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New data on the in situ position of the inactive X chromosome in the interphase nucleus of human fibroblasts

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Summary. The in situ spatial distribution of nucleolus-organizing-region (NOR) bearing chromosomes in relation to the inactive X chromosome was studied during interphase in human fibroblasts. The respective positions of these chromosomes were examined in 30 growing and 32 resting fibroblasts from reconstructed nuclei, using nucleoli and the Barr body as ultrastructural markers. Experimental values for the distance between the nucleoli and the Barr body were estimated by their coefficient of closeness and compared to the uniform distribution. The following results were obtained: (1) the distribution patterns for the two populations of nuclei were similar, (2) the distribution of the NOR-bearing chromosomes in relation to the inactive X chromosome varied and differed significantly from a uniform distribution, and (3) in many cases the Barr body was observed to be in a juxta-nucleolar position. The internal distribution revealed by this study is compared with the data in the literature, especially with the conflicting data obtained by other methods used to determine the interphase arrangement of chromosomes. The relationship between interphase and metaphase arrangements such as can be deduced with these methods, is discussed in relation to the mechanisms of the formation of metaphase plates or chromatid translocations.

Introduction

The idea that chromosomes are arranged in an orderly manner inside the interphase nucleus goes back to the work of Rabl (1885) and Boveri (1909). Although, since then evidence has accumulated showing that chromosomes are not randomly distributed, no definite conclusions were reached as to how they are arranged (for review see Comings 1968, 1980; Vogel and Schroeder 1974; Avivi and Feldman 1980). The results so far obtained are conflicting, chiefly due to the biological material itself. Clear identification of each individual chromosome in the interphase nucleus is impossible. Most studies of chromosome arrangement therefore use different indirect approaches whose findings are often contradictory. Consequently, the question arises of whether, for all chromosomes, methods like analysis of the spatial relationship between chromosomes in methaphase plates, or identification of the chromosomes involved in spontaneous or induced chromatid interchanges, actually reflect the position of the chromosomes inside the nucleus. This question is particularly relevant to the diametrically different positions reported for the sex chromosomes X and Y whether localized in situ or by indirect methods.

Studies of the in situ position of the X and Y chromosomes in the interphase nucleus, as determined by light microscopy, all show that both chromosomes are frequently located in a paranucleolar position (Petersen and Therkelsen 1962; Bobrow et al. 1971; Gagné et al. 1972; Wyandt and Iorio 1973; Goldgefter et al. 1973). Since, nucleoli are the site of in situ accumulation of the ribosomal gene transcription products, and are therefore the markers of the NOR-bearing chromosomes (for review see Stahl 1982), the positions found for the sex chromosomes in relation to the nucleoli by in situ analysis should be the same as those found by the other methods mentioned above, i.e. the sex chromosomes should in most cases be located either near the NOR-bearing chromosomes in metaphase plates, or involved with them in chromatid interchanges. However, this is not generally the case. Most studies based on metaphase plate analysis report a peripheral location for the sex chromosomes (Miller et al. 1963; Barton et al. 1964; Ockey 1969; Galperin 1969; Chandra et al. 1972; Hoehn and Martin 1973; Rodman et al. 1978; Wollenberg et al. 1981, 1982) and a more central location for the NOR-bearing chromosomes (Barton et al. 1964; Kowarzyk et al. 1965; Galperin 1968; Kirsch-Volders et al. 1977). In addition, reports concerning the chromosomes participating in spontaneous or induced chromatid interchanges show that the sex chromosomes are rarely involved (Vogel and Schroeder 1974; Bourgeois 1975; Hager et al. 1982), and it is even more rare to find sex and NOR-bearing chromosomes involved together when such interchanges do occur (Bourgeois 1974).

This is particularly true of the X chromosome, especially when the results obtained in situ are compared with those obtained by the two other methods. For instance, the position of the X chromosomes in relation to the nucleoli, i.e. to the NOR-bearing chromosomes, is not the same in human fibroblasts when determined in situ and in metaphase plates. The inactive X chromosome visualized by light microscopy after Feulgen staining was seen in situ near the nucleolus in at least one third of the nuclei examined (Petersen and Therkelsen 1962). The ^{3}H-thymidine-labeled inactive X chromosome, in contrast, was located at the periphery of spread metaphases

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while the NOR-bearing chromosomes occupied a central position (Ockey 1969).

To elucidate this problem, it therefore appears necessary to locate the inactive X chromosome and the nucleoli simultaneously in a straightforward way as suggested by Vogel and Schroeder (1974). Such in situ location in the interphase nucleus requires three-dimensional reconstitutions of complete nuclei from several sections observed in electron microscopy. Unlike the other chromosomes this is possible for the X chromosome, since it is inactive and can be unmistakably identified at the ultrastructural level. This chromosome is the only one found in a condensed form or Barr body in the interphase nucleus (Barr and Bertram 1949; Lyon 1962; Barr 1966; Moore 1966; Schellens et al. 1979). As already pointed out, the nucleoli served to identify the NOR-bearing chromosomes.

This study reports on the relative positions of the nucleoli in relation to the Barr body in two primary cultures of human fibroblasts with 45,XX/15 karyotype, one in the logarithmic growth phase and the other in the postlogarithmic phase. These cells were chosen since the inactive X chromosome is always the same one, as demonstrated by bromodeoxyuridine (BrdU) incorporation (personal communication of Dr. B. Dutrillaux). To elucidate the precise spatial arrangement of the nucleoli and Barr body, we used three-dimensional reconstructions of embedded in situ nuclei stained with osmiophilic oxidized diaminobenzidine (DAB), a method known to enhance the contrast of condensed chromatin (Roels and Goldfischer 1971). This technique was combined with statistical analysis of the distribution of the experimental values for the distance between the inactive X chromosome and the nucleoli, estimated by the method of Hemon et al. (1981).

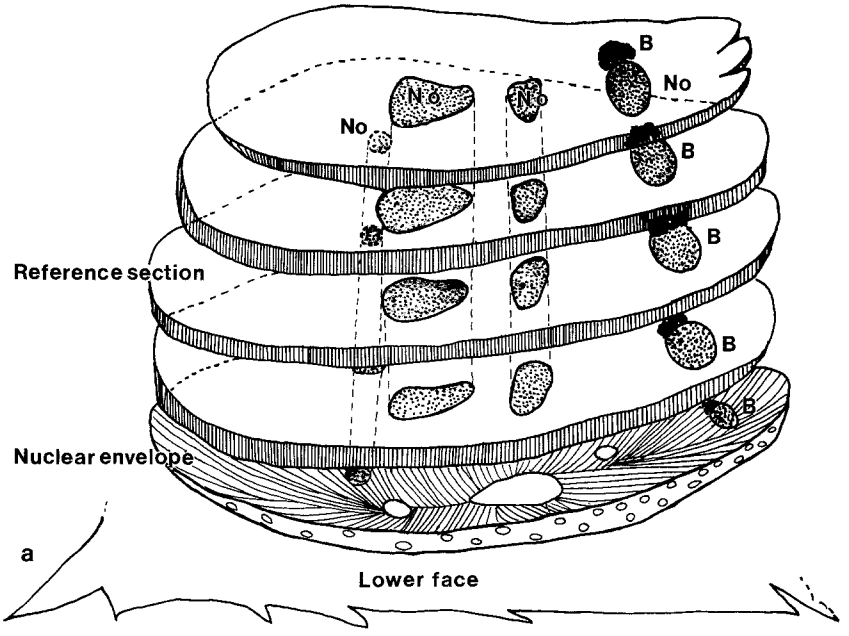
Materials and methods

Cultures

Primary cultures of human fibroblasts 45,XX/15 (kindly provided by Dr. B. Dutrillaux) were grown in 25 cm^2 plastic flasks in 10 cm^3 Eagle's minimum essential medium (MEM) supplemented with 18% heat-inactivated foetal calf serum and antibiotics. Cultures were usually trypsinized every five days. Half of the cultures were fixed in the logarithmic growth phase (GLP), and the other half were maintained in the postlogarithmic phase (PLP) for a week after complete confluency before being fixed. Fixation was done either in 1.6% glutaraldehyde in Sörensen buffer or in 4% formaldehyde.

Diaminobenzidine staining

Monolayers were washed in PBS and fixed for 3 h at 4°C in 4% formaldehyde in 0.1 *M* cacodylate buffer, pH 7.2, contain-



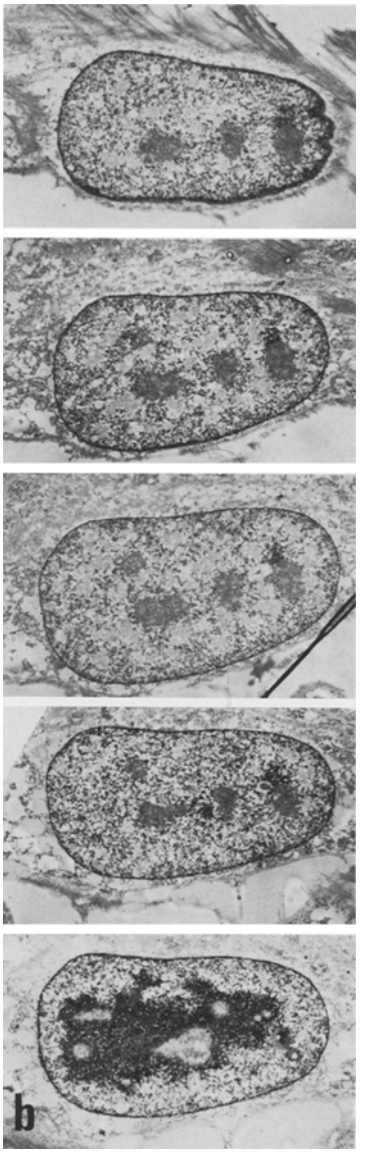


Fig. 1a and b. Three-dimensional reconstruction (**a**) of the serial sections of a nucleus (**b**) observed by electron microscopy. The cellular face of attachment to the glass *(lower face)* is easily distinghuished from the side in contact with the culture medium *(upper face)*. Here only five out of the nine serial sections of the whole nucleus are illustrated. The respective positions of the Barr body *(B)* and nucleoli *(No)* can clearly be followed from one section to the next one

ing 7.5% sucrose and 7 m*M* $CaCl_2$. Cells were washed overnight with this buffer, and then three times in 0.05 *M* CH_3COONa, pH 2.9. They were stained at 37°C for 3 h in DAB solution, as previously described by Anteunis and Pouchelet (1978). After three further washes, cells were treated for 90 min with 1% osmic acid in distilled water, dehydrated through a graded series of ethanols, and embedded in situ in epoxy resin.

Electron microscopy

Semi-thin serial sections 0.25–0.5 μm thick were obtained from the GLP and PLP fibroblasts embedded in situ. Serial sections were mounted on single-hole Formvar-coated grids. These sections were first examined at low magnification (× 600), in order to identify each nucleus and to eliminate all G_1 nuclei. Consecutive electron micrographs were then made of the same nucleus to determine the precise internal distribution of the Barr body and nucleoli.

Mathematical approach

1) Examination of three-dimensional reconstitutions of 34 GLP and 33 PLP nuclei, embedded in situ and cut parallel to the adherent side of the cells, revealed in each nucleus both the Barr body and the nucleoli extending from either the lower or upper side of the nucleus, as shown in Fig. 1. Therefore in this model, the three-dimensional distribution of the nucleoli in relation to the Barr body within the nuclear volume is comparable to the two-dimensional distribution of these structures in the nuclear section chosen as reference. For each nucleus studied, the section chosen as reference contained all the nucleoli and Barr body sections and was the median section of the nucleus.

2) To determine the distribution of the distance between the nucleoli and the Barr body taken as reference, we used the method of Hemon et al. (1981). This distance was estimated by the coefficient of closeness defined here as the s_{NU}/S_{NU} ratio. The area s_{NU} corresponds to the points of the nucleus located at a distance equal or smaller than the distance between the Barr body and the nearest nucleolus. The area S_{NU} corresponds to all the points of the nucleus at which nucleoli could be located if they were randomly distributed within the nucleus, as shown in Fig. 2.

3) To verify that the nucleoli were not distributed at random in relation to the Barr body taken as reference, we compared the distribution of the experimental values for the coefficient of closeness to the uniform distribution. The latter corresponded to the distribution of the theoretical values of this coefficient when the nucleoli are distributed at random in relation to the Barr body within the nucleus.

4) The observed distribution was compared to the theoretical distribution by the chi-square test.

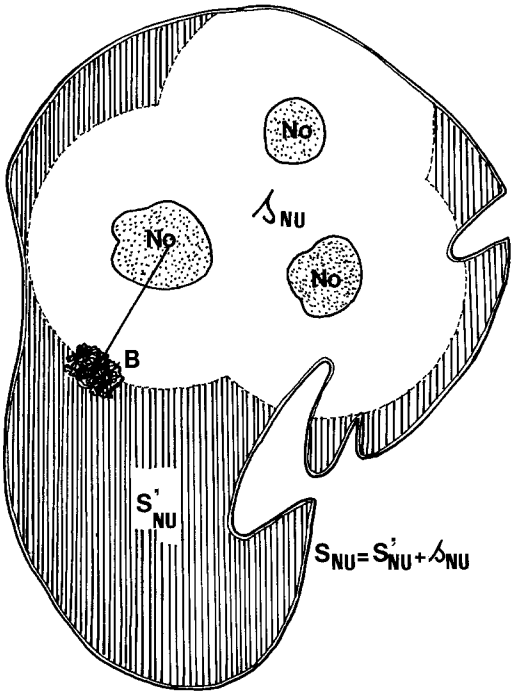


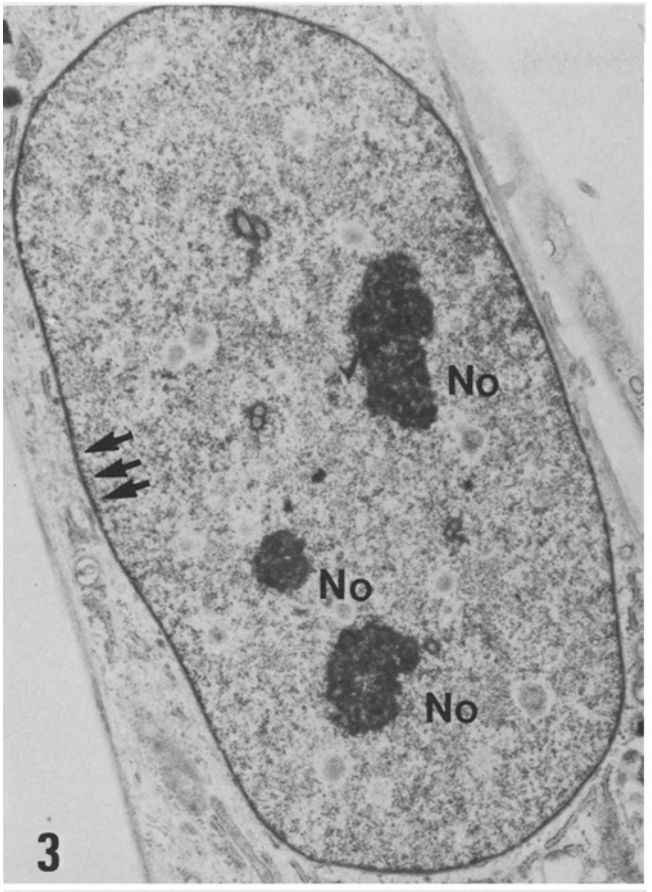
Fig. 2. Diagram showing the statistical method used to analyse the relative positions of nucleoli *(No)* in relation to the Barr body *(B)*. Within each reference section, the coefficient of closeness was measured by the ratio of the two area s_{NU}/S_{NU}, s_{NU} is the area of the discs delimited by the circles centered on each nucleolus and which diameters are equal to the distance of the nearest nucleolus to the Barr body. The area S_{NU} contains all the points of the nucleus at which nucleoli could be located if they were randomly distributed within the nucleus. This area corresponds to the total area of the nucleus less the areas of the Barr body and nucleoli, since these structures cannot be reduced to a single point

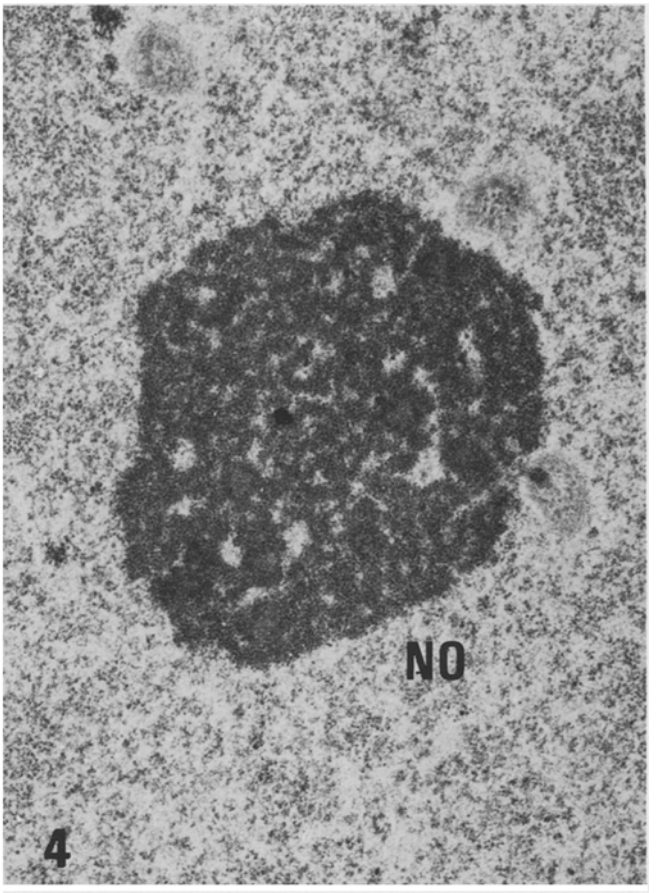
Results

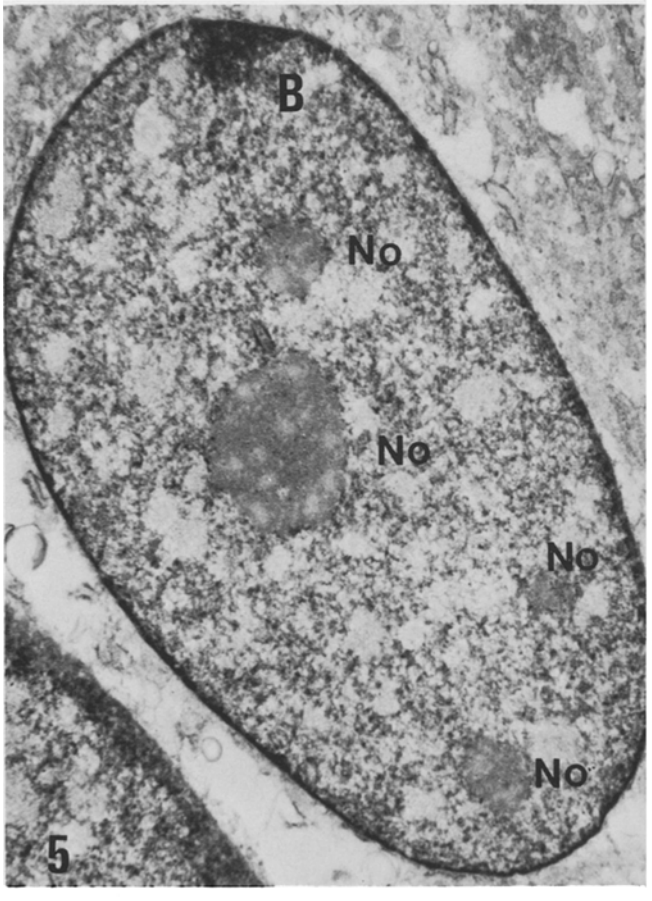
Electron microscopy

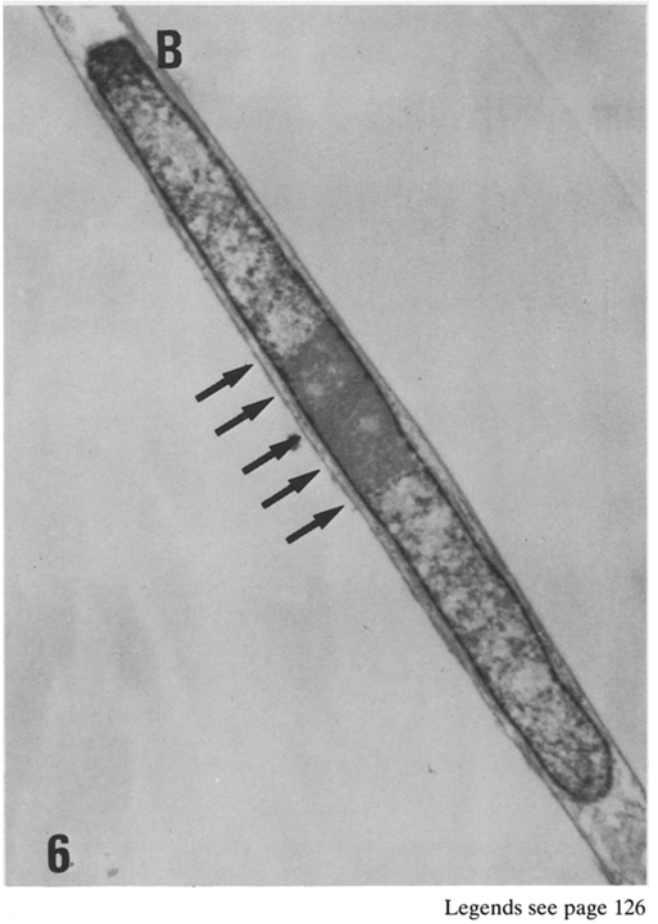
Observation of thin sections stained with uranyl acetate and lead citrate revealed that all the nuclei displayed only very small amounts of condensed chromatin, which was restricted to a thin layer underlying the nuclear envelope (Fig. 3). No perinucleolar condensed chromatin was present (Fig. 4), so that the only patch of light condensed chromatin was the Barr body (Fig. 7). After DAB staining the condensed chromatin of this body was better contrasted than with uranyl acetate and lead citrate staining (Figs. 5 and 6) and was easily recognizable even when located near the nucleolus (Fig. 1). All GLP and PLP fibroblasts embeeded in situ and cut parallel to the adherent side of the cell were reconstructed from an average number of eight semi-thin serial sections. The nuclei were shaped like well developed flat discs 2 μm thick, 16 μm wide, and 26 μm long (Fig. 6).

Three-dimensional electron microscope study of the nuclei clearly showed that all nucleoli were in contact with the nuclear envelope on one or both sides of the nucleus. They were shaped like small cylinders, and extended from the nuclear envelope towards the center of the nucleus (Figs. 1a and 6). The Barr body was observed to be adjacent to the periphery of the nuclear envelope in about 75% of the nuclei in both GLP and PLP fibroblasts (Table 1). In the other 25%, this body was more centrally located. Whatever its position, the Barr body was often adjacent to a nucleolus (Table 2). It always touched the nuclear envelope, at least in one point. In GLP nuclei, a Barr body was visualized in 32 out of the 34 nuclei analysed and in all the 33 PLP nuclei. One PLP and two GLP nuclei each contained two Barr bodies.









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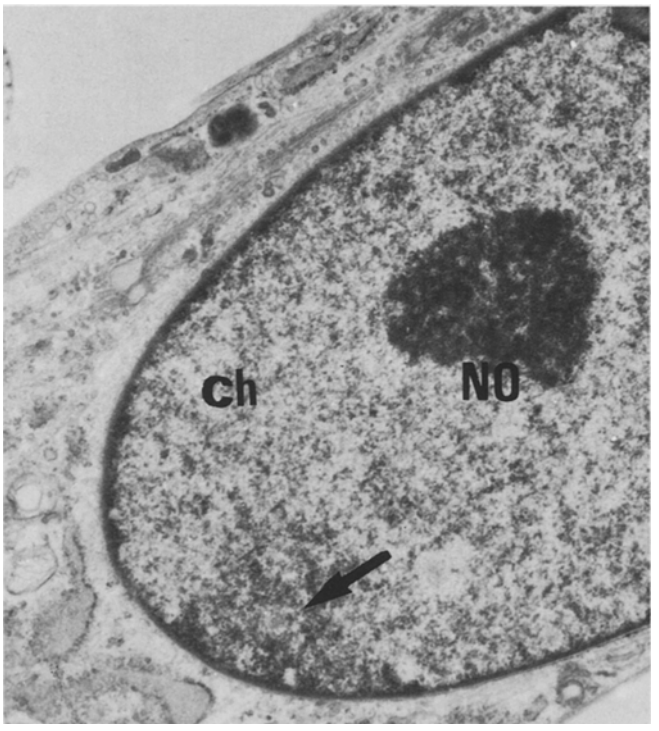

Fig. 7. Detailed ultrastructure of the Barr body in a section obtained by the classical procedure. In the human fibroblast, the whole chromatin is lightly condensed *(Ch)* including that of the Barr body. This low degree of condensation of the Barr body chromatin *(arrow)* rendered its identification very difficult without DAB staining. *No:* nucleolus. × 13000

Table 1. Juxta-nucleolar position of the Barr body in relation to its location within the nucleus

Coefficient of closeness	Position of the Barr body			
	Adjacent to the nuclear edge		Non-adjacent to the nuclear edge	
	Number of nuclei		Number of nuclei	
	PLP	GLP	PLP	GLP
1 –0.8	7	10	0	1
0.8–0.6	4	6	1	0
0.6–0.4	3	2	0	1
0.4–0.2	2	0	1	0
0.2–0.0	7	5	7	5

Quantitative analysis

The quantitative study was only performed in the nuclei containing one Barr body. Although the position of the nucleoli in relation to the Barr body varied from one nucleus to another, in both GLP and PLP fibroblasts the overall distribution pattern for the distance between the two was similar in both cell types (Table 2). The results of this statistical analysis also indicated that the distribution of the experimental values differed significantly from the distribution of the theoretical values that could be expected from a random location of the nucleoli in relation to the Barr body, with $P < 0.05$ by a χ^2 test. This analysis shows that the Barr body was very close or adjacent to a nucleolus in at least one third of the GLP cells and up to ⅔ of the PLP fibroblasts. The respective positions of the nucleoli and Barr body could not be related either to the number of nucleoli as shown Fig. 8, or to the position of the Barr body adjacent or not adjacent to the nuclear edge (Table 1).

Discussion

In the present work, we used an in situ approach to investigate the arrangement of the NOR-bearing chromosomes in relation to the inactive X chromosome inside the nucleus. This method gave us a constant reference plane as it was based on three-dimensional reconstitutions of cells embedded in situ. Our findings show that both the cell populations examined, displayed similar Barr body-nucleolus distribution patterns significantly different from a random distribution. In addition, they establish that the inactive X chromosome is often located near the nucleoli, i.e. near the NOR-bearing chromosomes, and that this location is not dependent on the number of nucleoli. The absence of any large blocks of condensed chromatin, especially perinucleolar chromatin in the nucleus of these human fibroblasts, as well as the DAB staining used, rule out the possibility of confusion between the Barr body and the nucleoli or any other blocks of condensed chromatin.

Previous studies have shown first that nucleolar fusion occurs in early G_1 (Bourgeois et al. 1982) and second that except for this short period, the position of the nucleoli is essential stable throughout interphase (Bourgeois et al. 1981). These data therefore imply that the position of the nucleoli we observed in GLP fibroblasts reflects the arrangement of the concerned chromosomes prevailing during the S and G_2 phases. This is confirmed by the fact that the PLP nuclei arrested in G_0 exhibited a similar distribution to that of the GLP nuclei.

Our quantitative ultrastructural data are in agreement with those obtained in situ by light microscopy which also showed first the Barr body at the nuclear edge in most of the nuclei and second Barr body in a juxta-nucleolar position in at least one third of the nuclei in bone marrow and embryo fibroblasts (Petersen and Therkelsen 1962). It should nevertheless be stressed that in the latter experiments, the Barr body could correspond to one or other of the two X chromosomes, where-

Fig. 3. Section of a human fibroblast embedded in situ in the Falcon flasks. Here, the sectioning was made parallel to the attachment side. The section was stained with uranyl acetate and lead citrate. The nucleus appears deprived on any condensed chromatin except for a thin layer *(arrows)* underlying the nuclear envelope. *No:* nucleolus. × 6600

Fig. 4. Detailed ultrastructure of a nucleolus. No condensed perinucleolar chromatin can be seen. *No:* nucleolus. × 17000

Fig. 5. Fibroblast section stained before embedding with DAB at pH 2.9. The condensed chromatin of the Barr body *(B)* is preferentially stained, and appears much more contrasted than the nucleoli *(No)*. × 6600

Fig. 6. Transverse section of a fibroblast embedded in situ then cut perpendicular to the attachment side *(arrows)*. This section illustrates the disc-like nucleus. The Barr body *(B)* located at the periphery of the nucleus appears strongly contrasted after DAB staining. × 6600

Table 2. Statistical analysis of the coefficient of closeness

Experiments		Coefficient of closeness					
		0.0–0.2	0.2–0.4	0.4–0.6	0.6–0.8	0.8–1	
GLP	Experimental number of nuclei	10	0	3	6	11	= 30
	Theoretical number of nuclei	6	6	6	6	6	= 30
	χ^2	$\chi^2_{ddl4} = 14.3$			$P < 0.01$		

Experiments		Coefficient of closeness					
		0.0–0.2	0.2–0.4	0.4–0.6	0.6–0.8	0.8–1	
PLP	Experimental number of nuclei	14	3	3	5	7	= 32
	Theoretical number of nuclei	6.4	6.4	6.4	6.4	6.4	= 32
	χ^2	$\chi^2_{ddl4} = 13.0$			$P < 0.05$		

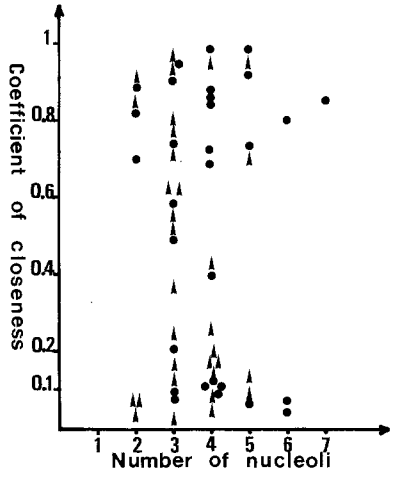

Fig. 8. Diagram showing the distribution of the coefficient of closeness in relation to the number of nucleoli

as in our experiments, this body always corresponded to the same inactive X chromosome. On the other hand the respective positions of the inactive X chromosome and the NOR-bearing chromosomes observed here in situ were quite different from those which can be inferred from analysis of induced or spontaneous chromatid interchanges or from metaphase spreads. However, the results on the individual position of the same chromosomes were similar whichever method was used. Therefore, the analysis of the discrepancies on the respective positions of those chromosomes between the different methods could be relevant to the understanding of the type of information obtained by these methods.

With regard to chromatid interchange analysis, all studies based on this method give concordant results for the X and NOR chromosomes. This remains true whatever the type of chromosomal damage studied and whatever the mathematical models used for this purpose (Caspersson et al. 1972; Morad et al. 1973; Vogel and Schroeder 1974; Bourgeois 1974; Hager et al. 1982). All studies show that the inactive X chromosome, the pair of X, and the single X chromosome of the male are not often involved in such interchange. Moreover, when the X chromosome is involved, the interchange rarely occurs with the NOR-bearing chromosomes as well. This is contrary to what could be expected from our in situ data, which indicate that in many cases the X and the NOR-bearing chromosomes are in close proximity.

Chromatid-interchange analysis is based on the assumption that two chromosomes have to be in proximity during interphase in order to be able to form an interchange. Accordingly, the lack of interchange shown by the X chromosome was interpreted as the result of its peripheral location. Also the data obtained by this method for the NOR-bearing chromosomes suggest that they are centrally located within the nucleus. However, the mechanisms leading to the formation of chromatid-interchanges require not only spatial proximity but also synchrony of DNA replication of the involved chromosomes (Natarajan and Ahnström 1969; Kihlman 1971; Brogger and Johansen 1972). From this point of view, the human inactive X chromosome is known to behave differently from all other chromosomes of the complement and to be the last to start DNA synthesis (for review see Lyon 1972). In particular, there is no synchrony of DNA replication between the X and NOR-bearing chromosomes (Giannelli 1974). Consequently, the X chromosome is less frequently involved in interchanges, so that the rarity of the interchanges between the X and NOR-bearing chromosomes is no longer incompatible with the proximity of these chromosomes observed here in situ.

As regards the position of the chromosomes as can be deduced from the analysis of metaphase spreads, the data are contradictory for the different cell types, the different techniques used, and from one experiment to another (for review see Comings 1980; Hens et al. 1982). Nevertheless, taken together they point to a preferential location of the X chromosome towards the periphery of the metaphase plates, and a position near the centre for the NOR-bearing chromosomes. Again, the individual positions of the inactive X- and NOR-bearing chromosomes directly observed in metaphase plates are similar to the ones obtained here in situ, while their respective positions are quite different.

Several factors which may influence this chromosomal arrangement in metaphase plates have been explored, including the effects of the presence or the absence of colchicine (Rodman et al. 1980), hypotonic treatment (Sele et al. 1977; Rodman et al. 1978), chromosome length (Hens 1976; Hens et al. 1982), and the timing of DNA synthesis (Ockey 1969; Hens et al. 1982). All these factors modify the disposition of chromosomes. However, intrinsic factors such as chromosomal length and the timing of DNA replication play an important part in metaphase spreading. From this point of view the X chromosome which is long and late replicating will

be expected to be more peripheral than the NOR-bearing chromosomes which are small and early replicating. However, this does not prove that the disposition of chromosomes in metaphase plates is the same as during interphase, chromosomal length and the timing of DNA replication might normally interfere, especially during in vivo formation of the equatorial plate.

The present study therefore indicates that although the results obtained in situ with electron microscopy are in agreement with those obtained in situ with light microscopy, they are quite different from those obtained by the two other methods as far as the respective position of the chromosomes is concerned. As already pointed out, this is because the spatial disposition of chromosomes during interphase is not the only factor affecting the findings given by metaphase spread and chromatid interchange analysis. In particular, as far as the inactive X chromosome is concerned, the asynchrony of its DNA replication with that of the other chromosomes is certainly the key factor for the results observed. However, it should be stressed that the existing agreement between the results obtained from analysis of chromatid interchanges and metaphase spreads, in particular for the inactive X chromosomes and NOR-bearing chromosomes, had led to the assumption that they were well suited to determine the position of the chromosomes within the interphase nucleus. Furthermore, in situ location of the Barr body along the nuclear envelope was considered as an additional argument in favour of the validity of the results obtained. Nevertheless, there is now growing evidence that in animal cells, all chromosomes are attached to the nuclear envelope (Franke 1974), and that their attachment is mediated by a peripheral scaffold associated with the inner membrane of the nuclear envelope (for review see Hancock and Boulikas 1982). In particular, the NOR-bearing chromosomes are attached to this scaffold (Hubert et al. to be published). This attachment is ensured by a specific segment of the chromatin bearing the ribosomal genes (Bourgeois et al. 1983). Thus, the NOR-bearing chromosomes and the X chromosomes are both located in the same position with reference to the nuclear envelope. Accordingly, the problem of the arrangement of chromosomes during interphase is no longer to determine which chromosome is the nearest to the nuclear envelope since these data demonstrate that all chromosomes are anchored to it. This problem now becomes to determine the respective positions between all the different chromosomes.

Therefore, in view of this attachment of all chromosomes to the nuclear periphery and the results obtained here in situ, it appears that (1) analysis of the chromosomes involved in spontaneous or induced interchanges only allows the study of homologous or heterologous chromosomes with synchronized DNA synthesis, and (2) analysis of spread chromosomes in metaphase figures only provides a partial picture of the in vivo disposition in the interphase nucleus, as admitted by some of the authors who use this method (Hens et al. 1982). In situ analysis of chromosomes by three-dimensional reconstitutions of interphase nuclei therefore seems the only possible approach (Bennett 1982). Such analysis is still limited to the chromosomes which can be identified by a morphological marker (Bourgeois et al. 1984). Nevertheless, it will be possible to determine the in situ position of each chromosome by molecular markers once in situ hybridization allows the accurate location of their position at the ultrastructural level as it has been obtained by light microscopy (Rappold et al. 1984).

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