot plot. Gates are set to discriminate the whole single nuclei population (R1) from debris, clumps and aggregates of nuclei (R2). The two major nuclei subsets (R1) are nuclei in the G_1 phase of the cell cycle, with a 2C-DNA content, and nuclei in the G_2 or M phases, with a 4C-DNA content. Region R3 illustrates the presence of numerous G_1-phase nuclei doublets, *i.e.* aggregates which have the same DNA content as single G_2M-nuclei, but a significant longer time of flight (pulse duration) through the laser beam. The DNA histogram (*B*) is obtained after selecting the whole single nuclei with gate R1 (*A*), which discards the majority of the debris and clumps. Residual, small debris (*arrow*) were discarded from subsequent analysis with the cell-cycle *ModFit* software.

Table 1. Comparison of frequency distribution [%] of nuclear DNA content of leaves (2 repetitions), HO (4 repetitions) and HNO (3 repetitions) calli within 1 or 2 populations and in each population within G_0G_1, G_2M and S phases of the cell cycle. DNA index was calculated after comparison (see Fig. 4, 5, 6), with leaves DNA index as reference (DNA index = 1).

	Population I			Population II		
	leaves	HO callus	HNO callus	leaves	HO callus	HNO callus
	100	75 ± 2	100	0	25 ± 2	0
G_0G_1	66 ± 5	76 ± 3	61 ± 2		63 ± 4	
G_2M	25 ± 3	12 ± 3	25 ± 2		12 ± 1	
S	9 ± 3	12 ± 2	14 ± 1		25 ± 5	
C.V.	8 ± 1	9 ± 1	9 ± 1		7 ± 1	
DNA index	1	1.04	3.48		2.09	

Ploidy level comparison between certified diploid leaves and HO callus: Nuclei from leaves and HO callus were prepared separately and then mixed in equal proportions before staining. The results (Fig. 4*A*) indicate the superposition of the 2C peaks of the two samples. Samples were also stained separately and analyzed under the same conditions and instrument settings (Figs. 4*B*,*C*). This superposition indicates that the

G_0G_1 (2C) and G_2M (4C) peaks for nuclei from leaves and HO callus represent the same, or two very similar, DNA contents.

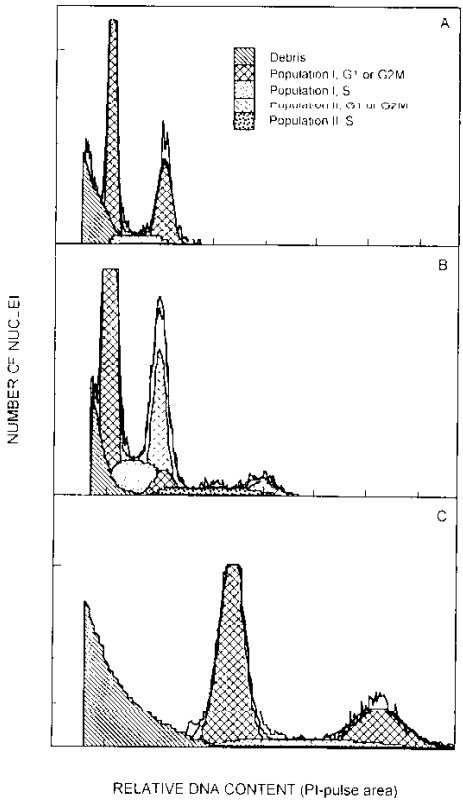

Fig. 3. Representative flow cytometric DNA distributions of propidium iodide (PI) stained nuclei isolated from certified diploid leaves (*A*), HO (*B*) and HNO (*C*) sugar beet cell line calli within 1 or 2 populations and in each population within G_0G_1, G_2M and S phases.

Ploidy level comparison between HO and HNO calli: Nuclei were prepared from the two callus types and mixed in equal amounts before PI-staining. By using bivariate dot plots of log forward scattered light versus log time of flight through the laser beam (Fig. 5*A*), or versus DNA content (Fig. 5*B*, linear scale), or by combination of DNA content and time of flight (Fig. 5*C*), it is possible to accurately discriminate HO and HNO nuclei in trivariate space. The logical gate W1 = (R1, R2 and R3) was restricted to HO nuclei and enclosed pure HO nuclei with 2C and 4C-DNA content, whereas the gate W2 = (R4, R5 and R6) contained all HNO nuclei which were characterized by considerably higher values in all parameters. The DNA histogram (Fig. 5*D*) was generated using W1 and W2 gates. Taking the first DNA peak to the left (HO nuclei) as a diploid landmark (2C or DNA index = 1; Fig. 4), the DNA index of the highly aneuploid HNO nuclei G_1 population was precisely calculated as

3.4 (6.8 C). Most small debris were discarded from the analysis by setting a threshold level on the low DNA content values (Fig. 5C).

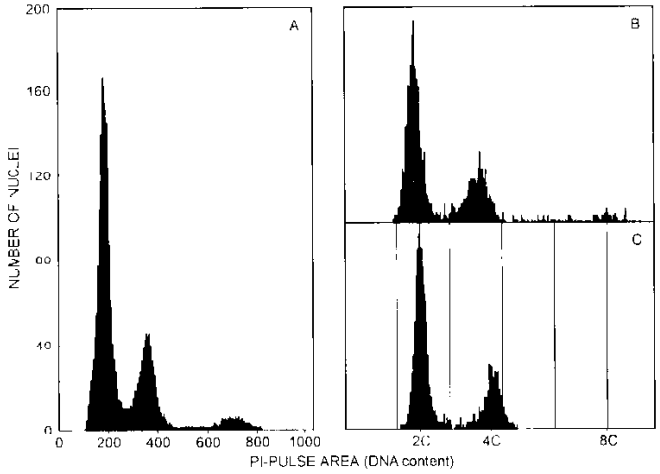

Fig. 4. Flow cytometric estimations of fluorescence distribution of nuclear DNA content: analysis was performed on a mixture of leaves and HO callus nuclei (*A*), on HO callus nuclei alone (*B*) and on leaves nuclei alone (*C*). All measurements were made with the same conditions and instrument settings.

The analysis by flow cytometry (Fig. 5*D*) indicates the presence of two very different populations. These two populations are distinguished by their DNA content. The population with high DNA content, large nuclear size and long time of flight is identified as belonging to the HNO callus nuclei. The ratio of the means of relative G_0G_1 nuclear content of each population was equal to 3.41.

Ploidy level comparison between certified diploid leaves and HNO callus: Flow cytometric DNA distributions of a mixture of HNO callus (Fig. 6*A*) and diploid leaf nuclei (Fig. 6*B*) used as an internal 2C landmark led to similar results: the size and the DNA content of the 2C peaks (Fig. 6) were very different. The DNA index of HNO nuclei was confirmed in the range of 3.3 - 3.6.

Discussion

Flow cytometry allows the analysis of nuclear DNA content in large populations of plant cells and so it can be used to study nuclear DNA content per genome, cell cycle parameters and ploidy changes (Galbraith *et al.* 1983). For nuclear DNA content analysis in plants, the measurement of isolated nuclei is generally accepted as a method of choice (Doležel *et al.* 1994). The chopping technique described by Galbraith *et al.* (1983) was used successfully for the isolation of intact cell nuclei of

leaves and calli of *Beta vulgaris*. However, optimum results (low C.V. of G_2/G_1 peaks and low background) were obtained only when the samples were fixed with

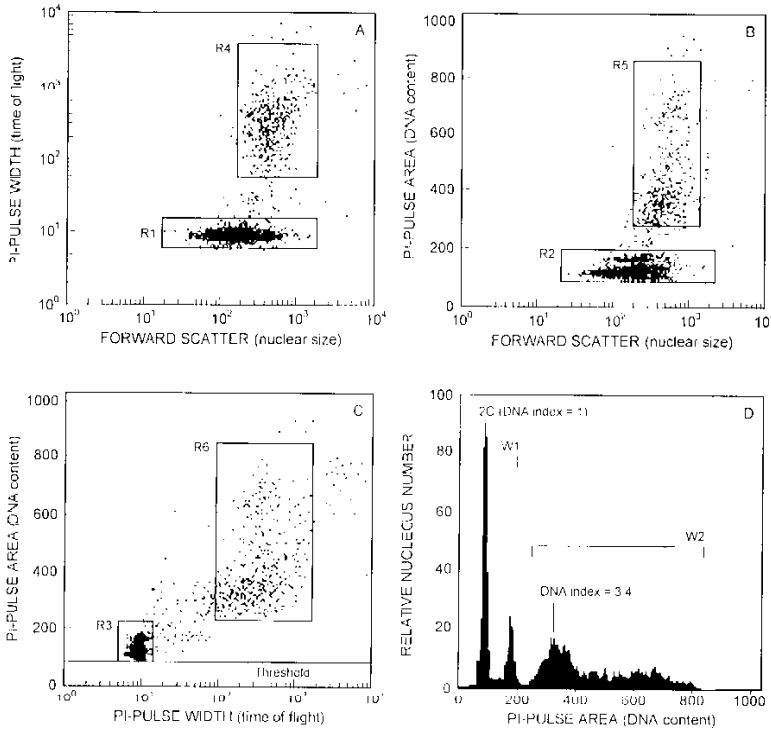

Fig. 5. Multiparameter flow cytometric analyses of HO and HNO callus nuclei mixed performed before PI-staining: *A* - the dot plot of the pulse width versus forward scatter; *B* - the dot plot of the DNA content versus forward scatter; *C* - the dot plot of the DNA content versus pulse width; *D* - the distribution of the nuclear DNA content of the two types of populations.

formaldehyde. In a DNA histogram of a specific sample (Fig. 2), nuclei were ordered according to their fluorescence intensity. For flow cytometry, it is generally assumed that a linear relationship exist between fluorescence intensity and DNA content of nucleus (Bennett and Smith 1991). The analyses made with leaf and HNO cells indicated the presence of only one population of cycling cells (Fig. 3). In HO cells two cycling populations of nuclei were detected. These two populations made a complete cell-cycle. The second population had a DNA content almost double that of the first (Table 1). It is difficult to assess whether this population (25 % of the nuclei) is aneuploid or tetraploid (obtained by endoploidisation). The difference measured was very small and not statistically significant. This second population probably includes the true callus cells; the first one might include the cells from the numerous shoot primordia which could not be discarded.

The comparison between the two types of HO callus cells and the certified diploid leaf cells indicated that the diploid content of the HO cells was similar to that of the certified diploid cells. However, the nuclear size (Figs. 1,5*A,B*) and the DNA content of the HNO callus (Fig. 5*C,D*) were higher. By different comparisons, it was confirmed that the DNA content of HNO nuclei was between 3.3 and 3.6 times higher than the diploid level (Table 1).

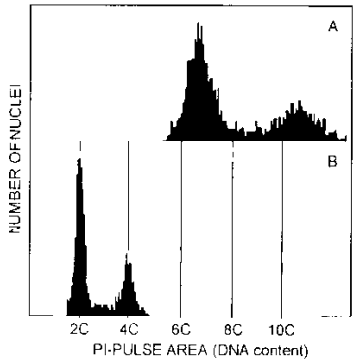

Fig. 6. Flow cytometric DNA distributions of HNO callus (*A*) and certified diploid leaf (*B*) nuclei analysed in the same conditions. Separate histograms were generated on the same DNA scale using logical gates as defined in Fig. 5.

The actual idea is that HNO cells might result from mutations which occurred in the HO callus under the effect of free radicals resulting from several stresses (Gaspar *et al.* 1995). The HNO cells indeed have been shown to be under permanent stress (Kevers *et al.* 1995). Cells with a 4C DNA content in the HO callus could be the origin of HNO cells after ploidization. Aneuploidy, chromosome rearrangement and instability may be associated with a gradual loss of regeneration ability (Winfield *et al.* 1993) as in HNO callus cells. In *Helianthus* calli derived from protoplasts, Keller *et al.* (1994) indicated that incomplete divisions (noncontinuous walls forming symplasts or syncytia, as observed in HNO callus) could be an obstacle to the establishment of all autonomous expression patterns and any subsequent regenerative process. The incomplete divisions presented by HNO cells are probably the origin of the polyploidization and aneuploidization of these cells and the subsequent loss of totipotency. All these results support the assumption that HNO callus cells are at the end of a neoplasic progression finishing with true cancerous cells (Gaspar *et al.* 1991).

Mitotic instability and chromosomal variability have been observed in several types of callus cultures (Novák 1980, Handro *et al.* 1993, Fluminhan and De Aguiar-Perecin 1998), including tumorous (Sacristan and Melchers 1969) and habituated (Butcher *et al.* 1975) tissues. One reason for the generation of polyploid cells in cultures, is the failure of spindle formation during mitosis or the occurrence of abnormal multipolar, instead of the usual bipolar, spindles. Where a spindle fails to form properly, two chromosome complements may combine resulting in a cell with a

doubled chromosome complement. Multipolar spindle formation or nuclear fusion may be the cause of the occasional appearance of cell lines with uneven ploidy (*e.g.* haploids or triploids). Another possible reason is that cells may become temporarily arrested in G2 but then be stimulated to re-enter the cell cycle at G1 without have completed normal mitosis.

Aneuploid cells result from other mistakes during nuclear division. For example, chromosomes may break into two or more fragments and chromosome lag at the anaphase stage of mitosis (mitotic non-disjunction) can lead to the formation of daughter cells, one with a lower, and the other with a higher than normal chromosome number (George 1993). Although in some species much of the genetic variation between cells can be explained by changes in chromosome number, in others most altered karyotypes are caused by the breakage and structural alteration of chromosomes or chromatids (half chromosomes during the early stages of mitosis). Chromosome breakage often occurs during the division of unorganised cells *in vitro* and can result in many different rearrangements of the karyotype. George (1993) and Fluminhan and De Aguiar-Perecin (1998) propose several mechanisms for such chromosome rearrangements.

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