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Genetics

Transformation with DNA from 5-azacytidine-reactivated X chromosomes

(methylation/X chromosome inactivation)

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ABSTRACT **It has been shown that 5-azacytidine (5-Aza-Cyd) can reactivate genes on the inactive human X chromosome. It is assumed that the 5-Aza-Cyd acts by causing demethylation of the DNA at specific sites, but this cannot be demonstrated directly without a cloned probe. Instead, we have utilized the technique of DNA-mediated transformation to show that the 5-Aza-Cyd-induced reactivation occurs at the DNA level. DNAs from various mouse–human or hamster–human hybrid cell lines, deficient for mouse or hamster hypoxanthine phosphoribosyltransferase (HPRT, EC 2.4.2.8) and varying in whether they contained either an active or inactive human X chromosome, were used in transformation of HPRT$^-$ cells. DNA from the active human X chromosome-containing cell lines yielded HPRT$^+$ transformants, whereas DNA from the inactive X chromosome-containing cell lines did not. The inactive X chromosomal DNA was able to transform thymidine kinase-deficient mouse cells, indicating that the DNA solution was normal. These results confirm that inactivation of the X chromosome involves a DNA modification. Furthermore, DNAs from three cell lines with a 5-Aza-Cyd-reactivated X chromosome also transform HPRT$^-$ cells, demonstrating that the 5-Aza-Cyd has altered the DNA structure and supporting the idea that methylation plays a role in X chromosome inactivation.**

Dosage compensation for most of the X chromosome is achieved in mammalian females early in development when one of the X chromosomes undergoes a condensation and ceases most transcriptional activity (1, 2). In eutherian mammals, either the maternal or paternal X chromosome may be inactivated in somatic cells, but once established, the pattern of inactivation remains the same for each cell and its descendants. It was originally proposed that the entire X chromosome is inactivated, but there is now evidence that the steroid sulfatase (STS; sterol-sulfate sulfohydrolase, EC 3.1.6.2) locus and the *Xg* locus to which it is linked escape inactivation (3–6).

Liskay and Evans (7) have shown that DNA from the inactive X chromosome will not function in DNA-mediated transformation of the hypoxanthine phosphoribosyltransferase (HPRT; EC 2.4.2.8) gene, suggesting that the inactive X chromosome has been modified at the DNA level. One possible DNA modification that has been proposed as a mechanism for X chromosome inactivation is methylation of cytosine residues (8–10). Recently, a number of reports have appeared correlating hypomethylation of specific sites with gene expression (for reviews see refs. 11 and 12). 5-Azacytidine (5-Aza-Cyd) is an analog of cytidine known to inhibit methylation of DNA (13). It has been used in a number of studies designed to investigate the relationship between DNA methylation and gene activity. Mohandas *et al.* (14) treated mouse–human hybrid cells, retaining an inactive human X chromosome, with 5-Aza-Cyd and were able to demonstrate reactivation of the human *HPRT* locus. Some of the HPRT$^+$ clones obtained also showed reactivation of other X chromosome-linked enzymes, glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49) and phosphoglycerate kinase (PGK; EC 2.7.2.3). This result suggests that DNA methylation plays a role in the maintenance of X chromosome inactivation but does not directly demonstrate an alteration of the *HPRT* gene at the DNA level. To obtain direct evidence that 5-Aza-Cyd modifies DNA when it reactivates the *HPRT* locus, we have carried out transformation experiments with DNA from the cells that had been reactivated by 5-Aza-Cyd. Our results confirm the findings of Liskay and Evans (7) and also show that the 5-Aza-Cyd-reactivated X chromosome is functional in HPRT transformation, supporting the methylation model of X chromosome inactivation.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. Isolation of the mouse–human and hamster–human hybrid lines from which the DNAs for this experiment were extracted has been described. For the mouse–human lines, one parent was the HPRT$^-$ mouse fibroblast, A9, and the other was a human fibroblast from a female carrying a balanced X autosome translocation. Two clones retaining the active human X chromosome but not the inactive one are designated 25-3 and 25-8 (4). The clone retaining the inactive human X chromosome but not the active one is designated 37-26R-D (5). 37-26R-D was treated with 5-Aza-Cyd as described (14) to yield two clones: 37-26R-D-1c expresses human HPRT and G6PD but not PGK, whereas 37-26R-D-1a expresses human HPRT and PGK but not G6PD. Prior to any DNA isolation, 37-26R-D was checked for the presence of the inactive X chromosome by assay for STS activity (4). The parents of the other hybrid lines used were the HPRT$^-$ hamster fibroblast, CHW 1102, and a human fibroblast from a female carrying a balanced X/9 translocation (15). Clone 11-10 retained the active and inactive human X chromosomes and, after selection in 8-azaguanine, it yielded clone 11-10R-a, retaining only the inactive human X chromosome. 11-10R-a was treated with 5-Aza-Cyd as above to yield a clone, 11-10R-a-9a, which expresses human HPRT and α-galactosidase but not G6PD or PGK.

Three cell lines were used as recipients in the transformation experiments. LTK$^-$ clone 1D has been described (16) and was obtained from M. Liskay. Cell line 613 is an HPRT$^-$ mutant derived from the LTK$^-$ line and provided by R. DeMars. It has a reversion frequency at the *HPRT* locus of $<1 \times 10^{-8}$ (17). Cell line 380-6 is an HPRT$^-$ Chinese hamster cell line provided

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Abbreviations: 5-Aza-Cyd, 5-azacytidine; HPRT, hypoxanthine phosphoribosyltransferase; G6PD, glucose-6-phosphate dehydrogenase; PGK, phosphoglycerate kinase.

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Table 1. Transformation of HPRT by hybrid cell DNA

Donor DNA	HPRT⁻ 613 cells: Positive per total plates	HPRT⁻ 613 cells: Colonies per cells treated	HPRT⁻ 613 cells: Overall frequency	HPRT⁻ 380-6 cells: Positive per total plates	HPRT⁻ 380-6 cells: Colonies per cells treated	HPRT⁻ 380-6 cells: Overall frequency
No DNA	0/26	$0/5.2 \times 10^7$	0	—	$0/1.5 \times 10^8$	0
25-3*	8/8	$40/1.6 \times 10^7$	2.5×10^{-6}	—	—	—
25-8*	—	—	—	3/30	$7/5.8 \times 10^7$	0.12×10^{-6}
37-26R-D†	0/53	$0/1.3 \times 10^8$	0	0/30	$0/2.9 \times 10^7$	0
37-26R-D-1c‡	12/12	$12/2.4 \times 10^7$	0.5×10^{-6}	5/30	$8/6.2 \times 10^7$	0.13×10^{-6}
37-26R-D-1a‡	12/15	$19/3 \times 10^7$	0.63×10^{-6}	—	—	—
11-10R-a†	0/15	$0/3 \times 10^7$	0	—	—	—
11-10R-a-9a‡	9/15	$9/3 \times 10^7$	0.30×10^{-6}	—	—	—

* Cell lines carrying an active human X chromosome.
† Cell lines carrying an inactive human X chromosome.
‡ Cell lines carrying a 5-Aza-Cyd-reactivated human X chromosome.

by U. Francke. When this last-named line was tested for reversion in the absence of DNA treatment, none was seen (Table 1). However, revertants did arise during transformation experiments with cell line 380-6 at a frequency of 1.5×10^{-8}, so all clones were examined electrophoretically to insure accurate scoring.

DNA Isolation. Isolation of DNA from frozen cell pellets was by a modification of the method of Blin and Stafford (18). After proteinase K/NaDodSO$_4$ treatment and phenol extraction, the DNA was either dialyzed against 0.05 M Tris, pH 8/0.01 M EDTA/0.01 M NaCl or was precipitated in isopropanol and dissolved in a similar buffer. The DNA was again dialyzed or precipitated after RNase A treatment and phenol extraction.

The DNA solutions used had A_{260}/A_{280} of 1.5–1.9. DNA lengths were shown to be >50 kilobases on 0.4% agarose gels.

DNA Transformations and Selection. Transformations were performed with the DNA/calcium phosphate precipitation technique of Wigler *et al.* (16). Twenty μg of DNA was added per plate. Cells of line 613 were additionally treated for 30 min with 10% dimethyl sulfoxide at the end of the DNA exposure period. After 24 hr posttransformation in nonselective media, the LTK⁻ cells were selected for TK⁺ transformants, and the cells of line 380-6 were selected for HPRT⁺ transformants by exposure to hypoxanthine/aminopterin/thymidine medium (19). Cells of line 613 were selected for HPRT⁺ transformants by using hypoxanthine/azaserine medium (17). Transformation frequencies were calculated by assuming one doubling of the 10^6 recipient cells prior to DNA addition.

Enzyme Electrophoresis. Apparent transformants were isolated and expanded for testing. Identification of HPRT activity was by starch gel electrophoresis (pH 6.8; gel buffer was 6.1 mM K_2HPO_4/1.2 mM citric acid; tank buffer was 167 mM K_2HPO_4/21.7 mM citric acid) or by isoelectric focusing and autoradiography (20). Analysis of G6PD activity was by cellulose acetate electrophoresis (21).

RESULTS

Transformation Experiments With Inactive X Chromosomal DNA. After 2–4 weeks of selection, cells of line 613 transformed with DNA from clone 25-3 (containing an active human X chromosome) yielded putative transformants at a frequency of 2.5×10^{-6} clones per cells treated (compared to the reversion frequency of $<1 \times 10^{-8}$). Similarly, cells of line 380-6 were transformed at a frequency of 0.12×10^{-6}. All transformation frequencies are summarized in Tables 1 and 2. Several of these transformants were shown by electrophoresis to express the human HPRT enzyme. In contrast, DNA from cells of clones 37-26R-D and 11-10R-a carrying a nonmutant *HPRT* gene on the inactive human X chromosome did not transform HPRT⁻ 613 or 380-6 cells to an HPRT⁺ state. In five separate experiments, 1.3×10^8 613 cells and 2.9×10^7 380-6 cells were exposed to the DNA, and no clones appeared after 4 wk of selection. These experiments were performed with coded DNAs to avoid biasing the scoring.

To determine that this failure to transform represents a difference in the structure of the inactive X chromosomal DNA, and not a poor DNA preparation, the same DNA preparations were used in LTK⁻ transformations. The DNA from cell line 25-3 transformed LTK⁻ to TK⁺ at a 1.9×10^{-6} frequency, whereas the 37-26R-D DNA yielded a TK⁺ transformation frequency of 0.67×10^{-6} and 11-10R-a yielded a frequency of 1.0×10^{-6}. Because reversion of the TK phenotype has not been observed in LTK⁻ cells (16), these clones can be assumed to be transformants.

Reactivated X Chromosomal DNA Does Transform for HPRT. DNA from clone 37-26R-D-1c carries the 5-Aza-Cyd-reactivated human *HPRT* and *G6PD* genes. In transformation of cells of line 613, it yielded an HPRT⁺ frequency of 0.5×10^{-6}. Nine of these clones have been examined electrophoretically, and all express the human HPRT enzyme, confirming that they are transformants (Fig. 1). The reactivated DNA also transformed cells of line 380-6 at a frequency of 0.13×10^{-6}. To rule out the possibility that 37-26R-D-1c had spontaneously reactivated, two independently reactivated lines, 37-26R-D-1a and 11-10R-a-9a, were also tested, and they successfully transformed cells of line 613 at 0.63×10^{-6} and 0.30×10^{-6} frequencies, respectively. Contamination by other human cells is ruled out by the failure to detect all human X chromosome-linked enzyme activities in these three lines. This result demonstrates that the 5-Aza-Cyd reactivation has altered the DNA structure of the *HPRT* gene.

Table 2. Transformation of TK by hybrid cell DNA

Donor DNA	TK⁻L cells: Positive per total plates	TK⁻L cells: Colonies per cells treated	TK⁻L cells: Overall frequency $\times 10^6$
No DNA	0/25	$0/5.2 \times 10^7$	0
25-3*	4/4	$15/8.0 \times 10^6$	1.9
37-26R-D†	13/15	$20/3.0 \times 10^7$	0.67
37-26R-D-1c‡	1/1	$3/2.0 \times 10^6$	1.5
37-26R-D-1a‡	8/11	$10/2.2 \times 10^7$	0.45
11-10R-a†	11/11	$16/2.2 \times 10^7$	0.73
11-10R-a-9a‡	12/13	$16/2.6 \times 10^7$	0.62

* Cell line carrying an active human X chromosome.
† Cell lines carrying an inactive human X chromosome.
‡ Cell lines carrying a 5-Aza-Cyd-reactivated human X chromosome.

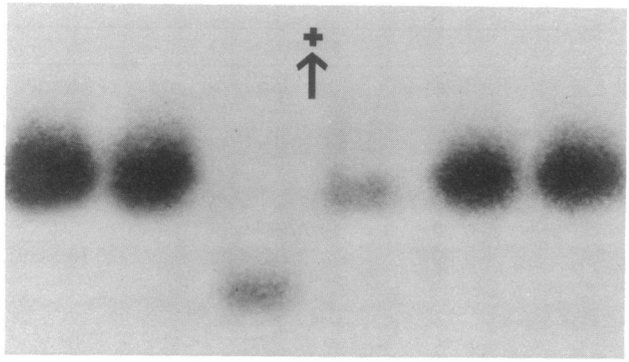

FIG. 1. Starch gel electrophoresis of the HPRT enzyme. Lanes 1, 2, 5, and 6 are extracts from 5-Aza-Cyd-reactivated transformants. Lane 3 is a normal mouse fibroblast extract, and lane 4 is a normal human fibroblast extract.

G6PD maps to the same region of the human X chromosome as does *HPRT*, and we were interested to see if cotransformation occurred for these two markers. We looked for cotransformation in 32 $HPRT^+$ clones transformed with normal human DNA and in 16 $HPRT^+$ 5-Aza-Cyd-reactivated transformants. No cotransformants were detected, and we conclude that the linkage between *HPRT* and *G6PD* is too loose for cotransformation to occur in either normal or 5-Aza-Cyd-reactivated DNA.

DISCUSSION

The ability of 5-Aza-Cyd-reactivated X chromosomal DNA to transform $HPRT^-$ cells to $HPRT^+$ cells gives strong support to the methylation model of X chromosome inactivation. 5-Methylcytosine is the only modified base in mammalian DNA, and the position of the methyl group in the DNA backbone is likely to affect protein interactions (11). It has been proposed that the presence of a maintenance methylase would provide the self-perpetuating feature necessary for inactivation of the X chromosome (11). The first evidence that DNA modification was a part of X chromosome inactivation came from the transformation experiments of Liskay and Evans (7). Their source of DNA was a CAK wild-type mouse line containing an active and an inactive X chromosome. Mutagenesis provided two separate $HPRT^-$ clones, presumably with the mutation in the active X chromosomes. DNA from neither of these clones would transform $HPRT^-$ recipients. This also was confirmed with a similarly derived hamster cell line as donor. Though unlikely, it was possible that their negative result was due to concomitantly mutagenizing the inactive *HPRT* locus or due to a spontaneous mutation at that locus. In our experiments, we are able to directly confirm the integrity of the *HPRT* locus on the inactive X chromosome by demonstrating successful transformation when the same chromosome is reactivated by 5-Aza-Cyd.

Our demonstration that 5-Aza-Cyd-reactivated X chromosomal DNA is able to transform $HPRT^-$ cells is also important in showing that the 5-Aza-Cyd reactivation previously reported (14) occurs at the DNA level. Furthermore, because the donor cells were grown for a number of generations in the absence of 5-Aza-Cyd prior to DNA isolation, these results indicate that a stable and heritable change in the *HPRT* gene has been induced.

It is believed that 5-Aza-Cyd substitutes for cytidine in DNA where the presence of nitrogen in place of carbon at the fifth position in the pyrimidine ring impairs methylation. We have shown directly that 5-Aza-Cyd reduces DNA methylation in line 37-26R-D (22). Thus, the ability of the reactivated DNA to transform $HPRT^-$ cells confirms that a structural change has been induced by 5-Aza-Cyd and that the most likely change is demethylation.

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