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# Incomplete X chromosome dosage compensation in chorionic villi of human placenta

**(X inactivation/glucose-6-phosphate dehydrogenase/DNA methylation/CpG dinucleotide clusters)**

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ABSTRACT **Studies of glucose-6-phosphate dehydrogenase (*G6PD*) in heterozygous cells from chorionic villi of five fetal and one newborn placenta show that the locus on the allocyclic X is expressed in many cells of this trophectoderm derivative. Heterodimers were present in clonal populations of cells with normal diploid karyotype and a late replicating X chromosome. The expression of the two X chromosomes was unequal, based on ratios of homodimers and heterodimers in clones. Studies of DNA, digested with *Hpa* II and probed with cloned genomic G6PD sequences, indicate that expression of the locus in chorionic villi is associated with hypomethylation of 3′ CpG clusters. These findings suggest that dosage compensation, at least at the *G6PD* locus, has not been well established or maintained (or both) in placental tissue. Furthermore, the active X chromosome in these human cells of trophoblastic origin can be either the paternal or maternal one; therefore, paternal X inactivation in extraembryonic lineages is not an essential feature of mammalian X dosage compensation.**

The mechanism(s) that has evolved in mammals to compensate for sex differences in X chromosome dosage results in silencing all but one X chromosome in somatic cells of the female (1). The maintenance of dosage compensation in human cells is remarkably stable. Attempts to derepress loci on the inactive X chromosome have been generally unsuccessful (2, 3) and only one reactivant has been found (4). In this case, the localized expression of the glucose-6-phosphate dehydrogenase (*G6PD*) locus on the allocyclic X occurred spontaneously and was associated with demethylation of two CpG dinucleotide clusters in 3′ coding sequences of *G6PD* (5).

DNA methylation is thought to have an important role in X chromosome dosage compensation (6–9) and has been implicated in the transmission of the single active X from one cell to its progeny. Patterns of methylation of *G6PD* (5, 10) and hypoxanthine phosphoribosyltransferase (*HPRT*) (11, 12) differ on the two X homologues. The CpG dinucleotide clusters, in the 5′ promoter region of *HPRT* and the 3′ coding region of *G6PD*, are hypomethylated on the active X in all tissues analyzed, whereas these clusters on the inactive X are extensively methylated in most tissues. An exception is placenta, as the *G6PD* and *HPRT* loci on the inactive X chromosome in this tissue are relatively undermethylated (5, 11); the inappropriate dosage of *Hpa* II cleavage products around CpG clusters in placenta from females raised the possibility that many inactive X chromosomes were methylated like active ones. Because some of these genes on the silent X might be functional, we examined the G6PD phenotype of chorionic villi, heterozygous for the common electrophoretic variants (13). Here we report our observations, which indicate that the stable dosage compensation, characteristic of the mammalian fetus proper, seems not to be a feature of the trophectoderm; many diploid cells from chorionic villi express the *G6PD* locus on the allocyclic X. The incomplete dosage compensation in this tissue is further evidence that methylation of CpG clusters in the *G6PD* gene has a role in maintaining X dosage compensation at this locus.

## MATERIALS AND METHODS

**Cells and Tissues.** Tissues were obtained for another study of first-trimester spontaneous abortions, occurring in potential *G6PD* heterozygotes (black females) from the Obstetrics Clinic at The Johns Hopkins Hospital. Available tissues included fetal lung and chorionic villi or smooth chorion from the placenta. Chorionic villi were purified from placental tissue by teasing out the villi with a forceps and washing three times in 0.9% NaCl. Aliquots of these tissues were analyzed for G6PD phenotype and, if heterozygous, were established in culture.

Cultures were established from separate pieces of villi (or lung), and four to six cultures were established from each specimen in 60-mm tissue culture dishes containing minimal essential medium (GIBCO) enriched with 1% nonessential amino acids (GIBCO) and 15% fetal calf serum. Clones were obtained from single cell suspensions of cultured cells in early subculture, plated 10 cells per 60-mm dish. Well-isolated clones were picked with cloning cylinders 10–14 days later and transferred to 35-mm dishes. Cloning efficiency was determined from a series of 35-mm Petri dishes, plated with 20–100 cells, fixed 2 weeks later and stained with methylene blue.

**Enzyme Assay.** The cells were washed in 0.9% NaCl, mechanically removed from the dish in NADP (0.1 mg/ml of $H_2O$), and lysed by sonication; tissues were washed and lysed by freeze-thawing two times, followed by sonication. Lysates were spun at 8800 × *g* and the resulting supernatant was used for analysis. Electrophoresis was carried out by using cellulose acetate gels, presoaked in electrophoresis buffer (17 mM Tris·HCl/0.43 mM $Na_2EDTA$/47 mM glycine/43 mM sucrose/0.1 mg of NADP per ml, pH 9.2), at 350 V for 21 min. The bands were visualized by soaking gels in reaction mix (10 mg of glucose 6-phosphate/2 mg of NADP/2 mg of *N*-methyl-5-thiotetrazole/1 mg of phenazine methosulfate in 10 ml of 0.1 M Tris·HCl, pH 8). The ratios of isozymes were determined from photographs of gels.

**Chromosome Studies.** The replication patterns of the X chromosomes were determined by using BrdUrd or thymidine incorporation and Hoechst/Giemsa to reveal substituted DNA (14). Metaphases stained with giemsa were used to determine if cultures were diploid or tetraploid.

**DNA Methylation.** DNA from placenta and cultured cells was digested with *Eco*RI and *Hpa* II, electrophoresed, blotted, and probed with pGD3 (10) as described (4, 5, 10).

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Abbreviations: G6PD, glucose-6-phosphate dehydrogenase; HPRT, hypoxanthine phosphoribosyltransferase.

Table 1. Characteristics of fetal specimens heterozygous for *G6PD*

| Specimen | Age, weeks | Origin | Karyotype | G6PD phenotype of cultures |
|---|---|---|---|---|
| Sp3 | 12–13 | Chorion | 46 XX | A, B, heterodimer |
| Sp9 | 13–16 | Membrane | 46 XX, t15/15 | A, B, heterodimer |
| Sp26 | 12–14 | Villi | 46 XX | A, B, heterodimer |
| Sp44 | 11 | Villi | 46 XX | A, B, heterodimer |
| Sp77 | | Villi | 46 XX | A, B, heterodimer |
| Sp61 | 18 | Lung | 46 XX | A, B |
| Sp73 | | Lung | | A, B |
| Sp82 | 10–11 | Lung | 46 XX | A, B |
| Sp86 | 8–9 | Lung | 47 XXX | A, B |
| Sp111 | | "Fetus" | | A, B |

## RESULTS

**Heterodimers in Chorionic Villi.** Nineteen of the 111 samples expressed both *G6PD A* and *G6PD B*, and multiple cell cultures (referred to as A, B, C, etc.) were initiated from these specimens. Four of the 19 did not proliferate, 1 was contaminated with bacteria, and the cultured cells originating from 4 other specimens expressed only a single *G6PD* isozyme. The remaining 10 specimens are characterized in Table 1. Five were derived from tissues of the fetus proper, all but one from fetal lung. The other 5 had no recognizable fetus, so that only chorionic villi or intact fetal membranes were available. The two groups of specimens were comparable with respect to developmental age. However, cell cultures derived from them differed in G6PD phenotype; although each culture was clearly heterozygous, having both *G6PD A* and *B* isozymes, only cultures of trophoblastic origin had, in addition to the parental homodimers, the G6PD AB hybrid dimer (Fig. 1). The heterodimers indicate that both *G6PD* alleles were expressed in the same cell, in at least some cells of the placental cultures.

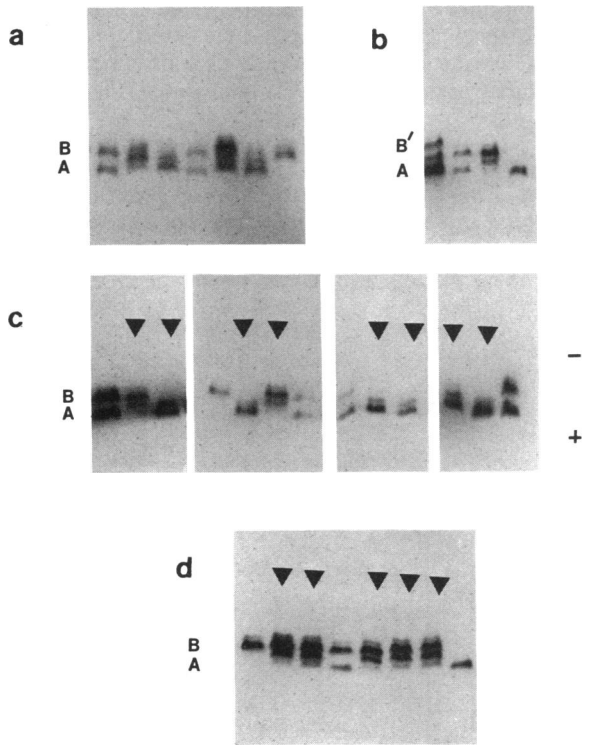


FIG. 1. G6PD phenotype of fibroblasts from chorionic villi. Lane numbers read from left to right. (*a*) Independent cultures of Sp26: Sp26A (lane 2), Sp26C (lane 3), Sp26D (lane 5), Sp26G (lane 6), and mixture of G6PD A + G6PD B fibroblasts (lanes 1 and 4). (*b*) Cultures of Sp9 and Nb1: Sp9A expressing G6PD B′, A, and heterodimer (lane 1), Nb1 (lane 3), mixture of G6PD A + G6PD B (lane 2), and G6PD A (lane 4). (*c*) Clones derived from Sp77E: clones (arrowheads) and mixtures of A + B (lanes 1, 7, 8, and 13). Clone 5d is in lane 9. (*d*) Clones derived from full-term placenta: Nb1B (arrowheads), mixture of A + B (lane 4), G6PD B (lane 1), and G6PD A (lane 8).

The distribution of heterodimers relative to homodimers is shown in Table 2. In only one specimen from Sp26 (Sp26A) were the isozymes distributed as expected if both homologous *G6PD* loci were fully expressed in each cell of the culture (i.e., 1 *A*:2 *AB*:1 *B*). In other cultures initiated from Sp26 and in cultures from the other four specimens, the heterodimer was not the most prominent isozyme; one of the homodimers (either *G6PD A* or *G6PD B*) was more prevalent. Therefore, both loci were not equally expressed and/or the cell population was heterogeneous with respect to *G6PD* expression. To determine the basis for the skewed distribution of isozymes we obtained clonal populations of cells from these specimens.

**Heterodimers in Diploid Clones.** Table 3 shows the G6PD phenotype of clones obtained from three of the five fetal chorion specimens. It is clear that the population of clones was heterogeneous: many clones from each specimen expressed only one isozyme, either *G6PD A* or *G6PD B*. However, seven clones from one culture of Sp77 (Sp77E, Fig. 1*c*) and one clone each from cultures of Sp9 and Sp26 expressed the heterodimer.

Hunt and Jacobs (15) find that ≈10% of cells of trophoblastic origin are tetraploid; our observations are generally comparable (Table 2), but some cultures are predominantly tetraploid (Sp26A and NB1E, Table 2). Therefore, some heterodimers observed in uncloned specimens may result from fusion of cells having different *G6PD* alleles on the active X. On the other hand, in most clones with the heterodimer, the hybrid molecules were not the result of cell fusion. Chromosome analysis could be carried out for five of the seven clones with the heterodimer (from Sp77E), and, in each, all of the metaphases examined were diploid (Fig. 2, Table 3). Even in clones that could not be karyotyped, the heterodimer most likely results from incomplete dosage compensa-

Table 2. Characteristics of chorionic villi cultures from fetal and newborn specimens

| Specimen | Subculture | Ratio of *G6PD* isozymes (*A*:*AB*:*B*) | Karyotype | | |
|---|---|---|---|---|---|
| | | | No. | % diploid | % tetraploid |
| Sp9A | 3 | 4:1:>1 | 101 | 98 | 2 |
| Sp9B | 3 | 4:1:2 | 54 | 98 | 2 |
| Sp9C | 3 | 1:<1:3 | 23 | 100 | 0 |
| Sp9D | 3 | 3:2:1 | 102 | 100 | 0 |
| Sp26A | 2 | 1:2:1 | 79 | 0 | 100 |
| Sp26C | 1 | 2:2:1 | 46 | 74 | 26 |
| Sp26D | 2 | 1:2:3 | 35 | 3 | 97 |
| Sp26G | 1 | 3:2:1 | 71 | 52 | 48 |
| Sp26H | 6 | 1:2:2 | | | |
| Sp44A | 3 | 2:2:1 | 59 | 93 | 7 |
| Sp44C | 1 | 1:1:2 | | | |
| Sp44D | 2 | 1:1:1 | 14 | 86 | 14 |
| Sp77D | 2 | 1:2:3 | 54 | 96 | 4 |
| | 5 | 1:2:4 | | | |
| Sp77E | 2 | 4:2:1 | | | |
| | 3 | 4:2:0 | | | |
| Nb1A | 3 | <1:1:4 | 15 | 94 | 6 |
| Nb1B | 3 | 1:2:4 | 30 | 100 | 0 |
| Nb1C | 3 | <1:1:2 | 59 | 98 | 2 |
| Nb1E | 3 | 2:1:2 | 53 | 32 | 67 |
| Nb1F | 3 | 2:3:3 | 34 | 77 | 23 |

Table 3. Characteristics of clones derived from chorionic villi cultures

| Specimen | Predominant G6PD dimer | Cloning efficiency, % | No. of clones with *G6PD* | | | No. of clones with karyotype | |
|---|---|---|---|---|---|---|---|
| | | | *A* | *B* | *A* + *B* + *AB* | Diploid | Tetraploid |
| Sp26D | B | 8.9 | 1 | 7 | 0 | 8 | 0 |
| Sp26G | A | 17.0 | 10 | 0 | 0 | 9 | 0 |
| Sp26H | B, AB | 1.3 | 0 | 0 | 1 | | |
| Sp77E | A | 21.8 | 15 | 0 | 7* | 8 | 0 |
| Sp9A | A | 2.8 | 14 | 0 | 1 | | |
| Nb1B | B | 7.7 | 0 | 3 | 9† | 9‡ | 0 |
| Nb1E | B, AB | 9.0 | 0 | 0 | 2§ | 0 | 2 |

*Five predominantly *G6PD A*; two predominantly *G6PD B*.
†All predominantly *G6PD B*.
‡Including five of the seven with heterodimer.
§Both predominantly *G6PD A*.

tion; no clone had the 1:2:1 ratio of *A*:*AB*:*B* isozymes expected for tetraploids resulting from cell fusion. The predominance of one homodimer (Fig. 1 *c* and *d*) suggests that one X allele is more active than the other.

**Incomplete Dosage Compensation in Full-Term Placenta.** To determine if dosage compensation was also incomplete in the placenta at term, we examined chorionic villi from a newborn heterozygous for G6PD. Amniotic fluid cells cultured from this normal female at 16 weeks of gestation expressed *G6PD A* and *B* but not the hybrid dimer. In contrast, cultures from the chorionic villi at term had the heterodimer (Fig. 1*b*, lane 3, and Table 2), and two clones derived from one culture and several from another (Fig. 1*d*, Table 3) expressed the locus on the inactive X. The clones from Nb1B could be karyotyped and were all diploid. Clones from the predominantly tetraploid culture Nb1E did not proliferate well, but two could be analyzed; each had the heterodimer, but the G6PD A homodimer predominated.

**Replication Is Allocyclic.** Clonal populations from fetal and newborn chorionic villi were exposed to BrdUrd or thymidine (or both) during DNA synthesis to determine if cells with the heterodimer had a late replicating chromosome. In both clones, the X chromosome remained allocyclic (Fig. 2) and seemed to replicate like an inactive X; more detailed studies of the sequence of late replication are necessary.

**DNA Methylation Studies.** The pattern of *G6PD* isozymes (with one parental homodimer predominant) in these exceptional diploid cells of trophoblastic origin is reminiscent of that of the spontaneous G6PD reactivant from skin fibroblasts (4) and reactivants induced in mouse–human hybrid cells by 5-azacytidine (5, 9, 11). In each case, expression of the locus on the inactive X was accompanied by hypomethylation of CpG clusters in the relevant gene (5, 11) and acquisition of nuclease hypersensitivity (16). We examined DNA from two of the diploid clones, which expressed the heterodimer (Sp77E 5b and 5d), for the pattern of methylation around *G6PD* loci, using genomic *G6PD* pGD3 (10) as probe (Fig. 3). In each case, we observed only the *Hpa* II fragments characteristic of DNA from the active X (5, 10). In cultures from which these clones were obtained, both *G6PD* alleles resembled active alleles (77E, lane 5), not surprising because of the large number of clones with heterodimers derived from this culture. On the other hand, another culture from this specimen did not express the heterodimer, and the A-1 CpG cluster on the inactive X chromosome in cells of this culture was more extensively methylated (Fig. 3, lane 7).

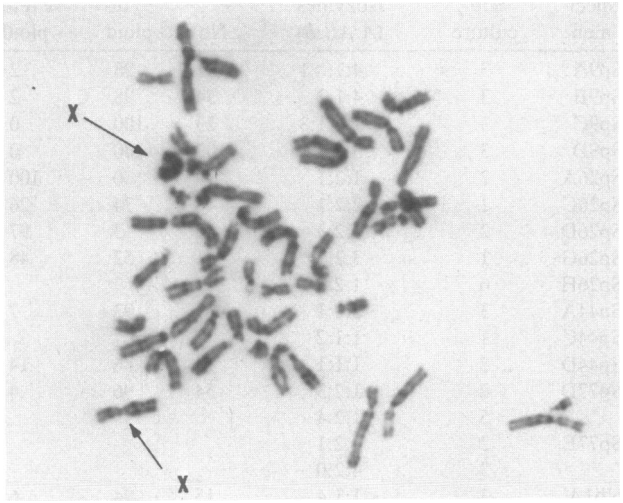


FIG. 2. Metaphase from clone 5d (from chorionic villi specimen 77E) that expressed the heterodimer (see Fig. 1*d*), showing diploid karyotype and allocyclic X (long arrow). BrdUrd, Hoechst/Giemsa stain, terminal pulse of thymidine (14).

## DISCUSSION

**Extent of the Expression of the Allocyclic X.** Observations of five fetal and one newborn specimen of trophoblastic tis-

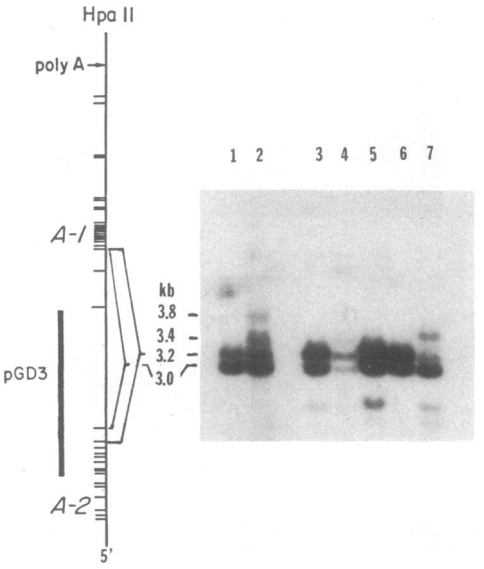


FIG. 3. Southern blot of DNA from chorionic villi cultures of Sp77 and diploid clones that express the heterodimer, showing DNA methylation at the *G6PD* locus and location of CpG clusters and probe. Uncultured chorionic villi from male (lane 1) and female (lane 2); clones 5d (lane 3) and 5b (lane 4) derived from Sp77E (lane 5); Sp77D also having the heterodimer (lane 6) and Sp77B without the heterodimer (lane 7). DNA was digested with *Eco*RI and *Hpa* II and probed with pGD3. kb, Kilobases.

sue indicate that some, but not all, cells in each specimen express the *G6PD* locus on two X chromosomes. This population is heterogeneous, consisting of tetraploid cells with two *active* X chromosomes and diploid cells that express the locus on both the active and inactive X. Because of this heterogeneity, it is difficult to determine the level of expression of the locus on the inactive X. The inequality of gene product in diploid clones suggests that one chromosome is more active than the other; however, these clonal populations could be heterogeneous, if the locus on the allocyclic X were unstable. On the other hand, the expression of the allocyclic X seems relatively stable; the heterodimer in clones that expressed it was present for several subcultures (data not shown). Two subclones were isolated from Nb1B and the G6PD phenotype of both subclones resembled that of the progenitor clone. Furthermore, the frequency of clones with heterodimers in chorionic villi from the full-term specimen seemed greater than that in first-trimester specimens (Table 3), unlikely if the locus on the allocyclic X were in process of being silenced. Hybrid cells derived from these clones will provide the means to determine if the *G6PD* locus on the allocyclic X is underexpressed and if other loci on the chromosome are expressed. Even though the chromosome remains late replicating, we suspect that *G6PD* is not the only active locus (see below).

**Inactivation Is Random in Human Placenta.** The ratio of parental isozymes, not obviously unequal in tissues prior to culture, was significantly skewed in cells cultured from these specimens (Table 2). Therefore, it is not surprising that clones derived from the same culture frequently expressed the same G6PD phenotype (Table 3). Such phenotypic homogeneity most likely reflects the small number of progenitor cells originating these cultures; trophoblastic cells proliferate less well, senesce earlier, and clone less well than fetal lung cells (unpublished observations). On the other hand, either G6PD variant might predominate in independent cultures from a specimen (Fig. 1*a* and Table 2: compare Sp9A vs. Sp9C, Sp26D vs. Sp26G, Sp44A vs. Sp44C, and Sp77D vs. Sp77E), and clones of both A and B type are found within a single specimen (Table 3—i.e., Sp26D and Sp26G). Therefore, these observations of cultured cells confirm previous ones of fresh tissues (17, 18) showing that the active X in chorionic villi can come from either parent. The random choice of active X in human chorionic villi contrasts with the situation in all trophectoderm derivatives and the yolk sac endoderm of the mouse, in which the active X is always maternal (19–21). Species differences in origin of the active X may reflect species differences in the origin of these trophectoderm derivatives. On the other hand, human cells of trophectoderm derivation clearly differ from those of the fetus proper, with respect to dosage compensation, presumably because they differentiate earlier. In any event, paternal X inactivation is not an essential feature of X dosage compensation in extraembryonic membranes of mammals.

**Relation to Competence of Inactive X DNA in Gene Transfer.** Kratzer *et al.* (22) have reported that DNA from the inactive X in mouse yolk sac endoderm can complement *HPRT* activity of mouse cells in DNA-mediated gene transfer, whereas similarly prepared DNA from tissues of the embryo proper cannot. They interpreted their findings to indicate that the DNA modification (presumably methylation) responsible for noncompetence of DNA from the allocyclic X in the embryo is less prevalent in extraembryonic tissues. Our demonstration that some placental cells express the *G6PD* locus on the inactive X provides an explanation for their observations. They did not observe activity of the *HPRT* locus on the inactive X in mouse yolk sac endoderm, but these assays were carried out in murine tissues—significant as we have observed the heterodimer in only one sample of uncultured tissue—perhaps for technical reasons. Examination of cultured cells from these extraembryonic tissues may reveal that dosage compensation is incomplete in murine tissues as well.

**Hypothesis: Partial Dosage Compensation Is Attributable to Incomplete Lock-In Mechanism.** The expression of the locus on the allocyclic X results from either failure to establish dosage compensation at the onset or faulty maintenance of the process. The absence of clones with the precise 1:2:1 ratio of *A:AB:B* isozymes that characterizes two fully expressed loci in 69 XXY triploid cells (23) or hybrids derived from diploid cells (24) may indicate that these trophoblastic cells did not fully escape X inactivation. Furthermore, the studies of *HPRT* (11) and *G6PD* loci (5) on the human X suggest that the role of methylation in X dosage compensation is to maintain the dimorphic state by promoting transcription of the locus on the active X and inhibiting transcription of the inactive one. Conceivably, in trophoectoderm, among the earliest tissues to differentiate (25), the CpG clusters in the *G6PD* gene on the inactive X fail to be methylated or are methylated less than those in later differentiating tissues; as a consequence, the silence of the *G6PD* gene on the allocyclic X is not well maintained.

The association of hypomethylation of the 3′ CpG clusters at the *G6PD* locus with expression of the locus on the allocyclic X in chorionic villi is further evidence that CpG clusters have a role in transcription of this locus and maintenance of dosage compensation of this "housekeeping" gene. Because the 5′ CpG cluster at the *HPRT* locus is also undermethylated in female placenta (11), it is likely that this locus on the allocyclic X in placenta is expressed as well. In fact, hypomethylation is not limited to *G6PD* or to X-linked genes, as globin sequences (26) and repetitive DNA sequences (27) are also undermethylated in trophoectoderm; some autosomal genes, presumed to be silent in this tissue, may also be expressed if hypomethylation plays a significant role in their regulation. Further studies of this extraembryonic tissue should provide the means to obtain new insights into early events that determine methylation patterns as well as other determinants of gene activity.

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