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Nuclease Sensitivity of the Mouse *HPRT* Gene Promoter Region: Differential Sensitivity on the Active and Inactive X Chromosomes

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We investigated the conformation of the X-linked mouse hypoxanthine-guanine phosphoribosyltransferase gene (*HPRT*) promoter region both in chromatin from the active and inactive X chromosomes with DNase I and in naked supercoiled DNA with S1 nuclease. A direct comparison of the chromatin structures of the active and inactive mouse *HPRT* promoter regions was performed by simultaneous DNase I treatment of the active and inactive X chromosomes in the nucleus of interspecies hybrid cells from *Mus musculus* and *Mus caroli*. Using a restriction fragment length polymorphism to distinguish between the active and inactive *HPRT* promoters, we found a small but very distinct difference in the DNase I sensitivity of active versus inactive chromatin. We also observed a single DNase I-hypersensitive site in the immediate area of the promoter which was present only on the active X chromosome. Analysis of the promoter region by S1 nuclease digestion of supercoiled plasmid DNA showed an S1-sensitive site which maps adjacent to or within the DNase I-hypersensitive site found in chromatin but upstream of the region minimally required for normal *HPRT* gene expression.

The mouse hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.8) gene (*HPRT*) is an X-linked, constitutively expressed gene which codes for an enzyme involved in the metabolic salvage of purines. The levels of expression of *HPRT* are modulated in a tissue-specific manner (27), and in females the gene is subject to differential control via mammalian X-chromosome inactivation (4, 7, 27). Thus, female somatic tissues present an opportunity to analyze the transcriptionally active and inactive states of the *HPRT* gene within the same nucleus, one allele on the active X chromosome and the other on the inactive X chromosome.

Furthermore, the mouse *HPRT* gene possesses an unusual promoter region which is highly G+C rich (14). Unusual features of the *HPRT* promoter include the apparent absence of TATA and CAAT box sequences in the immediate 5′ flanking region and the high frequency of the dinucleotide CpG. This G+C-rich character and lack of TATA or CAAT boxes or both are shared by the promoters of several genes, all of which are constitutively expressed (12, 17, 19, 23, 24, 28).

DNase I analysis of chromatin. A characteristic feature of transcriptionally active chromatin is its greater susceptibility to digestion by DNase I relative to that of inactive chromatin (5, 29). In addition, certain sites in the chromatin of active genes show a significantly enhanced susceptibility to DNase I (5, 6, 31). These DNase I-hypersensitive sites (DHSs) tend to be localized near or within the promoter or control regions of eucaryotic genes (5, 6), although DHSs are also found at other locations within and adjacent to active genes (1, 3, 20–22). Experiments were performed to determine whether chromatin from the mouse *HPRT* gene contains a DHS within or near the promoter region. Intact nuclei from male mouse livers were prepared by a modified procedure of Zimmer and Schwartz (W. G. Zimmer, Ph.D. thesis, Baylor College of Medicine, Houston, Tex., 1985). Fresh mouse livers were minced and then homogenized in cold 0.5 M sucrose in TEKSS (25 mM KCl, 1 mM EDTA, 1 mM ethylene bis(oxyethylenenitrilo)tetraacetic acid (EGTA), 0.15 mM spermine, 1.5 mM spermidine, 50 mM Tris hydrochloride [pH 7.5]). The homogenate was spun at 5,000 × g, suspended in 0.5 M sucrose in TEKSS plus 0.5% Triton X-100, and rehomogenized. Nuclei were sedimented as before and rehomogenized as required. The final nuclear pellet was resuspended in sucrose–TEKSS–Triton X-100, layered onto a solution of 0.88 M sucrose in TEKSS, and spun as before. The resulting pellet was suspended in DNase I digestion buffer (31) at a concentration of 0.5 to 2.75 mg of DNA per ml. Various concentrations of DNase I were added to 100 μl of nuclear suspensions and digested for 15 to 20 min at 37°C. DNA was prepared from each reaction mixture as described by Yang et al. (32). The DNA was digested with several restriction enzymes (Fig. 1), fractionated in agarose gels, and blotted onto nitrocellulose. The filters were then hybridized (33) with a nick-translated 230-base-pair (bp) *Bst*NI-*Nae*I fragment probe which spans the junction between the first exon and first intron (Fig. 1). The results are shown in the autoradiograms in Fig. 1. With each of the restriction enzymes used, a single subband was seen, typical of those generated by a single DHS. The DHS was mapped by correlating the size of the single hypersensitive subband (seen after each of the different restriction enzyme digestions) with the relative positions of the restriction sites in the promoter region (Fig. 1). These mapping data demonstrate the presence of a single DHS in the 14-kilobase (kb) region between the 5′ *Xmn*I site and the 3′ *Bam*HI site (Fig. 1). This site is located in the immediate 5′ flanking region of the gene, which includes the functional promoter (14, 15) and perhaps a very small portion of the first exon. The width of the subband seen after *Xmn*I digestion suggests that the size of the hypersensitive site is approximately 200 bp, in agreement with that in other genes (5). This DHS includes part or all of a so-called CG island (2) which is rich in the dinucleotide CpG.

We next sought to make a direct comparison between the DNase I sensitivity of the mouse *HPRT* gene in its expressed and nonexpressed states. For this study we used two mouse

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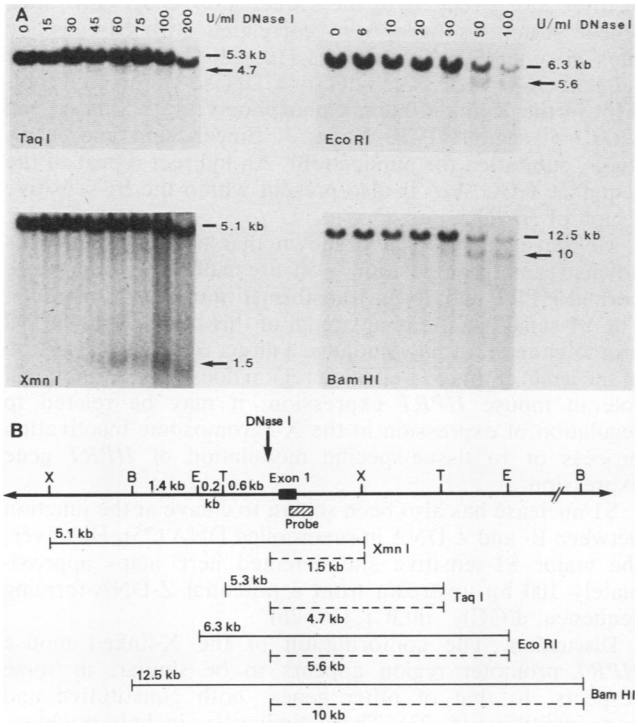

FIG. 1. DHS in promoter region of mouse *HPRT* gene. (A) Southern blots of DNA from DNase I-treated nuclei from male mouse livers. The arrows show the positions of hypersensitive subbands. (B) Restriction map showing position of DHS in chromatin from the mouse *HPRT* promoter region. The dotted lines represent hypersensitive subband fragments. X, *Xmn*I; B, *Bam*HI; T, *Taq*I; E, *Eco*RI.

cell lines derived from female embryos of *Mus musculus-Mus caroli* hybrids (4). Two clonally isolated cell lines from these interspecies hybrids, C3 and B5, contain both an *M. musculus* and an *M. caroli* X chromosome within each nucleus. In the C3 line, the *M. caroli* X chromosome is active and the *M. musculus* X chromosome is inactive, whereas in the B5 line, the *M. musculus* X chromosome is active and the *M. caroli* X chromosome is inactive (4). Furthermore, Lock et al. (9) have described a restriction fragment length polymorphism (RFLP) which can be used to distinguish between the *M. musculus* and *M. caroli HPRT* genes. This RFLP occurs in a *Taq*I fragment which spans the mouse *HPRT* promoter region in both species (Fig. 2). Thus, in these hybrid cell lines, it is possible to distinguish between the active and inactive *HPRT* promoter regions within the same nucleus by Southern blot analysis. The C3 line carries a 5.3-kb *Taq*I *M. musculus* inactive *HPRT* promoter fragment and a 3-kb *M. caroli* active *HPRT* promoter fragment; the B5 line has the converse, a 3-kb *M. caroli* inactive *HPRT* promoter and a 5.3-kb *M. musculus* active *HPRT* promoter. Using this RFLP, we performed a direct internally controlled comparison of the DNase I sensitivity of the active and inactive mouse *HPRT* promoter regions within the same nucleus. Nuclei prepared from B5 and C3 cells were treated with increasing concentrations of DNase I. DNA prepared from DNase I-treated nuclei was then digested with *Taq*I and fractionated, blotted, and hybridized as described above. The DNase I sensitivity of the *HPRT* promoter regions on the active and inactive X chromosomes is shown in Fig. 2. In the C3 line, which carries an active *M. caroli* X chromosome, the rate of digestion of the 3-kb *M. caroli* promoter band was relatively greater than the rate of digestion of the companion 5.3-kb inactive *M. musculus* promoter band. In the B5 line, in which the active X chromosome is derived

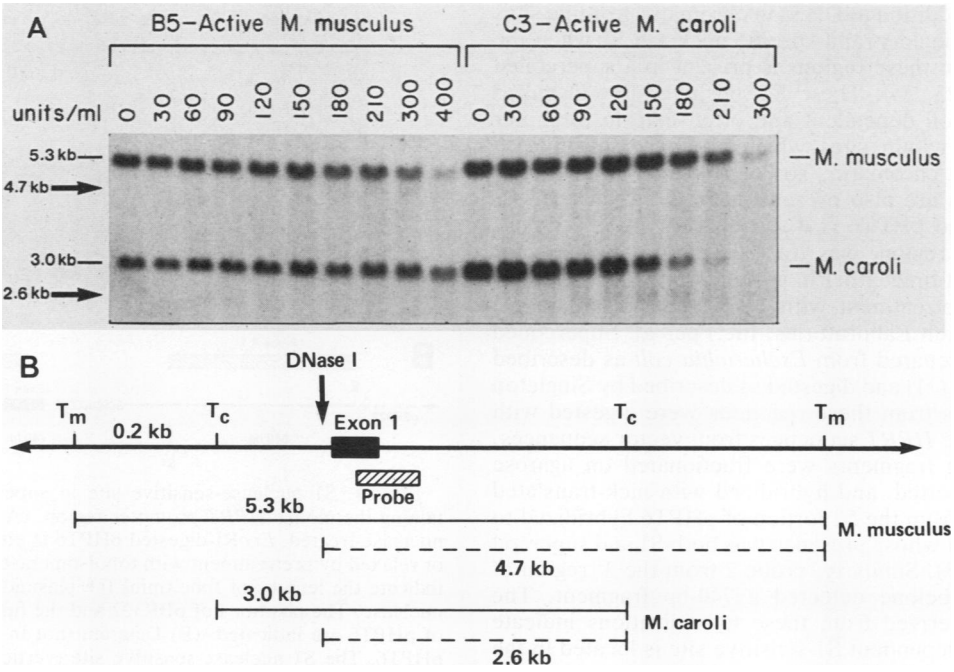

FIG. 2. Differential DNase I sensitivity of chromatin from active and inactive mouse *HPRT* promoter regions. (A) Southern blot of DNA from DNase I-treated nuclei of *M. musculus-M. caroli* hybrid cell lines. (B) Restriction map and diagram of mouse *HPRT* gene promoter region. The arrows in the Southern blot and the dotted lines in the diagram indicate hypersensitive subbands. T_m, *M. musculus Taq*I sites; T_c, *M. caroli Taq*I sites.

from *M. musculus*, the active 5.3-kb *M. musculus* band was digested by DNase I at a greater rate than was the inactive 3-kb *M. caroli TaqI* band. The data in Fig. 2 permit, for the first time, an approximate quantitation of the difference in DNase I susceptibility between alleles on the active and inactive X chromosomes. The intensity of the 5.3-kb *M. musculus* band at 300 U/ml and that of the 3.0-kb *M. caroli* band at 210 U/ml in the C3 cell line appear to be equivalent. Thus, the difference between the DNase I sensitivity of the promoter regions of the active and inactive *HPRT* genes is, at most, twofold (for the active and inactive promoters, 210 and 300 U of DNase I per ml, respectively, were required to achieve similar levels of digestion). Apparently, the condensation of the inactive X chromosome into the Barr body (7, 16) does not render its overall chromatin structure unusually resistant to DNase I, compared with the euchromatic active X chromosome. Thus, the conformational feature(s) which allows DNase I to distinguish between active and inactive chromatin is probably not the primary feature(s) responsible for the cytological staining properties and condensation of the inactive X chromosome. The data in Fig. 2 also permit an analysis of the state of the DHS on the active and inactive X chromosomes. The polymorphic hypersensitive subbands which would be expected in these two cell lines after DNase I treatment of nuclei and *TaqI* digestion of purified DNA are shown in Fig. 2. Assuming the DHS occurs in the same region in both species, *M. musculus* would yield a 4.7-kb hypersensitive subband and *M. caroli* would yield a 2.6-kb hypersensitive subband. The C3 hybrid line with the active *M. caroli* X chromosome displayed only the 2.6-kb *M. caroli* hypersensitive subband. Conversely, the B5 line with the active *M. musculus* X chromosome showed only the *M. musculus* 4.7-kb hypersensitive subband. Thus, the DHS in the promoter region of the mouse *HPRT* gene is present only on the active X chromosome.

S1 nuclease sensitivity of supercoiled promoter DNA. Nuclease digestion of the 5′ flanking regions of several genes has revealed, in addition to DHSs in chromatin, specific sites sensitive to the single-strand-specific nuclease S1 when the cloned DNA from these regions is present in a supercoiled plasmid (8, 13, 23, 34). These S1-sensitive sites in naked DNA are supercoil dependent and often map in promoter regions near or within sites which are hypersensitive to DNase I in intact chromatin. To determine whether similar S1-sensitive sites are also present in the G+C-rich *HPRT* promoter, plasmid pHPT6 (14), which contains the mouse *HPRT* promoter region, was treated with S1 nuclease for various lengths of time either in its supercoiled form or after relaxation by pretreatment with 10 U of topoisomerase I (Bethesda Research Laboratories, Inc.) per μl. Supercoiled plasmids were prepared from *Escherichia coli* as described by Maniatis et al. (11) and digested as described by Singleton et al. (25). DNAs from these reactions were digested with *Eco*RI to separate *HPRT* sequences from vector sequences, and the resulting fragments were fractionated on agarose gels, Southern blotted, and hybridized with nick-translated probes. Probe 1 from the 5′ portion of pHPT6 hybridized to a 640-bp fragment whose presence was both S1 and supercoil dependent (Fig. 3). Similarly, probe 2 from the 3′ region of the promoter subclone detected a 740-bp fragment. The fragment sizes derived from these hybridizations indicate that a supercoil-dependent S1-sensitive site is located in the region surrounding position −200 of the mouse *HPRT* promoter. This site is located very near or within the 5′ end of the DHS in chromatin. The DNA sequence in the region surrounding position −200 shows a notable feature: a series of three direct repeats of the sequence CTGGG (15). Such repeat structures have been correlated with S1-sensitive sites in other DNA sequences (10, 34). Interestingly, the sequence CTGGG is included in a DNase I-protected footprint in the X-linked human phosphoglycerate kinase gene (*PGK*) 5′ region (T. P. Yang, J. Singer-Sam, and A. D. Riggs, submitted for publication). An indirect repeat of the sequence GGGAGA is also present within the S1-sensitive region of *HPRT*.

Melton et al. (15) have shown that flanking sequences downstream from position −50 are sufficient to produce normal HPRT activity in transformed minigene constructs. The S1-sensitive site is upstream of this minimal functional promoter and thus may not have a direct role in transcription of the gene. If this S1-sensitive region does have a functional role in mouse *HPRT* expression, it may be related to regulation of expression in the X-chromosome inactivation process or to tissue-specific modulation of *HPRT* gene expression.

S1 nuclease has also been shown to cleave at the junction between B- and Z-DNA in supercoiled DNA (25). However, the major S1-sensitive site detected here maps approximately 100 bp upstream from a potential Z-DNA-forming sequence, $d(GC)_5 \cdot d(GC)_5$ (18, 26).

Discussion. The conformation of the X-linked mouse *HPRT* promoter region appears to be similar, in some respects, to that of other genes, both constitutive and nonconstitutive (5, 23). These similarities include preferential DNase I digestion of active chromatin versus inactive chromatin, a DHS near or containing the functional promoter, and an S1-sensitive site in supercoiled DNA which maps near or within the DHS. The localization of a DHS to a 5′ flanking CpG-rich island is also seen in the human dihydrofolate reductase gene (23) and the X-linked human

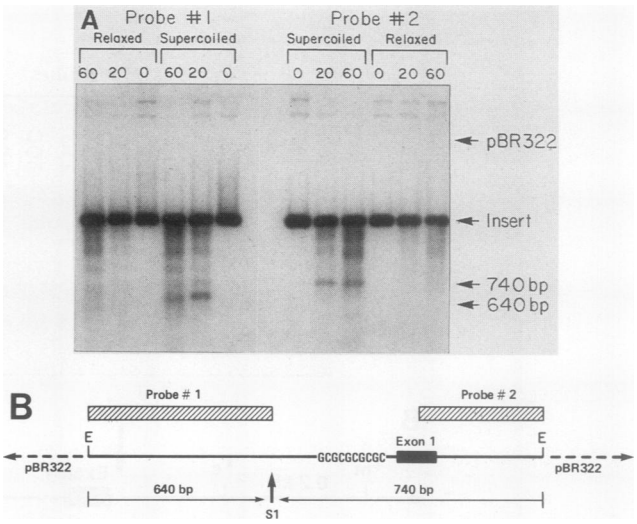

FIG. 3. S1 nuclease-sensitive site in supercoiled plasmid containing the mouse *HPRT* promoter region. (A) Southern blot of S1 nuclease-treated, *Eco*RI-digested pHPT6 in either supercoiled form or relaxed by pretreatment with topoisomerase I. The lane numbers indicate the lengths of time (min) the plasmid was treated with S1 nuclease. The positions of pBR322 and the full-length *Eco*RI insert of pHPT6 are indicated. (B) Diagram (not to scale) of insert from pHPT6. The S1 nuclease-sensitive site (vertical arrow) is approximately 200 bp upstream from the 5′ end of exon 1. $(GC)_5$ indicates a potential Z-DNA-forming sequence within the promoter region. E, *Eco*RI site. Probe 1 is a 648-bp *Eco*RI-*Bst*NI fragment of pHPT6. Probe 2 is a 914-bp *Nae*I-*Eco*RI fragment of pHPT6.

HPRT gene (30). Furthermore, the latter work (30) demonstrates that human *HPRT* chromatin from the active X chromosome appears to be more susceptible to cleavage by the restriction endonuclease *Msp*I than is inactive X chromatin, in agreement with our results with DNase I and the mouse *HPRT* gene. Riley et al. (20, 21) have also shown a similar difference in DNase I sensitivity in X-linked *PGK* chromatin, although they did not state that their analysis was localized to the far 5′ region of the gene within or adjacent to the promoter. Nevertheless, these results extend the observation of differential DNase I sensitivity to other regions of X-linked genes. However, unlike results with most other genes, their data also suggest an unexpectedly small difference in the DNase I sensitivity between active and inactive human *PGK* chromatin, in concordance with our results with mouse *HPRT*. It is possible that the small difference observed in the DNase I susceptibility between the active and inactive X chromosomes could reflect a novel chromatin conformation of constitutive genes expressed at low levels (20) or the chromatin conformation of GC islands.

It has been proposed (23, 30) that the correlation between promoter regions, CpG islands, and nuclease-sensitive sites may be important for the expression of constitutive genes. However, the mouse *HPRT* gene requires only 49 bp of 5′ flanking DNA sequences to permit normal expression in minigene transformation of cultured cells (15). This 49 bp does not include the S1-sensitive site or approximately one-half of the highly CpG-rich island (in the immediate 5′ flanking region) characteristic of the promoter region of constitutive genes. Thus, the interaction of these features in the expression, modulation, and regulation of constitutive genes remains unclear. Furthermore, no conformation or DNA sequence motif(s) characteristic of active or inactive X-linked genes is apparent at present.

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