Inactive X chromosome has the highest concentration of unmethylated *Hha* I sites

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ABSTRACT A procedure enabling the highly sensitive detection of accessible restriction endonuclease sites on metaphase chromosomes is described. The procedure is based on the following: (i) a terminal deoxynucleotidyltransferase is used to add a biotinylated nucleotide (Bio-11-dUTP) tail to the 3' hydroxyl terminus generated by the action of a restriction enzyme and (ii) the biotinylated oligonucleotide is detected by a peroxidase-based immunocytochemical method. When used with the 5-methylcytosine-sensitive enzyme *Hha* I, it gives rise to a pattern close to R and T banding on autosomes. In addition, the staining of one X chromosome in females appears very unusual by its pattern and its strong intensity. This procedure, as applied on a case with a polysomy X chromosome, provides direct evidence of an overall hypomethylation of the inactive X chromosomes.

In mammals, the two X chromosomes are not equivalent, one of them being largely transcriptionally inactivated early in development (1, 2). Despite a considerable amount of work devoted to the elucidation of the molecular mechanism of this inactivation, a fully comprehensive view has not yet been obtained. The direct correlation between hypermethylation and inactivation (3-13) does not withstand a detailed analysis, and this oversimplified point of view is now being reconsidered. On female metaphase spreads, reduced accessibility of the inactive X chromosome DNA was demonstrated by using *in situ* nick-translation procedures, a result interpreted as indicative of reduced transcriptional activity (14-16). In contrast, anti-5-methylcytosine antibodies failed to show any difference between the two X chromosomes (17). DNA methylation can also be studied with restriction endonucleases. Indeed, some of these enzymes have within their recognition sequence a dinucleotide, CpG. The restriction enzyme *Hha* I belongs to this group and is unable to cut the DNA if the cytosine is methylated on the 5 position of the pyrimidine ring. We have examined the possibility of using this property to study DNA methylation on metaphase chromosomes. To monitor the action of the enzyme, an immunocytochemical method for detection of the 3' hydroxyl terminus of DNA was developed. The method reveals a high concentration of unmethylated sites on the inactive late-replicating X chromosome in females, thus providing direct evidence of an overall hypomethylation of the DNA of this chromosome.

MATERIAL AND METHODS

Principle of the Method. The method is based on the ability of the enzyme terminal deoxynucleotidyltransferase (18) to use the 3' hydroxyl terminus generated by restriction endonucleases as a primer for polymerizing Bio-11-dUTP. The biotinylated nucleotide tails are then detected immunocytochemically by using first a polyclonal antibody and then an

IgG-peroxidase conjugate. The peroxidase substrate diaminobenzidine (DAB) was chosen to reveal the immunocomplexes since it provides a highly sensitive and well-contrasted signal after an intensification step involving gold reaction and silver precipitation (19, 20).

Chromosome Preparation. Metaphase spreads from male and female cells, including a case with a 48,XXXX karyotype, were obtained from blood lymphocytes or fibroblasts according to published procedures (21). Slides were either kept at -18°C or used at most 24 hr after the spreading.

Restriction Endonucleases and Methylases Reactions. Slides were first incubated for 10 min in the restriction enzyme buffer recommended by the supplier. Five units of *Hha* I restriction enzyme (New England Biolabs) diluted to 50 μ l in the same buffer was added over the slides, which were then covered with coverslips and incubated 30 min at 37°C. For control experiments, in some cases, instead of *Hha* I, the restriction enzyme *Pst* I (with its own buffer) was used at a concentration ranging from 0.1 unit/ μ l to 0.01 unit/ μ l under otherwise identical conditions. Also in one instance, an *Hha* I methylase step was included before any endonuclease reaction. It was performed with 50 units of *Hha* I methylase (New England Biolabs) dissolved in 50 μ l of the recommended buffer at 37° for 30 min.

Bio-11-dUTP Tailing. After restriction endonuclease reaction, slides were then rinsed twice for 5 min with restriction enzyme buffer and once with terminal deoxynucleotidyltransferase buffer (100 mM sodium cacodylate, pH 7.0/1 mM CoCl₂/0.2 mM dithiothreitol). Eleven units of terminal deoxynucleotidyltransferase (New England Biolabs) dissolved in 50 μ l of the same buffer complemented with 40 μ M Bio-11-dUTP was then added to each slide. Incubation at 37° was resumed for 30 min. Slides were washed for 30 min in 0.3 M NaCl/30 mM sodium citrate at room temperature, 30 min in 15 mM NaCl/1.5 mM sodium citrate at 42°C, and again 15 min in 0.3 M NaCl/30 mM sodium citrate at room temperature.

Immunoperoxidase Detection of Bio-dUTP Tails. Prior to immunostaining, slides were incubated for 10 min in a modified phosphate-buffered saline (PBS) solution (PBS/0.1% Tween 20/0.15% bovine serum albumin). Then 50 μ l of a goat anti-biotin IgG (Vector Laboratories, Burlingame, CA) diluted (1:500) in the modified PBS was added and the slides were incubated at 37°C for 1 hr. Slides were rinsed twice for 10 min each in modified PBS. Fifty microliters of the second antibody, a peroxidase-labeled anti-goat IgG (Tebu-Nordic, France) diluted 1:40 in modified PBS, was added, and incubation was resumed for 1 hr at 37°C. Slides were rinsed once in modified PBS and once in unmodified PBS, 10 min each. Slides were then developed by dipping for 4-7 min in 100 ml of DAB solution (0.5 mg/ml) in PBS to which 100 μ l of 30% hydrogen peroxide was added immediately before use. Slides were washed in distilled water.

DAB Intensification. DAB intensification was performed according to Gallyas *et al.* (19) as modified by Burns *et al.*

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Abbreviation: DAB, diaminobenzidine.

(20). To obtain the optimal contrast, the final silver reaction was performed stepwise. After each step the intensification was controlled for each slide under the microscope.

RESULTS

When lymphocytes from either males or females cultured under standard conditions were subjected to our procedure using the *Hha* I restriction nuclease and omitting the last step (i.e., DAB intensification), a weakly contrasted R/T-like banding pattern (22) was obtained (Fig. 1). A major difference from the standard R/T banding was the strong staining of the short arm of a number of acrocentrics, comparable to a nucleolar organizing region (NOR) staining pattern (23). This contrasted with the poor staining of constitutive heterochromatin. When we proceeded to the DAB intensification, the general staining was progressively reinforced. For male metaphases after a short intensification time, a preferential darkening of the short arms of some acrocentrics and of R-band-rich chromosomes was observed (Fig. 1). With longer times, chromosomes became homogeneously dark and the banding vanished. In the female, the same sequence of events was observed with one striking exception: after a short period of intensification one chromosome appeared much darker than all the others (Figs. 1 and 2). Its intermediate size, the position of its centromere, and its absence in

male mitosis led us to hypothesize that it was the inactive and late-replicating X chromosome.

In several cases, reassessment of metaphases before intensification allowed us to identify the chromosomes by their R-banding-like pattern. In females the two X chromosomes differed. One was pale, with an R banding comparable to the one obtained for autosomes. The other X chromosome was slightly darker, with the exception of a pale region extending from q21 to q23-q24. This banding pattern differed from a characteristic R banding. Controlled intensification showed that the chromosome with this atypical pattern was precisely the one that becomes very dark. Except after long intensification times, the pale q21 to q23-q24 segment and the distal portion of the short arm remained usually unchanged (Fig. 2 *b, c, and e*).

To demonstrate definitively the identification of the inactivated X chromosome, we performed the experiment on metaphases from 48,XXXX female fibroblasts. The banding pattern obtained on the autosomes with the fibroblasts showed no marked difference from that obtained previously with peripheral lymphocytes. The DAB intensification induced the same modifications as for lymphocytes, but three of the four X chromosomes became very dark (Fig. 3). Reassessment of the metaphases without DAB intensification showed that for these three chromosomes the pattern is

FIG. 1. Immunodetection of accessible unmethylated *Hha* I sites. (*a* and *b*) Female metaphase before (*a*) and after (*b*) the DAB intensification step. The inactive late-replicating X chromosome (X_1), much darker than the active early-replicating X chromosome (X_e), can easily be identified in *b* (arrow). No chromosome with equivalent behavior is observed in male cells [before (*c*) and after (*d*) DAB intensification].

FIG. 2. Typical aspects of inactive X chromosomes. (a) A single female metaphase was chosen in which, prior to DAB intensification, an inactive late-replicating X chromosome (X_1) and chromosome 13 could be unambiguously identified. It was photographed at 0, 2, and 4 min (left to right) in the course of DAB intensification. (b-f) Regions including one inactive X chromosome (arrow) for different female metaphases were photographed after the same time of DAB intensification (4 min). A discrete R banding is exhibited by autosomes.

similar to that of the presumed inactive X chromosome from 46,XX lymphocytes.

We verified that the observed staining was related to the presence of unmethylated *Hha* I sites. Indeed when an *Hha* I methylase step was added before the endonuclease restriction or when the *Hha* I restriction step was omitted, chromosomes remained extremely pale and the DAB intensification step was ineffective. Subsequent staining with Giemsa exhibited normal chromosomes.

A methyl-insensitive restriction endonuclease was also tested. In a series of experiments *Hha* I was replaced by the

Pst I endonuclease. For equivalent enzyme concentrations, the staining of chromosomes from lymphocytes was much more intense than with *Hha* I and there was no obvious banding pattern. For lower concentrations (0.01 unit/ μ l), however, the chromosomes were paler and a poor R-banding pattern appeared, but, in contrast to *Hha* I digestion, there was no preferential staining of the short arms of acrocentrics nor differentiation of X chromosomes in female metaphases.

DISCUSSION

A method for the detection of accessible restriction endonuclease sites on metaphase chromosomes has been previously proposed (24-27). It was based on a differential Giemsa staining resulting in a differential loss of DNA induced by the action of the restriction endonuclease. Enzymes such as *Hae* III having frequent recognition sites on human DNA were able to reveal an identifiable pattern close to G banding. However, enzymes with rare cutting sites and particularly the methyl-sensitive enzymes *Hpa* II and *Hha* I were ineffective in generating a banding pattern even when they were used at concentrations of 1-5 units/ μ l (27). Our method compared to this previous one provides a dramatic increase in sensitivity.

The R banding obtained here when *Hha* I is used demonstrates the presence of unmethylated CpG in these regions. However this pattern may theoretically result from two factors: the local concentration of cutting sites on DNA and the accessibility of the DNA in these regions. Indeed, *in situ* nick-translation (14, 15), *in situ* restriction enzyme digestion of metaphase spreads (24-27), and our results with low concentration of *Pst* I converge to show that DNA accessibility is nonuniform along the chromosome. We can however conclusively discuss two particularities observed in the *Hha* I banding pattern.

FIG. 3. Immunodetection of accessible unmethylated *Hha* I sites in an XXXX female metaphase. The arrows point to the three inactive X chromosomes. The active X chromosome cannot unambiguously be identified in this metaphase.

First, the short arms of the acrocentrics showed neither heterogeneity nor heavy labeling when we used the *Pst* I restriction enzyme. Their respective degrees of accessibility are thus approximately equivalent. The heterogeneous labeling of these regions when *Hha* I was used revealed, therefore, a differential methylation apparently not correlated with any drastic change in DNA accessibility. Differential labeling of the short arms of acrocentrics has also been obtained with anti-5-methylcytosine antibodies (17) and NOR staining (23).

Second, *in situ* restriction enzyme digestion of metaphase spreads or our procedure with *Pst* I do not reveal differences in the staining of the two X chromosomes in females. Interestingly, the reduced DNA accessibility for the inactive X chromosome found with the *in situ* nick-translation procedure (14, 15) was not observed here. Thus the increased labeling of the inactive X chromosome when *Hha* I was used in our procedure is most probably due to hypomethylation. Our result generalizes the observation, made through the use of a few X chromosome-specific probes, of hypomethylation in defined regions of the inactive X chromosome (8, 28–31). In contrast to the method used by Miller *et al.* (17), the present method detects positively some unmethylated CpG and is therefore well suited to the detection of small variations in the level of these unmethylated dinucleotides. It appears that most of the biotin-labeled regions on the autosomes and the active X chromosome are the R bands, in which possibly the CpG islands reside. These islands represent only $\approx 1\%$ of the genome (11). In the known cases they are hypomethylated on the inactive X chromosome (32) and therefore are not prone to labeling with the present technique. This is consistent with the unusual banding pattern of the inactive X chromosome obtained here. Thus, it appears that the strong labeling of the inactive X chromosome is possibly due to a relative hypomethylation elsewhere in the DNA sequence. It would be interesting to establish the functional significance of this hypomethylation.

1. Lyon, M. F. (1961) *Nature (London)* **190**, 372–373.
2. Lyon, M. F. (1972) *Biol. Rev.* **47**, 1–35.
3. Liskay, R. M. & Evans, R. J. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 4895–4898.
4. Mohandas, T., Sparkes, R. S. & Shapiro, L. J. (1981) *Science* **211**, 393–396.
5. Venolia, L., Gartler, S. M., Wassman, E. R., Yen, P., Mohandas, T. & Shapiro, L. J. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 2352–2354.
6. Lester, S. C., Korn, N. J. & DeMars, R. (1982) *Somatic Cell Genet.* **8**, 265–284.
7. Graves, J. A. M. (1982) *Exp. Cell Res.* **141**, 99–105.
8. Monk, M. (1986) *BioEssays* **4**, 204–208.
9. Toniolo, D., D'Urso, M., Martini, G., Persico, M., Tufano, V., Battistuzzi, G. & Luzzatto, L. (1984) *EMBO J.* **3**, 1987–1995.
10. Wolf, S. F., Dintzis, S., Toniolo, D., Persico, G., Lunnen, K. D., Axelman, J. & Migeon, B. R. (1984) *Nucleic Acids Res.* **12**, 9333–9348.
11. Bird, A. P. (1986) *Nature (London)* **321**, 209–213.
12. Riggs, A. D., Singer-Sam, J. & Keith, D. H. (1985) in *Biochemistry and Biology of DNA Methylation*, eds. Cantoni, G. L. & Razin, A. (Liss, New York), pp. 211–222.
13. Gartler, S. M., Dyer, K. A., Graves, J. A. M. & Rocchi, M. (1985) in *Biochemistry and Biology of DNA Methylation*, eds. Cantoni, G. L. & Razin, A. (Liss, New York), pp. 223–238.
14. Kerem, B., Goitein, R., Richler, C., Marcus, M. & Cedar, H. (1983) *Nature (London)* **304**, 88–90.
15. Kerem, B., Goitein, R., Diamond, G., Cedar, H. & Marcus, M. (1984) *Cell* **38**, 493–499.
16. Jablonka, E., Goitein, R., Marcus, M. & Cedar, H. (1985) *Chromosoma* **93**, 152–156.
17. Miller, D. A., Okamoto, E., Erlanger, B. F. & Miller, O. J. (1982) *Cytogenet. Cell Genet.* **33**, 345–349.
18. Deng, G. R. & Wu, R. (1983) *Methods Enzymol.* **100**, 96–116.
19. Gallyas, F., Gorcs, T. & Merchenthaler, I. J. (1982) *J. Histochem. Cytochem.* **30**, 183–184.
20. Burns, J., Chan, V. T. W., Jonasson, J. A., Fleming, K. A., Taylor, S. & McGee, J. O'D. (1985) *J. Clin. Pathol.* **38**, 1085–1092.
21. Dutrillaux, B. & Couturier, J. (1981) *La Pratique de l'Analyse Chromosomique* (Masson, Paris), pp. 34–36.
22. International System for Human Cytogenetic Nomenclature (1978) *Cytogenet. Cell Genet.* **21**, 309–404.
23. Goodpasture, C. & Bloom, S. E. (1975) *Chromosoma* **53**, 37–50.
24. Mezzanotti, R., Bianchi, U., Vanni, R. & Ferrucci, L. (1983) *Cytogenet. Cell Genet.* **36**, 562–566.
25. Bianchi, N. O., Bianchi, M. S. & Cleaver, J. E. (1984) *Chromosoma* **90**, 133–138.
26. Kaelbling, M., Miller, D. A. & Miller, O. J. (1984) *Chromosoma* **90**, 128–132.
27. Bianchi, N. O., Vidal-Rioja, L. & Cleaver, J. E. (1986) *Chromosoma* **94**, 362–366.
28. Wolf, S. F., Jolly, D. J., Lunnen, K. D., Friedmann, T. & Migeon, B. R. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 2806–2812.
29. Yen, P. H., Patel, P., Chinault, A. C., Mohandas, T. & Shapiro, L. J. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 1759–1763.
30. Lindsay, S., Monk, M., Holliday, R., Huschtscha, L., Davies, K. E., Riggs, A. D. & Flavell, R. A. (1985) *Ann. Hum. Genet.* **49**, 115–127.
31. Starr, T. & Wood, S. (1988) *Am. J. Hum. Genet.* **42**, 267–270.
32. Bird, A. (1987) *Trends Genet.* **3**, 342–347.