Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 5357–5361, September 1982
Genetics

Evidence for DNA modification in the maintenance of X-chromosome inactivation of adult mouse tissues

(Searle's translocation/DNA-mediated cell transformation/hypoxanthine phosphoribosyltransferase)

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Communicated by Clement L. Markert, June 1, 1982

ABSTRACT **The role of DNA modification in the maintenance of mammalian X-chromosome inactivation was investigated by using the technique of DNA transformation in mammalian cells. The ability of inactive X-chromosome DNA from adult mouse tissues to act in transformation for the X-linked hypoxanthine phosphoribosyltransferase gene (*Hprt*) could be ascertained by utilizing a recently discovered electrophoretic variant form of the hypoxanthine phosphoribosyltransferase enzyme and a previously available X:autosome translocation. Our findings indicate that inactive X-chromosome DNA from several tissues of adult female mice is strikingly inefficient, in comparison to active X-chromosome DNA, in eliciting genetic transformation for hypoxanthine phosphoribosyltransferase. These results provide *in vivo* evidence that is consistent with DNA modification playing an important role in the maintenance of X-chromosome inactivation.**

The somatic cells of normal diploid female mammals have two X chromosomes but only one X chromosome is active in each cell (1). The available evidence suggests that the single-active X is derived for most tissues by a process of random inactivation of either the paternal or maternal X and that once the process is initiated the inactivated state of that X chromosome is maintained through successive cell divisions (2–7). The mechanism(s) responsible for maintaining a difference between the active and inactive states could involve theoretically alterations in the structure of DNA itself (8–10), chromatin structure (11), or chromosomal proteins (12, 13), or any combination thereof.

Experimental attempts to reactivate the inactive X chromosome have been employed to provide a basis for defining the molecular mechanisms that are responsible for maintaining the inactive X. In general, attempts to select for reactivation of the X-linked hypoxanthine phosphoribosyltransferase gene (*Hprt*) suggest that random reactivation is a relatively rare event (5, 6). Recently, reactivation of human X-chromosome genes has been observed at relatively high frequency after 5-azacytidine treatment of human–mouse somatic cell hybrids containing an inactive human X chromosome (14–16). These findings suggest that DNA methylation may play a significant role in the maintenance of the inactive X.

The role of DNA modification in the maintenance of the inactive X has also been examined by asking whether active and inactive X chromosomal DNA could function in DNA-mediated gene transfer (17). In brief, DNA was extracted from mutant cell lines carrying a defective *Hprt* gene on the active X chromosome and retaining an intact, inactive X chromosome. The DNA from these cells did not produce transformants that express hypoxanthine phosphoribosyltransferase (HPRT; EC 2.4.2.8), whereas control cells with a functional *Hprt* gene did. These authors argued that the failure of such DNA to function in HPRT transformation implied a difference between inactive and active X-chromosome DNA. It is important to note that they assumed but could not prove that the *Hprt* gene on the inactive X chromosome was intact.

An electrophoretic variant for HPRT recently discovered in feral trapped mice provides a direct method of identifying active and inactive X-chromosome genes in DNA-mediated gene transfer (unpublished data). This variation segregates as an X chromosome-linked gene in F_1 matings and in backcrosses. It maps to a position on the mouse X chromosome which is consistent with somatic genetic analyses for the position of the *Hprt* structural locus (18). We have produced females that are genetically heterozygous at the *Hprt* locus but who express only one and the same allelic form of the enzyme in their tissues. To achieve this, we crossed males carrying the variant $Hprt^a$ allele with $Hprt^b/b$ females that were heterozygous for the X:autosome translocation T(X;16)16H. Chromosomally balanced female progeny carry the $Hprt^b$ allele on the translocated and active portion of the X chromosome and the $Hprt^a$ allele on the intact and inactive X chromosome. Therefore, these nonmosaic females express only the $Hprt^b$ allele in their somatic tissues. These matings also produce females heterozygous for $Hprt^a/b$ with two intact, normal X chromosomes (Fig. 1). DNA was extracted from several tissues of the translocation-bearing females and used in a HPRT cell transformation system. If the inactive X allele of HPRT is efficient in transformation, then both HPRT-A- and HPRT-B-expressing transformant colonies should be produced. However, if inactive X-chromosome DNA does not function efficiently, then all or a large majority of transformants should express only the HPRT-B enzyme. As a control, DNA from normal X/X females heterozygous for HPRT was used in parallel transformations. DNA from these phenotypically mosaic females should yield approximately equal numbers of HPRT-A and HPRT-B transformants.

Our findings indicate that inactive X-chromosome DNA of several adult mouse tissues is not efficient in HPRT transformation. These results strongly suggest that active and inactive X-chromosome DNA sequences are different at or near the *Hprt* locus and provide *in vivo* evidence that is consistent with DNA modification playing a significant role in the maintenance of X-chromosome inactivation.

MATERIALS AND METHODS

Mice. The HPRT-A electrophoretic variant used for these studies came from an Asian house mouse *Mus castaneus*. Our colony was derived from samples originally trapped in Bangkok,

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Abbreviations: HPRT, hypoxanthine phosphoribosyltransferase; PGK, phosphoglycerate kinase.

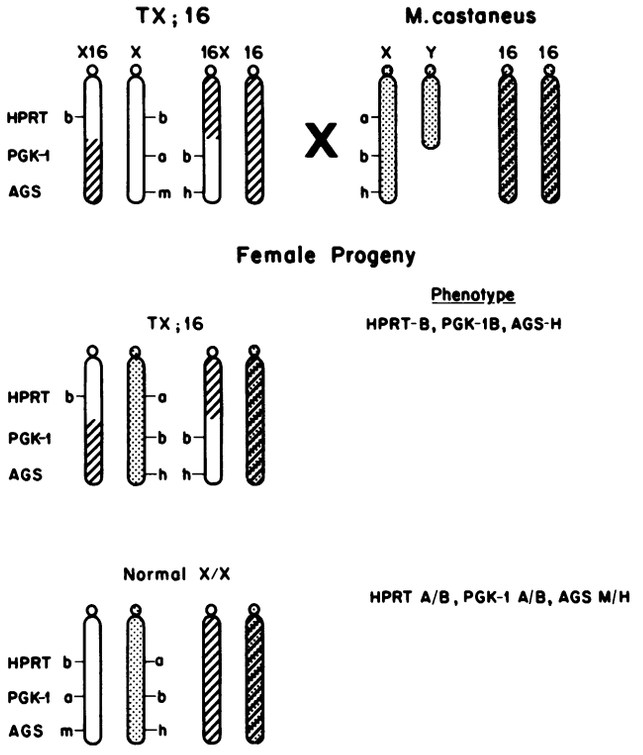

FIG. 1. Diagrammatic representation of autosome 16 and X chromosomes in TX;16 translocation females, *Mus castaneus* males, and in female progeny used for these studies. The relative positions of the genes for the enzymes HPRT, phosphoglycerate kinase (PGK), and α-galactosidase (AGS) on the intact and translocated X chromosome are shown.

Thailand, and sent to us in 1972. The variant forms of HPRT segregate as an X-chromosome gene in F_1 and backcross matings (unpublished data).

The HPRT-B electrophoretic form is common to inbred strains of mice and is present in the T(X;16)16H stock and in C3H/HeHa. *Pgk-1*^{a}, a C3H/HeHa congenic strain carrying an electrophoretic variant of another X-chromosome gene product phosphoglycerate kinase (*Pgk-1*).

The T(X;16)16H females are heterozygous for a reciprocal X:autosome translocation involving chromosome 16. The *Hprt* locus is present on the centromeric segment of the X chromosome and the breakpoint of the translocation occurs between the *Hprt* and *Pgk-1* loci (18). The T(X;16)16H females heterozygous for X-chromosome genes do not exhibit the normal mosaic phenotype observed for X-chromosome genes (19, 20). Instead, only the allele on the translocated X chromosome can be detected in our assays.

We have used the nonrandom expression to maintain and identify females heterozygous for the translocation. The original T(X;16)16H stock received from E. Eicher (The Jackson Laboratory) was *Pgk-1*^{b}/*b*. We crossed these mice to C3H/*Pgk-1*^{a} males to produce *Pgk-1*^{a}/*b* female progeny. Half of the female progeny were PGK-1A/B and half were phenotypically PGK-1B. The latter females were presumptive T(X;16) carriers and were mated with C3H/*Pgk-1*^{a} males. These females produced female progeny that were either PGK-1A or PGK-1B. Approximately 3% of the progeny were PGK-1AB, which represented recombination between *Pgk-1*^{b} and the breakpoint of the translocation. The PGK-1B (*Pgk-1*^{a}/*b*) females (*Hprt*^{b}/*b*) were used in this study as T(X;16)16H carrier females.

DNA Extraction and Transformation. DNA was extracted from mouse tissues as described elsewhere (21). The V79 Chinese hamster cell line used as the HPRT recipient and the procedures used in DNA transformation have been described (17). The spontaneous reversion frequency of this V79 line is 10^{-8}–10^{-9} (ref. 17; unpublished observations). During the transformation protocol, cells are *not* subcultured subsequent to DNA exposure and prior to selection. Therefore, colonies arising on different plates must each represent independent transformation events.

Isoelectric Focusing. Horizontal isoelectric focusing gels were used following the procedure described by Chasin and Urlaub (22).

Tissue samples were homogenized in a 0.25 M sucrose/0.02 M imidazole buffer, pH 7.4 (10% wt/vol) with a Polytron homogenizer. The samples were centrifuged at 15,000 × *g* for 20 min and the supernatants were removed and applied to filter paper wicks for isoelectric focusing.

Transformant cells were harvested, pelleted, and stored frozen at −20°C. For isoelectric focusing assay, the pellets were suspended in 0.9% saline and freeze-thawed two times. The homogenate was centrifuged 65,000 × *g* for 20 min and the supernatant was applied to wicks.

HPRT activity in the gel was identified isotopically by applying a reaction mixture over the gels following procedures described by Chasin and Urlaub (22).

RESULTS

Experimental Rationale. We have exploited a well-studied balanced X:autosome translocation (TX;16) and a recently available electrophoretic variant of HPRT in the mouse to ask whether inactive X-chromosome DNA is efficient in transformation for the X-linked gene *Hprt*. Adult female mice carrying a translocation between the X and chromosome 16 in the heterozygous condition detectably express in their somatic tissues only those X-linked genes residing on the translocated portions of the X chromosome (refs. 19, 20; see Fig. 1). The normal X chromosome is the inactive X and is not expressed at a detectable level. Therefore, such translocation-carrying females are not mosaic for X-chromosome expression. Females heterozygous for an electrophoretic variant of HPRT and the translocation were constructed from a cross of the TX;16 stock with *M. castaneus*. A diagrammatic scheme of the X chromosome and autosome 16 in the parents and female progeny is shown in Fig. 1. The TX;16 female progeny express only the HPRT-B form in >95% of their somatic cells while carrying the *Hprt*^{a} allele on the inactive X chromosome. DNA from such TX;16 females was extracted and used in a DNA-mediated gene transfer system for HPRT. Individual transformant lines were isolated and tested for their HPRT electrophoretic type. The ability of inactive X-chromosome DNA to function in transformation can be determined by the frequency of transformant clones expressing the HPRT coded for by the allele (*Hprt*^{a} in these experiments) carried on the inactive X chromosome.

To serve as a control, DNA was taken from sib females that were X/X and expressed both HPRT-A and HPRT-B in their tissues. With this DNA, both HPRT-A and HPRT-B-expressing transformants were expected, assuming that both alleles, when present on an active X chromosome, are functional in transformation.

Isoelectric-Focusing Patterns of HPRT. The isoelectric-focusing patterns of HPRT in brain, liver, and kidney for C3H/HeHa *Pgk-1*^{a} (HPRT-B), *M. castaneus* (HPRT-A), a 1:1 mixture of the HPRT-A and HPRT-B homogenates, and an F_1 female are shown in Fig. 2. The difference between these strains segregates as an X chromosome-linked gene that maps to the X chromosome in the same relative position as previously sug-

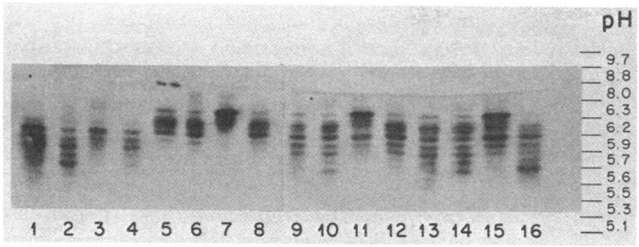

FIG. 2. Isoelectric-focusing phenotypes of mouse tissue HPRT in a pH 5.0–8.0 gradient. Lanes 1, 2, 3, and 4, *M. castaneus* (HPRT-A) brain, liver, kidney, and spleen, respectively; lanes 5, 6, 7, and 8, C3H/HeHa (HPRT-B) brain, liver, kidney, and spleen, respectively; lanes 9, 10, 11, and 12, 1:1 mixture of HPRT-A and HPRT-B brain, liver, kidney, and spleen, respectively; lanes 13, 14, 15, and 16, (C3H/HeHa × *M. castaneus*) F_1 female (HPRT-A/B) brain, liver, kidney, and spleen, respectively. The pH gradient across the gel is shown on the right.

gested by somatic cell analysis (18). Tissue-specific variation in the isoelectric-focusing pattern of HPRT was observed between brain, liver, and kidney. However, the relative difference between HPRT-A and HPRT-B is retained for each tissue. These results suggest that the difference between tissue forms of HPRT is the consequence of tissue-specific post-transcriptional modification, whereas the HPRT-A and HPRT-B difference represents an alteration at the DNA level.

HPRT Expression in X/X and T(X;16) Females. We have examined the HPRT expression in eight T(X;16) female progeny that were *Hprt*^{a}/*b* heterozygotes. No HPRT-A form could be detected in brain, liver, spleen, kidney, or bone marrow cells of these mice (Fig. 3). By contrast, normal X/X females from the same cross were phenotypically HPRT-A/B and PGK-1A/B.

We have examined the HPRT phenotypes of the progeny produced by one of the TX;16 (*Hprt*^{a}/*b*) females used for DNA-mediated gene transfer to verify that these females are capable of transmitting an intact *Hprt*^{a} allele. We obtained five progeny from this female which included two HPRT-A/B females, one HPRT-B female, one HPRT-A male, and one HPRT-B male. These results are consistent with segregation of the two *Hprt* alleles in the *Hprt*^{a}/*b* T(X;16) × *M. castaneus* F_1 female.

We also examined the chromosome number of bone marrow cells to determine whether these cells have the normal diploid chromosomal number (Table 1). These results indicate that the expression of the HPRT-B phenotype in TX;16 females is not simply a consequence of somatic X-chromosome loss.

Expression of HPRT Differences in Transformant Cells. Because the HPRT-A and HPRT-B electrophoretic difference most likely represents a structural gene alteration, this difference should be expressed in cells transformed with the appropriate DNA. Normal X/X *Hprt*^{a}/*b* heterozygous females will be a mosaic mixture of cells expressing HPRT-A and HPRT-B. DNA from the tissues of these females should be capable of producing transformants expressing either HPRT-A or HPRT-B. Brain, kidney, and liver DNA from two X/X females heterozygous for HPRT was used to transform V79 hamster cells. The HPRT phenotypes of 35 independent transformant colonies were examined and a summary of these results can be seen in Table 2. Only two HPRT phenotypes were observed among these transformant colonies and the difference between the major staining bands in these transformant phenotypes was similar to the charge difference observed between HPRT-A and HPRT-B mouse tissues. The pattern of transformant HPRT differs slightly from the HPRT pattern observed in mouse tissues but the major charge forms seem to be similar to those observed in mouse kidney homogenates (see Fig. 4). Significantly, the HPRT-A and HPRT-B isoelectric-focusing patterns of the transformant clones derived from brain, liver, and kidney DNA are identical. These results are consistent with a structural gene difference between HPRT-A and HPRT-B that can be transferred by DNA. Furthermore, the similarity of HPRT phenotypes in transformants from DNA derived from different tissues suggests that the differences in HPRT gel phenotypes of brain, liver, and kidney tissue homogenates are a consequence of a tissue-specific post-transcriptional modification of HPRT. Tissue-specific post-transcription and difference between V79 cells and mouse tissue cells could explain the slight difference in electrophoretic pattern observed. Finally, and most important, both the A and B alleles are capable of eliciting HPRT transformation.

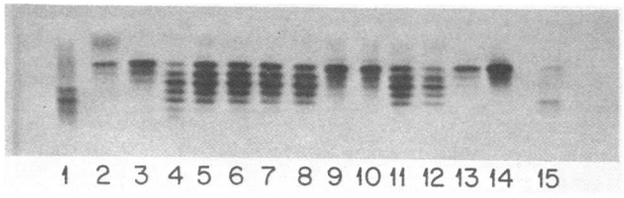

FIG. 3. Isoelectric-focusing phenotypes of T(X;16) × *M. castaneus* F_1 female HPRT-B, (*Hprt*^{a}/*b*) tissues. Lane 1, HPRT-A hemolysate; lane 2, HPRT-B hemolysate; lane 3, brain; lane 4, heart; lanes 5–8, liver; lane 9, right kidney; lane 10, left kidney; lane 11, spleen; lane 12, lung; lane 13, submandibular gland; lane 14, bone marrow; lane 15, 1:1 mixture of HPRT-A and HPRT-B kidney homogenates. Compare with tissue distributions of HPRT-A and HPRT-B tissue forms in Fig. 2.

Table 1. Chromosome number in bone marrow cells of HPRT-A/B (X/X) and HPRT-B (TX;16) female progeny of the T(X;16) × *M. castaneus* mating

Female	Chromosomes, no.			
	38	39	40	41
HPRT-A/B (XX)	1	0	12	0
HPRT-B (TX;16)	0	1	21	0
HPRT-B (TX;16)	2	4	13	0

Transformation Efficiency of Active and Inactive X-Chromosome Alleles. DNA extracted from the brain, liver, and kidney of three adult TX;16 females heterozygous for *Hprt*^{a}/*b* was applied to recipient cells to test whether the *Hprt*^{a} allelic form on the inactive X chromosome is efficient in transformation for HPRT. Fifty-nine independent transformant lines were tested for their HPRT electrophoretic phenotypes. As seen in Fig. 5 and Table 2, 58 of 59 expressed only HPRT-B. It is important to note that 24 of the transformants were induced with DNA from a female that successfully transmitted the *Hprt*^{a} to three of five progeny tested, thus indicating that she carried an intact *Hprt*^{a} allele. Therefore, the inactive X-chromosome *Hprt*^{a} allele of the TX;16 females appears to be *inefficient* in gene trans-

Table 2. HPRT electrophoretic phenotypes of transformants derived from X/X *Hprt*^{a}/*b* (HPRT-A/B) and T(X;16)16H *Hprt*^{a}/*b* (HPRT-B) female tissue DNAs

	X/X			T(X;16)		
	n	HPRT-A	HPRT-B	*n*	HPRT-A	HPRT-B
Brain	17	4	13	13	0	13
Kidney	7	4	3	27	0	27
Liver	11	1	10	19	1	18
Total	35	9	26	59	1	58

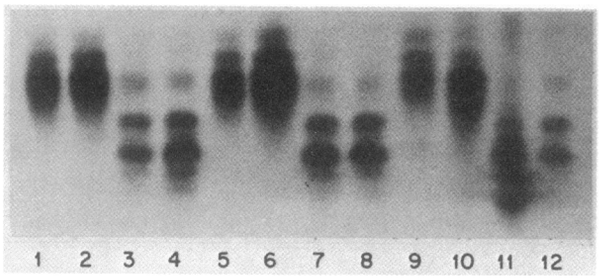

FIG. 4. pH 5.0–7.0 isoelectric-focusing phenotypes of HPRT$^+$ transformants from X/X HPRT-A/B DNA extracted from brain (lanes 1–4), liver (lanes 5–8), and kidney (lanes 9–12). HPRT-B transformants (lanes 1, 2, 5, 6, 9, and 10) and HPRT-A transformants (lanes 3, 4, 7, 8, 11, and 12).

fer. The appearance of the one exceptional HPRT-A-expressing transformant will be discussed later. It is noteworthy that the overall transformation frequency was approximately 1 in 10^6 cells (10^{-6}) treated with DNA, whereas the reversion frequency of our HPRT hamster recipient is $<10^{-8}$. Furthermore, the migration of hamster cell HPRT enzyme on our gel system approximates the mobility of mouse B form (data not shown).

DISCUSSION

We have examined the ability of inactive X-chromosome DNA from adult tissues to function in cell transformation for the *Hprt* gene utilizing an electrophoretic variant of HPRT and an X:autosome translocation in the mouse. Females heterozygous for this translocation show X-linked expression in their tissues of only those genes on the translocated X chromosome. Exploiting this feature, we constructed females that expressed only the *Hprt^b* allele and carried an inactive *Hprt^a* allele. DNA from brain, liver, and kidney of these females was used in HPRT gene transfer. Only 1 of 59 independent transformant lines expressed the HPRT-A allele from the inactive X chromosome. As a control, we used DNA from normal *Hprt^a/b* X/X females that were phenotypically HPRT-A/B. Both electrophoretic forms of HPRT were represented among 35 independent transformants from these controls. In addition, DNA from three tissues (brain, liver, and kidney) yielded transformants with identical HPRT phenotypes.

Three conclusions—two minor and one major—can be drawn from these experiments. First, the HPRT-A and HPRT-B electrophoretic variation must involve alteration of the *Hprt* structural gene because the "A" and "B" phenotypes can be transferred with DNA. Second, the variation in the isoelectric-focusing pattern seen between different tissues of the same individual is probably the consequence of tissue-specific post-

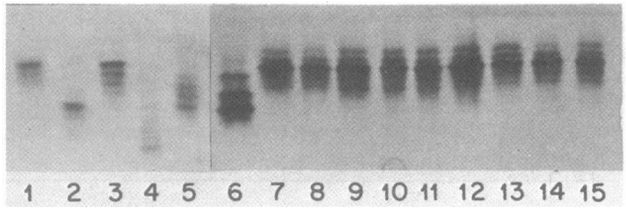

FIG. 5. pH 5.0–8.0 isoelectric-focusing HPRT phenotypes of HPRT$^+$ transformants from (TX;16 × *M. castaneus*)F$_1$ HPRT-B (*Hprt^a/b*) female DNA. Lane 1, hamster V79 HPRT$^+$; lane 2, *M. castaneus* kidney (HPRT-A); lane 3, C3H/HeHa kidney (HPRT-B); lane 4, *M. castaneus* liver (HPRT-A); lane 5, C3H/HeHa liver (HPRT-B); lane 6, HPRT-A control transformant; lanes 7–15, HPRT-B transformants from TX;16 DNA.

transcriptional modification of the primary *Hprt* gene product because these observed tissue differences are not transmitted with DNA. Third, and most important, these findings indicate that inactive X-chromosome DNA from adult tissue is *not* efficient in cell transformation for the *Hprt* gene.

It is noteworthy that one transformant expressing the HPRT-A form was obtained with DNA from the translocation heterozygotes. This transformant could represent a rare transformation event with inactive X-chromosome DNA. Alternatively, a small percentage of cells in such females could contain inactive translocated X material and an active intact X and thus, an active *Hprt^a* allele. Although we cannot detect expression of HPRT-A in such females, we cannot rule out that a small percentage of cells (<3%) indeed expresses *Hprt^a* and therefore has an intact active X chromosome. Other investigators using time of replication as an assay for inactive X-chromosome material suggest that up to 7% of the cells of an adult TX;16 female contain an active intact X chromosome (20).

Our findings are consistent with a difference between active and inactive X-chromosome DNA at or near the *Hprt* locus in somatic tissue of the adult mouse. Although we do not know at what level this difference interferes with DNA-mediated transfer of HPRT, we believe that this difference is related to the mechanism of X-chromosome inactivation. More specifically, we favor the idea that the maintenance of X-chromosome inactivation involves some form of DNA modification. Of course, DNA modification could also be involved in the random initiation event of inactivation that occurs in the embryo. However, our findings do not speak to that issue directly. This supposed modification must be reversible in light of observed X-chromosome reactivation in the female mammalian germ line (23, 24). Independent evidence for DNA modification in X-chromosome inactivation comes from the observation that human inactive X genes can be reactivated at high frequency after treatment of mouse–human cell hybrids with the DNA methylation inhibitor 5-azacytidine (14, 16). Because available evidence in other more defined systems suggests that 5-azacytidine "derepresses" genes by causing hypomethylation of the DNA (21), these findings are consistent with DNA methylation acting in the X-inactivation process. Recent evidence from studies that used clonally derived HPRT$^+$ and HPRT$^-$ human cells suggests that 5-azacytidine derepression of the inactive X chromosome may not occur in all cells at similar frequencies (25). During this study we were informed of two investigations in which DNA from cells before and after 5-azacytidine treatment had been used in transformation for HPRT (15, 26). Efficient transformation by the inactive X-chromosome allele was only achieved with DNA from cells treated with 5-azacytidine that were reactivated for HPRT. These results strongly suggest that demethylation of inactive X-chromosome DNA *per se* is a prerequisite for efficient transformation (15, 26).

There are several important features of the experimental approach used in this study that differ from previous work on gene transfer with active and inactive X-chromosome DNA (17). A major advantage was that the use of the electrophoretic variant for HPRT and a translocation allowed the examination of active- and inactive-X-chromosome *Hprt* DNA from *in vivo* sources. It seems reasonable that the genetic makeup of somatic cells *in vivo* is less susceptible to the kinds of genetic alterations that occur in cells maintained in culture. In this regard, a limitation of one previous work (17) was the inability to convincingly demonstrate that the inactive X chromosome carried an intact X-chromosome *Hprt* gene. We have shown that the inactive *Hprt^a* allele carried on the normal X chromosome of translocation heterozygotes can be genetically transmitted to progeny and therefore remains intact. One additional advantage of this system is

that we can obtain DNA that can be assayed from the active and inactive X-chromosome in a single preparation. Finally, while our experiments were in progress, another laboratory has reported on DNA-mediated transfer of HPRT using DNA from a Lesch–Nyhan carrier female-derived cell line (27). DNA from $HPRT^+$ and $HPRT^-$ clones of this cell line transformed a mouse $HPRT^-$ cell line with equal efficiency. The $HPRT^-$ (L–N) clones presumably carried an intact but inactive $Hprt^+$ gene which accounts for the HPRT transformants. We cannot easily account for these contrasting results. It is possible either that the genetic lesion of this particular Lesch–Nyhan mutation was not directly in the coding region for *Hprt* and that the functional *Hprt* gene could be separately transmitted in DNA-mediated gene transfer (27) or that simian virus 40 transformation that was used to establish the human Lesch–Nyhan carrier cell line altered the DNA of the inactive X chromosome.

In conclusion, we have used DNA-mediated gene transfer and electrophoretic variation at the *Hprt* locus to analyze the state of active and inactive X-chromosome DNA of adult mouse tissues. The results suggest that inactive X-chromosome DNA may be different from active X-chromosome DNA, thus further supporting DNA modification as an important feature in the maintenance of X-chromosome inactivation. Coupling DNA transformation and electrophoretic variation of HPRT should provide a powerful system for examining the state of inactive X-chromosome DNA from other tissues and stages of development.

We thank Melanie Murawski and Mary Kay Gruschka for their excellent technical help and Dr. T. B. Shows for the use of his Zeiss Photoscope.

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