PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **186**, 223–228 (1987)

Immunocytochemical Evidence for Methylation of the Inactive X Chromosome in Human Fetal Oogonia (42606)

GEORGIANA M. JAGIELLO,* UMADEVI TANTRAVAHI,† MERCEDES B. DUCAYEN,* AND BERNARD F. ERLANGER‡

*Department of Obstetrics and Gynecology, Department of Genetics and Development, and Center for Reproductive Sciences of the International Institute for the Study of Human Reproduction, and ‡Department of Microbiology and Cancer Center, College of Physicians and Surgeons of Columbia University, New York, New York 10032, and †Department of Medicine and Genetics, Children's Hospital, Harvard Medical School, Boston, Massachusetts 02115

Abstract. The state of DNA methylation of the X chromosomes of human interphase oogonia from a 46,XX and a 46,XX/47,XXX fetus at 17 weeks of gestation was tested immunocytochemically with an antibody to 5-methylcytosine (5MeC). Of 1637 oogonial nuclei from the 46,XX fetal ovary, 313 (19.1%) contained Barr bodies, of which 93.6% were positive for 5MeC. Of 1780 oogonia from the 46,XX/47,XXX fetus 327 (18.4%) contained Barr bodies; 175 oogonia had one Barr body and 152 had two. Of the single Barr bodies 145 (82.8%) had positive 5MeC reaction product. Of the 152 oogonia from the XXX line, 97 (63.8%) had positive 5MeC on both Barr bodies, 35 (23%) had one positive and one negative, and 20 (13.1%) had no product on either Barr body. This immunocytochemical evidence supports the hypothesis that the DNA of the inactive X-chromosome of the human 17-week gestation oogonium is methylated. © 1987 Society for Experimental Biology and Medicine.

As originally proposed, the Lyon hypothesis (1) postulated random clonal inactivation of one of the two X-chromosomes in female mammalian somatic cells, thus resulting in dosage compensation with reference to the male. The cytologic marker for the inactive human X-chromosome has been well documented to be the Barr body (2). Since the original enunciation of this hypothesis, modifications of the state of inactivity of the X-chromosome have been suggested (3–5) as well as numerous theories on the mechanism and maintenance of inactivation (6–8). One of the models which has been proposed implicates methylation of the X-chromosome DNA as a repressor of gene activity (9, 10) as measured, for example, by X-linked enzymes (11, 12), or inhibition of methylation by 5-azacytidine with resultant expression of genes (13). However, when testing the methylation of the two X-chromosomes of human leucocyte metaphase preparations by immunocytochemical localization, Miller *et al.* (14) were unable to detect a difference in reaction product between these chromosomes at this stage of mitosis. It was the purpose of the present work to examine a sample of human fetal oogonia with similar methodology for the possibility that the inactive X observed cytologically as a Barr body was significantly methylated during oogenesis.

Materials and Methods. Two aborted human female feti, aged 17 weeks, provided oogonia for study. Mitotic metaphases in the first case revealed it to be a 46,XX and the second case to be a 46,XX/47,XXX mosaic. The specimens were recovered within an hour of passage, and ovaries were rapidly removed and processed for cytologic examination of oogonia and oocytes by a modification of the method of Fang and Jagiello (15). Fetal ovaries were cut into 1-mm cubes and placed in 0.07 *M* KCl at 38°C for 5 min. Cubes were fixed in fresh 3:1 absolute ethanol:glacial acetic acid and squashed and smeared on clean, cold, wet slides. Slides were air dried in a desiccator. Interphase oogonial nuclei were located by the criteria of Baker (16) with a 100× Zeiss Planapo phase lens for identification of the absence or precise location of Barr bodies and verniers were recorded. The Barr body was defined for this scoring as a dark planoconvex structure of approximately 1 μm in diameter resting against the nuclear membrane (2). Slides were replaced in a desiccator for 48 hr and then processed for the immunocytochemical localization of 5-methylcytosine (5MeC) using a rabbit anti-5-methylcytidine. This antibody has been characterized in publications by Schreck *et al.* (17), Lubit *et al.* (18, 19), and Tantravahi *et al.* (20, 21), and is the same antibody as used by Miller *et al.* (14). Slides were

0037-9727/87 $1.50
Copyright © 1987 by the Society for Experimental Biology and Medicine.
All rights reserved.

rinsed in filtered PBS, pH 7.2, placed in petri dishes containing PBS, and irradiated at a distance of 30 cm from a 30-W uv germicidal lamp (General Electric Co.) for 8 hr at ambient temperature. They were rinsed with a spray of PBS, washed in a Coplin jar containing PBS for 5 min, drained, and covered with 0.3 ml of a 1:75 cold dilution of the anti-5MeC in PBS. Slides were incubated in a high humidity chamber at room temperature for 40 min, drained of antibody, and rinsed once with PBS as before. A second goat antibody against rabbit immunoglobulin tagged with peroxidase (Miles Laboratories) was layered on the slides at a dilution of 1:50 and incubated at room temperature for 40 min. Slides were again rinsed with PBS, incubated at room temperature for 20 min in 50 ml of a filtered solution of 50 mg 3,3',diaminobenzidine (Sigma) dissolved in 100 μl dimethylsulfoxide (Fisher Scientific Co.) and 0.003% hydrogen peroxide in PBS, and then rinsed two times with PBS. Slides were dehydrated in successive passages of 2 min each through 50% to absolute alcohol and air dried. Control slides of human leucocyte metaphases and oogonial preparations omitting the first or second antibody were run simultaneously. Oogonial nuclei were relocated on the slides using the previously recorded verniers and immunoperoxidase-treated slides scored for the presence of reaction product on Barr bodies at 100× with a Zeiss Neofluar objective. Barr bodies were scored as postive for 5 MeC if they revealed significant overlying brown reaction product (Figs. 1b–1h). Somatic cell nuclei adjacent to the oogonial nuclei were scored for reaction product in the same manner.

Results. The human leucocyte metaphases revealed positive reaction product on chromosomes 1, 9, 15, and 16 as previously reported by Lubit *et al.* (18). Negative controls displayed no reaction product on somatic or oogonial nuclei. Methylation of the Barr bodies in the 46XX and the mosaic 46,XX/47,XXX cases was seen in a significant percentage of oogonia (Table I). In 1637 oogonia from the 46,XX fetal ovary, 313 or 19.1% contained Barr bodies (Fig. 1a). Of these, 93.6% revealed immunoperoxidase reaction product on the Barr body (Fig. 1b). Oogonia (1780) from the 46,XX/47,XXX fetus were studied, of which 327 (18.4%) contained Barr bodies: 175 oogonia had a single Barr body and 152 had two Barr bodies (Table I); 145 (82.8%) had single Barr bodies with overlying 5MeC reaction product. Oogonia from the 47,XXX line demonstrated 97 (63.8%) with both Barr bodies positive, 35 (23%) with one Barr body reactive and 20 (13.1%) oogonia where neither Barr body was positive (Figs. 1c–1h). Of all oogonia from both cases wherein 5MeC positive Barr bodies were scored, 98% had reaction product in the nuclei presumed to be on one or more of the known autosomal (No. 1, 9, 15, or 16) sites (Figs. 1b–1h).

Scores of 197 fibroblast/somatic nuclei from the 46,XX case, 99 of which had Barr bodies, revealed 94 (95%) had 5MeC reaction product, while 5 (5%) were negative. The 46,XX/47,XXX case provided 260 somatic nuclei for scoring, of which 119 (46%) had one or two Barr bodies. In the 46,XX line, 79 (79%) of 100 Barr bodies had 5MeC product and 21 (21%) did not, while the 47,XXX line provided a total of 19 somatic nuclei with double Barr bodies: 13 (68%) of these had 5MeC on both Barr bodies, 2 (11%) had one positive and one negative, and 4 (21%) had no detectable reaction product on either Barr body.

Discussion. The question of inactivation of the sex chromosomes during mammalian oogenesis presents an especially interesting example of the more complex issues of when and how X inactivation occurs and is maintained, the possible relationship to chromatin condensation, essentiality to X-linked gene expression, and the possibility of an X inactivation–reactivation cycle (3). The present study addresses one aspect of these points, the possibility that fetal human oogonia at 17 weeks gestation contain an inactive X chromosome detectable as a Barr body in which the DNA is methylated. No accurate predictions about effects of hyper- or hypomethylation of the inactive X chromosome can be made, however.

The gestational age of the fetuses studied in the present work was accurately determined by ultrasonography of biparietal diameter and femur length. A significant population of oogonia were available for analyses in which 19.1% of the 46,XX cells from the first fetus contained a Barr body and 18.4% of the 46,XX/47,XXX contained one or two Barr bodies. The presence of oogonia in these fetal

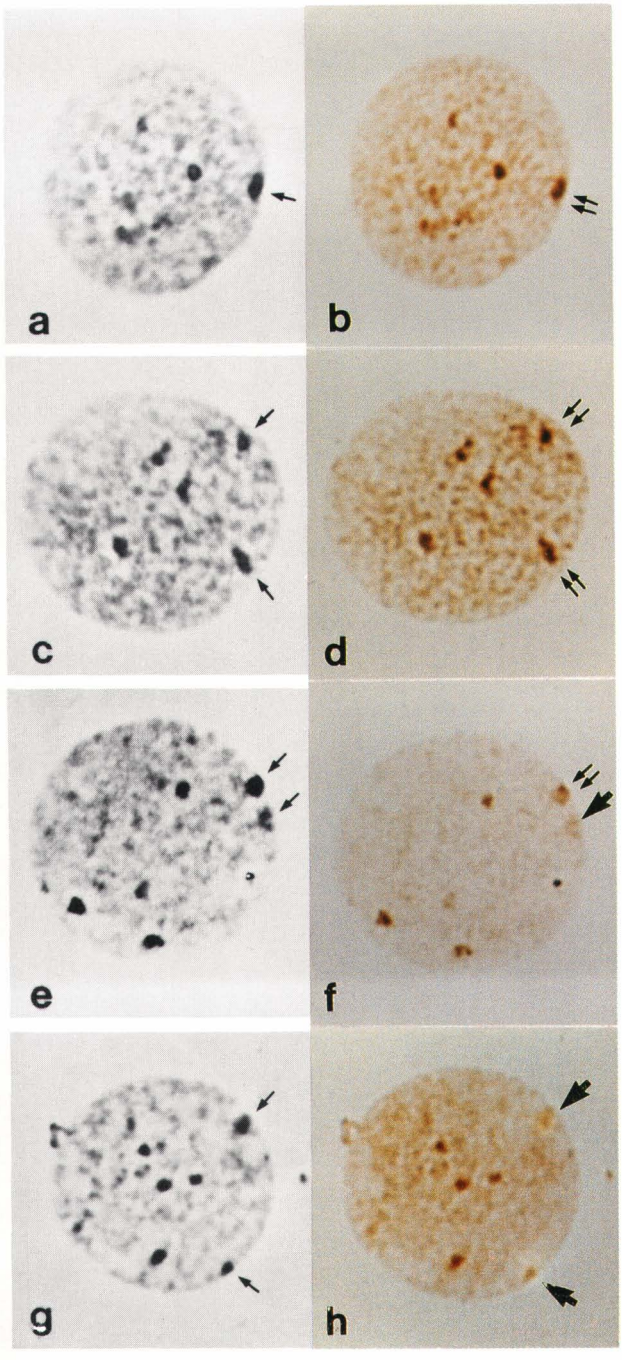

FIG. 1. Immunocytochemical staining for 5-methyl cytosine of Barr bodies in human interphase oogonia. (a, c, e, g) Phase micrographs showing Barr bodies in nuclei (small arrow). (b, d, f, h) Positive 5-methyl cytosine reaction product (double small arrows); negative (large arrow). (b–h) Reaction product on putative autosomes seen. 1500×.

TABLE I. IMMUNOCYTOCHEMICAL REACTIVITY WITH ANTI-5MeC OF HUMAN INTERPHASE OOGONIA NUCLEI

Karyotype	Total oogonia	Oogonia with Barr bodies (%)	Barr body:n 5MeC (%)		Two Barr bodies 5MeC:n (%)		
			+	−	(++)%	(+−)	(−−)
46,XX	1637	313 (19.1)	293 (93.6)	20 (6.4)	—	—	—
46,XX/47,XXX	1780	327 (18.4)	145 (82.8)	30 (17.1)	97 (63.8)	35 (23)	20 (13.1)

Note. (+) One with 5MeC reaction product; (++) both with product; (+−) one with product, one without; (−−) both without product.

ovaries was consonant with the report of Baker (16) who quantified 33.8% of the germ cells in the human ovary as oogonia at 4 months postconception. This author did not score oogonia for the inactive X, but Ohno *et al.* (22) found human oogonia at 3 months gestation to be sex chromatin negative, and had earlier suggested that reactivation of the inactive X, as diagnosed by the Barr body, occurred at the time of differentiation of the primordial germ cell into an oogonium upon entry into the gonad (23). Since the number of oogonia sampled in the present study was sizable, it is probable that the difference from the Ohno study represents sample size or variable gestational dating.

The immunocytochemical detection of 5-methylcytosine in the Barr bodies of both sets of fetal oogonial specimens with the specific antibody used was definitely positive when compared with control oogonia. However, Miller *et al.* (14) studying leucocyte mitotic metaphases of female human and owl monkey using the same antibody and method for testing methylation of the two X chromosomes observed that the antibody bound lightly to the arms of all chromosomes and the two X chromosomes did not differ from each other. It was concluded that the inactive X at the metaphase stage in the leucocyte cell cycle was not hypermethylated and thus X inactivation did not involve methylation of a majority of potential sites on the inactive X. In the oogonial nuclei studied in the present experiment positivity for 5MeC was detected over the inactive X seen as the Barr body and four nuclear sites which could represent the centromeric heterochromatin of chromosomes 1, 9, 15, and 16. The presumptive active X chromosome, had it been equally methylated as reported by Miller *et al.*, could be expected to be observed, but was not. Though not absolute proof of an inequality of methylation of inactive and active X chromosomes, the finding of definite reaction product over the Barr body suggests that, unlike the X chromosomes of leucocytes at metaphase, its state of methylation in oogonia varied from the active X.

It should be noted that in these studies the criterion for positivity was a strong concentration of reaction product (illustrated in Fig. 1) that was clearly distinguishable from both the pale stain observed in chromatin and the total absence of reaction product in the preparations with primary or secondary antibody omitted. Although it is hazardous to attempt quantitative observations at the light microscopic level under these experimental conditions, it is possible that the weak reaction in chromatin represents localization of scattered 5MeC sites in the DNA of the genome. It is evident that accurate quantitation in such studies demands—in addition to precise morphometry—the use of methods in which there is a defined and measurable relationship between antibody-binding sites and signal (e.g., gold particles) rather than immunoenzymatic methods where the signal is the reaction product of an enzymatic reaction. Nevertheless, given the previously demonstrated specificity of this antibody (14, 17–21), the described positive results adequately support the qualitative observation of concentrated 5MeC-binding sites in Barr bodies of fetal oogonia and somatic cells.

As in all immunocytochemical studies, alternative explanations for the observed negative result must be entertained, as for example total destruction or inaccessibility of the antigenic sites recognized by the antibody in

some Barr bodies. The former is unlikely in these preparations, processed under conditions favorable to the localization of 5MeC (14). The latter possibility is more difficult to discard since the accessibility of 5MeC theoretically can be influenced by the steric configuration of chromatin and chromosomes.

Analysis of 5MeC-positive Barr bodies found in oogonia from the 47/XXX line of Case No. 2 demonstrated three different states of methylation as judged cytologically. A pattern of methylation was observed ranging from a state wherein the two inactive X-chromosomes were methylated to a condition where one or the other was methylated to a final condition where neither was methylated. Of interest was the persistent condensed state of the Barr body in all three combinations, an observation which might represent the reversal process of Gartler's "two-step" hypothesis of inactivation (24) which proposes an initial heterochromatinization of sex chromatin followed by methylation as a second repressor of expression. Conceivably the present findings could represent sequential or simultaneous methylation of each Barr body from none to both. However, this interpretation would not be supported by any of the currently available data on gene expression and the timing of reactivation of the X chromosome in proximity to the entry into meiosis (4). Since the inactive X of somatic cells does not, in most tissues, revert to an active state, the findings of comparatively few 5MeC-negative Barr bodies in the fibrobalst/somatic cell nuclei adjacent to the oogonia in the present study would suggest that persistent X inactivity was associated with methylation.

Taken together, the present data suggest that X chromosome condensation and methylation are characteristic of the X inactive state of oogonia during early human oogenesis.

This work was supported by NIH Grants HD18735 and AI-06860. We thank Mr. Wilbur Bowne and Mr. Adi C. C. Lu for their technical assistance in the photographic work and Dr. R. Mesa-Tejada for his comments on immunocytochemical methods.

1. Lyon MF. Gene action in the X chromosome of the mouse (*Mus musculus* L.) Nature (London) **190**:372–373, 1961.
2. Mittwoch U. Sex chromatin. J Med Genet **1**:50–76, 1964.
3. Gartler S, Riggs AD. Mammalian X-chromosome inactivation. Annu Rev Genet **17**:155–190, 1983.
4. Graves JAM. Inactivation and reactivation of the mammalian X chromosome—On the threshold of molecular biology. In: Johnson MH, Ed. Development in Mammals. Amsterdam, Elsevier, Vol 5, chap. 8, 1983.
5. Lyon MF. The X chromosomes and their levels of activation. In: Cytogenetics of the Mammalian X Chromosome: Basic Mechanisms of X Chromosome Behavior. New York, Alan R. Liss, Pt A, chap. 8: pp187–204, 1983.
6. Comings DE. The rationale for an ordered arrangement of chromatin in the interphase nucleus. Amer J Hum Genet **20**:440–460, 1968.
7. Graves JAM, Garler SM. Mammalian X chromosome inactivation: Testing the hypothesis of transcriptional control. Somatic Cell Mol Genet **12**:275–280, 1986.
8. Grumbach MM, Morishima A, Taylor JH. Human sex chromosome abnormalities in relation to DNA replication and heterochromatinization. Proc Natl Acad Sci USA **49**:581–589, 1983.
9. Holliday R, Pugh JE. DNA modification mechanisms and gene activity during development. Science **187**: 226–232, 1975.
10. Riggs AD. X-inactivation, differentiation and DNA methylation. Cytogenet Cell Genet **14**:9–25, 1975.
11. Lock LF, Melton DW, Caskey CT, Martin GR. Methylation of the mouse hprt gene differs on the active and inactive X chromosomes. Mol Cell Biol **6**: 914–924, 1986.
12. Migeon BR, Wolf SR, Mareni C, Axelman J. Derepression with decreased expression of the G6PD locus on the inactive X chromosomes in normal human cells. Cell **29**:595–600, 1982.
13. Mohandas T, Sparkes RS, Shapiro LJ. Reactivation of an inactive human X chromosome: Evidence for X-inactivation by DNA methylation. Science **211**: 393–396, 1981.
14. Miller DA, Okamoto E, Erlanger BF, Miller OJ. Is DNA methylation responsible for mammalian X chromosome inactivation? Cytogenet Cell Genet **33**: 345–349, 1982.
15. Fang JS, Jagiello GM. Complete chromomere map of mid/late pachytene human oocytes. Amer J Hum Genet **35**:879–888, 1983.
16. Baker TG. A quantitative and cytological study of germ cells in human ovaries. Proc R Soc London Ser B **158**:417–433, 1963.
17. Schreck RR, Erlanger BF, Miller OJ. The use of antinucleotide antibodies to probe the organization of chromosomes denatured by ultraviolet irradiation. Exp Cell Res **88**:31–39, 1974.
18. Lubit BW, Schreck RR, Miller OJ, Erlanger BF. Human chromosome structure as revealed by an im-

munoperoxidase staining procedure. Exp Cell Res **89**: 426–429, 1974.
19. Lubit BW, Pham TD, Miller OJ, Erlanger BF. Localization of 5-methylcytosine in human metaphase chromosome by immunoelectron microscopy. Cell **9**: 503–509, 1976.
20. Tantravahi U, Breg WR, Werteleicki V, Erlanger BF, Miller OJ. Evidence for methylation of inactive human rRNA genes in amplified regions. Hum Genet **56**:315–320, 1981.
21. Tantravahi U, Guntaka RV, Erlanger BF, Miller OJ. Characterization of ribosomal RNA genes in mammalian cells. 1. Amplified ribosomal RNA genes are enriched in 5-methylcytosine. Proc Natl Acad Sci USA **78**:489–493, 1981.
22. Ohno S, Klinger HP, Atkin NB. Human oogenesis. Cytogenetics **1**:42–51, 1962.
23. Ohno S. Life history of female germ cells in mammals. In: Second International Conference on Congenital Malformations. New York, The International Medical Congress, Ltd., pp36–40, 1963.
24. Gartler SM, Dyer KA, Graves JAM, Rocchi M. A two step model for mammalian X-chromosome inactivation. In: Cantoni GL, Razin A, Eds. Progress in Clinical and Biological Research. New York, Alan R. Liss, Vol 198:pp223–235, 1985.

Received March 4, 1987. P.S.E.B.M. 1987, Vol. 186.
Accepted July 9, 1987.