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# DNA methylation of the X chromosomes of the human female: an in situ semi-quantitative analysis

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**Abstract.** We present an in situ semi-quantitative analysis of the global DNA methylation of the X chromosomes of the human female using antibodies raised against 5-methylcytosine. The antibodies were revealed by immunofluorescence. Images were recorded by a CCD camera and the difference in intensity of fluorescence between active (early replicating) and inactive (late-replicating) X chromosomes was measured. Global hypomethylation of the late-replicating X chromosomal DNA was observed in three cases of fibroblast primary cultures that were characterized by numerical and structural aberrations of the X chromosomes [46,X,ter rea(X;X), 48,XXXX and 46,X,t(X;15)]. In these cases, the difference between early and late-replicating X chromosomes was significantly greater than the intra-metaphasic variations, measured for a pair of autosomes, that result from experimental procedures. In cells with normal karyotypes, the differences between the two X chromosomes were in the range of experimental variation. These results demonstrated that late replication and facultative heterochromatinization of the inactive X are two processes that are not related to global hypermethylation of the DNA.

## Introduction

Mammals have heteromorphic sex chromosomes, XX for females and XY for males. Gene dosage equivalence in males and females is achieved by random inactivation with respect to parental origin, early in development, of one of the X chromosomes in females (Lyon 1961).

The inactive X chromosome is characterized by several features when compared with its active homologue. In interphase, it is highly condensed and forms the Barr body located at the nuclear periphery (Bourgeois et al. 1985; Dyer et al. 1989). The X-inactivation process results in its allocyclic replication at the end of the S-phase. The inactive X chromosome can be demonstrated at metaphase by hypotonic heat treatment (Kanda 1973). In addition, it is not labelled with antibodies raised against acetylated histone H4 (Jeppesen and Turner 1993).

The initiation of X-chromosome inactivation is under the control of an inactivation centre (*XIC*) localized in Xq13 (Brown et al. 1991b). The gene *XIST*, exclusively expressed in the inactive X chromosome, was mapped in this region (Brown et al. 1991a, b). It encodes an untranslated large RNA, which is localized close to the inactive X chromosome (Brown et al. 1992). Considerable evidence now exists to support a role for *XIST* in the initiation of inactivation. Spreading and maintenance of the inactivation status from *XIC* to the whole chromosome involve additional processes that are as yet poorly understood.

Numerous studies have established that the methylation in C5 of cytosines (5mC) within the DNA is an important component of mammalian X-chromosome inactivation. Thus in mouse, as for most autosomal genes, CpG islands are unmethylated in the active X chromosome whereas those in the inactive chromosome are extensively methylated (for review see Migeon 1994). However in human, the situation is more complex; for example, pseudoautosomal genes lack a methylated CpG island either because the island is unmethylated, or because there is no island (Kaslow and Migeon 1987; Mondello et al. 1988). Some coding regions are methylated similarly in the two X chromosomes (Wolf et al. 1984; Migeon et al. 1991) or are even hypomethylated only in the inactive X chromosome (Lindsay et al. 1985). Several loci that escape X inactivation are characterized by a lack of methylated CpG islands (Migeon 1990; Kaslow and Migeon 1987; Mondello et al. 1988). Specific gene expression from the inactivated X chromosome is also associated with hypomethylation of the corresponding region (Boyd and Fraser 1990; Heard and Avner 1994). The actual role of methylation in gene inactivation is not well established, but methylation is gen-

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erally considered to be involved in the stabilization of the inactive status rather than as part of the initial events (Lyon 1993; Migeon 1994).

The data available on the process of methylation/inactivation of the X chromosome are limited to a few loci, the methylation status of the whole chromosome rarely being investigated. Conflicting results have been obtained in three in situ studies on metaphase chromosomes by digestion with methylation-sensitive restriction enzymes: either no difference between the two X chromosomes was found (Adolph and Hameister 1990) or overall hypomethylation was reported for the inactive X chromosome (Viegas-Pequignot et al. 1988) or the active X chromosome (Prantera and Ferraro 1990). The different methodologies used in these studies may explain these contradictions. Using antibodies against 5mC, Miller et al. (1982) found no labelling difference between the two X chromosomes of metaphase lymphocytes. More recently, Miniou et al. (1994) suspected undermethylation of the inactive X chromosome in a patient with ICF syndrome. Thus, the overall DNA methylation status of inactive versus active X chromosomes of females remains controversial.

Recently, we have reinvestigated the possibility of using antibodies against 5mC (anti-5mC) for the in situ study of DNA methylation. We used an improved method (Montpellier et al. 1994; Barbin et al. 1994), in association with image cytometry, that offers the possibility of establishing both the methylation profile of chromosomes and the DNA methylation level within nuclei (Veilleux et al. 1995). In the present study, the global methylation of active and inactive X chromosomes was investigated in fibroblast primary cultures and in lymphocytes from human female donors.

## Material and methods

*Cell cultures and chromosomal preparations.* Short-term lymphocyte cultures were developed from two female donors (LF1) and (LF2). Fibroblast primary cultures from the 4th to the 10th passage were obtained from two females with normal karyotype (NF1 and NF2), and three patients with numerical or structural chromosomal anomalies: case 1: 46, X,ter rea(X;X)(p22.3;p22.3); case 2: 48, XXXX; case 3: 46,X, t(X;15)(q10;q10)(p10;p10).

Lymphocytic and fibroblast cultures were prepared according to Dutrillaux and Couturier (1981). Chromosomal preparations were obtained by usual method, with some modifications: hypotonic shock was achieved with a solution of human plasma and KCl (v/v) and fixation was in 3:1 methanol:acetic acid (v/v). Primary fibroblast cultures from cases 1, 2 and 3 were synchronized by a fluorodeoxyuridine block, 24 h prior to harvest. Ten hours before harvest, the block was removed by the addition of thymidine at 10 mM, and bromodeoxyuridine was added, at a final concentration of 5 µg/ml, only to the cultures used for fluorescence plus Giemsa staining (FPG). Metaphases were accumulated by adding Colcemid 3 h before harvest. For karyotyping or identification of the late-replicating X chromosome, preparations were submitted to R-banding or FPG treatment, respectively, as described in Dutrillaux and Couturier (1981).

*Antibodies and immunostaining.* The production and characterization of monoclonal antibodies against 5mC have been described previously (Reynaud et al. 1991). Rabbit polyclonal antibodies against single-stranded DNA were purified on protein A-Sepharose (Millipore). The specificity of the anti-DNA antibodies was assessed on chromosomal preparations after preincubation in the presence of single-stranded DNA as a competitor. Complete inhibition of chromosomal labelling was achieved in the presence of 50 µg/ml of single-stranded DNA.

Immunostaining was performed after pepsin digestion and HCl treatment (Barbin et al. 1994) or UV irradiation (Miller et al. 1982). For fibroblasts, pepsin digestion and HCl treatment were shortened to 10 and 12 min, respectively. Treatment of the slides for the identification of the late- or early replicating X chromosomes by FPG and for measurement of 5mC content by immunostaining were found to be incompatible. It was thus impossible to obtain both types of information on the same metaphase.

Two types of control were performed. The first was done in order to detect any differences in morphology after pepsin-HCl treatment, using Giemsa staining or orange acridine. The second was done using antibodies against DNA to evaluate the accessibility to antibodies along the chromosomes and in particular along the active and inactive X chromosomes.

*Fluorescent image acquisition and analysis procedure.* Observations were performed with an epifluorescence microscope (Axiophot, Zeiss). We used a 100X magnification, 0.7–1.3 numerical aperture, oil immersion objective lens (44 04 86, Zeiss). Images were recorded with a dual-mode cooled CCD (charge coupled device) camera (C4880, Hamamatsu). The intregration time of the fluorescent signal was about 1 s. Conversion of the digitalized images from 14 to 8 bits was performed, after background substraction, with a linear function allowing for each 8192 grey-level image to be transformed to a 255 grey-level image. These images were analysed with SAMBA 2640 software (Alcaltel-TITN). Chromosomes were identified by their R-like banding after anti-5mC labelling (Barbin et al. 1994). For each metaphase, chromosomal outlines were established by thresholding and manual correction when necessary. For each chromosome, the integrated intensity of fluorescence (expressed in arbitrary units) and area (in pixels) were measured. The intensity of fluorescence per pixel (IF) was calculated, for each chromosome, using STAT 2005 software (Alcatel - TITN).

*Data analysis.* Metaphases were analysed when both pairs of chromosomes X and 6 or 8 were clearly identifiable and not overlapping. The principle used in this quantitative study was to calculate the difference in intensity of fluorescence between the X chromosomes within each metaphase. For cells with two X chromosomes, the relative fluorescence ratio was defined as:

$$FR(X) = \frac{IF(X1) - IF(X2)}{IF(Xm)}$$

IF(Xi) being the intensity of fluorescence for chromosome Xi and IF(Xm) the mean value of IF(Xi) for the two X chromosomes.

When it was possible to distinguish structurally the two X chromosomes (cases 1 and 3), X1 was the early replicating and X2 the late-replicating chromosome. In case 3, only the contribution of the long arms was considered for both chromosomes. In normal metaphases, where it was not possible to differentiate early and late-replicating chromosomes, X1 was the X chromosome with the highest IF.

For case 2, where it was also not possible to distinguish between the early replicating and the three late-replicating chromosomes, the four chromosomes were classified from 1 to 4 in decreasing order of IF value. The relative fluorescence ratio was defined as:

$$FRX(i-f) = \frac{IF(Xi) - IF(Xf)}{IF(Xm)}$$

with IF(Xi) always being greater than IF(Xf). IF(Xm) was the mean value of IF(X) for the four X chromosomes of the metaphase. It was then possible to calculate FRX(1–2), FRX(1–3) and FRX(1–4) between the X chromosomes with the highest IF and

the three others. In the same way, we calculated FR X(2–3), FRX(2–4) and FRX(3–4) between the three X chromosomes presenting the lowest IF.

For each donor, in order to take into account the experimental variations within metaphases, FR(6) and FR(8) were also calculated between the chromosome 6 and 8 homologues, respectively. These chromosomes were chosen because their length and IF are similar to those of the X chromosomes. The statistical relevance of the results was tested first using the Mann-Whitney U-test so as to compare the FRX distibution with that of FR(6) and FR(8), and secondly using the Wilcoxon signed-rank test so as to determine whether there was any significant difference between the IF values of early and late-replicating X chromosomes.

## Results

### *Fluorescent patterns of X chromosomes*

Metaphase chromosomes from lymphocytes and fibroblasts, after pepsin followed by HCl treatment and incubation in the presence of anti-5mC antibodies, displayed a repetitive banding pattern (Barbin et al. 1994). This pattern was characterized by brightly fluorescing juxtacentromeric heterochromatin, medium fluorescence of T-bands, and slightly duller fluorescence of R-bands (Fig. 1a). No, or almost no, fluorescence was observed for G-bands. This pattern enabled the recognition of all chromosomes. Among the middle-sized chromosomes, the Xs always exhibited fairly dull fluorescence.

When the pattern of X-chromosome fluorescence was examined, 13/20 of LF1 and LF2 metaphases and 24/30 of NF1 and NF2 metaphases exhibited an X with pale fluorescence, in particular for the q arm. The short arms of both the chromosomes often had apparently similar fluorescence of the Xp11 and Xp22 bands. Differences between homologous chromosomes were also occasionally observed for other middle-sized chromosomes such as chromosomes 6 or 8.

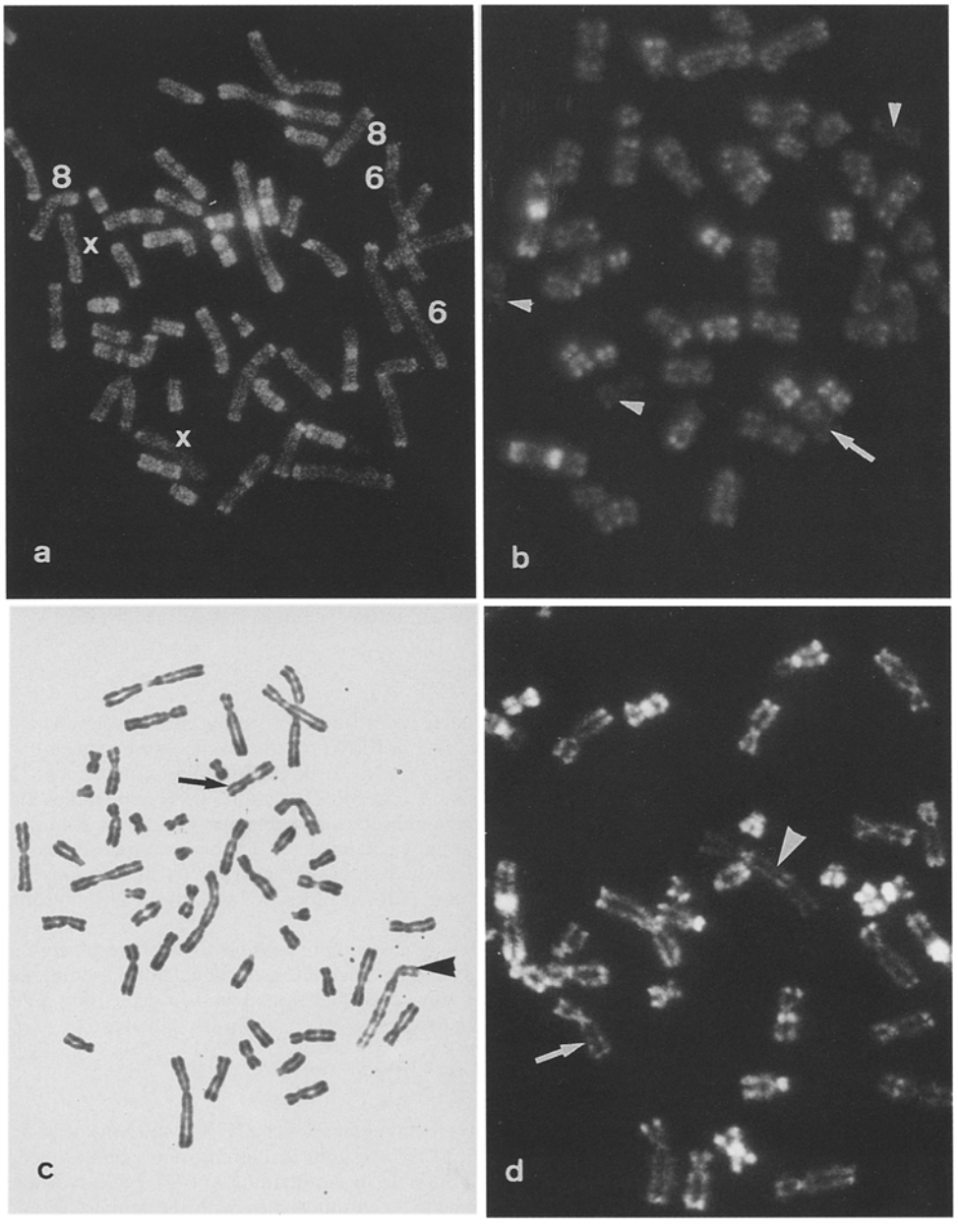


**Fig. 1a–d.** Digitized images from lymphocyte and fibroblast metaphases after immunostaining with antibodies against 5-methylcytosine (5mC). **a** Metaphase from a lymphocyte culture (LF1); the "R-band-like" fluorescent pattern enables the identification of chromosomes. In this metaphase a difference in intensity of fluorescence is detected between the two X chromosomes. No visual difference is observed between the homologues of chromosomes 8 or 6. In this case, differences in the intensity of fluorescence between the two X chromosomes and the two chromosomes 8 can be measured, while this is not possible between chromosomes 6 because one of them crosses other chromosomes. **b** Metaphase from case 2 with a 48,XXXX karyotype; three of the X chromosomes (*arrowheads*) exhibit very low fluorescence and the fourth (*arrow*) X chromosome, the most intense fluorescence. **c, d** Metaphase from case 1: 46,X ter rea (X;X). **c** After incorporation of 5-bromo-2'-deoxywidine (BrdU) and fluorscence plus Giemsa (FPG) treatment, the rearranged pseudodicentric X chromosome (*arrowhead*) is very pale compared with the normal one (*arrow*). This indicates that the rearranged chromosome is the late-replicating chromosome and the normal, the early one. **d** Metaphase after immunostaining with anti-5mC; the inactive pseudodicentric X (*arrowhead*) shows dull fluorescence in comparison with the active X chromosome (*arrow*)

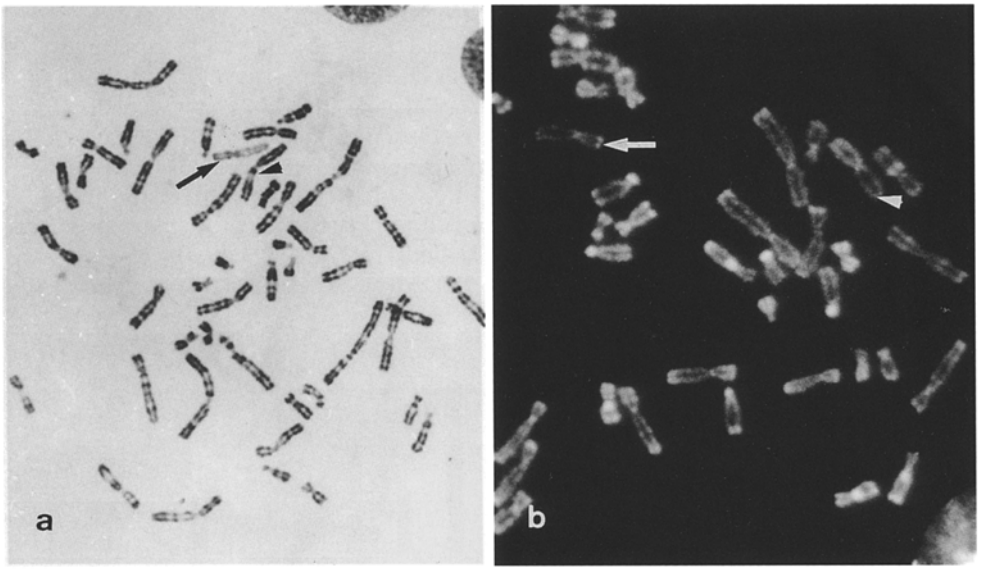


**Fig. 2a, b.** Metaphases from case 3 (46, X t(X;15)). **a** After FPG treatment showing the early replication of the rearranged chromosome (*arrowhead*) and the late replication of the normal X (*arrow*). **b** After immunostaining with anti-5mC, the q arm of the normal X chromosome appears pale in comparison with the intensity of fluorescence displayed by the X q arm of the early replicating one

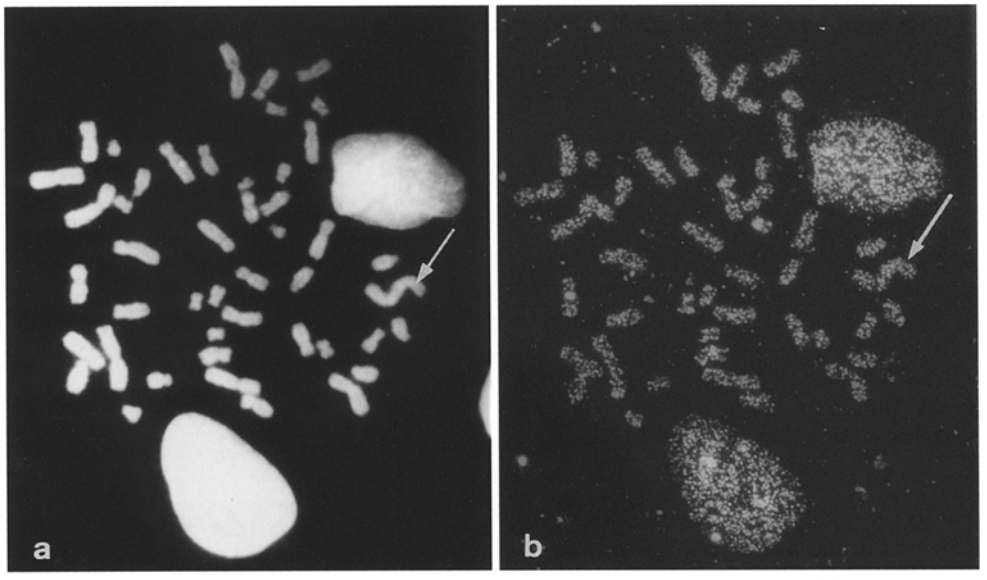


**Fig. 3a, b.** Metaphases from case 1 (46, X,ter rea(X;X)(p22.3;p22.3)). **a** Staining with 4',6-diamidino-2-phenylindole (DAPI). **b** The same metaphase after pepsin digestion, HCl treatment and treatment with a polyclonal anti-DNA revealed by a fluoresceinated second antibody. The inactive rearranged X chromosome (*arrow*) exhibits the same pattern of fluorescence as the other chromosomes within the metaphase, suggesting similar accessibility of the antibodies to all chromosomes. Its morphology appears similar to that observed with DAPI before treatment

The difference in fluorescence between the early and late-replicating X chromosomes appeared to be greater for fibroblasts with numerical or structural abnormalities of the X chromosomes.

For case 1, 46,X,ter rea(X;X), the unambiguously recognizable abnormal X chromosome (Fig. 1c) was shown to be late replicating. After immunofluorescent labelling, in the 18 metaphases examined, this chromosome always exhibited pale fluorescence compared with that of the normal X chromosome (Fig. 1d). For case 2, 48, XXXX, each of the 23 metaphases studied contained three X chromosomes with dull fluorescence and one that appeared brighter than the others (Fig. 1b). For case 3, 46, X, t(X;15), with an early replicating rearranged X chromosome (Fig. 2a), only the Xq arms of both late- and early-replicating chromosomes were examined. In the majority of the metaphases analysed (80%–85%), the long arm of the normal X appeared by weakly fluorescent when compared with its counterpart the der (Xq;15q) chromosome (Fig. 2b).

Experiments were also performed after UV irradiation of metaphase preparations from lymphocytes and fibroblasts with normal or abnormal karyotypes. The pattern of fluorescence of the early or late-replicating X was similar to those described above. However, the difference in intensity of fluorescence was always less than after pepsin and HCl treatment (results not shown).

To determine whether the weak labelling of the late-replicating X chromosomes could be due to a difference in accessibility of antigenic sites, chromosomal preparations were treated in parallel with anti-DNA antibodies. As illustrated for case 1 (Fig. 3), the level of fluorescence of the rea(X;X) chromosome was similar to that presented by the other chromosomes of the metaphase. Other controls using Giemsa staining or orange acridine did not reveal morphological alterations or changes in the DNA pat-

tern of the inactive X chromosomes (results not shown). The low intensity of fluorescence detected for the inactive X chromosomes in cases 1, 2 and 3 is therefore, interpreted to reflect hypomethylation rather than an artefact.

*Semi-quantitative analysis of the difference in methylation between early and late-replicating X chromosomes*

Semi-quantitative analysis was performed on chromosomal preparations following pepsin digestion, HCl treatment and incubation in the presence of antibodies.

For fibroblasts with a normal karyotype (NF1 and NF2), the values of the relative FR were calculated and represented on a histogram for chromosomes X, 6 and 8 (Fig. 4). These distributions were not statistically different for FR(X), FR(6) and FR(8), using the Mann-Whitney U-test. Similar results were obtained with lymphocytes cultures LF1 and LF2 (results not shown).

The results differed for fibroblasts that were characterized by structurally or numerically abnormal X chromosomes. In case 1, with a late-replicating pseudodicentric X chromosome, the IF measured for the active X chromosome was 1.4±0.2 times the value measured for the inactive X chromosome. The FR(X) values were systematically higher than the FR(6) and FR(8) values (Fig. 5), with significantly different distributions ($P < 0.005$). In case 2, with a 48, XXXX karyotype, no morphological criteria could distinguish the three late-replicating chromosomes from the early replicating one. The IF values were measured in 15 metaphases for the four X chromosomes. For each metaphase, the IF value of the more heavily labelled X chromosome was 1.9±0.5 times higher than the mean IF value for the three others.

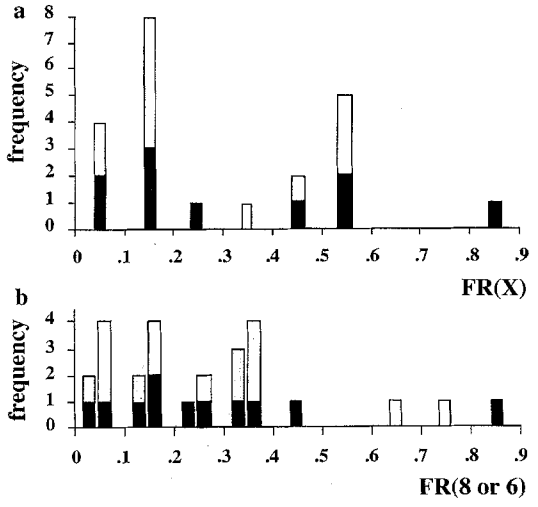


**Fig. 4a, b.** Distributions of the relative fluorescence ratio (FR) for fibroblast primary cultures with normal karyotype. **a** FR(X) for the two X chromosomes; *black boxes* NF1, *white boxes* NF2; **b** FR for the two chromosomes 8 (*boxes on the left in each class, dark grey* NF1, *pale grey* NF2) and chromosomes 6 (*boxes on the right in each class, black* NF1, *white* NF2) taken as controls for intrametaphase variations resulting from experimental procedures

The FRX(1–2), FRX(1–3) and FRX(1–4) values calculated between the X chromosome with the highest IF and the three other chromosomes were higher than those [FRX(2–3), FRX(2–4) and FRX(3–4)] between the three X chromosomes presenting the lowest IFs ($P < 0.0001$; Fig. 6). The histograms obtained for FRX(2–3), FRX(2–4) and FRX(3–4) were not significantly different from those obtained for FR(6) and FR(8). We also compared FRX(1–2) with FRX(2–4), which represents the smallest difference between the first two more heavily

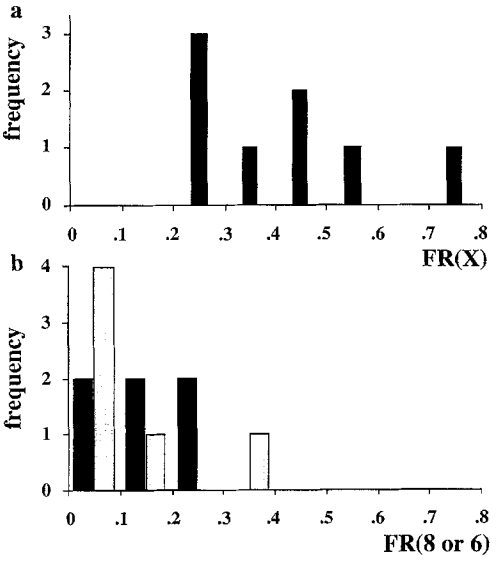


**Fig. 5a, b.** FR distributions for case 1 with a pseudodicentric X. **a** FR(X) values, **b** FR(8) and FR(6) values, represented by the *left black boxes* and the *right grey ones*, respectively

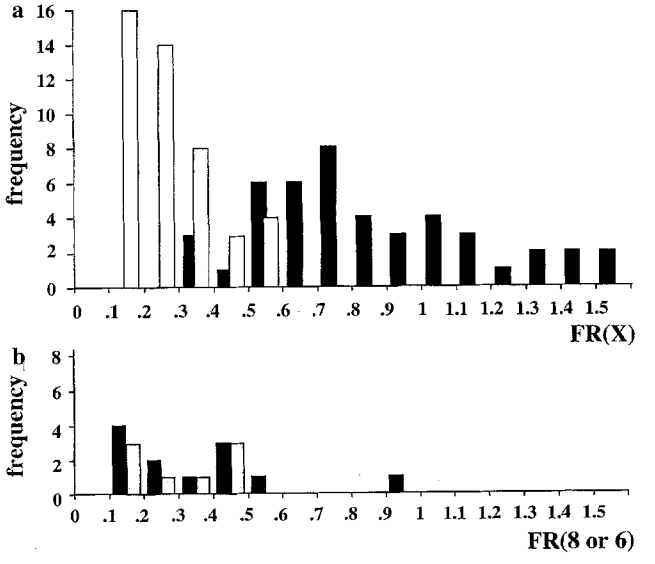


**Fig. 6a, b.** Case 2 with a 48, XXXX karyotype. **a** Histogram of FR(X) values, calculated between the X chromosome with the highest fluorescence intensity (IF) and the three others [FRX(1–2), FRX(1–3), FRX(1–4)] is represented in *black*. In *white* is the histogram of FR(X) values calculated between the X chromosomes with the lowest IF taken two by two [FRX(2–3), FRX(2–4), FRX(3–4)]. **b** Histograms of FR(8) (*black boxes*) and FR(6) (*pale grey boxes*) values calculated for the same metaphases

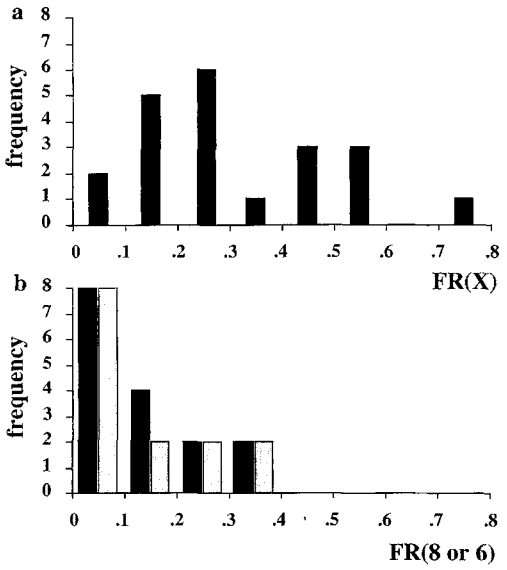


**Fig. 7a, b.** Case 3 with a der(X;15)(q10;q10) chromosome. **a** Histogram of FR(X) values calculated only for the Xq arms of the der(X;15)(q10;q10) and the normal X chromosome. **b** Histograms of FR(8) and FR(6) values, respectively represented by *black* and *pale grey boxes*

labelled X chromosomes in comparison with the greater difference found between the three less-labelled X chromosomes. Results show that the difference remained statistically significant ($P = 0.0001$) using the Mann Whitney U-test or the Wilcoxon signed-rank test. When comparing FRX(2–3) with FRX(2–4) or FRX(3–4), the difference was never significant. Taken together, these data suggest first that the more labelled chromosome is the early replicating X chromosome, and secondly that its level of methylation differs from the three other late replicating X chromosomes. For case 3, characterized by a t(X;15) chromosome, 26 metaphases were analysed. Statistical calculations using the Wilcoxon signed-rank test showed significant difference between the IF for the Xq part of the t(X;15) chromosome and the IF for the normal Xq ($P$=0.0002). In 21 metaphases, the Xq arm of the rearranged early replicating chromosome displayed a 1.4 ± 0.3 times higher IF than the late-replicating one. In the five other metaphases, the long arm of the late-replicating chromosome displayed the greatest fluorescence. Analysis by the Mann Whitney U-test of the distribution of FR(X), FR(6) and FR(8) values of the 21 metaphases for which the early replicating q arm is more labelled than the late replication, showed that the FR(X) distribution is different from that of FR(6) and FR(8) ($P = 0.002$) (Fig. 7). Thus, as in the previous examples, the late-replicating X chromosome always shows a significant difference in methylation level in comparison with the early replicating one.

## Discussion

A recently developed immunofluorescent method (Barbin et al. 1994), combined with image cytometry, was used to compare DNA methylation between the late- and early replicating X metaphase chromosomes from lymphocyte and fibroblast primary cultures in human females. The presence of 5mC was detected by specific antibodies. Control experiments indicated that the treatment conditions produced no change that could result in differential accessibility of the antibodies to chromosomal DNA.

For normal female lymphocytes or fibroblasts visual inspection suggested that differences in anti-5mC labelling could differentiate early and late-replicating X chromosomes. However, it was not possible to correlate timing of replication and methylation status. The semi-quantitative comparison between labelling of the X chromosomes shows that the differences were not statistically significant. This apparent contradiction could indicate that the visually observed undermethylation of one X chromosome is in the same range of magnitude as the relatively high experimental intrametaphasic variations also observed for autosomal chromosomes.

The lack of detectable difference between the global DNA methylation level of the active and inactive chromosomes of cells with normal karyotypes is in opposition to the known hypermethylation of genes borne by the inactive X chromosome (Razin and Cedar 1991; Singer-Sam and Riggs 1993). However, the coding regions correspond to only a small part of the genome, which is essentially constituted of non-coding repeated or non-repeated sequences. The present results suggest that the hypermethylation of the CpG islands of the repressed genes does not reflect the overall status of the inactive X chromosomal DNA.

The situation was clearer for cases with numerical or structural X chromosomal anomalies. For the donor with four Xs, in all the metaphases examined, a group of three X chromosomes was poorly labelled by anti-5mC antibodies, suggesting a low methylation level. In contrast, the fourth X chromosome was quite different, and had a labelling intensity comparable to that of autosomes. Although it was impossible to distinguish directly early and late-replicating X chromosomes, our results suggest that, in these cells containing three late- and one early replicating X, the late-replicating Xs corresponded to the three hypomethylated X chromosomes.

The case of the pseudodicentric X is quite informative. In all the metaphases analysed for replication, the normal X was always found to be early replicating. The binding difference between the normal X and its pseudodicentric counterpart was significantly higher than that observed between homologous autosomes, demonstrating hypomethylation of the late-replicating X.

The results obtained for the case with t(X;15) are comparable, but less simple to explain. The replication study of this case showed late replication of the normal X in about 90%, and of the Xq arm of the t(X;15) in about 10% of the metaphases (Biemont et al. 1978). In the present study, low binding of anti-5mC antibodies was observed in about 80% and 20% of metaphases for the normal X and Xq arm of the t(X;15), respectively. Here again, there is a strong indication that the late-replicating X is hypomethylated, the difference in percent-

age possibly being explained by the difference in tissue: replication studies were performed on lymphocytes and methylation studies on fibroblasts.

Analysis of the data shows that, in the cases of the fibroblasts with abnormal karyotypes, hypomethylation of inactive X chromosomes is clearly established. Several parameters could be involved in this phenomenon such as DNA demethylation due to cell culture (Baylin et al. 1991). The global DNA methylation status of the studied fibroblasts is not known. However, since the comparison was made between the two X chromosomes of the same cell, the observed differences cannot correspond only to an eventual non-specific decrease in methylation of the cell DNA. Moreover, differences between the X chromosomes are not detectable with fibroblasts of normal karyotype grown in the same conditions. A specific mechanism seems to exist in the abnormal cells that induces or amplifies the undermethylation of the inactive X chromosome. The possible existence of a correlation between abnormal cells and the presence of detectable methylation differences between the two X chromosomes was supported by an observation cells of a patient with ICF syndrome. The difference in the interaction of the anti-5mC antibodies with the two X chromosomes was visually distinguishable in the context of a heavily hypomethylated genome (Miniou et al. 1994). It could be hypothesized that in the pathological cases studied, changes in the quantity, activity and/or timing of expression of the DNA methyltransferase during the cell cycle interfere with the ability of the enzyme efficiently to methylate the replicated DNA.

In this context, at least three parameters favour the hypomethylation of the inactive X chromosome: (1) the DNA of the inactive X chromosome, which replicates at the end of the S-phase, could be a hypersensitive target for an altered DNA methylation mechanism; (2) the condensed structure of the heterochromatin could limit the accessibility of the DNA methyltransferase to the DNA; (3) the chromosomal condensation that occurs shortly after the replication of the inactive X chromosome could be an additional limitation for a weakly efficient methylation process by DNA packing in a poorly accessible structure.

Finally, because regions of constitutive heterochromatic in human were shown to be highly methylated, a general belief associated the facultative heterochromatinization of the inactive X chromosome with hypermethylation of DNA. Until now, however, no study has supported this idea (Miller et al. 1982; Viegas-Pequignot et al. 1988; Prantera and Ferraro 1990; Adolph and Hameister 1990). In addition, it is know that, in pachytene, the X is inactivated, although CpG islands have been shown to be hypomethylated (Driscoll and Migeon 1990). These results taken together with our data indicated that heterochromatinization of the inactive X chromosome corresponding to its inactivation and late replication is independent of the global level of DNA methylation.

The heterochromatinization could be more related to other properties of the constitutive and facultative heterochromatin such as the underacetylation of the histone H4 (Jeppesen and Turner 1993) or specific structural proteins (Shaffer et al. 1993).

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