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Methylation of the Mouse *hprt* Gene Differs on the Active and Inactive X Chromosomes

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It has been proposed that DNA methylation is involved in the mechanism of X inactivation, the process by which equivalence of levels of X-linked gene products is achieved in female (XX) and male (XY) mammals. In this study, Southern blots of female and male DNA digested with methylation-sensitive restriction endonucleases and hybridized to various portions of the cloned mouse *hprt* gene were compared, and sites within the mouse *hprt* gene were identified that are differentially methylated in female and male cells. The extent to which these sites are methylated when carried on the active and inactive X chromosomes was directly determined in a similar analysis of DNA from clonal cell lines established from a female embryo derived from a mating of two species of mouse, *Mus musculus* and *Mus caroli*. The results revealed two regions of differential methylation in the mouse *hprt* gene. One region, in the first intron of the gene, includes four sites that are completely unmethylated when carried on the active X and extensively methylated when carried on the inactive X. These same sites are extensively demethylated in *hprt* genes reactivated either spontaneously or after 5-azacytidine treatment. The second region includes several sites in the 3′ 20kilobases of the gene extending from exon 3 to exon 9 that show the converse pattern; i.e., they are completely methylated when carried on the active X and completely unmethylated when carried on the inactive X. At least one of these sites does not become methylated after reactivation of the gene. The results of this study, together with the results of previous studies by others of the human *hprt* gene, indicate that these regions of differential methylation on the active and inactive X are conserved between mammalian species. Furthermore, the data described here are consistent with the idea that at least the sites in the 5′ region of the gene play a role in the X inactivation phenomenon and regulation of expression of the mouse *hprt* gene.

In mammals, female and male cells express equivalent levels of most X-linked gene products despite the fact that females have twice the number of X-linked genes per diploid complement as do males. This "dosage compensation" of X-linked genes in mammals results from the inactivation of one of the two X chromosomes in each female cell (16) concomitant with cellular differentiation in the early embryo (24, 25, 32). Once it is established, inactivity of the X chromosome is stably inherited through successive mitotic divisions. In germ cells, however, the inactive X is reactivated just prior to meiosis (4, 8, 12). X chromosome inactivation and reactivation thus represent stable changes in gene expression that are developmentally regulated.

The mechanisms by which X inactivation and reactivation occur are unknown, but it has been suggested that X inactivation occurs in several phases, i.e., the primary inactivation event, spreading of inactivation from the site(s) of the initial event, and a process that results in maintenance of the inactive state. It has been proposed that DNA methylation has a role in the process of X inactivation (5, 7, 26, 28). Several studies of the expression of X-linked genes, including the *hprt* gene, provide indirect evidence that DNA methylation plays a role in at least the maintenance of inactivation. The *hprt* allele carried on the inactive X in fibroblasts is inefficient in transformation of hypoxanthine phosphoribosyltransferase (HPRT)-deficient cells to an HPRT-positive phenotype, whereas the *hprt* allele carried on the active X can transform these cells efficiently (2, 13, 14, 35). This fact suggests that DNAs of the inactive and active X chromosomes are differentially modified. DNA methylation is thought to be involved, since hypomethylation of the DNA after incorporation of the cytidine analog, 5-azacytidine, can result in reactivation of genes carried on the inactive X (6, 13, 23), and DNA isolated from such reactivants can transform HPRT-deficient cells efficiently (13, 36). These observations suggest that a functionally significant difference in cytosine methylation exists between the *hprt* gene carried on the inactive, as opposed to the active, X chromosome.

Studies of the methylation patterns of two human X-linked genes, *hprt* and *g6pd*, also suggest that there is differential methylation of inactive- and active-X DNAs. The human *hprt* gene was characterized by comparison of restriction patterns obtained by the digestion of female and male genomic DNAs with methylation-sensitive restriction endonucleases and hybridization to cloned DNA probes (38, 41). In addition, DNAs isolated from various mouse-human somatic-cell hybrids containing an active human X chromosome, an inactive human X chromosome, or an inactive human X carrying a reactivated *hprt* gene were similarly analyzed. A general negative correlation between activity and methylation of the 5′ region of the gene was observed, although methylation of specific sites tended to be variable. In the human *g6pd* gene, a similar analysis revealed several sites that generated female-specific restriction fragments which were also present in mouse-human somatic-cell hy-

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brids that contained only the inactive human X chromosome (33, 37). In contrast, no correlation between activity and methylation of two unidentified human X-linked sequences was found (39). However, it is not clear that these sequences are part of genes that are subject to X inactivation or what regions of the genes were analyzed.

The goal of the present study was to characterize the pattern of methylation of the mouse *hprt* gene when it is carried on the inactive and active X chromosomes. One reason for carrying out this study on the mouse gene was to begin to determine whether the observed negative correlation between activity and methylation of specific regions of the human genes is also observed in these genes in other mammals. *hprt* was chosen for study because it is the only X-linked mouse gene known to undergo X inactivation for which both full-length cDNA and genomic clones are presently available. In addition, selective and counterselective techniques are available for the isolation of rare HPRT-deficient and reactivant subclones of cultured cell lines, and thus it is possible to obtain material for the study of the gene after alterations in its activity.

MATERIALS AND METHODS

Cell lines. Clonal F_1 hybrid fibroblastic cell lines B5 and C3 were kindly provided by T. Shows (3). A subclone of B5, NLRX, containing an active *Mus musculus* X chromosome and lacking a *Mus caroli* X chromosome, was isolated by the single-cell cloning technique described by MacPherson (17). The HPRT-deficient subclone B5TGA2 was isolated by culturing B5 cells in medium containing 20 μm 6-thioguanine. Reactivants of the B5TGA2 HPRT-deficient subclone were isolated by culturing B5TGA2 cells in medium containing hypoxanthine, aminopterin, and thymidine (HAT; 15) either directly or after a 24-h treatment with 8.2 μM 5-azacytidine. The cultures were then passaged, and stocks were established. The replication pattern of the X chromosomes was determined by a bromodeoxyuridine-labeling and acridine orange-staining procedure (32).

Phosphoglycerate kinase (PGK), glucose-6-phosphate dehydrogenase (G6PD), and HPRT isozyme analyses were performed with cell lysates prepared as previously described (19). Electrophoretic separations were performed with Cellogel (Kalex Scientific). For PGK, separation and detection of the isozymes were accomplished by the procedure described by Bucher et al. (1). For G6PD, separation of isozymes was accomplished under the same conditions as for PGK, and detection of isozymes was accomplished as described by Mathai et al. (20). HPRT isozymes were separated by electrophoresis at 200 V for 3 h at 4°C with a 0.02 M phosphate buffer (pH 6.75). HPRT activity was visualized by incubating the gel for 30 min in 50 mM Tris (pH 7.5), 5 mM $MgSO_4$, 0.3 mg of phosphoribosylpyrophosphate per ml, and 50 mM [^{14}C]hypoxanthine at room temperature. The gel was blotted to a polyethyleneimine cellulose sheet (CEL 300 PEI; Brinkmann Instruments, Inc.), and the sheet was rinsed in water, dried, and exposed to film.

DNA isolation and analysis. High-molecular-weight DNA was isolated from various mouse tissues and cultured cells by the procedures described by Silver (29). Digestions with methylation-sensitive restriction endonucleases were carried out for 12 to 16 h under conditions recommended by the manufacturers (Bethesda Research Laboratories, Inc.). Completeness of digestion was monitored, in most cases, with supercoiled plasmid DNA. Gel electrophoresis was performed with 1% agarose gels, unless otherwise stated, and TBE buffer. DNA was transferred to nitrocellulose, and hybridizations were then done in a solution of 50% formamide, 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.0), 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 2× Denhardt solution (18), and 165 μg of sheared salmon testes DNA per ml at 42°C for 2 to 3 days. Filters were washed at room temperature in 2× SSC–1× Denhardt solution for 20 to 30 min, at 50°C in two changes of 1× SSC–0.1% sodium dodecyl sulfate for 40 min, in two changes of 0.1× SSC–0.1% sodium dodecyl sulfate for 40 min, and then in two changes of 0.1× SSC for 40 min. Densitometry was performed with a soft-laser scanning densitometer (LKB Instruments, Inc.).

Probes. The exon 1 subclone was obtained by isolating the *Dde*I-*Hga*I fragment of the full-length cDNA clone of the mouse *hprt* gene, pHPT5 (11), self-ligating this 57-base-pair (bp) fragment, and subcloning into pUC8. pHPT5-ex1 contains a trimer of the *Dde*I-*Hga*I fragment. Additional exon-specific subclones were obtained by digesting the 1.4-kilobase (kb) *Pst*I fragment of pHPT5 with *Ava*I, *Dde*I, *Hga*I, and *Sau*3A and subcloning into pUC12. Resultant exon-specific subclones were identified by restriction site analysis and hybridization to genomic DNA digested with *Eco*RI. The exon 3 subclone, pHPT5-ex3, contains the 123-bp *Ava*I-*Sau*3A fragment, and the exon 5 to 9 subclone, pHPT5-ex5.9, contains the 453-bp *Sau*3A-*Hga*I fragment. The intron 1-specific subclone, pHPTλ13-in1, was constructed from the mouse *hprt* genomic clone 13 (22) by digesting with *Hin*dIII and *Eco*RI and subcloning the 1.8-kb fragment into SP65. Whole plasmids or isolated fragments were nick translated for hybridization. Sequence analysis was done as previously described (22).

RESULTS

Identification of sites in the *hprt* gene that are differentially methylated in female and male. As a first step towards identifying methylation differences between sites on the inactive and active X chromosomes, comparisons were made of the restriction patterns of female and male genomic DNA digested with methylation-sensitive restriction endonucleases and hybridized to a cloned probe of the X-linked gene *hprt*. Since female somatic cells contain both an inactive and an active X chromosome, whereas male somatic cells contain only a single active X, any female-specific restriction fragments observed are likely to reflect a difference in methylation between the *hprt* gene on the inactive, compared with the active, X chromosome.

Initially, the full-length cDNA clone of the mouse *hprt* gene, pHPT5 (Fig. 1), was used to screen for such differences in DNA that had been isolated from pools of adult female and adult male mouse tissues, including brain, kidney, liver, and spleen, and digested with one of the following cytosine methylation-sensitive restriction endonucleases in combination with *Eco*RI: *Ava*I, *Hae*II, *Hha*I, or *Hpa*II. The recognition sequences of these enzymes contain the dinucleotide CG. In each case, when the cytosine is methylated, cleavage of the recognition site does not occur. In addition, a similar analysis was performed with *Msp*I, an isoschizomer of *Hpa*II, that cleaves regardless of the methylation of such cytosine residues. A total of four female-specific restriction fragments were identified: 6.3- and 1.2-kb fragments in DNA digested with *Ava*I-*Eco*RI, an 8.7-kb fragment in DNA digested with *Hpa*II-*Eco*RI, and a 7.6-kb fragment in DNA digested with *Hha*I-*Eco*RI (data not shown). In contrast, identical restriction patterns were observed in female and

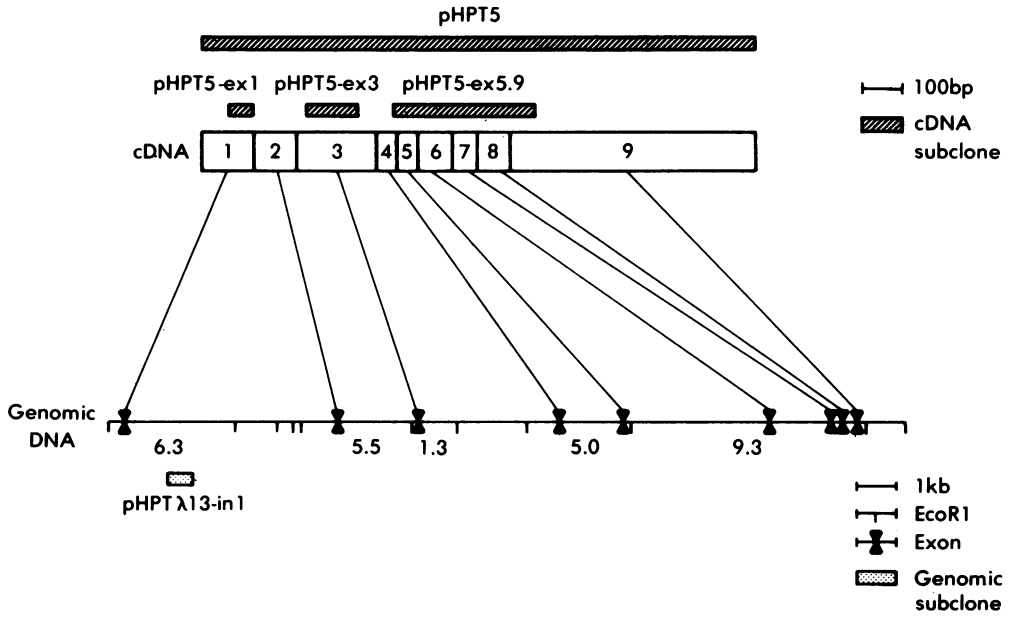

FIG. 1. Structure of the mouse *hprt* gene and cloned probes derived from it. The *hprt* gene has nine exons that are contained within almost 40 kb of genomic DNA. Its structure, including the placement of exons and a restriction map of *Eco*RI sites, is shown (from reference 22). The sizes of *Eco*RI fragments that contain exons are indicated. The full-length cDNA clone pHPT5 and subclones of it, i.e., pHPT5-ex1, pHPT5-ex3, and pHPT5-ex5.9, are depicted by hatched boxes. The genomic subclone pHPTλ13-in1 is depicted by a dotted box.

male genomic DNAs digested with *Hae*II or *Msp*I in combination with *Eco*RI or with *Eco*RI alone (data not shown). Regional localization of the restriction sites that generate these four female-specific restriction fragments identified in the initial screening with the full-length cDNA clone was accomplished by the use of the subclones illustrated in Fig. 1.

(i) Sites in the 5′ region of the gene. The 6.3-kb female-specific fragment observed in *Ava*I-*Eco*RI digests was localized by using an exon 1 subclone, pHPT5-ex1. Exon 1 is contained within a 6.3-kb *Eco*RI fragment, as evidenced by the presence of a single 6.3-kb band in female and male DNAs digested with *Eco*RI and hybridized to the exon 1 probe (data not shown). In DNA digested with *Ava*I and *Eco*RI, the exon 1 probe hybridized to 6.3- and 1.0-kb fragments in female DNA but to only a 1.0-kb fragment in male DNA (Fig. 2A). These observations in conjunction with other data (not shown) from restriction maps and sequence analysis of genomic clones (22) indicate that an *Ava*I site approximately 100 bp 3′ of exon 1 (A1.1) is differentially cut in female and male DNAs. (A1.1 refers to *Ava*I [A], exon-intron 1 region [1], and the first site 5′ to 3′ [.1] [Fig. 2C].) In female DNA this site is not cut in all cases, suggesting that it is present in both methylated and unmethylated forms, whereas in male DNA this site is always cut and thus appears to be completely unmethylated.

The exon-intron 1 region was further analyzed with an intron 1 subclone designated pHPTλ13-in1 (Fig. 1). As expected from the restriction map shown in Fig. 2C, in *Ava*I-*Eco*RI digests, two bands of 6.3 and 5.3 kb in female DNA and a single band of 5.3 kb in male DNA were detected (Fig. 2B). With the intron 1 probe, the following four additional female-specific restriction fragments, not previously observed when the full-length cDNA was used as a probe, were detected: *Hae*II-*Eco*RI digests of female DNA contained two hybridizing fragments of 6.3 and 5.3 kb in addition to the 4.9-kb fragment also present in male DNA, and *Hpa*II-*Eco*RI digests of female DNA contained two hybridizing fragments of 6.3 and 5.6 kb in addition to the 5.1-kb fragment also present in male DNA. Female and male DNAs showed identical restriction patterns when digested with *Msp*I in combination with *Eco*RI or with *Eco*RI alone (Fig. 2B). From these data it appears that in the first intron of the *hprt* gene, there is at least one *Ava*I site, two *Hae*II sites, and two *Hpa*II sites that are differentially cut in female and male. Additional Southern blot analyses with *Hin*dIII or *Taq*I instead of *Eco*RI in combination with restriction mapping and sequence analysis of genomic clones (22; data not shown) indicate that the five sites in this region that generate female-specific restriction fragments correspond to A1.1, H1.1, H1.2, M1.4-11, and M1.12 (H, M, and .4-11 refer respectively to *Hae*II, *Msp*I-*Hpa*II, and clustered sites that are treated as a single site for the purposes of this study; Fig. 2C; see also Fig. 5A). In all cases, these sites are present in both methylated and unmethylated forms in female DNA and are not methylated in male DNA.

(ii) Sites in the 3′ portion of the gene. The restriction sites that generate the 1.2-kb *Ava*I-*Eco*RI, 8.7-kb *Hpa*II-*Eco*RI, and 7.6-kb *Hpa*I-*Eco*RI female-specific restriction fragments observed in the initial screening with the full-length cDNA clone, pHPT5, were also localized, in this case to the 3′ 20 kb of the gene, with subclones of pHPT5. For example, the 1.2-kb fragment was localized with an exon 3 subclone, pHPT5-ex3 (Fig. 1). Exon 3 is contained within a 1.3-kb *Eco*RI fragment, as determined by restriction mapping of genomic clones (22) and confirmed by hybridization of the exon 3 probe to genomic DNA digested with *Eco*RI (Fig. 3A). Sequencing data show an *Ava*I site (A3.1) present within exon 3 that divides the 1.3-kb *Eco*RI fragment into two fragments of 0.1 and 1.2 kb (11). Since the exon 3 probe hybridizes to 1.2- and 1.3-kb fragments in female DNA but to only the 1.3-kb fragment in male DNA digested with *Ava*I and *Eco*RI (Fig. 3A), the 1.2-kb female-specific restriction fragment must result from differential cutting of A3.1 in female and male. The *Ava*I site is thus present in both methylated and unmethylated forms in female DNA. How-

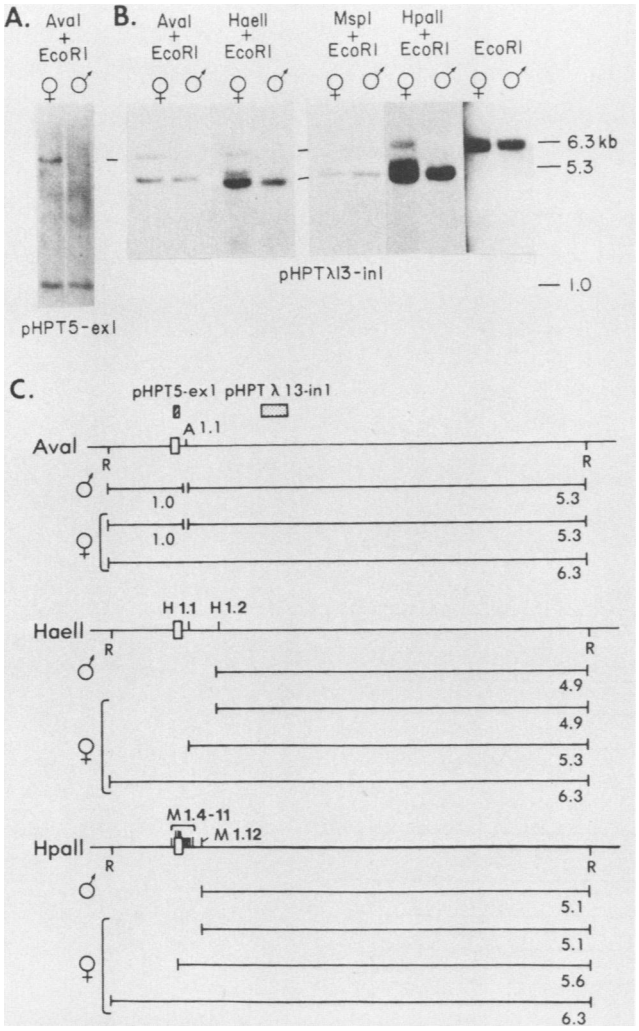

FIG. 2. Female-specific restriction fragments in the 5′ region of the mouse *hprt* gene. (A) Southern blots of female and male genomic DNAs digested with *Ava*I and *Eco*RI and hybridized to pHPT5-ex1, an exon 1 subclone. Fragment sizes were estimated from DNA markers. (B) Southern blots of female and male genomic DNAs digested with *Eco*RI alone or in combination with *Ava*I, *Hae*II, *Hpa*II, or *Msp*I and hybridized to pHPTλ13-in1, an intron 1 subclone. (C) For each enzyme (*Ava*I, *Hae*II, and *Hpa*II-*Msp*I), a map of the restriction sites present within the *Eco*RI fragment containing exon 1 is shown. The open box represents exon 1. Below each map is a depiction of the fragments detected in the Southern blots shown in panel A or B, illustrating how the differences in restriction patterns between females and males arise.

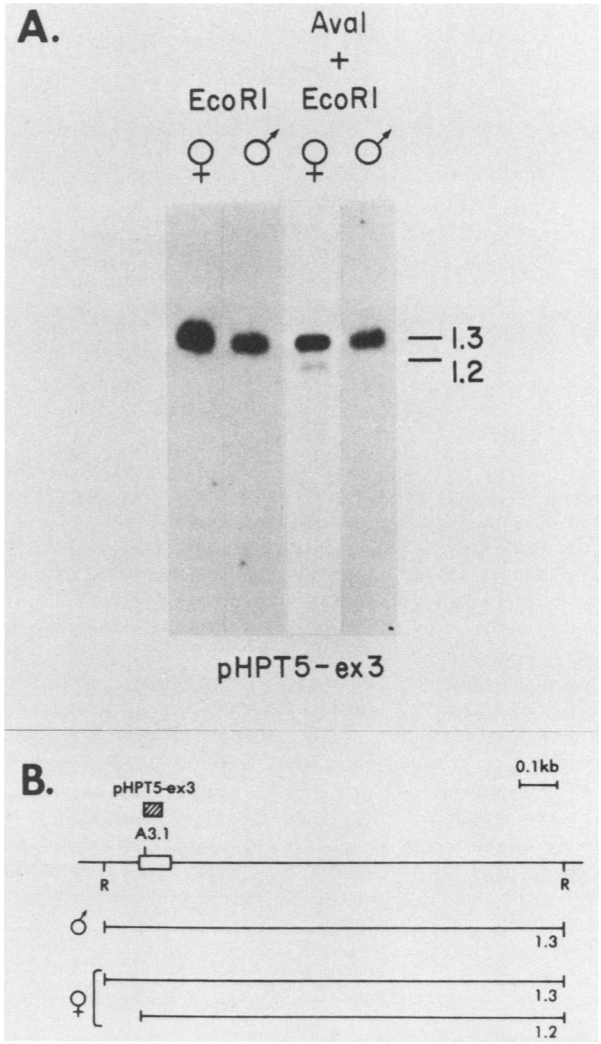

FIG. 3. A female-specific restriction fragment in the exon 3 region of the mouse *hprt* gene. (A) Female and male genomic DNAs were digested with either *Eco*RI alone or *Ava*I and *Eco*RI, separated by electrophoresis in a 1.2% agarose gel, transferred to nitrocellulose, and hybridized to pHPT5-ex3, an exon 3 subclone. Fragment sizes (in kilobases) were estimated from DNA markers. (B) A restriction map showing the *Eco*RI fragment containing exon 3, which is represented by the open box, and the location of the *Ava*I site, whose placement was determined from a published sequence of the cDNA (11). Beneath this map is a depiction of the fragments detected in the Southern blot shown in panel A, illustrating how the differences in restriction pattern between females and males arise.

ever, in contrast to the results obtained for the sites in the 5′ region, A3.1 appears to be completely methylated in male DNA.

Regional localization of the 8.7-kb *Hpa*II-*Eco*RI female-specific fragment was accomplished with a subclone of exons 5 to 9, pHPT5-ex5.9 (Fig. 1). Exons 5 and 6 through 9 are contained within 5.0- and 9.3-kb *Eco*RI fragments, respectively. However, in this study, only the 9.3-kb fragment was detectable with the exon 5 through 9 probe (Fig. 4A). This result is presumably because the 18-bp exon 5 component of the probe hybridizes weakly under the conditions used in this analysis. In genomic DNA digested with *Hpa*II and *Eco*RI, this probe hybridized to 8.7-, 3.9-, and 9.3-kb fragments in female DNA but to only the 9.3-kb fragment in male DNA (Fig. 4A). Presumably the female-specific 3.9-kb fragment was not detected in the preliminary screening with the full-length cDNA probe, pHPT5, because it comigrates with a pseudogene fragment of similar size which hybridizes to pHPT5 due to homology to the 3′ region of exon 9 (22). When *Msp*I was used in place of *Hpa*II, two fragments of 4.8 and 3.9 kb were observed in both female and male DNAs (Fig. 4A). These data, as well as information from a similar analysis with *Hin*dIII in place of *Eco*RI (data not shown) and known map positions of *Eco*RI and *Hin*dIII sites in genomic clones (22), indicate that the female-specific fragments of 8.7 and 3.9 kb were generated by differential cutting of *Hpa*II (*Msp*I) sites M5.1 (approximately 0.6 kb 3′

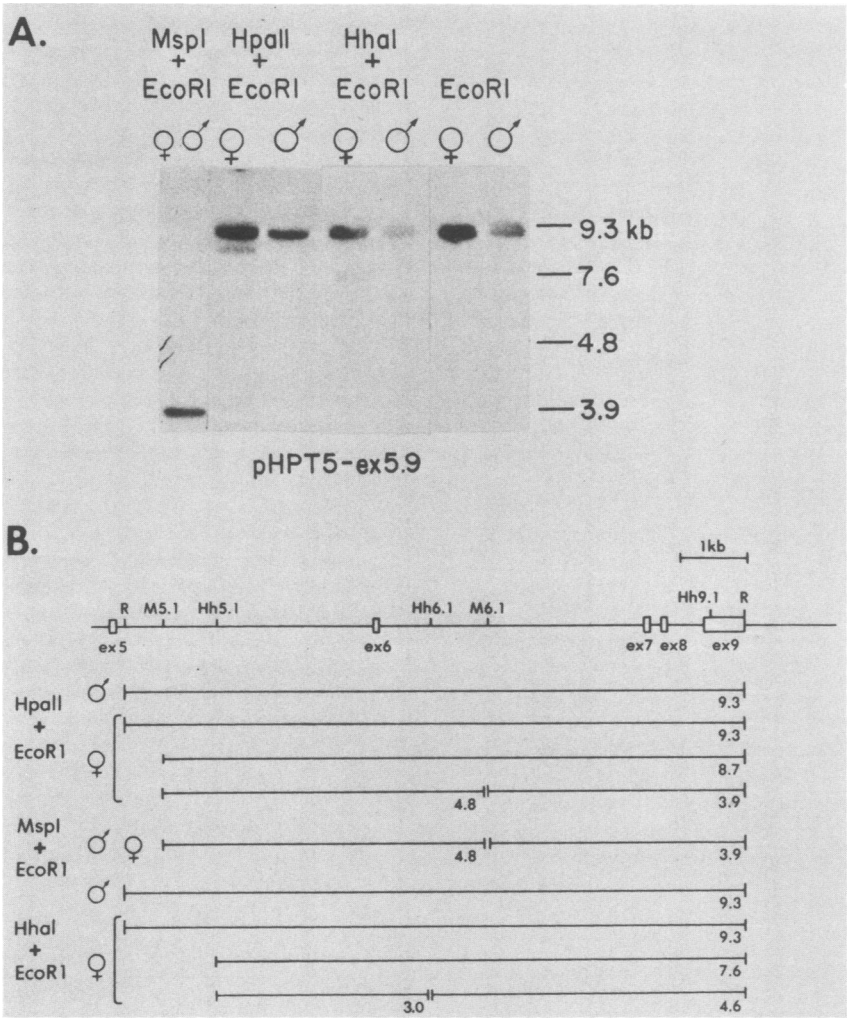

FIG. 4. Female-specific restriction fragments in the 3′ region of the mouse *hprt* gene. (A) Southern blots of female and male genomic DNAs digested with *Eco*RI alone or in combination with *Msp*I, *Hpa*II, or *Hha*I and hybridized to pHPT5-ex5.9, a cDNA subclone containing exons 5 through 9. Fragment sizes were estimated from marker DNA. (B) A map of the *Hpa*I (Hh) and *Hpa*II-*Msp*I (M) restriction sites present in the *Eco*RI fragment containing exons 6 to 9 is shown. The placement of each site, except Hh9.1, was deduced from fragment sizes observed in digests of genomic DNA hybridized to pHPT5-ex5.9 and represents the minimum number of sites present in this region. The position of Hh9.1 was determined from the sequence of the cDNA (11). Below the map is a depiction of the fragments detected in the Southern blot shown in panel A, illustrating how the differences in restriction pattern between female and male arise.

of exon 5) and M6.1 (1.5 kb 3′ of exon 6) (Fig. 4B). A 4.8-kb fragment would also be expected to be present in *Hpa*II-*Eco*RI digests of female genomic DNA in which M6.1 is cut. Presumably this fragment was not observed, since it contains only exon 6, and hybridization of exon 6 is relatively weak (see the relative intensities of the 3.9- and 4.8-kb fragments in *Msp*I-*Eco*RI digests, which contain exons 7 through 9 and exon 6, respectively). These *Hpa*II sites also appear to be present in both methylated and unmethylated forms in female DNA and in a completely methylated form in male DNA.

In *Hha*I-*Eco*RI digestions of female genomic DNA, the exon 5 through 9 probe hybridized to 7.6-, 4.6-, and 9.3-kb fragments, but to only the 9.3-kb fragment in male DNA (Fig. 4A). These data, plus additional analysis with *Hin*dIII (data not shown), indicate that these female-specific restriction fragments result from differential cutting of *Hha*I sites in intron 5 (Hh5.1) and intron 6 (Hh6.1) (Fig. 4B). A 3.0-kb fragment would also be expected to be present in *Hha*I-*Eco*RI digests of female genomic DNA in which Hh6.1 is cut. Presumably this fragment was not observed due to the relatively weak hybridization of exon 6, as previously discussed. Both of these sites appear to be present in both methylated and unmethylated forms in female DNA and in a completely methylated form in male DNA.

Direct determination of the relationship between gene expression and methylation of sites in the *hprt* gene. Assuming that active X chromosomes in female and male cells have identical patterns of methylation, the differential cleavage of recognition sites by methylation-sensitive restriction endonucleases in female and male cells suggests that the methylation of these sites differs on the inactive and active X chromosomes. However, to prove this possibility definitively, methylation of these sites on the inactive and active X chromosomes must be analyzed directly. One means of accomplishing this analysis involves the use of clonal fibroblastic cell lines isolated from a *M. musculus* × *M. caroli* F_1 hybrid female embryo. Such clones fall into two catagories; one group contains an inactive *M. musculus* X and an active *M. caroli* X, and the other group contains the converse.

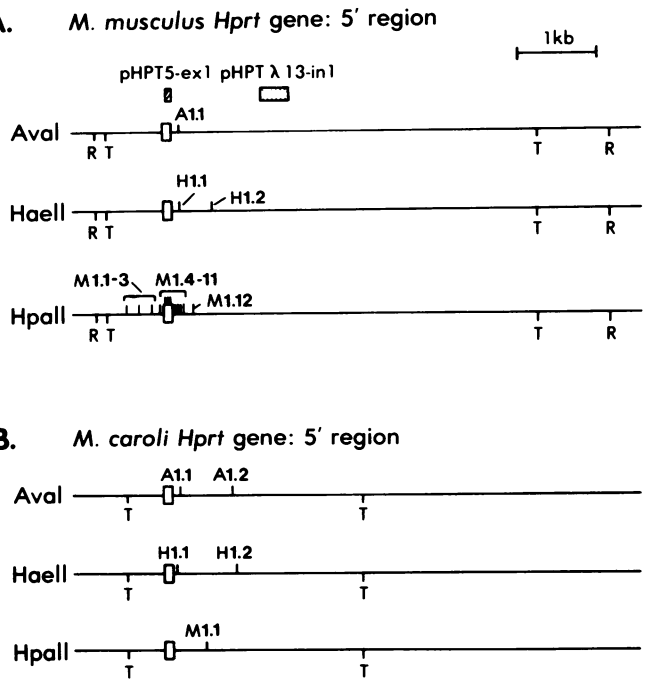

FIG. 5. The 5' region of the *M. musculus* (A) and *M. caroli* (B) *hprt* genes. The position of restriction sites for the methylation-sensitive enzymes *Ava*I, *Hae*II, and *Hpa*II, as discussed in the text, are indicated. R, *Eco*RI; T, *Taq*I. The open boxes represent exon 1.

These experiments were undertaken on the assumption that the *M. caroli hprt* gene has regions, similar to those found in the *M. musculus hprt* gene, that are differentially methylated. If this assumption is correct, direct determination of the extent of methylation of *hprt* genes carried on the inactive and active X chromosomes in clonal female F_1 hybrid embryonic cells should be possible, providing that restriction fragment length polymorphisms exist between *M. musculus* and *M. caroli* such that fragments from the *hprt* gene of each species can be distinguished when the sites of interest are either methylated or not methylated.

M. caroli mice identical to those used to produce the F_1 hybrid cells are no longer available. Therefore the positions of all restriction sites in the *M. caroli hprt* gene present in the F_1 hybrid cells had to be deduced by determining the restriction patterns of the *hprt* genes in hybrid cells and comparing them with those of the *M. musculus* gene. From such data, it is apparent which fragments in the F_1 hybrid cells are of *M. caroli* origin. For example, DNA from the F_1 hybrid cell clones digested with *Taq*I was found to contain both 5.3- and 3.0-kb fragments when hybridized to the intron 1 probe (data not shown). Since similarly digested and hybridized genomic DNAs from *M. musculus* males and females contained only the 5.3-kb hybridizing fragment, it was deduced that the 3.0-kb fragment was generated from the *M. caroli hprt* gene. The validity of the deduction was confirmed by the absence of the 3.0-kb fragment in a subclone of the F_1 hybrid cell line B5, NLRX, that contains only a single *M. musculus* X chromosome.

This and similar deductive analyses made it possible to construct the restriction map of the *M. caroli hprt* gene shown in Fig. 5B. The 5' region of the *M. caroli hprt* gene contains a minimum of two *Ava*I sites (c[A1.1] and c[A1.2]), two *Hae*II sites (c[H1.1] and c[H1.2]), and one *Hpa*II site (c[M1.1]). (c[ ] refers to sites in the *M. caroli hprt* gene, whereas m[ ] will hereafter be used to designate sites in the *M. musculus* gene.) Additional restriction sites for these methylation-sensitive endonucleases could be present in this region of the *M. caroli hprt* gene, since any sites that are completely methylated when carried on both the active and inactive X chromosomes would not be detected in this analysis. Similarly, the map of the *M. musculus hprt* gene shows the minimum number of sites located in the region between m[M1.12] and the 3' *Eco*RI restriction site, since this map is based on data obtained by hybridizing the intron 1 probe to *M. musculus* genomic DNA digested with the appropriate restriction endonucleases. However, the restriction map of the 5' region of the *M. musculus hprt* gene (Fig. 5A) is more complete because the location of sites between the 5' *Eco*RI site and m[M1.12] was determined from sequence data (22; data not shown). The existence of the *Taq*I restriction fragment length polymorphism between *M. musculus* and *M. caroli*, and other restriction fragment length polymorphisms, made it possible to carry out the analyses described below.

(i) Sites in the 5' region of the gene. To determine the state of methylation of intron 1 *Ava*I sites, genomic DNAs from the clonal F_1 hybrid cell lines C3 and B5 were digested with *Ava*I and *Taq*I and hybridized to the intron 1 probe (Fig. 6). The clonal cell line C3 contains an active *M. caroli* X and an inactive *M. musculus* X. A single *M. caroli* fragment of 1.7 kb (c[A1.2-T]) was detected, indicating that the c[A1.2] site is not methylated when it is carried on the active X. In contrast, two *M. musculus* fragments of 5.3 and 4.4 kb (m[T-T] and m[A1.1-T], respectively) were detected. The major fragment, of 5.3 kb, constitutes about 95% of the total *M. musculus* hybridization as determined by densitometric analysis. These data demonstrate that the m[A1.1] site is methylated in almost all cells in which it is carried on the inactive X. Complementary results were obtained when the B5 cell line, which contains an active *M. musculus* X chromosome and an inactive *M. caroli* X, was analyzed. A single *M. musculus* fragment of 4.4 kb (m[A1.1-T]) was detected, indicating that the m[A1.1] site in the *M. musculus hprt* gene is not methylated when carried on the active X. In contrast, three *M. caroli* fragments were detected, of 3.0 (c[T-T]), 2.3 (c[A1.1-T]), and 1.7 (c[A1.2-T]) kb. The major hybridizing fragment, of 3.0 kb, constitutes about 98% of the total *M. caroli* hybridization as determined by densitometric analysis. Since the vast majority of *M. caroli* hybridization was localized in the 3.0-kb fragment, which is generated when neither *Ava*I site is cut, it is apparent that sites c[A1.1] and c[A1.2] in the *M. caroli hprt* gene are almost always methylated when carried on the inactive X chromosome. These data are summarized in Table 1.

The extent of methylation of *Hae*II sites in the 5' region of the gene when it is carried on the inactive and active X chromosomes was determined in a similar way. When genomic DNA from the clone C3 was digested with *Hae*II and *Taq*I and hybridized to the intron 1 probe, a single *M. caroli* fragment of 1.6 kb (c[H1.2-T]) was observed (Fig. 7). Since this clone contains an active *M. caroli* X, it thus appears that c[H1.2] is not methylated when carried on the active X. In contrast, two *M. musculus* fragments, of 5.3 (m[T-T]) and 4.1 (m[H1.2-T]) kb, were detected, and the 5.3-kb fragment constituted about 75% of the total *M. musculus* hybridization. This result demonstrates that the m[H1.2] site is methylated in about 75% of the cells in which it is carried on the inactive X and that, in those cells in which site m[H1.2] is methylated, the m[H1.1] site is always

methylated also. From this analysis with the intron 1 probe, it is not possible to determine whether m[H1.1] is methylated in the 25% of cases in which m[H1.2] is not methylated. Complementary results were obtained from studies of genomic DNA from the B5 clone digested with *Hae*II and *Taq*I and hybridized to the intron 1 probe. A single *M. musculus* fragment of 4.1 kb (m[H1.2-T]) and three *M. caroli* fragments of 3.0 (c[T-T]), 2.2 (c[H1.1-T]), and 1.6 (c[H1.2-T]) kb were observed (Fig. 7). Since this clone contains a *M. musculus* active X, the presence of a single 4.1-kb *M. musculus* fragment indicates that m[H1.2] is not methylated when carried on the active X. Of the *M. caroli* fragments that hybridized with the intron 1 probe, the 3.0-kb fragment constituted 98% of the total *M. caroli* hybridization; thus, c[H1.1] and c[H1.2] are methylated in almost all cells in which they are carried on the inactive X. The results of this study as well as a similar analysis of *Hpa*II sites (data not shown) are summarized in Table 1. It should be noted that the values shown in Table 1 define the proportion of cells in the clonal F_1 hybrid fibroblast cultures in which a given site is methylated. It is apparent, however, from a consideration of the data shown in Fig. 2, that the proportion of cells in which a given site on the inactive X chromosome is methylated might not be the same in a cell population derived from a pool of adult tissues. Data to be presented elsewhere indicate that this result is due to tissue-specific variation in the extent of methylation of a given site on the inactive X (L. F. Lock, N. Takagi, and G. R. Martin, manuscript submitted).

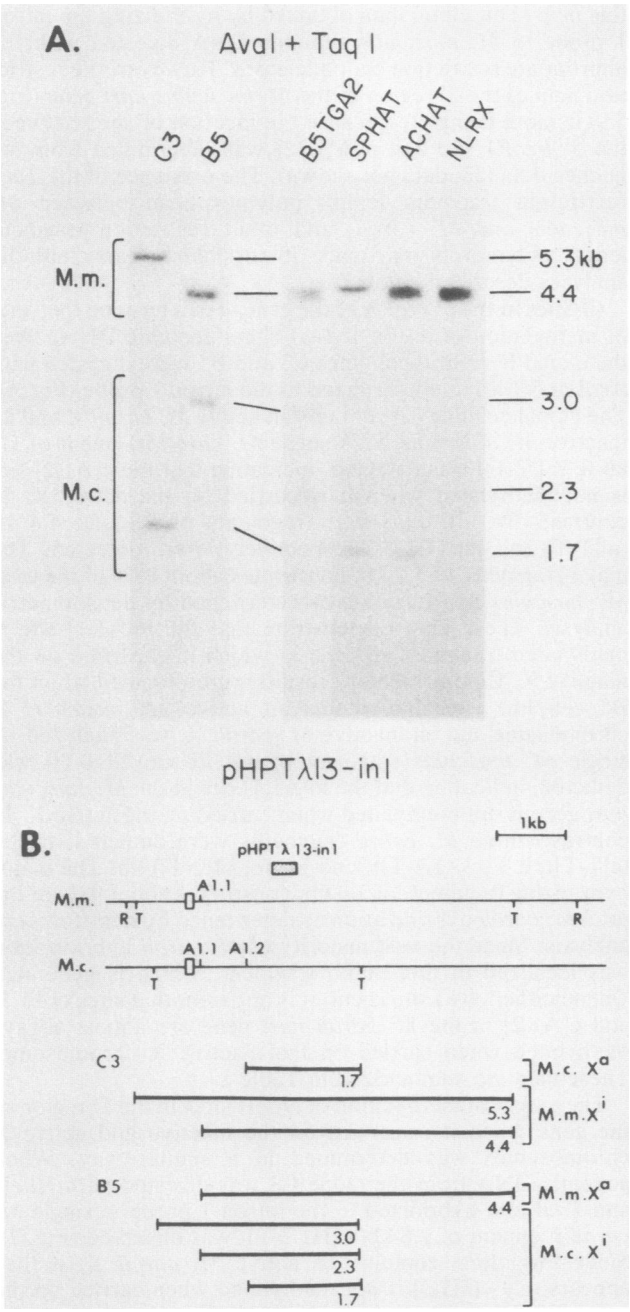

FIG. 6. Direct determination of the methylation of *Ava*I sites in the 5′ region of the *hprt* gene when carried on the active and inactive X chromosomes. (A) Southern blots of genomic DNA from the F_1 hybrid clonal cell lines C3 and B5, the HPRT-deficient subclone B5TGA2, the reactivant cultures SPHAT and ACHAT, and the subclone of B5 that lacks the *M. caroli* X (NLRX) digested with *Ava*I and *Taq*I and hybridized to pHPTλ13-in1. Fragment sizes were estimated from DNA markers. (B) For both the *M. musculus* (M.m.) and *M. caroli* (M.c.) *hprt* genes, the *Ava*I sites located in *Taq*I fragments containing exon 1, which is represented by the open box, are shown. The fragments detected in the Southern blot shown in panel A are depicted, illustrating how the differences in restriction pattern between the active X (X^a) and inactive X (X^i) arise.

In all of these studies employing the intron 1 probe, the extent of methylation of sites c[A1.1], c[H1.1], m[H1.1], m[M1.1-3], and m[M1.4-11] when carried on the active X could not be determined, because in each case a completely unmethylated site is located between these sites and genomic DNA homologous to the probe (Fig. 5). To determine the extent of methylation of these sites when carried on the active X, additional analysis was performed with the exon 1 probe (data not shown). The results of all these studies are summarized in Table 1. Within a 400-bp region of intron 1, there are four sites in the *M. musculus* and four sites in the *M. caroli hprt* gene that are invariably unmethylated when present on an active X but are extensively methylated when present on an inactive X. Sites adjacent to this region either do not differ in methylation when present on the active or inactive X, e.g., c[H1.1], or are unmethylated on the active X but considerably less extensively methylated on the inactive X, e.g., m[M1.4-11].

(ii) Sites in the 3′ portion of the gene. The female-specific restriction fragments that are generated as a result of differential cleavage by methylation-sensitive restriction endonuclease digestion of sites in the 3′ region of the *M. musculus* gene, i.e., m[A3.1], m[Hh5.1], m[Hh6.1], m[M5.1], and m[M6.1], all appeared to be completely methylated in male and variably methylated in female. Because of a lack of appropriate restriction fragment length polymorphisms, direct determination of the relationship between X-chromosome activity and methylation of only one of these sites, A3.1, could be accomplished with the F_1 hybrid clonal cell lines (Fig. 8). Because of an *Eco*RI restriction fragment length polymorphism between *M. musculus* and *M. caroli*, digestion with *Eco*RI alone results in a *M. musculus* fragment of 1.3 kb and a *M. caroli* fragment of 1.5 kb. In double digestions with *Eco*RI and *Ava*I, the same fragments are observed when the A3.1 site is methylated, but a 1.2-kb *M. musculus* fragment and a 1.35-kb *M. caroli* fragment are

TABLE 1. Extent of methylation of sites in the 5′ region of the *hprt* gene when carried on the active and inactive X chromosomes in fibroblasts

Type of X chromosome	Extent of methylation[a] (%)										
	M. musculus						*M. caroli*				
	M1.1-3	M1.4-11	A1.1	H1.1	M1.12	H1.2	H1.1	A1.1	M1.1	A1.2	H1.2
Active	—[b]	0	0	0	0	0	100	0	0	0	0
Inactive	≥20	20	95	≥75	100	75	98	98	100	98	98

[a] For each species, sites read from 5′ (left) to 3′ (right).
[b] —, Not assayable with the exon 1 probe because m[M1.4-11], which is completely unmethylated, lies between M1.1-3 and the genomic sequences that hybridize to the exon 1 probe.

observed when the A3.1 site is not methylated. When genomic DNA from the clone C3 was digested with *Ava*I and *Eco*RI and hybridized to the exon 3 probe, two fragments were observed, i.e., a *M. musculus* fragment of 1.2 kb (m[A3.1-R]) and a *M. caroli* fragment of 1.5 kb (c[R-R]). In a similar analysis of the B5 clone, two fragments were observed, i.e., a *M. caroli* fragment of 1.35 kb (c[A3.1-R]) and a *M. musculus* fragment of 1.3 kb (m[R-R]). These results demonstrate that the A3.1 site is methylated when carried on the active X and not methylated when carried on the inactive X.

Alterations in methylation pattern after reactivation of the *hprt* gene on the inactive X chromosome. The relationship between methylation and activity of the gene was further investigated by analysis of the extent of methylation of sites in inactive *hprt* genes that had been reactivated. An HPRT-deficient subclone of the B5 cell line was isolated by selection in 6-thioguanine-containing medium. This subclone, B5TGA2, was characterized biochemically and cytologically (data not shown). It was found to have an active *M. musculus* X, as evidenced by the expression of the *M. musculus* forms of G6PD and PGK; as expected, these cells do not express the *M. musculus* form of HPRT. A *M. caroli* inactive X chromosome is present in these cells, as evidenced by the observation of a late-replicating X in about 70 to 90% of metaphases examined and by the absence of expression of the *M. caroli* forms of HPRT, G6PD, and PGK.

When B5TG-A2 cells were selected for *hprt* gene expression by plating in HAT-containing medium, they formed HAT-resistant colonies at a frequency of 10^{-6}. The frequency of colony formation was increased 16-fold by treatment with 8.2 μM 5-azacytidine for 24 h before plating in selective medium. Colonies that formed spontaneously or after 5-azacytidine treatment were pooled separately and expanded. Both types of cultures expressed only the *M. caroli* form of HPRT. Thus, the ability of these cultures to grow in HAT-containing medium is a consequence of the reexpression of the *M. caroli hprt* allele on the inactive X. The spontaneous reactivant culture contained a late-replicating X in 96% of metaphases analyzed, whereas the 5-azacytidine-induced reactivant culture did not contain a late-replicating X in any of the metaphases examined. This result suggests that the *M. caroli hprt* gene expressed in the spontaneous reactivant culture is still associated with the late-replicating X chromosome, whereas in the 5-azacytidine-induced culture, it is not. Presumably, either the 5-azacytidine-reactivated *hprt* gene is still carried on the *M. caroli* X but the chromosome is no longer late replicating, or the reactivated gene is no longer associated with the late-replicating *M. caroli* X, which has been lost.

(i) Sites in the 5′ region of the gene. The inverse correlation between gene activity and methylation of intron 1 sites in the *M. caroli hprt* gene was studied in both spontaneous and 5-azacytidine-induced reactivant cultures. DNA from the HPRT-deficient cells, B5TGA2, from which the reactivants were derived, has the same restriction pattern as does DNA from the B5 cells when digested with *Taq*I and either *Ava*I (Fig. 6), *Hae*II (Fig. 7), or *Hpa*II (data not shown) and hybridized with the intron 1 probe. DNA from the B5TGA2 and B5 cells contains hybridizing *M. musculus* fragments of 4.4 (m[A1.1-T]), 4.1 (m[H1.2-T]), or 4.2 (m[M1.12-T]) kb in *Taq*I and *Ava*I, *Hae*II, or HpaII digests, respectively, and a major *M. caroli* fragment of 3.0 kb (c[T-T]) in all digestions. DNAs from the spontaneous (SPHAT) and 5-azacytidine-induced (ACHAT) reactivant cultures contain the same hybridizing *M. musculus* fragments of 4.4, 4.1, and 4.2 kb in *Taq*I-*Ava*I, -*Hae*II, and -*Hpa*II digests, respectively. However, DNAs from SPHAT and ACHAT cultures contain no 3.0-kb c[[T-T] fragment; only smaller *M. caroli* fragments could be detected. DNAs from both cultures contained hybridizing fragments of 1.7 kb (c[A1.2-T]) in *Ava*I-*Taq*I digestions (Fig. 6), 1.6 kb (c[H1.2-T]) in *Hae*II-*Taq*I digestions (Fig. 7), and 2.0 kb (c[M1.1-T]) in *Hpa*II-*Taq*I digestions (data not shown). However, in the *Ava*I-*Taq*I digestion, the 2.3-kb (c[A1.1-T]) fragment, and in the HaeII-TaqI digestion, the 2.2-kb (c[H1.1-T]) fragment, which would be expected if there was incomplete demethylation of the A1.2 and H1.2 sites, respectively, were each detected in only one of the two reactivants, and not in both. That these fragments are generated from the *M. caroli hprt* gene is confirmed by their absence from the DNA of NLRX cells, a subclone of the B5 cells that has spontaneously lost the late-replicating *M. caroli* X. In summary, the absence of the 3.0-kb *M. caroli* fragment in the reactivant cultures indicates that the *M. caroli* sites c[A1.1], c[A1.2], c[H1.1], c[H1.2], and c[M1.1] undergo extensive demethylation when the *M. caroli hprt* gene is reactivated either spontaneously or after 5-azacytidine treatment.

(ii) A site in the 3′ portion of the gene. The direct correlation between activity and methylation observed for the A3.1 site was further investigated by analysis of the extent of methylation of this site in DNA from the spontaneous and 5-azacytidine-induced reactivant cultures (Fig. 8). With respect to the c[A3.1] site in the *M. caroli hprt* gene that is reactivated in the SPHAT and ACHAT cells, no differences in restriction pattern could be detected after reactivation; the same 1.35-kb *M. caroli* fragment was detected in DNA from B5TGA2, SPHAT, and ACHAT cells, and no larger fragment was observed. This result demonstrates that the *M. caroli* gene can be expressed in the absence of methylation of the c[A3.1] site.

Interestingly, there was one difference detected in this study, reflecting a change in methylation of the m[A3.1] site on the active *M. musculus* chromosome after 5-azacytidine treatment. In DNA from the parental B5TGA2 cells as well

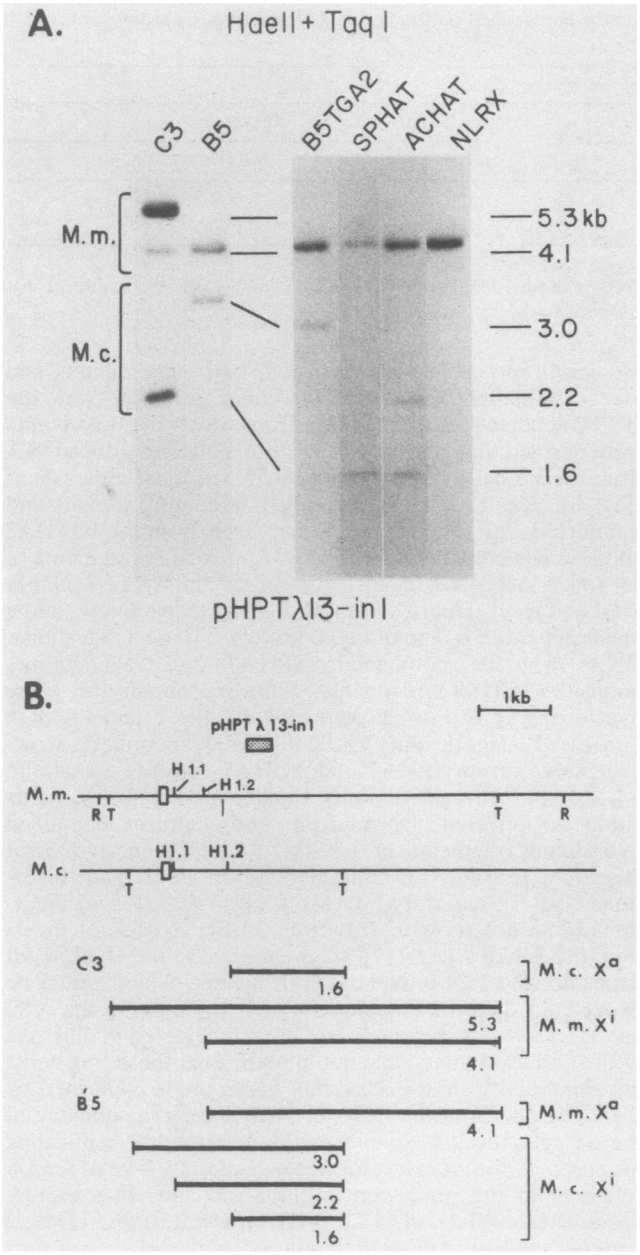

FIG. 7. Direct determination of the methylation of *Hae*II sites in the 5′ region of the *hprt* gene when carried on the active and inactive X-chromosomes. (A) Southern blots of genomic DNA from the F_1 hybrid clonal cell lines C3 and B5, the HPRT-deficient subclone B5TGA2, the reactivant cultures SPHAT and ACHAT, and the subclone of B5 that lacks the *M. caroli* X (NLRX) digested with *Hae*II and *Taq*I and hybridized to pHPTλ13-in1. Fragment sizes were estimated from DNA markers. (B) For both the *M. musculus* (M.m.) and *M. caroli* (M.c.) *hprt* genes, *Hae*II sites located in *Taq*I fragments containing exon 1, which is represented by the open box, are shown. The fragments detected in the Southern blot shown in panel A are depicted, illustrating how the differences in restriction pattern between the active X (X^a) and inactive X (X^i) arise.

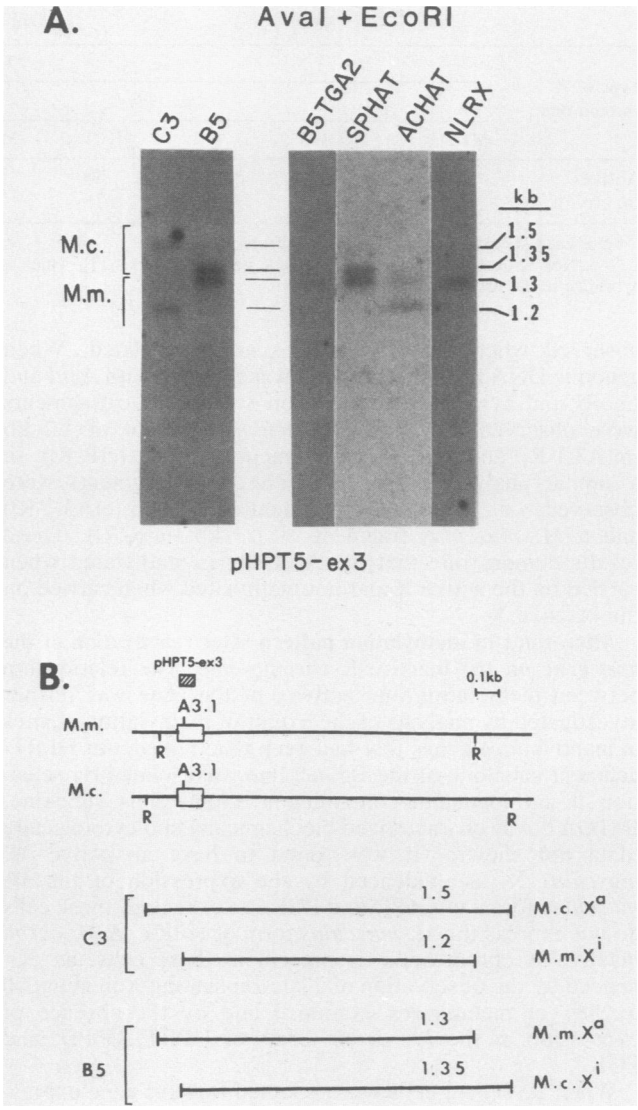

FIG. 8. Direct determination of the methylation of the *Ava*I site in exon 3 of the *hprt* gene when carried on the active and inactive X chromosomes. (A) Southern blots of genomic DNA from the F_1 hybrid clonal cell lines C3 and B5, the HPRT-deficient subclone B5TGA2, the reactivant cultures SPHAT and ACHAT, and the subclone of B5 that lacks the *M. caroli* X (NLRX) digested with *Ava*I and *Eco*RI and hybridized to pHPT5-ex3. Fragment sizes were estimated from DNA markers. (B) For both the *M. musculus* (M.m.) and *M. caroli* (M.c.) *hprt* genes, the *Ava*I sites located in *Taq*I fragments containing exon 3, which is represented by the open box, are shown. The fragments detected in the Southern blot shown in panel A are depicted, illustrating how the differences in restriction pattern between the active X (X^a) and inactive X (X^i) arise.

as from spontaneous reactivants, only the 1.3-kb *M. musculus* fragment was observed, but in DNA from ACHAT cells, the 1.2-kb fragment was also observed. Presumably this decrease in size reflects a demethylation of the m[A3.1] site as a consequence of the 5-azacytidine treatment, and this change is independent of any alteration in the activity of the *M. musculus hprt* gene. Taken together, these data indicate that there is no strict correlation between gene activity and methylation of the A3.1 site.

DISCUSSION

The characterization of the methylation of the mouse *hprt* gene reported here directly demonstrates that the 5′ region of the gene, in which there are 11 restriction sites assayable by the methods employed here involving the use of methylation-sensitive restriction endonucleases, contains a cluster

of four sites that are completely unmethylated when carried on the active X and extensively methylated when carried on the inactive X. This region extends about 400 bp 3′ of exon 1 into the first intron. Sites bordering this region either show a similar, although not as extensive, negative correlation between activity and methylation or are completely methylated irrespective of whether they are on the active or inactive X. This inverse correlation between activity and methylation of the 5′ region of the mouse *hprt* gene has been extended by analysis of the methylation of reactivated *hprt* alleles; i.e., the sites in the 5′ region of the *hprt* gene in both spontaneous and 5-azacytidine-induced reactivants are extensively demethylated.

The data described here also demonstrate that a second region of differential methylation is present in the 3′ 20 kb of the gene which spans exons 3 to 9. This region of the gene contains six sites assayable by the methods employed here. The relationship between gene activity and methylation in this region is the converse of what was observed for the sites in the 5′ region of the gene; i.e., the sites in the 3′ region are completely methylated when carried on the active X and always unmethylated when carried on the inactive X. However, this direct correlation between activity and methylation of these sites does not extend to reactivated *hprt* alleles. At least one of these sites remains unmethylated after either spontaneous or 5-azacytidine-induced reactivation of the *hprt* gene. In view of this finding, it seems unlikely that the methylation state of sites in the 3′ region of the gene is involved in the mechanism by which inactivity is maintained. The findings in this study thus focus attention on the possible role of the sites in the 5′ region in the regulation of the gene's activity, although they do not preclude the possibility that other regions of the gene are also differentially methylated and play a role in the control of its expression.

The 5′ region of the mouse *hprt* gene, extending beyond exon 1 into the first intron, is known to have a relatively unusual structure. The promoter region of this gene lacks the characteristic regulatory signals TATA and CAT (22), although it contains a nuclease-hypersensitive site when carried on the active X (40). In addition, it contains a very high proportion of the dinucleotide 5′-CG-3′ (11, 22). This high level is even more striking in view of the fact that, in general, CG dinucleotides are fivefold less frequent in the DNA of higher vertebrates than would be expected if the distribution of nucleotides were completely random (10, 27, 31). Interestingly, the homologous 5′ portion of the human *hprt* gene shares these features (9; P. Patel and A. C. Chinault, personal communication). It thus appears that the structure of the region surrounding the first exon has been conserved between mouse and human, possibly in the absence of strict sequence conservation.

The results described here, in conjunction with the observation that sites in the 5′ region of the human *hprt* gene are unmethylated when carried on the active X and methylated when carried on the inactive X (38, 41), are the first to demonstrate that not only the structure per se, but also the pattern of differential methylation in the 5′ region is conserved between human and mouse. Since evolutionary conservation often implies functional significance, this fact adds weight to the argument that methylation of sites in the CG clusters in the 5′ region of the gene play an important role in the control of its expression. Interestingly, the data reported here, in conjunction with the finding by Yen et al. (41) of one site near exon 3 of the human *hprt* gene that is methylated when carried on the active X and unmethylated when carried on the inactive X, suggest that there has also been conservation of the pattern of differential methylation in the portion of the gene containing exons 3 to 9. The functional significance of such conservation is unclear in view of the lack of a strict correlation between gene activity and methylation of the 3′ sites.

The results of studies of the human *g6pd* gene provide a second example of an X-linked gene in which there is a CG-rich region, in this case at the 3′ region of the gene, in which there are sites that are unmethylated when carried on the active X and methylated when carried on the inactive X (33, 37). This fact raises the possibility that such differential methylation plays a role not only in the regulation of expression of the gene, but also in the X inactivation phenomenon per se. However, similar CG-rich regions have also been found in a number of genes that are not X linked (21, 30, 34). Observation of such regions in non-X-linked genes does not rule out a role in the phenomenon of X inactivation. Rather, it has been suggested that, whereas the initial event leading to coordinate inactivation of genes on one of the two X chromosomes in female cells involves a unique X inactivation-specific regulatory function, the mechanism used to maintain differential expression of genes carried on the active and inactive X chromosomes might not be unique to the X inactivation process (37, 38). Thus, regulatory strategies, such as differential methylation of CG-rich regions in the *hprt* and *g6pd* gene, although not unique to the X inactivation process, could still have a role in that process.

A complete understanding of the role, if any, DNA methylation plays in the X inactivation process will be difficult to obtain solely from studies on the phenomenon ex post facto. Although the analysis of cells in which some of the genes on the inactive X have been derepressed has provided a useful approach to the problem, it will be even more efficacious to study methylation of the relevant sites on the X chromosome before, during, and immediately after X inactivation. It should then be possible to determine whether methylation is most likely associated with the process of initiation, spreading, or maintenance of repression. The data described here, identifying sites of differential methylation in the mouse *hprt* gene of possible relevance to the X inactivation process, are a prerequisite for studies designed to determine the temporal relationship between DNA methylation and the biochemical and cytogenetic manifestations of X inactivation that are feasible in the mouse. These studies might ultimately help clarify the role of DNA methylation in the process of X inactivation.

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LITERATURE CITED

1. **Bucher, T., W. Bender, R. Fundele, H. Hofrer, and I. Linker.** 1980. Quantitative evaluation of electrophoretic allo- and isozyme patterns. FEBS Lett. **115**:319–324.
2. **Chapman, V. M., P. G. Kratzer, L. D. Siracusa, B. A. Quarantillo, R. Evans, and R. M. Liskay.** 1982. Evidence for DNA modification in the maintenance of X-chromosome inactivation of adult mouse tissues. Proc. Natl. Acad. Sci. USA **79**:5357–5361.
3. **Chapman, V. M., and T. B. Shows.** 1976. Somatic cell genetic

evidence for X-chromosome linkage of three enzymes in the mouse. Nature (London) **259**:665–667.
4. **Gartler, S. M., R. Andina, and N. Gant.** 1975. Ontogeny of X-chromosome inactivation in the female germ line. Exp. Cell Res. **91**:454–457.
5. **Gartler, S. M., and A. D. Riggs.** 1983. Mammalian X-chromosome inactivation. Annu. Rev. Genet. **17**:155–190.
6. **Graves, J. A. M.** 1982. 5-Azacytidine-induced re-expression of alleles on the inactive X chromosome in a hybrid mouse cell line. Exp. Cell Res. **141**:99–105.
7. **Holliday, R., and J. E. Pugh.** 1975. DNA modification mechanisms and gene activity during development. Science **187**:226–232.
8. **Johnston, P. G.** 1981. X chromosome activity in female germ cells of mice heterozygous for Searle's translocation T(X;16)16H. Genet. Res. **37**:317–322.
9. **Jolly, D. J., H. Okayama, P. Berg, A. C. Esty, D. Filpula, P. Bohlen, G. G. Johnson, J. E. Shirely, T. Hunkapillar, and T. Friedmann.** 1983. Isolation and characterization of a full-length expressible cDNA for human hypoxanthine phosphoribosyltransferase. Proc. Natl. Acad. Sci. USA **80**:477–481.
10. **Josse, J., D. A. Kaiser, and A. Kornberg.** 1961. Enzymatic synthesis of deoxyribonucleic acid. VII. Frequencies of nearest neighbor base sequences in deoxyribonucleic acid. J. Biol. Chem. **236**:864–875.
11. **Konecki, D. S., J. Brennand, J. C. Fuscoe, C. T. Caskey, and A. C. Chinault.** 1982. Hypoxanthine-guanine phosphoribosyltransferase genes of mouse and Chinese hamster: construction and sequence analysis of cDNA recombinants. Nucleic Acids Res. **10**:6763–6775.
12. **Kratzer, P. G., and V. M. Chapman.** 1981. X chromosome reactivation in oocytes of *Mus caroli*. Proc. Natl. Acad. Sci. USA **78**:3093–3097.
13. **Lester, S. C., N. J. Korn, and R. DeMars.** 1982. Derepression of genes on the human inactive X chromosome: evidence for differences in locus-specific rates of derepression and rates of transfer of active and inactive genes after DNA-mediated transformation. Somatic Cell Genet. **8**:265–284.
14. **Liskay, R. M., and R. J. Evans.** 1980. Inactive X chromosome DNA does not function in DNA-mediated cell transformation for the hypoxanthine phosphoribosyltransferase gene. Proc. Natl. Acad. Sci. USA **77**:4895–4898.
15. **Littlefield, J.** 1964. Selection of hybrids from matings of fibroblasts in vitro and their presumed recombinants. Science **145**:709–711.
16. **Lyon, M. F.** 1972. X-chromosome inactivation and developmental patterns on mammals. Biol. Rev. **47**:1–35.
17. **MacPherson, I.** 1973. Microdrop techniques, p. 241–244. *In* P. F. Kruse and M. K. Patterson (ed.), Tissue culture methods and applications. Academic Press, Inc., New York.
18. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Martin, G. R., C. J. Epstein, B. Travis, G. Tucker, S. Yatziv, D. W. Martin, S. Clift, and S. Cohen.** 1978. X-chromosome inactivation during differentiation of female teratocarcinoma stem cells *in vitro*. Nature (London) **271**:329–333.
20. **Mathai, C. C., S. Ohno, and E. Beutler.** 1966. Sex-linkage of glucose-6-phosphate dehydrogenase gene in *Equidae*. Nature (London) **210**:115–117.
21. **McKeon, C., H. Ohkubo, I. Pastan, and B. de Crombrugghe.** 1982. Unusual methylation pattern of the α2(1) collagen gene. Cell **29**:203–210.
22. **Melton, D. W., D. S. Konecki, J. Brennand, and C. T. Caskey.** 1984. Structure, expression, and mutation of the hypoxanthine phosphoribosyltransferase gene. Proc. Natl. Acad. Sci. USA **81**:2147–2151.
23. **Mohandas, T., R. S. Sparkes, and L. J. Shapiro.** 1981. Reactivation of an inactive human X chromosome: evidence for X inactivation by DNA methylation. Science **211**:393–396.
24. **Monk, M.** 1978. Biochemical studies on mammalian X-chromosome activity, p. 189–223. *In* M. H. Johnson (ed.), Development in mammals, vol. 3. Elsevier/North-Holland, Inc. Amsterdam.
25. **Monk, M., and M. I. Harper.** 1979. Sequential X chromosome inactivation coupled with cellular differentiation in early mouse embryos. Nature (London) **281**:311–313.
26. **Riggs, A. D.** 1975. X inactivation, differentiation and DNA methylation. Cytogenet. Cell Genet. **14**:9–15.
27. **Russell, G. J., P. M. B. Walker, R. A. Elton, and J. H. Subak-Sharpe.** 1976. Doublet frequency analysis of fractionated vertebrate nuclear DNA. J. Mol. Biol. **108**:1–23.
28. **Sager, R., and R. Kitchin.** 1975. Selective silencing of eukaryotic DNA. Science **189**:426–433.
29. **Silver, L.** 1982. Genomic analysis of the H-2 complex region associated with mouse t haplotypes. Cell **29**:961–968.
30. **Stein, R., N. Sciaky-Galli, A. Razin, and H. Cedar.** 1983. Pattern of methylation of two genes coding for housekeeping functions. Proc. Natl. Acad. Sci. USA **80**:2423–2426.
31. **Swartz, M. N., T. A. Trautner, and A. Kornberg.** 1962. Enzymatic synthesis of deoxyribonucleic acid. XI. Further studies on nearest neighbor base sequences in deoxyribonucleic acids. J. Biol. Chem. **237**:1961–1967,
32. **Takagi, N., O. Sugawara, and M. Sasaki.** 1982. Regional and temporal changes in the pattern of X-chromosome replication during the early post-implantation development of the female mouse. Chromosoma **85**:275–286.
33. **Toniolo, D., M. D'Urso, G. Martini, M. Persico, V. Tufano, G. Battistuzzi, and L. Luzzatto.** 1984. Specific methylation pattern at the 3′ end of the human housekeeping gene for glucose-6-phosphate dehydrogenase. EMBO J. **3**:1987–1995.
34. **Tykocinski, M. L., and E. C. Max.** 1984. CG dinucleotide clusters in MHC genes and in 5′ demethylated genes. Nucleic Acids Res. **12**:4385–4396.
35. **Venolia, L., and S. M. Gartler.** 1983. Comparison of transformation efficiency of human active and inactive X-chromosomal DNA. Nature (London) **302**:82–83.
36. **Venolia, L., S. M. Gartler, E. R. Wassman, P. Yen, T. Mohandas, and L. J. Shapiro.** 1982. Transformation with DNA from 5-azacytidine-reactivated X chromosomes. Proc. Natl. Acad. Sci. USA **79**:2352–2354.
37. **Wolf, S. F., S. Dintzis, D. Toniolo, G. Persico, K. D. Lunnen, J. Axelman, and B. R. Migeon.** 1984. Complete concordance between glucose-6-phosphate dehydrogenase activity and hypomethylation of 3′ CpG clusters: implications for X chromosome dosage compensation. Nucleic Acids Res. **12**:9333–9348.
38. **Wolf, S. F., D. J. Jolly, K. D. Lunnen, T. Friedmann, and B. R. Migeon.** 1984. Methylation of the hypoxanthine phosphoribosyltransferase locus on the human X chromosome: implications for X-chromosome inactivation. Proc. Natl. Acad. Sci. USA **81**:2806–2810.
39. **Wolf, S. F., and B. R. Migeon.** 1982. Studies of X chromosome DNA methylation in normal human cells. Nature (London) **295**:667–671.
40. **Wolf, S. F., and B. R. Migeon.** 1985. Clusters of CpG dinucleotides implicated by nuclease hypersensitivity as control elements of housekeeping genes. Nature (London) **314**:467–469.
41. **Yen, P. H., P. Patel, A. C. Chinault, T. Mohandas, and L. J. Shapiro.** 1984. Differential methylation of hypoxanthine phosphoribosyltransferase genes on active and inactive human X chromosomes. Proc. Natl. Acad. Sci. USA **81**:1759–1763.