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Differential Methylation of the Ornithine Carbamoyl Transferase Gene on Active and Inactive Mouse X Chromosomes

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Ornithine carbamoyl transferase (*Oct*) is an X-linked gene which exhibits tissue-specific expression. To determine whether methylation of specific CpG sequences plays a role in dosage compensation or tissue-specific expression of the gene, 13 potentially methylatable sites were identified over a 30-kilobase (kb) region spanning from approximately 15 kb upstream to beyond exon II. Fragments of the *Mus hortulanus Oct* gene were used as probes to establish the degree of methylation at each site. By considering the methylation status in liver (expressing tissue) versus kidney (nonexpressing tissue) from male and female mice, the active and inactive genes could be investigated on active and inactive X-chromosome backgrounds. One *Msp*I site, 12 kb 5′ of the *Oct*-coding region, was cleaved by *Hpa*II in liver DNA from males but not in kidney DNA from males and thus exhibited complete correlation with tissue-specific expression of the gene. Six other sites showed partial methylation, reflecting incomplete correlation with tissue-specific expression.

A growing body of evidence indicates that methylation is involved in the stable maintenance of X-chromosome inactivation, although whether it is a cause or an effect of inactivation remains unclear (11, 16). DNA-mediated gene transfer has shown that the hypoxanthine phosphoribosyl transferase (*Hprt*) gene on the active X chromosome of embryonic lineages and adult organs, but not on the inactive X, is competent in transferring the Hprt$^+$ phenotype to Hprt$^-$ recipient cells (5). Also, the potent methylation inhibitor 5-azacytidine can reactivate *Hprt* and other genes on an inactive human X chromosome in somatic cell hybrids (17).

Studies of X-linked housekeeping genes have revealed CpG dinucleotide clusters located at either 5′ end (e.g., *Hprt* [27] and phosphoglycerate kinase [*Pgk*; 10]) or the 3′ end (e.g., *G6pd* [26]) which are differentially methylated on the active and inactive X chromosome. Hypomethylation of the G+C-rich islands has been perfectly correlated with the activity of the genes, whereas CpG clusters on the inactive X chromosome are methylated and nuclease insensitive (21, 22). Studies on methylation of the bodies of these genes have been more difficult to interpret. However, it appears that tissue-specific patterns, including both methylation and nonmethylation of critical sites, may be involved in the control of transcription of housekeeping genes in different tissues (1).

Ornithine carbamoyl transferase, an enzyme of the urea cycle, is expressed primarily in the liver, and the *Oct* gene is X linked. Evidence to date suggests that it is not a housekeeping gene, however, but is regulated developmentally and in a tissue-specific manner. The *Oct* promoter contains the CAAT and TATAA boxes characteristically absent from promoter regions of housekeeping genes (15), it lacks the CpG cluster (although these are not found exclusively in housekeeping genes [3, 4]), and its expression is below detectable limits in such tissues as the mouse kidney (D. A. Stephenson, unpublished data). *Oct* is, therefore, a potential model for determining if the expression of an X-linked, nonhousekeeping gene is controlled by methylation in different organs.

To determine whether the *Oct* gene is unmethylated at specific sites in liver compared with kidney and whether these sites are differentially methylated in inactive X chromatin, we analyzed potentially methylatable sites upstream and in the first intron of the *Oct* gene isolated from *Mus hortulanus*. The study revealed a number of sites which were differentially methylated in expressing versus nonexpressing tissue and two sites which exhibited sex-specific differences.

MATERIALS AND METHODS

Mice. Mice used for these studies came from our colony of *M. hortulanus*, originally derived from samples trapped in Yugoslavia.

Isolation and characterization of genomic clones. The probes used for the initial screening of a mouse genomic library were a full-length *Oct* cDNA clone and a 760-base-pair (bp) genomic subclone covering the promoter region of the gene, both inserted into pUC9 (G. Veres, unpublished data). Genomic clones were isolated from a *M. hortulanus* genomic library constructed by P. Howles, Roswell Park Memorial Institute, Buffalo, N.Y., as a *Sau*3A partial digest of *M. hortulanus* genomic DNA in λ EMBL4 (8). Bacteriophage were plated on lawns of LE392 (18), and those with homology to *Oct* were identified by using the phage hybridization procedure of Benton and Davis (2). DNA was prepared from purified clones by liquid culture (14) and was mapped with restriction endonucleases by the rapid mapping technique (20) under conditions recommended by the suppliers (New England BioLabs, Inc.).

Construction and handling of all recombinant bacteriophage and plasmids were carried out in accordance with the National Institutes of Health guidelines for recombinant DNA research.

Isolation of DNA fragments and ^{32}P labeling. All inserts from plasmids and DNA fragments from genomic clones were isolated by restriction enzyme digestion and purified on

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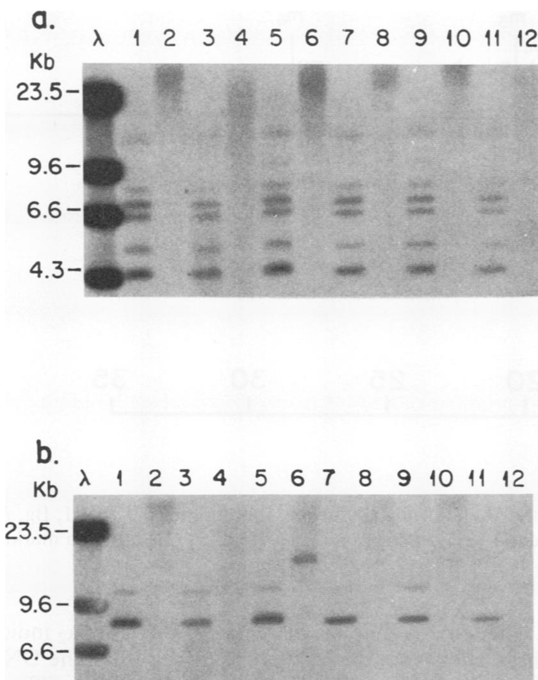

FIG. 1. (a) Southern analysis of tissues from a *M. hortulanus* adult male by using full-length *Oct* cDNA probe. Samples were digested with *Msp*I (odd-numbered lanes) and *Hpa*II (even-numbered lanes). Lanes: 1 and 2, kidney; 3 and 4, gut; 5 and 6, liver; 7 and 8, lung; 9 and 10, brain; 11 and 12, heart. *Hin*dIII-digested λ DNA size markers are indicated. (b) As for panel a, but probed with the 760-bp promoter probe.

a 1% low-melting-point SeaKem (FMC Corp., Marine Colloids Div.) agarose gel. The relevant fragments were cut out and labeled directly by using the oligonucleotide random priming method of Feinberg and Vogelstein (7).

Genomic DNA isolation. High-molecular-weight DNA was prepared from mouse liver and kidney by the protocol described for isolation of DNA from mouse tails (9). Briefly, tissue was diced in buffer (50 mM Tris, pH 8, 100 mM EDTA, 100 mM NaCl, 1% sodium dodecyl sulfate [SDS]) and incubated with proteinase K (0.5 mg/ml) at 55°C overnight. After RNase A treatment, the DNA was extracted several times with phenol and phenol-chloroform and was then ethanol precipitated twice.

DNA methylation analysis. Complete restriction digests of genomic DNA were carried out according to the instructions of the manufacturer by using 10 U of DNA per μg for methylation-sensitive enzymes and 1 to 5 U of DNA per μg for the rest with overnight incubation.

Southern analysis. DNA fragments obtained by restriction enzyme digestion were separated by gel electrophoresis on a 0.8% agarose gel and transferred to Zetabind (AMF Cuno) or nitrocellulose (Schleicher & Schuell, Inc.) by Southern blotting (22). Zetabind blots were prewashed in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% SDS and then prehybridized in 6× SSC–5× Denhardt–0.1% sodium PP_i–0.1% SDS–salmon sperm DNA (150 μg/ml). Hybridization was carried out at 65°C overnight with labeled probe at 10^6 cpm/ml of hybridization solution. On the next day, the blots were washed two times in 2× SSC–0.1% SDS–0.1% sodium PP_i at 65°C and then extensively washed in the same buffer with 1× SSC. The blots were exposed to Kodak XAR or XRP film at −80°C with Cronex Lightning-Plus (Du Pont Co.) intensifying screens for 48 to 72 h.

Densitometry. Negatives of gel photographs taken before and after Southern blotting were scanned by using an LKB Ultrascan XL laser densitometer to check DNA concentration from lane to lane and to monitor transfer efficiency. After the autoradiographs were analyzed densitometrically and were corrected for any variation in DNA concentration, the intensities of bands within a lane or between any two lanes could be compared.

RESULTS

Tissue-specific methylation sites of the *Oct* gene were initially determined by comparing the *Hpa*II cleavage of *Msp*I sites of several tissues of an adult *M. hortulanus* male. (*Hpa*II is the methylation-sensitive isoschistomer of *Msp*I.) The methylation of sites identified in genomic DNA by a full-length *Oct* cDNA probe is shown in Fig. 1a. We uniformly observed seven *Msp*I fragments in the DNA from each of the tissues examined, but no comparable fragments were cleaved by *Hpa*II in any of the tissues. This suggests that none of the *Msp*I sites identified by the full-length cDNA probe are hypomethylated tissue specifically. The 760-bp promoter probe specifically identified a 16-kilobase (kb) fragment in *Hpa*II-digested liver DNA (Fig. 1b, lane 6), suggesting that hypomethylation of *Msp*I sites flanking the 16-kb fragment is important in tissue-specific expression.

A *M. hortulanus* genomic library was screened with both the full-length *Oct* cDNA probe and the 760-bp promoter probe to isolate clones at the 5′ end of the gene and determine the location of the *Msp*I sites flanking the 16-kb fragment. Three overlapping clones were identified which together cover nearly 35 kb, extending from 15 kb upstream of the gene through exon II. A composite map of the three clones (Fig. 2) indicates all CpG sequences which are cleaved by *Msp*I, *Hha*I, or *Ava*I and which can be tested for methylation. The position of the promoter probe and the locations of exons I and II are also indicated.

We specifically asked whether the cleavage of genomic DNA by methyl-sensitive restriction endonucleases differs in DNA taken from the primary expressing tissue, i.e., liver, and a nonexpressing tissue, i.e., kidney. Moreover, the cleavage of these sites in DNA of *M. hortulanus* males was compared to that in females to ascertain whether the inactive X chromosome of females is differentially methylated. The strategy for estimating the degree of methylation at each potential methylation site involved the subcloning of genomic sequences adjacent to each site to use as probes. Genomic DNA was cleaved by using an appropriate restriction enzyme, which recognized sequences flanking the methylatable site, to yield a fragment of known size on Southern analysis. If methylation-sensitive endonucleases cleaved additional sites within this fragment, then a decrease in fragment size was readily identified. Partial methylation at a site was indicated by the occurrence of smaller fragments in addition to the full-length fragment on double digestion.

Probe M_1-M_2. The relative positions of sites M_1, M_2, and M_3, along with the flanking *Eco*RI sites, are shown in Fig. 3a. Probe M_1-M_2 hybridized to the expected 2.8-kb fragment in *Msp*I-digested liver and kidney genomic DNA from both sexes (Fig. 3a, lanes 1 to 4). The appropriate dosage difference is readily observed between DNAs from males and females. The same probe detected the 16-kb fragment in male and female liver DNAs (i.e., DNAs from males and

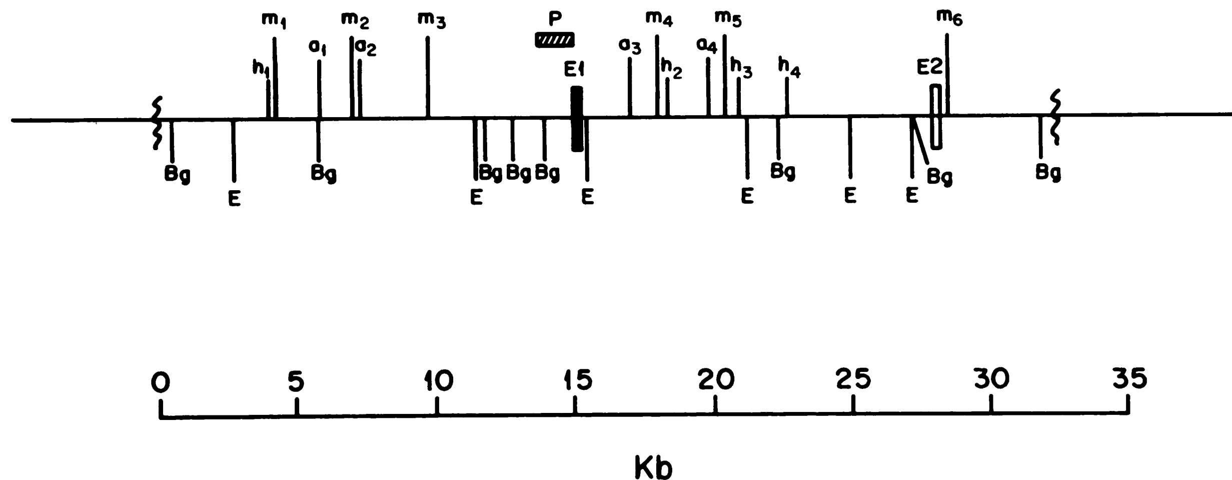

FIG. 2. Composite restriction map of overlapping genomic clones covering the 5' end of the *Oct* gene. Abbreviations: a, *Ava*I; Bg, *Bgl*II; E, *Eco*RI; h, *Hha*I; m, *Msp*I. The positions of the promoter probe (P: ▨), exon I (E1; ■), and exon II (E2; ▭) are also marked.

females) digested with *Hpa*II but not in kidney DNAs (lanes 5 to 8). If M_1 was unmethylated, we would expect to observe a 7.0-kb fragment on *Hpa*II-*Eco*RI double digestion, and in fact, this was the fragment size observed in liver DNA. A second fragment of 8.6 kb was seen in female liver DNA suggesting that the M_1 site is unmethylated on the active X chromosome but methylated on the inactive X in females.

Probe M_4-M_5. Probe M_4-M_5 hybridized to a 2.4-kb fragment on *Msp*I digestion of both liver and kidney DNA in the expected ratio of 2:1 for females to males (Fig. 3b, lanes 1 to 4). A similar 2.4-kb fragment was observed in *Hpa*II digests of liver DNA but not of kidney DNA (lanes 5 to 8). Moreover, the equivalent intensity of hybridization in lanes 5 and 6 (ratio of 1:1) suggests that only the active X chromosome is unmethylated at M_4 and M_5. An *Hpa*II-*Bgl*II double digestion of male liver DNA (lane 9) produced additional fragments of 6.5 kb (M_5 unmethylated) and 8.5 kb (both M_4 and M_5 methylated). The proportion of M_4 and M_5 sites which were methylated in the tissue was estimated by quantitative analysis of the relative abundance of the different restriction fragments. Densitometric analysis of the autoradiograph (assuming complete digestion of the genomic DNA and comparable transfer and hybridization of each fragment) indicates that the amount of 2.4-kb fragment was equivalent to the amount of 6.5- and 8.5-kb fragments combined. This suggests that approximately 50% of male liver cells are unmethylated at both M_4 and M_5. Double digestion of kidney DNAs (lanes 11 and 12) produced a prominent 8.5-kb fragment with less abundant fragments of 6.5 and 4.5 kb, reflecting subpopulations of cells methylated at either *Msp*I site M_4 or M_5.

Probe A_3-A_4. Probe A_3-A_4 hybridized to a 2.9-kb fragment in *Ava*I-digested liver DNA (Fig. 3c, lanes 1 and 2), indicating that both sites were unmethylated in a proportion of cells in the active tissue. *Ava*I-*Eco*RI double digestion of male liver DNA (lane 5), revealed additional fragments of 4.4 kb (A_3 unmethylated) and 6.6 kb (both methylated). Densitometric analysis indicated that only 25% of the DNA was unmethylated at both A_3 and A_4. The presence of a female-specific 4.6-kb fragment (A_4 unmethylated; lanes 6 and 8) suggested that it is characteristic of the inactive X chromosome, but densitometric analysis indicated that it only occurred in a small proportion of cells (approximately 10 to 15%). The overall analysis of *Ava*I sites A_1 and A_2 indicates that these sites were methylated equivalently in the DNA of both tissues and that there was no significant difference between males and females.

Probe BglII-H_1. Site H_1 was investigated by using a 3.5-kb *Bgl*II-*Hha*I genomic fragment. *Bgl*II-*Hha*I double digestion of liver DNA (Fig. 3d, lane 5) gave a predominant 3.5-kb fragment, indicative of nonmethylation of H_1 in approximately 75% of cells. H_1 was totally methylated in kidney samples, as indicated by the presence of the 5.0-kb fragment.

Probe H_2-H_3. Probe H_2-H_3 hybridized to 4.0- and 8.5-kb fragments in male liver DNA digested with *Hha*I and *Bgl*II but to only the 8.5-kb fragment in male kidney DNA (Fig. 3e, lanes 5 to 8). These results indicate that H_2 was methylated about 50% of the time in tissues which express *Oct* and was fully methylated in nonexpressing tissues. The absence of a 7.2-kb fragment in any lanes when H_2-H_3 was the probe indicates that H_3 was totally methylated. In contrast, the 4.0-kb fragment was observed in female kidney DNA (lane 8), which suggests that H_2 might be hypomethylated on the inactive X chromosome. Finally, by use of the H_3-H_4 probe, no differences were found in DNAs from the different tissues or from males and females (data not shown).

The methylation status of each of the 13 sites studied in the different X chromosome configurations is shown in Fig. 4. In liver from a male, in which the *Oct* gene is expressed, eight sites exhibited some degree of hypomethylation. Two of these sites are located approximately 12 kb upstream from the first exon, and five are clustered in a 5-kb region of intron I. A comparison of the methylation patterns of these sites in expressing versus nonexpressing tissue from males identified changes in methylation which may be correlated with the expression of the *Oct* gene. Seven of the above sites showed some degree of hypermethylation, four of them (H_1, M_1, H_2, and A_4) being fully methylated in the inactive gene. A comparison of male and female patterns of methylation in kidney and liver provides an estimate of the sites which were differentially methylated on the inactive X chromosome (assuming that the gene on the active X is methylated to the same extent in both male and female tissue). The methylation patterns are similar except for hypomethylation at H_2 and A_4 in 10 to 15% of kidney cells from females. Hypomethylation of these sites may indicate that the inactive X

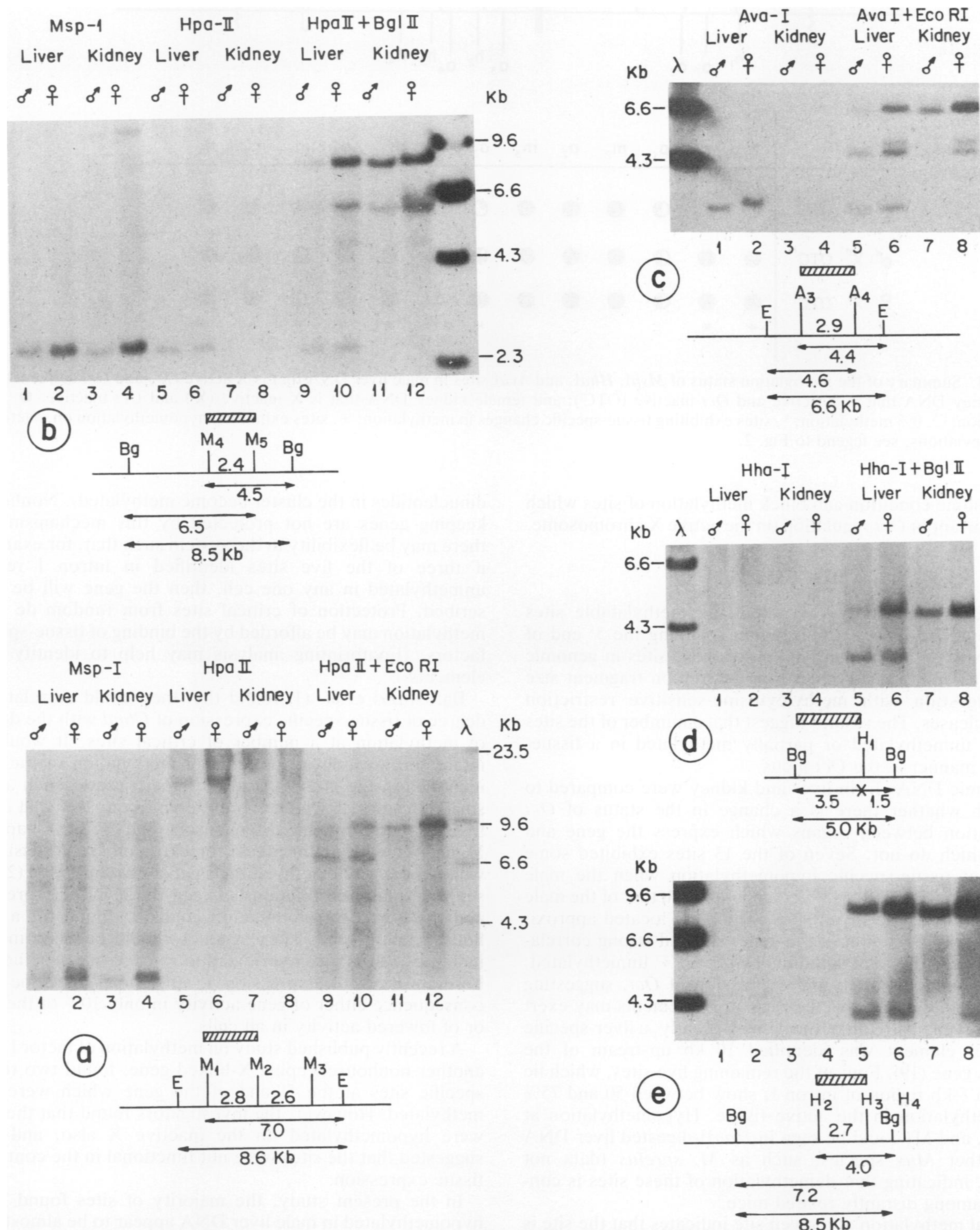

FIG. 3. Southern analysis of liver and kidney DNAs from male and female adult *M. hortulanus* mice. The line drawing beneath each autoradiograph indicates the positions of probes (▨) and flanking restriction sites used to predict the fragment sizes. For abbreviations, see legend to Fig. 2. (a) DNAs digested with *Msp*I, *Hpa*II, or *Hpa*II-*Eco*RI and probed with M_1-M_2. (b) DNAs digested with *Msp*I, *Hpa*II, or *Hpa*II-*Bgl*II and probed with M_4-M_5. (c) DNAs digested with *Ava*I or *Ava*I-*Eco*RI and probed with A_3-A_4. (d) DNAs digested with *Hha*I or *Hha*I-*Bgl*II and probed with *Bgl*II-H_1. (e) DNAs digested with *Hha*I or *Hha*I-*Bgl*II and probed with H_2-H_3.

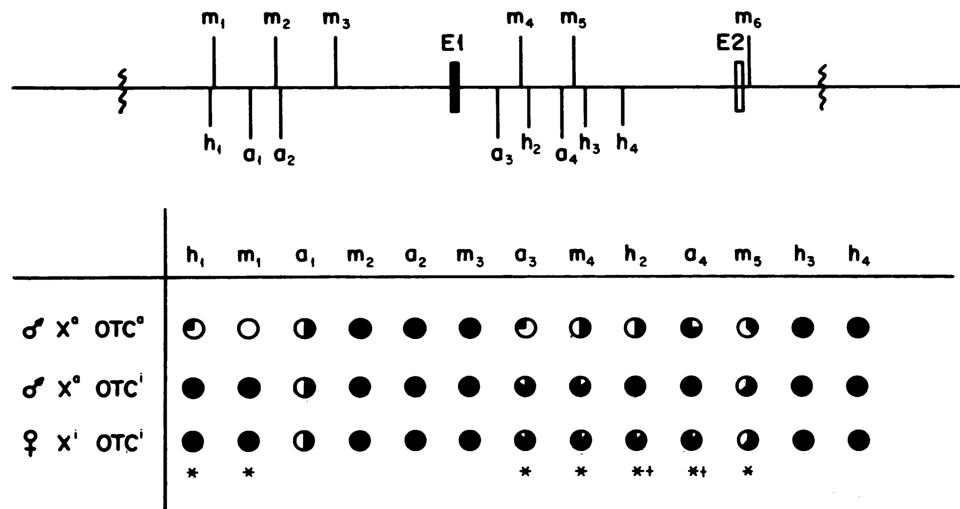

FIG. 4. Summary of the methylation status of *Msp*I, *Hha*I, and *Ava*I sites in male liver DNA that is X active (X^a) and *Oct* active (OTC^a); male kidney DNA that is X active and *Oct* inactive (OTC^i); and female kidney DNA that is X inactive (X^i) and *Oct* inactive. ●, 100% methylation; ○, 0% methylation; *, sites exhibiting tissue-specific changes in methylation; +, sites exhibiting hypomethylation on inactive X. For abbreviations, see legend to Fig. 2.

chromosome condition can block methylation of sites which are important in *Oct* regulation on the active X chromosome.

DISCUSSION

In the present study, 13 potentially methylatable sites were identified over a 30-kb region spanning the 5′ end of *Oct*. A survey of the methylation of these sites in genomic DNA was achieved by measuring restriction fragment size after digestion with methylcytosine-sensitive restriction endonucleases. The results suggest that a number of the sites may be unmethylated or partially methylated in a tissue-specific manner at the *Oct* locus.

Genomic DNAs from liver and kidney were compared to establish whether there is a change in the status of *Oct* methylation between organs which express the gene and those which do not. Seven of the 13 sites exhibited some degree of tissue-specific hypomethylation when the male liver methylation pattern was compared with that of the male kidney. Two of the seven sites, which are located approximately 12 kb upstream of the gene, showed strong correlation between hypomethylation (M_1, >95% unmethylated; H_1, 75% unmethylated) and expression of *Oct*, suggesting that *cis*-acting tissue-specific regulatory elements may exert an effect well upstream of the gene. Recently, a liver-specific enhancer element was identified 10 kb upstream of the albumin gene (19). Four of the remaining five sites, which lie within a 5-kb region of intron I, show between 50 and 75% hypomethylation in the active tissue. Hypomethylation at M_1, M_4, and M_5 could be seen in *Hpa*II-digested liver DNA from other *Mus* species, such as *M. spretus* (data not shown), indicating that demethylation of these sites is conserved among distantly related mice.

Partial methylation at a given site indicates that the site is methylated in some cells and not in others. This may reflect heterogeneity in the cell populations (e.g., parenchymal versus nonparenchymal tissue in the liver) or different functional states within the same cell population (23). Wolf and Migeon (28) suggested that CpG clusters in housekeeping genes might prevent inactivation of the genes by random de novo methylation (assuming that not all of the CpG dinucleotides in the cluster become methylated). Nonhousekeeping genes are not protected by this mechanism, but there may be flexibility in the system such that, for example, if three of the five sites identified in intron I remain unmethylated in any one cell, then the gene will be transcribed. Protection of critical sites from random de novo methylation may be afforded by the binding of tissue-specific factors. (Footprinting analysis may help to identify such elements.)

Battistuzzi et al. (1) found that they could correlate the degree of tissue-specific expression of *G6pd* with the degree of methylation at a number of critical sites. It would be interesting to look at the level of methylation of the sites identified in the present study in other tissues, such as the small intestine, in which expression is about 10% that of the liver (S. Grant, D. A. Stephenson, and V. M. Chapman, Mouse News Lett. in press), or extraembryonic tissue, in which the level of methylation is generally depressed (21), to see if a similar correlation between the level of expression and the degree of methylation could be found for a nonhousekeeping gene. These studies should be done in conjunction with in situ hybridization to ascertain whether the lowered level of expression in the small intestine is a consequence either of gene activity in only 10% of the cells or of lowered activity in all cells.

A recently published study on methylation of factor IX (6), another nonhousekeeping X-linked gene, found two tissue-specific sites at the 5′ end of the gene which were 50% methylated. However, the investigators found that the sites were hypomethylated on the inactive X also, and they suggested that the sites were not functional in the control of tissue expression.

In the present study, the majority of sites found to be hypomethylated in male liver DNA appear to be almost fully methylated on the inactive X chromosome in female liver DNA (with the exception of site A_1). Since hypomethylation of A_4 and H_2 on the inactive X chromosome was present in only a subset of cells, it is unlikely to be important in maintaining X dosage compensation and may reflect the fact that the inactive X chromosome is methylated or demethylated independently of alterations which govern the expres-

sion of the gene on the active X chromosome. No sex-specific methylation sites were found in the factor IX study (6) or in a recent report on the methylation of human *Oct* (M. C. Hannibal, C. Ruta Cullen, D. C. Kaslow, K. E. Davies, and B. R. Migeon, Abstr. Am. J. Human Genet. **39**:A201, 1986). Lock et al. (12) showed that for the *Hprt* locus, inactive X-specific methylation sites do not become methylated until several days after the primary X-inactivation event. It would be interesting to determine when the sites identified in the present study become methylated in postimplantation embryos and if those sites are unmethylated on the active X-chromosome gene before expression is initiated in development.

The mosaic pattern of *Oct* expression in females heterozygous for the sparse fur (*Spf*) mutation (25) clearly indicates that *Oct* is subject to X inactivation and that liver-specific expression does not override the inactive state. However, when the *Oct* locus is physically separated from the X inactivation center by X-autosome translocation, as in T37H, it can escape the inactivation process (13). The mechanism by which nonhousekeeping genes are silenced on the inactive X remains obscure but could reflect inaccessibility of inactive chromatin to the tissue-specific demethylation process.

Veres et al. (24) showed that an 800-bp fragment containing the 5′ promoter region of the *Oct* gene was sufficient to direct the expression of a fused chloramphenicol acetyltransferase gene in Hep-G2 cells but not in mouse fibroblast NIH 3T3 cells. Numerous transgenic mouse studies have indicated that tissue-specific expression is more complicated in vivo. It should be possible to breed transgenic mice carrying the 12-kb region, with or without sites H_1 and M_1, upstream of a suitable marker gene to confirm that the sites are important for tissue-specific expression of *Oct*. The results of this study suggest that hypomethylation of sites 12 kb away can affect the ability of tissue-specific elements to react with the promoter or that hypomethylation may act in concert with other changes in methylation status and chromatin structure to effect the expression of the gene.

To distinguish between methylatable sites on the active and inactive X chromosomes and to determine their relationship to activation or inactivation of the *Oct* locus, mice are currently being bred which carry the T(X;16)16H translocation and different polymorphic forms of *Oct* on each X chromosome. It should then be possible to measure the methylation status on each chromosome at different times during early development, to follow the establishment of the inactive gene methylation pattern, and to determine what alterations effect the expression of *Oct*.

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