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Multiple In Vivo Footprints Are Specific to the Active Allele of the X-Linked Human Hypoxanthine Phosphoribosyltransferase Gene 5′ Region: Implications for X Chromosome Inactivation

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Dosage compensation of X-linked genes in male and female mammals is accomplished by random inactivation of one X chromosome in each female somatic cell. As a result, a transcriptionally active allele and a transcriptionally inactive allele of most X-linked genes reside within each female nucleus. To examine the mechanism responsible for maintaining this unique system of differential gene expression, we have analyzed the differential binding of regulatory proteins to the 5′ region of the human hypoxanthine phosphoribosyltransferase (HPRT) gene on the active and inactive X chromosomes. Studies of DNA-protein interactions associated with the transcriptionally active and inactive HPRT alleles were carried out in intact cultured cells by in vivo footprinting by using ligation-mediated polymerase chain reaction and dimethyl sulfate. Analysis of the active allele demonstrates at least six footprinted regions, whereas no footprints were detected on the inactive allele. Of the footprints on the active allele, at least four occur over canonical GC boxes or Sp1 consensus binding sites, one is associated with a potential AP-2 binding site, and another is associated with a DNA sequence not previously reported to interact with a sequence-specific DNA-binding factor. While no footprints were observed for the HPRT gene on the inactive X chromosome, reactivation of the inactive allele with 5-azacytidine treatment restored the in vivo footprint pattern found on the active allele. Results of these experiments, in conjunction with recent studies on the X-linked human PGK-1 gene, bear implications for models of X chromosome inactivation.

The random inactivation of a single X chromosome during normal mammalian female embryogenesis results in a unique system of differential gene expression in which a transcriptionally active X chromosome and transcriptionally inactive X chromosome occupy the same nucleus. The inactivation of genes on one X chromosome in female somatic cells compensates for the dosage imbalance of X-linked genes between the sexes (8, 9). The molecular mechanisms responsible for initiating, spreading, and maintaining X chromosome inactivation are unknown. However, DNA-protein interactions (8, 28), chromatin structure (20, 34, 36), DNA replication (7, 47), and DNA methylation (19, 24, 25, 31, 38, 55, 57) have all been postulated to be involved. Though X inactivation is a chromosome-wide phenomenon and process, some degree of regulation at the level of individual X-linked genes must also be involved, as indicated by the ability to independently reactivate individual genes on the inactive X chromosome by 5-azacytidine (5-azaC) (12, 13, 31, 49, 50).

The differential expression of genes on the active and inactive X chromosomes is manifested by a difference in nuclease sensitivity of chromatin from the active and inactive alleles of the X-linked hypoxanthine-guanine phosphoribosyltransferase (HPRT) and phosphoglycerate kinase (PGK-1) genes (12, 23, 39, 40, 54, 56). Furthermore, the presence of DNase I hypersensitive sites in the 5′ region of the active HPRT and PGK-1 alleles (23, 40, 55, 56) and the absence of these hypersensitive sites on the inactive alleles suggest differential binding of regulatory proteins to genes on the active and inactive X chromosomes (4, 10). McBurney (28) has proposed that differential expression of genes on the active and inactive X chromosomes involves specific DNA-binding proteins that bind to *cis*-acting regulatory sequences near or within the promoter region of each X-linked gene that is subject to inactivation. This hypothesis predicts the existence of a sequence-specific DNA-binding repressor protein that silences genes on the inactive X chromosome and activator proteins that bind to regulatory regions of genes on the active X chromosome and activate transcription. Recently, in vivo footprint analysis of the human PGK-1 gene has revealed multiple DNA-protein interactions in the 5′ region specific to the active allele (36, 38); no in vivo footprints were detected on the inactive allele.

HPRT (EC 2.4.2.8) catalyzes the salvage of hypoxanthine and guanine to their respective nucleotides, IMP and GMP. HPRT is present in all cells and tissues, with elevated mRNA levels and enzymatic activity in the central nervous system, particularly the basal ganglia (46). The mammalian HPRT gene is X linked and constitutively expressed except on the inactive X chromosome, where it is transcriptionally silenced by X chromosome inactivation. As commonly seen in constitutively expressed genes, the HPRT promoter region lacks canonical TATA or CAAT sequences, uses multiple transcription start sites, and is extremely GC rich with multiple GC box sequences (5′-GGGCGG-3′) which are

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potential binding sites for the transcription factor Sp1 (3, 21, 35). Primer extension and nuclease protection analyses of the human HPRT promoter region (21, 35) have demonstrated multiple sites of transcription initiation in the region from −104 to −169 (relative to the translation start site). Furthermore, the human promoter is capable of functioning bidirectionally in vitro (18, 41), and a minimal region from −219 to −122 appears to be sufficient for normal levels of HPRT gene expression (41). A putative negative regulatory element has been reported in the region from position −570 to −388 (41).

We now report in vivo footprint analysis of the human HPRT gene 5′ region on the active and inactive X chromosomes by using the ligation-mediated polymerase chain reaction (LMPCR) (32, 37). We demonstrate multiple DNA-protein interactions specific to the active human HPRT allele and the absence of detectable DNA-protein interactions on the inactive allele. One unique footprinted region appears to define a novel regulatory element(s). These results, in conjunction with similar analysis of the human PGK-1 gene (36, 38), have implications for potential models that describe the molecular basis of X chromosome inactivation.

MATERIALS AND METHODS

Cell lines. GM00468 (NIGMS Human Genetic Mutant Cell Repository, Camden, N.J.) is a normal human 46, XY male fibroblast cell line containing an active X chromosome. Cell line 4.12 (33) (generously provided by David Ledbetter) is a hamster-human somatic cell hybrid containing only the active human X chromosome in the HPRT-deficient hamster cell line RJK88. RJK88 is a derivative of the V79 Chinese hamster fibroblast cell line and carries a deletion of the endogenous hamster HPRT gene (6). Cell line 8121-6TG D, hereafter referred to as 8121, is a hamster-human somatic cell hybrid containing an inactive human X chromosome in a RJK88 hamster cell background (provided by David Ledbetter). The human HPRT gene in 8121 cells was confirmed to be inactive by Northern (RNA) blot analysis with a human HPRT cDNA probe by the inability of these cells to grow in hypoxanthine-aminopterin-thymidine (HAT)-containing medium, by the growth of these cells in the presence of 6-thioguanine, and by the ability to reactivate the human HPRT gene in these cells by 5-azaC treatment (see below). HeLa S3 cells were grown in suspension culture and contain at least one active HPRT gene. GM05009b (NIGMS Human Genetic Mutant Cell Repository) is a human 49, XXXXX female fibroblast cell line carrying a single active X chromosome and four inactive X chromosomes (8, 11, 48).

In vivo footprint analysis was also carried out on the human HPRT gene of hybrid line 8121 in which the HPRT gene on the inactive human X chromosome was reactivated by treatment with 5-azaC. Cell line 8121R9a is a HPRT reactivant of 8121 grown from a single HAT-resistant colony after treatment with 5-azaC essentially as described by Hansen et al. (12). Cell line M22 is a 5-azaC-treated HPRT reactivant of a mouse-human somatic cell hybrid containing an inactive human X chromosome in a murine A9 cell background (this hybrid generously provided by Barbara Migeon).

All somatic cell hybrids containing an active HPRT gene were cultured by using standard techniques in Dulbecco's modified Eagle's medium (GIBCO) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin supplement (P-S; GIBCO) and supplemented with 1× HAT (0.1 mM hypoxanthine, 0.4 μM aminopterin, 0.016 mM thymidine). Cultures of cell line 8121 were maintained as described above but without HAT. Human fibroblasts were maintained in Ham's F-12 (GIBCO) with 10 to 20% FBS and 1% P-S. HeLa cells were grown in suspension with suspension modified essential media with 5% FBS and 1% P-S.

Preparation of DNA—in vivo DMS treatment and DNA isolation. Growth media were aspirated from nearly confluent T-150 flasks or 150-mm plates, and cells were washed once with 37°C phosphate-buffered saline (PBS). Twenty microliters of dimethyl sulfate (DMS) was then added to 20 ml of 37°C PBS (to a final DMS concentration of 0.1%) and mixed vigorously, and the final solution was gently layered over the cells in each culture flask. Initially, optimal DMS concentration and duration of DMS treatments were empirically determined; all subsequent experiments were carried out by using a 5-min treatment with 0.1% DMS. After treatment with DMS, the DMS-containing PBS was quickly aspirated, and the cells were washed twice with 50 ml of ice-cold PBS. Then 5 to 10 ml of lysis solution (50 mM Tris [pH 8.5], 50 mM NaCl, 25 mM EDTA, 0.5% sodium dodecyl sulfate [SDS], 300 μg of proteinase K per ml) was added to each flask or plate and incubated overnight at room temperature. Sodium chloride was added to a final concentration of 200 mM, and the lysate was extracted once with phenol, twice with phenol-chloroform-isoamyl alcohol (25:24:1), and once with chloroform. DNA in the final aqueous phase was then precipitated with 2 volumes of ethanol and sedimented at 4,000 × *g* for 45 min. The supernatant was decanted, and the pellet was washed with 80% ethanol. After air drying, the pellet was resuspended in either TE (10 mM Tris-HCl [pH 8], 1 mM EDTA) or water.

Occasionally, purified genomic DNA was digested with restriction enzymes (*Eco*RI or *Bam*HI, which do not cut within the region of the human HPRT gene to be analyzed) to reduce viscosity. After restriction enzyme digestion, the DNA was extracted twice with phenol-chloroform-isoamyl alcohol and ethanol precipitated as above.

Purified in vivo DMS-treated DNA was chemically cleaved at DMS-modified guanine residues by using standard Maxam-Gilbert piperidine treatment (27). DNA dissolved in water was first brought to a final concentration of 1 M piperidine with a concentrated stock solution of piperidine. DNA in TE was first ethanol precipitated and then redissolved in 1 M piperidine. Purified DNA dissolved in 1 M piperidine was incubated at 90 to 95°C for 30 min. Samples were then placed on ice, precipitated in 0.3 M sodium acetate (pH 5.2) and 2 volumes of ethanol, and sedimented at 14,000 × *g*. The resulting pellets were washed twice with 80% ethanol and dried overnight in a vacuum concentrator. Dried DNA pellets were then resuspended in TE and stored at −20°C. To obtain similar signal intensities among different samples in the final autoradiogram, DNA concentrations were determined spectrophotometrically. In order to confirm that equal amounts of DMS-treated genomic DNA were used in the subsequent LMPCR reactions and that the size distribution of piperidine-cleaved fragments was within the desired size range (average length of 600 bases for in vivo DMS-treated samples), a small aliquot of each sample was fractionated on alkaline agarose minigels (43) and stained with ethidium bromide.

In vitro DMS treatment. Control samples of purified genomic DNA were subjected to Maxam-Gilbert chemical modifications in vitro and then to piperidine cleavage. Unmodified genomic DNA was prepared as described above (without prior in vivo DMS treatment) and resuspended in water. For each base-specific cleavage reaction, 50 μg of

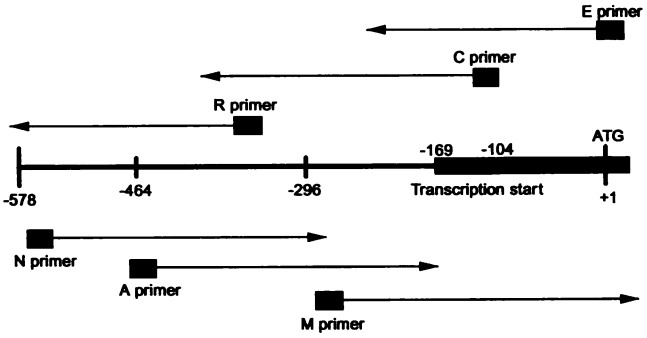

FIG. 1. Location of primers used in LMPCR analysis of the human HPRT 5′ region. The numbered line represents the human HPRT gene 5′ region with positions numbered relative to the translation initiation codon. The large rectangle represents the first exon, with the cross-hatched portion signifying the region of multiple transcription initiation sites (21, 35). The smaller rectangles above and below the numbered line indicate positions of the PCR primer sets used in the LMPCR footprinting analysis. Primer sets N, A, and M are complementary to the lower-strand sequence, and primers E, C, and R are complementary to the upper-strand sequence. Lines with arrowheads indicate the region and direction resolved by each primer set.

genomic DNA was dried and resuspended in 5 μl of sterile water. In the guanine-specific cleavage reactions, purified genomic DNA was modified with 0.5% DMS for 1 min at room temperature and processed as described by Maxam and Gilbert (27). Subsequent piperidine cleavage and DNA precipitation were performed as described above.

In order to provide a complete DNA sequencing ladder of the region of interest on each autoradiogram, plasmid pλ4×8-RB1.8 (kindly provided by P. Patel) containing a 1.8-kb *Eco*RI-*Bam*HI fragment of the human HPRT gene 5′ region in pUC8 (35) was linearized with *Eco*RI, and 2.5 μg of plasmid DNA was chemically modified and cleaved by the standard G, G+A, T+C, and C Maxam-Gilbert reactions. The chemically cleaved plasmid DNA was then diluted appropriately to produce autoradiogram signals equivalent to the genomic DNA samples following LMPCR and hybridization with a labelled probe.

LMPCR. Chemically modified and cleaved DNA was then subjected to amplification by LMPCR essentially as described by Mueller and Wold (32) and Pfeifer et al. (37). The oligonucleotide primers were synthesized (University of Florida Oligonucleotide Synthesis Facility) and used for LMPCR reactions to amplify and analyze specific regions of the human HPRT gene 5′ region. For in vivo footprint analysis of the lower strand, the following primer sets were used: set N, primer 1, GATGTGTACCCTGATCTG, and primer 2, GGGTGACTCTAGGACTCTAGGTCTCA; set A, primer 1, AATGGAAGCCACAGGTAGTG, and primer 2, AGGTCTTGGGAATGGGACGTCTGGT; and set M, primer 1, GAATAGGAGACTGAGTTGGG, and primer 2, GGAG CCTCGGCTTCTTCTGGGAGAA. For analysis of the upper strand, the following primer sets were used: set E, primer 1, AGCTGCTCACCACGACG, and primer 2, CCAG GGCTGCGGGTCGCCATAA; set C, primer 1, AGGCGGA GGCGCAGCAA, and primer 2, GGGAAAGCCGAGAGGT TCGCCTGA; and set R, primer 1, CCAACTCAGTCTCCT ATTCA, and primer 2, GAGGGCTCCCTGATTCCCAAAC CTA. The region covered by each primer set and the relative positions of the primer sets are diagrammed in Fig. 1.

After annealing of primer 1 to chemically cleaved genomic or plasmid DNA, primer extension of the HPRT-specific oligonucleotides with Sequenase (U.S. Biochemicals) was performed as described by Pfeifer et al. (37) except that 7-deaza-dGTP was substituted in a 3:1 molar ratio with dGTP. A total of 5 μg of chemically cleaved genomic DNA was used for each Sequenase reaction. Following extension of primer 1 by Sequenase, blunt-end ligation of the asymmetric double-stranded linker was performed as described by Mueller and Wold (32). Ligated DNA was ethanol precipitated in 2.5 M ammonium acetate and redissolved in 20 μl of sterile water. The appropriate region of the human HPRT gene was then amplified by PCR with *Taq* DNA polymerase (Perkin-Elmer Cetus) with primer 2 from each primer set and the longer oligonucleotide of the asymmetric linker as primers (37). Again, 7-deaza-dGTP was substituted for dGTP in a 3:1 molar ratio with dGTP to allow the amplification of regions with extremely high G+C contents. After 18 cycles of PCR (using a Coy Tempcycler), the DNA was extracted once with phenol-chloroform-isoamyl alcohol and once with chloroform, and precipitated with ammonium acetate and ethanol as before. The resulting pellet was washed with 1 ml of 80% ethanol, dried in a vacuum concentrator, resuspended in 20 μl of water, and stored at −20°C. Each of the HPRT-specific primer sets was used individually for LMPCR because multiplex analysis (37, 38) using two or more primer sets in each LMPCR reaction occasionally yielded artifacts or variability between experiments.

Gel electrophoresis and electrotransfer. A total of 5 μl of each PCR mixture was dried and resuspended in 2.0 μl of formamide-dye solution (98% formamide, 0.25% xylene cyanol, 0.25% bromophenol blue, 10 mM EDTA [pH 8]). The redissolved samples were denatured at 95°C for 5 min and quenched on ice. Denatured samples were then loaded onto a 0.04-cm-thick, 8.3 M urea–6% polyacrylamide (29:1 acrylamide-bis-acrylamide) DNA sequencing gel in 1× TBE (50 mM Tris, 50 mM boric acid, 2 mM EDTA [pH 8.3]). After electrophoresis at 40 to 50°C, the gel was transferred to Whatman 541 SFC paper. DNA in the gel was then electrotransferred to Hybond N+ nylon membrane (Amersham) by using an electroblotting apparatus (Polytech Products) at 110 V and 2 A in transfer buffer (40 mM Tris, 40 mM boric acid, 1.6 mM EDTA [pH 8.3]) for 45 min as described by Church and Gilbert (2). After transfer, the nylon membrane was rinsed briefly in transfer buffer and then dried in a vacuum oven at 80°C for 1 h.

Probe synthesis, hybridization, and washing. The ^{32}P-labelled hybridization probes used to visualize the DNA sequencing ladder and in vivo footprints were synthesized from a single-stranded M13 template by using a modification of the procedure described by Church and Gilbert (2). To generate the appropriate single-stranded HPRT-specific templates for probe synthesis, the 1.8-kb *Eco*RI-*Bam*HI human HPRT 5′ genomic fragment of plasmid pλ4×8-RB1.8 (35) was cloned into the *Eco*RI-*Bam*HI sites of both M13mp18 and M13mp19, yielding two subclones with the insert in different orientations, with each single-stranded template carrying a different strand of the human HPRT gene 5′ region. Large-scale preparations of each single-stranded M13 template DNA were performed as described by Sambrook et al. (43).

Synthesis of the labelled single-stranded hybridization probe from the appropriate M13 template was similar to that described by Church and Gilbert with one notable exception. Synthesis of the labelled probe was primed using primer 2

from the appropriate HPRT-specific LMPCR primer set rather than priming with the M13 universal primer. This modified procedure for probe synthesis was performed as follows. One-half picomole of the appropriate purified M13 template (containing one strand of the human HPRT 5′ region), 5 μl of a 1-pmol/μl solution of the appropriate primer 2 (which is complementary to the M13 template), and 2.5 μl of 10× Klenow buffer (10× buffer is 2 M NaCl, 500 mM Tris [pH 8]) were combined in a 1.5-ml microcentrifuge tube. The mixture was denatured at 95°C for 5 min and then incubated at 50°C for 30 min. Following annealing, 5 μl of 50 mM $MgCl_2$, 5 μl of 0.1 M dithiothreitol, 2 μl of a 3 mM solution (each) of dATP, dGTP, and dTTP, 10 μl of [α-^{32}P]dCTP (Amersham, 3,000 Ci/mmol, 10 μCi/μl), and 2 μl of Klenow fragment (5 U/μl) (Ambion) were added and incubated at 37°C for 45 min. Next, 120 μl of formamide-dye solution was added and the mixture denatured at 95°C for 10 min, quenched on ice, and loaded onto a 1.5-mm-thick 6% denaturing polyacrylamide gel (6% acrylamide, 40:1 acrylamide-bisacrylamide, 8.3 M urea) in 2× TBE (100 mM Tris, 100 mM boric acid, 4 mM EDTA). Electrophoresis was continued until the xylene cyanol and bromophenol blue markers were separated by 4 to 5 cm, and then labelled probe was excised from the gel. The optimal probe length is just above the xylene cyanol dye, though shorter and longer probes have been used with success. The probe length is controlled by adjusting the ratio of template DNA to radiolabelled dCTP. The portion of the acrylamide gel containing the probe was cut from the remainder of the gel with a razor blade, crushed into a fine paste with a glass rod, and suspended in 4 to 6 ml of hybridization solution (0.25 M Na_2HPO_4 brought to pH 7.2 with phosphoric acid, 7% SDS, 1% fraction V bovine serum albumin [Sigma], 1 mM EDTA, as described by Church and Gilbert [2]) at 65°C. Simultaneously, the nylon blot was prehybridized for 10 to 15 min with 15 ml of hybridization solution at 65°C in the siliconized glass tube of a hybridization chamber (Robbins Scientific). After 15 min, the prehybridization solution was discarded and the slurry containing the labelled probe was added directly to the hybridization tube. The blot was hybridized for 6 to 8 h at 68°C, the hybridization solution was discarded, and the blot was quickly and vigorously rinsed three or four times with 50 to 100 ml of wash solution (20 mM Na_2HPO_4 brought to pH 7.2 with phosphoric acid, 1% SDS, 1 mM EDTA as described by Church and Gilbert) at 65°C in the hybridization tube. The blot was transferred to a shaking water bath (Bellco) containing wash solution at 65°C, and the wash solution was exchanged every 10 to 15 min until nonspecific background was removed. The blot was then covered with plastic wrap and exposed to either Kodak X-OMAT AR film or Amersham Hyperfilm MP without intensifying screens for 3 h to several days.

RESULTS

The 5′ region of the human HPRT gene on the active and inactive X chromosomes was examined in vivo for sequence-specific DNA-protein interactions. The region spanning positions −530 to −14 (relative to the translation initiation codon) was subjected to in vivo footprint analysis by using a modification of the LMPCR technique described by Mueller and Wold (32) and Pfeifer et al. (37).

This analysis was performed on seven different cell lines to examine the in vivo footprint pattern of either the active or the inactive HPRT allele. Hybrid cell line 4.12 contains only the active human X chromosome in hamster cell line RJK88, which carries a deletion of the hamster HPRT gene (6). Thus, any in vivo footprint detected on the HPRT gene will be specific to the active human HPRT allele. Similarly, cell line 8121 is a human-hamster hybrid that contains the inactive human X chromosome in a RJK88 hamster cell background. Footprints detected on the HPRT gene in this cell line will be associated with the inactive human allele. Since sequence-specific DNA-binding proteins in cell lines 4.12 and 8121 will most likely be of hamster origin and will be bound to heterologous human HPRT DNA sequences, normal human male fibroblasts (GM00468) and HeLa cells were included in the analysis as controls. Both of these cell lines carry an active human HPRT gene interacting with endogenous human DNA-binding proteins and were useful for identifying footprints that may have been due to artifacts of a heterologous human-hamster hybrid system. To confirm that in vivo footprints on the inactive HPRT allele in hybrid 8121 are also present in intact female human cells, a human fibroblast cell line carrying five X chromosomes (49, XXXXX) was also analyzed (GM05009b). Because this cell line carries four inactive human X chromosomes and a single active X chromosome (8, 11, 48), the predominant in vivo footprint pattern from the human HPRT gene will be derived from the inactive allele. Therefore, analysis of these cells will confirm results from hybrid cell line 8121 (carrying the inactive X chromosome).

In addition to the in vivo footprint pattern on the active and inactive X chromosomes, the footprint pattern of 5-azaC-reactived HPRT genes on the inactive X chromosome was examined. Cultures of 8121 cells (carrying an inactive X chromosome) were plated at low density, grown in the presence of 5-azaC, and selected for reactivation of the human HPRT gene in HAT-containing medium. Cells that carried a reactivated HPRT gene were HAT resistant and isolated as single cell-derived colonies. The isolate that displayed the highest level of HPRT mRNA (cell line 8121R9a; data not shown) was used for in vivo footprint analysis. In vivo footprint analysis was also performed on cell line M22, a 5-azaC-reactivated human HPRT gene in a HPRT-deficient mouse A9 cell background (54).

Figure 1 shows the relative location of the oligonucleotide primer sets used for LMPCR in vivo footprinting of the 5′ region of the human HPRT gene. The region from positions −530 to −14 was analyzed for sequence-specific DNA-protein interactions on both strands. More extended analysis of the lower strand of the region spanning −13 to +42 and the upper strand of the region spanning −531 to −580 was also possible by using primer sets M and R, respectively.

Results of LMPCR in vivo footprinting of the upper strand in the region of the multiple transcription start sites (21, 35) with primer set E is shown in Fig. 2A. A single guanine showing strong enhanced reactivity to DMS is detected at position −91 in all samples prepared from cells treated in vivo with DMS that carry an active X chromosome or a 5-azaC-reactivated human HPRT gene. This enhanced cleavage site is not detected in purified DNA samples (from the same cell lines) that were treated with DMS after DNA isolation, nor is it detected in the in vivo-treated sample of cell line 8121 which contains the inactive human X chromosome. Very weak protection from DMS is also observed at the guanine residue at position −93. These features are the only evidence for a footprint on the upper strand between positions −14 and −162, and all samples with an active human HPRT gene display the identical footprint pattern. This includes samples in which the human HPRT gene is active in human, hamster, and mouse cell backgrounds as

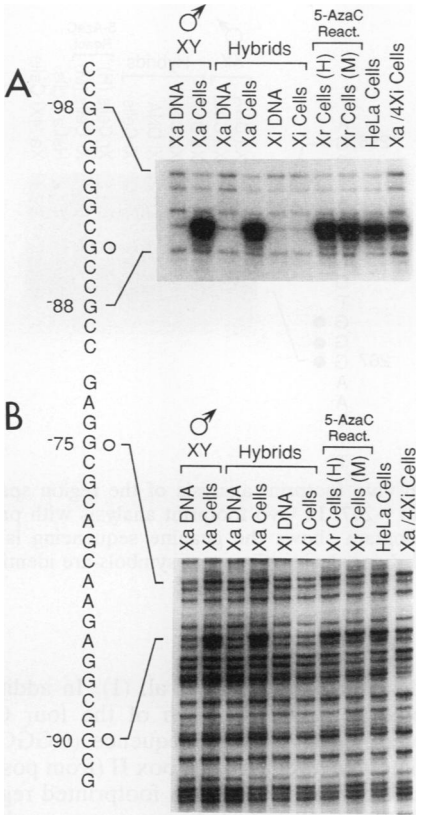

FIG. 2. In vivo footprints in the region spanning positions −75 to −98. (A) In vivo footprint analysis with primer set E. This autoradiogram shows the guanine-specific cleavages and sequencing ladder from the upper strand. (B) In vivo footprint analysis with primer set M. This autoradiogram shows the guanine-specific cleavages and sequencing ladder from the lower strand. The nucleotide sequence in the region of each footprint and the position of each nucleotide relative to the translation initiation codon are shown to the left of each sequencing ladder. Open circles to the right of the nucleotide sequence represent the sites of enhanced DMS reactivity, and solid circles represent sites of protected guanine nucleotides. For the gel lane designations, DNA denotes purified naked DNA isolated from the appropriate cell line and treated with DMS in vitro; Cells, samples that were obtained from intact cells treated in vivo with DMS; Xa, samples containing the active human X chromosome; Xi, samples containing the inactive human X chromosome; Xr and 5-AzaC, samples from rodent-human hybrid cell lines containing a 5-azaC-reactivated human HPRT gene on the inactive X chromosome in either a hamster (lane H; cell line 8121R9a) or mouse (lane M; cell line M22) cell background; XY, samples prepared from normal diploid male human fibroblasts (cell line GM00468); Hybrids, samples prepared from hamster-human somatic cell hybrids containing either the active (cell line 4.12) or inactive (cell line 8121) human X chromosome; HeLa, HeLa cells; Xa/4Xi, samples from a 49, XXXXX female fibroblast cell line (cell line GM05009b).

well as those reactivated with 5-azaC. Interestingly, a palindrome of the sequence GCGGC, with a dyad axis of symmetry between positions −92 and −91, includes both the site of strong enhanced DMS reactivity and the weakly protected guanine residue. However, because this footprint is not detected in purified DNA treated with DMS (in vitro-treated samples), it is very likely that the footprint is due to binding of a protein in vivo rather than secondary structure in purified DNA. Because of the strength of this enhanced DMS reactivity at position −91, the sample from the 49, XXXXX human fibroblast cell line also shows a readily detectable signal despite the presence of only a single active HPRT gene among four inactive genes.

Analysis of this same region on the opposite strand (lower strand) was carried out by using PCR primer set M; the results are shown in Fig. 2B. Comparison of the cleavage patterns and relative band intensities between DMS-treated purified DNA samples and in vivo DMS-treated samples reveals two enhanced DMS-reactive sites, one at position −75 and another single enhancement at position −90. As with the footprint in this region on the upper strand, these enhanced cleavages occur only in samples where intact cells carrying an active human X chromosome or active human HPRT gene were treated in vivo with DMS prior to DNA purification. One site of enhanced reactivity (at position −90) occurs within the immediate region of the footprint observed on the opposite (upper) strand (at the strong enhancement at position −91). The enhancement at position −75 on the lower strand is 16 nucleotides downstream of the other protection and enhancements in this region, and it is unclear whether this single site of enhanced reactivity represents a separate footprint (i.e., different DNA-binding protein) or is part of the DNA-protein interaction occurring around position −91. The DNA sequence containing the −91 footprint has not been reported to be a site for binding of a transcription factor (5, 26).

Proceeding upstream from position −91, no evidence for footprints on either strand is detected in any of the samples until position −159 is analyzed with primer set M on the lower strand. In all samples carrying an active human HPRT gene that were DMS treated in vivo, the guanine nucleotide at position −159 shows enhanced DMS reactivity followed by protected guanines at positions −160 and −165 (Fig. 3A). Again, no evidence for a corresponding footprint is detected in in vivo-treated samples from the somatic cell hybrid 8121 containing the inactive X chromosome. Similarly, the cleavage pattern of the 49, XXXXX sample was comparable to the pattern seen with both naked DNA and hybrid 8121. Further evidence for a footprint in this region from samples containing an active HPRT gene is detected on the upper strand by using primer set C. As shown in Fig. 3B, enhanced DMS reactivity at the guanine residue in position −163 is followed by four protected guanine residues (positions −164 to −168). Weaker (but significant) protection is observed in the 5-azaC-reactivated human HPRT gene in the mouse cell background (cell line M22); this appears to be true for nearly all of the footprints detected in this cell line, and the reason for this is unclear. This footprinted region (from position −159 to −168) contains a canonical GC box (GGGCGG; designated GC box I in Fig. 3) suggestive of binding in vivo of the transcription factor Sp1 (1,3)—or a rodent homolog of Sp1—to the active human HPRT allele and in 5-azaC-reactivated HPRT genes.

The in vivo footprint associated with GC box I on the active HPRT gene is followed in these same samples by a series of DMS-protected sites and enhanced reactivity sites immediately upstream at guanines in three additional GC box sequences (designated GC boxes II, III, and IV) amplified by primer set C. As seen in Fig. 3, in vivo footprints are detected on both strands between positions −172 to −190 (that includes GC box II), −194 to −205 (that includes GC box III), and −207 to −215 (that includes GC box IV). Each of these in vivo footprints is detected only in samples containing an active or reactivated human HPRT gene. However, only the sequence surrounding GC box III (GGGGCGGGGC) conforms to the consensus Sp1 binding

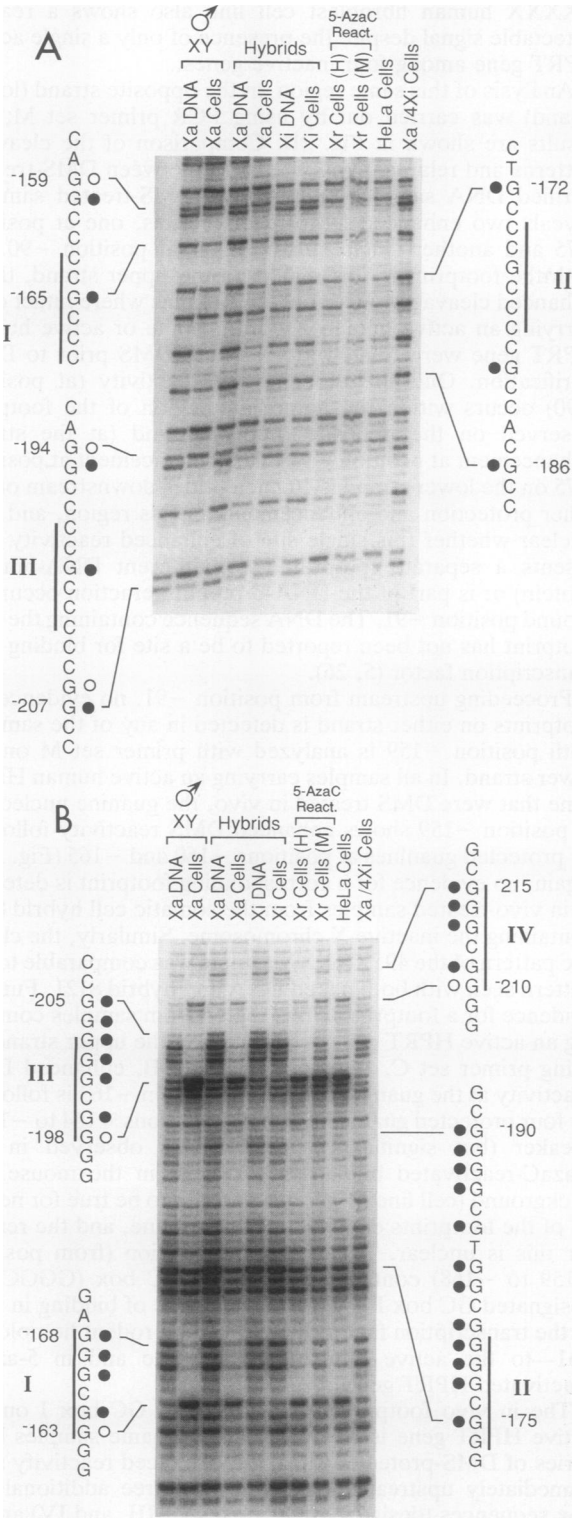

FIG. 3. In vivo footprint analysis of the region spanning positions −159 to −215. (A) In vivo footprint analysis with primer set M. The autoradiogram shows the guanine sequencing ladder of the lower strand. (B) In vivo footprint analysis with primer set C. The autoradiogram shows the guanine-specific sequencing ladder of the upper strand. Lane designations and symbols are identical to those in Fig. 2. Solid vertical lines indicate the position of GC boxes, and roman numerals adjacent to GC boxes correspond to positions of GC boxes indicated in Fig. 5 and discussed in text.

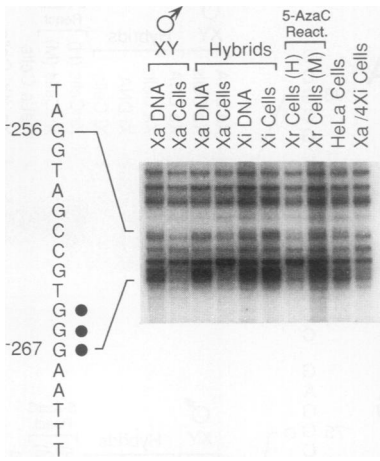

FIG. 4. In vivo footprint analysis of the region spanning positions −256 to −267. In vivo footprint analysis with primer set A. The autoradiogram shows the guanine sequencing ladder of the lower strand. Lane designations and symbols are identical to those in Fig. 2.

sequence described by Briggs et al. (1). In addition to the potential binding of Sp1 at each of the four GC boxes, another potential Sp1 binding sequence (GGGGCGTGGC [1]) immediately upstream of GC box II (from position −181 to −190) is also included within a footprinted region on the active HPRT gene, though it does not carry a classical GC box sequence. Thus, the active (and 5-azaC-reactivated) human HPRT promoter region exhibits in vivo footprints over five potential Sp1 binding sites. Interestingly, the region surrounding the footprint between positions −175 and −190 contains a direct repeat of the sequence GCGGGGCG.

Further upstream from the multiple footprints associated with GC boxes I to IV, primer set A detects a series of three protected guanine residues on the active HPRT alleles between positions −265 and −267 on the lower strand (Fig. 4), though the degree of protection appears to vary according to the cell line analyzed. The footprint is readily detected in diploid male human fibroblasts (GM00468), hybrid cell line 4.12 containing the active human X chromosome, and a 5-azaC-reactivated human HPRT gene in a hamster-human hybrid (cell line 8121R9a), while it is clearly not present in hamster-human hybrid 8121 carrying the inactive human X chromosome. However, the three guanine residues are only weakly protected, if at all, in two other cell lines containing an active human HPRT gene, the 5-azaC-reactivated HPRT gene in a mouse-human hybrid (cell line M22), and HeLa cells. The basis of the weak protection of this region in HeLa cells is unknown, particularly since HeLa cells show strong footprints at all of the other footprinted regions, and a factor binding to this DNA sequence (5′-TGGGAATT-3′) has been reported in HeLa cell extracts (17) (see Discussion). The reason for very weak protection at this position in the mouse-human hybrid reactivant is also unknown; this cell line also shows slightly weaker protections in the region of the GC boxes (Fig. 3). No footprint of this region is observed in any cell line on the upper strand by using primer set C, perhaps because this region on the upper strand is deficient in guanine residues. Curiously, unlike all of the other footprints observed in this study, this region does appear to demonstrate full protection in the 49, XXXXX human fibroblast cell carrying four inactive X chromosomes (Fig. 4, lane

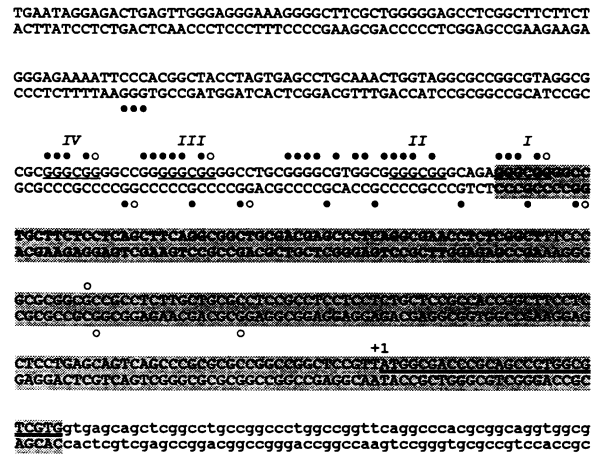

FIG. 5. Summary of in vivo footprint analysis of the human HPRT gene 5′ region. The sequence of the human HPRT 5′ region indicating positions of in vivo footprints on the active HPRT allele. Numbering of nucleotides begins with +1 at the translation initiation codon. The shaded region indicates the first exon. The nucleotides shown in boldface within the first exon represent the region of multiple transcription initiation sites (21, 35). The double underlined region denotes the protein coding region within exon 1. The region shown in lowercase letters indicates nucleotides within the first intron. The regions underlined with a single line indicate the positions of GC boxes. Each of the four GC boxes is numbered with a roman numeral that corresponds to the roman numerals indicating GC boxes in Fig. 3. Closed circles indicate the position of protected guanine residues, and open circles indicate the position of enhanced DMS reactivity. Circles above the nucleotide sequence indicate footprints detected on the upper strand and circles below the sequence indicate footprints detected in the lower strand.

Xa/4Xi), suggesting that this region may be bound by a protein on most or all of the multiple inactive X chromosomes as well as the active X chromosome.

No evidence for any other footprints in the region from −580 to +42 is detected on either strand. Figure 5 shows the nucleotide sequence of the 5′ region from the human HPRT gene and summarizes the DMS in vivo footprint data by indicating the position of all DMS-protected sites and sites of enhanced DMS reactivity detected in this study.

DISCUSSION

In vivo DMS footprint analysis of the immediate 5′ region of the human HPRT gene in a variety of cell lines carrying active and/or inactive human X chromosomes has revealed multiple footprints specifically on the transcriptionally active allele. At least six in vivo footprints are located on the active, or 5-azaC-reactivated, HPRT gene and are presumed to indicate sites of sequence-specific DNA-protein interactions. The footprint patterns in cell lines carrying an active human HPRT gene are essentially identical despite differences in the species of the background cell line (human, hamster, or mouse), suggesting the DNA-binding proteins from the rodent species are interacting with the human HPRT DNA sequences in a manner identical to that of the human binding proteins seen in normal human male cells. The appearance of these footprints correlate with transcriptional activity of the human HPRT gene and the presence of a nuclease-hypersensitive site in the 5′ region of the transcriptionally active gene (23, 55). In contrast, the HPRT gene on the inactive X chromosome—with a single apparent exception in the 49, XXXXX female cell line (see below)—appears to be devoid of detectable sequence-specific in vivo footprints. Furthermore, the DMS reactivity pattern of the inactive HPRT gene in hybrid 8121 is essentially indistinguishable from that of naked DNA.

DNA-protein interactions specific to the active HPRT allele. The DNA sequence contained within (or immediately adjacent to) the footprint associated with the strong DMS-reactive sites at position −91 on the upper strand and at −90 and −75 on the lower strand (termed the −91 footprint) appears to represent a new *cis*-acting regulatory element and a target sequence for a new DNA-binding protein(s). A DNA data search with the DNA sequence from the immediate region containing the enhanced DMS-reactive sites at position −91 to position −75 did not yield clear sequence identity with any previously described regulatory elements among vertebrate control DNA sequences (5, 26). The position of this footprinted region just 3′ to the multiple sites of transcription initiation (−104 to −169) suggests the protein(s) associated with this DNA sequence may function in transcription initiation as has been postulated for other DNA-binding regulatory factors located in a similar position. These factors include HIP-1 (29), Inr (45), YY1 (44), and TFII-I (42). Comparison of the DNA sequence in the −91 footprint with the DNA sequences bound by these initiation factors yielded no significant sequence similarity between these *cis*-acting elements and the −91 footprint. This suggests that the DNA-protein interaction(s) in the −91 footprint may represent a new regulatory element involved in transcription initiation.

Recently, Rincon-Limas et al. (41) have reported that promoter DNA sequences between −219 to −122 are necessary and sufficient for normal expression levels of the human HPRT gene by DNA transfection and transient expression assays. However, the region spanning this promoter fragment does not include the region carrying the −91 in vivo footprint. More recent studies of the −219 to −122 promoter fragment in transgenic mice indicate additional DNA sequences from the HPRT gene 5′ region are required for normal promoter function (41a).

Upstream of the −91 footprint, a closely spaced cluster of at least four in vivo footprints is observed between positions −159 to −215 in the HPRT gene only on active human X chromosomes and on 5-azaC-reactivated HPRT genes. The close proximity of the footprints in this region makes it difficult to infer the actual number of discrete protein binding sites. However, this region contains four copies of the hexanucleotide GC box sequence 5′-GGGCGG-3′, each of which is included in regions that exhibit an in vivo footprint on the active human HPRT gene. This sequence is the core sequence of the binding site for the transcription factor Sp1 (1), suggesting a role for multiple Sp1 molecules (and its rodent homolog in somatic cell hybrids) in transcription of the human HPRT gene in vivo. However it is possible that a protein(s) other than Sp1 may be interacting at these apparent Sp1 binding sites. Nonetheless, the footprints associated with GC boxes I, III, and IV exhibit a very similar footprint pattern, suggesting that the same protein(s) is bound in vivo at these three sites. The in vivo footprint that includes GC box II (from positions −172 to −190) is larger and displays a slightly different pattern of DMS protection and enhanced reactivity from GC boxes I, III, and IV. Closer examination of the DNA sequence in this region reveals another potential Sp1 binding site between positions −181 to −190 that does not contain a classical GC box. Of the five potential Sp1 binding sites, only GC box III and the potential Sp1 binding

site upstream of GC box II match the reported consensus binding site for Sp1 (1). Thus, the DNA sequences containing GC boxes I, II, and IV may represent additional degeneracy in the binding site sequence for Sp1 (or binding of a protein[s] other than Sp1).

Further upstream of the GC boxes in a region from position −265 to −267, three adjacent guanine nucleotides exhibit some degree of protection from DMS in vivo in all cell lines carrying an active human HPRT gene. The DNA sequence including and surrounding the protected guanine residues contains a potential binding site for the transcription factor AP-2 (52), as well as factors E2aE-CB and E4F2, cell-encoded factors that bind to this sequence in the adenovirus E2A and E4 genes, respectively (17, 26). This in vivo footprinted region in the human HPRT gene is also not included within the minimal promoter fragment (from −219 to −122) previously identified as having full promoter function in transient expression assays (41). Furthermore, the 49, XXXXX human female cells carrying a single active X chromosome and four inactive X chromosomes appear to display full protection in this region (see Results) (Fig. 4). This would suggest that this factor is bound to most, if not all, of the HPRT gene copies in this cell line, regardless of whether they are on the active or inactive X chromosomes. The role of this factor in the differential expression of the HPRT gene on the active and inactive X chromosomes is unclear.

No other in vivo footprints in the immediate 5′ region on either the active or inactive human HPRT alleles are detected in this study. This includes the region from −570 to −388 reported to contain a negative regulatory element (41). However, DNA-binding proteins that are weakly associated with guanine residues, or that bind DNA sequences lacking guanines, may not be detected by DMS footprinting. Binding of such proteins may be revealed by in vivo footprinting with DNase I (36).

Comparison of in vivo footprints in the human HPRT and PGK-1 genes. In vivo footprint analysis of the human HPRT gene now permits a comparison with similar studies of the human PGK-1 gene on the active and inactive X chromosomes by Pfeifer et al. (36, 38). These studies reveal both significant similarities and differences. The promoter regions of both genes are GC rich, lack TATA boxes, and display multiple in vivo footprints only on the active X chromosome and 5-azaC-reactivated genes. The promoter regions of both genes on the active X chromosome also exhibit in vivo footprints associated with multiple GC boxes, suggesting that the ubiquitous transcription factor Sp1 is involved in the transcriptional activation of both genes. No in vivo footprints are detected by using DMS on the inactive HPRT allele (with one possible exception in 49, XXXXX cells; see above) or with DMS and DNase I on the inactive PGK-1 allele (36, 38).

Other than the presumptive Sp1 in vivo footprints associated with the multiple GC boxes and/or Sp1 consensus sequences in each gene, no DNA sequences common to both genes are footprinted. For instance, the human PGK-1 gene does not display a footprint in the region equivalent to the −91 footprint region in human HPRT (just downstream of the multiple transcription start sites in both genes). Thus, there appears to be no novel DNA-binding regulatory factor or *cis*-acting regulatory sequence that is specific for X-linked genes (or even to X-linked housekeeping genes) either on the active or inactive X chromosomes.

Implications for X chromosome inactivation. In vivo footprinting studies of the X-linked human HPRT and PGK-1 genes do not appear to support the hypothesis that X inactivation is a process regulated by a specific DNA sequence that binds either activator or repressor proteins within the promoter region of each X-linked gene subject to inactivation (28). The absence of an in vivo footprint on the inactive allele of the HPRT and PGK-1 genes argues against a sequence-specific repressor protein binding to each X-linked gene subject to X inactivation which coordinately silences genes on the inactive X chromosome. These data also argue against models for X inactivation that require a unique activator protein(s) that specifically and coordinately potentiates transcription of X-linked genes (on the active X chromosome) since a novel in vivo footprinted DNA sequence common to both HPRT and PGK-1 has not been identified on the active allele of both genes. However, it is possible that binding sites for important regulatory proteins may be located outside of the 5′ region analyzed in these studies.

A role for DNA methylation in X inactivation has been suggested, in part, by the relative hypermethylation of cytosine residues in the GC-rich island in the 5′ region of X-linked housekeeping genes on the inactive allele compared with that on the active allele (19, 38, 53, 57). Meehan et al. (30) and Huang et al. (16) have described DNA-binding proteins that preferentially bind to methylated DNA. These proteins could potentially play a role in silencing transcription of housekeeping genes by specifically binding to hypermethylated GC-rich promoter regions (or GC islands) on the inactive X chromosome. No evidence for such proteins has been detected in the 5′ region of either the HPRT or PGK-1 (36, 38) genes by in vivo footprinting of the inactive alleles. However, it is possible that these proteins are not detected by these studies because of a lack of DNA sequence specificity or weak binding to DNA.

The presence of multiple footprints on the active X chromosome and the lack of footprints on the inactive X chromosome suggest that transcription factors in female nuclei—while able to bind and activate transcription of genes on the active X chromosome in the same nucleus—may be unable to gain access to their target DNA sequences on the inactive X chromosome or are unable to form stable sequence-specific DNA-protein complexes on the inactive X chromosome. One possibility for preventing binding of factors on the inactive allele of X-linked genes is that DNA methylation may interfere directly with formation of stable sequence-specific DNA-protein complexes (22, 51). However, this may not be a general mechanism for preventing stable binding of transcription factors to the inactive X chromosome because binding of at least one potential factor identified by in vivo footprinting on the active X chromosome—Sp1—is not affected by methylation within its binding site when assayed in vitro (14, 15). An alternative mechanism for the differential binding of transcription factors to the active and inactive alleles of X-linked genes involves chromatin structure. The presence of nucleosomes at DNA-binding sites (36) or the higher-order chromatin structure on the inactive X chromosome may prevent access of transcription factors to their binding sites. It is also possible that hypermethylation of the 5′ region of housekeeping genes on the inactive X chromosome may have a role in establishing or stabilizing local chromatin structure of 5′ *cis*-acting regulatory sites (and/or GC islands).

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