

Chromatin structure analysis of the mouse *Xist* locus

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ABSTRACT The *Xist* gene is expressed exclusively from the inactive X chromosome and plays a central role in regulating X chromosome inactivation. Here we describe experiments aimed at defining the extent of the active chromatin domain of the expressed *Xist* allele. By using an allele-specific general DNaseI sensitivity assay we show that there is preferential digestion of the expressed allele at sites within the transcribed locus but not in flanking sites located up to 70 kb 5'. A putative proximal boundary for the *Xist* domain is located within 10 kb upstream of promoter P₁. Chromatin in the expressed domain was found to be acetylated at H4 in XX somatic cells but also in XY cells, where *Xist* is never expressed. A single clear exception to this was the *Xist* promoter, which is acetylated only in XX cells. These observations concur with the view that H4 acetylation may not be a general marker of active chromatin domains and further support data implicating local promoter acetylation as being of primary functional significance *in vivo*.

In female mammals, dosage compensation is achieved by the transcriptional silencing of one of the two X chromosomes, a process known as X inactivation (1). Features of the inactive X chromosome (Xi) that distinguish it from its active counterpart (Xa) are that it is condensed during interphase, replicates late in S phase, and is underacetylated at histones H3 and H4 (reviewed in ref. 2). More recently it has been shown that chromatin on Xi is highly enriched for the variant histone macroH2A (3).

The classically defined X inactivation center (Xic) is required both for initiation of X inactivation in early development and for propagation of the inactivation signal in *cis* (reviewed in ref. 4). Initiation of random X inactivation involves determining how many (counting) and which (choosing) X chromosome to inactivate. It has been suggested that this is achieved by cells blocking a single Xic and thus marking that chromosome as the active X chromosome. X inactivation then proceeds in *cis* from unblocked Xics at the onset of cellular differentiation (5).

The X inactive specific transcript (*Xist*) gene, originally identified as a candidate for the Xic (6–9), produces a large RNA with no apparent protein coding potential (10, 11). *Xist* RNA “coats” the inactive X chromosome domain in the interphase nucleus, suggesting that it provides the primary signal for in *cis* propagation of X inactivation (11, 12). A requirement for *Xist* in propagation of X inactivation in *cis* has been demonstrated by using targeted deletion of transcribed regions (13, 14). Importantly, the counting function of the Xic was unaffected in these experiments. A subsequent gene-targeting experiment indicated that sequences distal to *Xist* are important in counting (15). Analysis of XY embryonic stem (ES) cells bearing a 450-kb *Xist* yeast artificial chromosome transgene demonstrated that *Xist* is sufficient for X inactivation

(16) and that transgenic loci recapitulate both counting and propagation functions (16, 17). This result was subsequently shown by using a much smaller 35-kb transgenic *Xist* construct that encompasses *Xist*, 9 kb of upstream sequence, and 6 kb of downstream sequence (18).

It has recently been shown that developmental up-regulation of *Xist* on the inactive Xi allele is attributable to RNA stabilization (19, 20) and that this in turn results from a developmentally regulated promoter switch (21). An upstream promoter (P₀) transcribes an unstable isoform of *Xist* RNA before X inactivation. As cells differentiate, there is a switch to downstream promoters (P₁, P₂) and *cis* accumulation of stable *Xist* RNA on Xi. The Xa allele continues to transcribe unstable RNA from P₀ for a short period and is then transcriptionally silenced.

Here we describe experiments directed toward defining the chromatin domain structure of the *Xist* locus by assaying both general nuclease sensitivity of Xi and Xa *Xist* alleles and by determining histone acetylation levels in XY and XX cells. The study was undertaken to identify boundaries at which there is a transition from the expressed *Xist* locus to flanking silent inactive X chromatin. Such boundaries could theoretically be important for insulating the *Xist* locus. In addition, we wished to define the probable maximal region encompassing regulatory elements required for *Xist* expression in XX somatic cells. Results from the DNaseI sensitivity assay indicate that the expressed locus lies in a relatively compact domain with a proximal limit located within 10 kb upstream of promoter P₁. Analysis of H4 acetylation levels is consistent with acetylation/deacetylation playing a role in regulating initiation of transcription but not in defining the active chromatin domain.

MATERIALS AND METHODS

Chromatin Acetylation Assay. Chromatin acetylation assays were carried out as described (22). In all experiments, we used an antibody mixture with specificity to highly acetylated H4 isoforms.

The following modifications were used to isolate nuclei from mouse tissue; thymus from 6- to 8-week-old BALB/c mice were removed into ice-cold PBS containing 5 mM sodium butyrate (Na butyrate) and gently teased apart to release cells. Cell pellets were resuspended in 10 ml of ice-cold 0.32 M sucrose/5 mM MgCl₂/10 mM Tris-HCl, pH 7.5/0.2 mM PMSF/5 mM Na butyrate and the nuclei released by Dounce homogenization. Homogenates were centrifuged at 1,000 × *g* (10 minutes, 4°C), and the pellet was resuspended in 5 ml of ice-cold 2.2 M sucrose/5 mM MgCl₂/10 mM Tris-HCl, pH 7.5/0.2 mM PMSF/5 mM Na butyrate and layered onto 5 ml of the same sucrose solution. Nuclei were pelleted at 50,000 × *g* (1 hr, 4°C) and resuspended in 1 ml of digestion buffer (0.32

Abbreviations: Xi, X inactive; Xa, X active; Xic, X inactivation center; SNUPE, single-nucleotide primer extension.

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M sucrose/50 mM Tris-HCl, pH 7.5/4 mM MgCl₂/1 mM CaCl₂/5 mM Na butyrate/0.1 mM PMSF).

DNA Probes. GPT1 is a 1-kb *Xba*I fragment located 3 kb downstream of *Xist* exon VII. MP1 is a 500-bp PCR fragment spanning the P₁ promoter. HP is a 1-kb *Hind*III-*Pst*I fragment 2 kb upstream of P₁; HH1.5 is a 1.5-kb *Hind*III fragment 3 kb upstream; 2.1(2)P is a 2-kb *Pst*I fragment 17 kb upstream; E(2.3-1) is a 1-kb *Eco*RI fragment 20 kb upstream; 19E(2) is 2-kb *Eco*RI fragment 40 kb upstream, and E55(13) and E55(16) are *Eco*RI subclones (700 bp and 2 kb) located ≈70 kb upstream. 33H(2) is a 2-kb *Hind*III genomic fragment located between the E55(16) probe and *DXCrc318*. Other probes are as described; *Xist* cDNA clones mXist1, w5i and w7d (8, 10); NM18B (23); *Cdx4* exon 3 region (24); *Tsx* (25); actin cDNA clone (26); R198 mouse minor satellite probe (27); *DXSmh141* probe (28).

DNase I Sensitivity Assay. Livers of adult mice were removed and immediately Dounce-homogenized in 20–40 ml of ice-cold buffer S [buffer A (15 mM Tris-HCl, pH 7.5/15 mM NaCl/60 mM KCl/0.5 mM spermidine/0.15 mM spermine) containing 0.3 M sucrose/0.5 mM EGTA/2 mM EDTA/0.5 mM 2-mercaptoethanol] to make a single-cell suspension. Further homogenization using a tighter fitting pestle was carried out to release nuclei. The homogenate was centrifuged at 1,000 × *g*, (10 min, 4°C), and the pellet was gently resuspended in 5 ml of buffer S. An additional 5 ml of buffer S was added, followed by 10 ml of buffer S containing 0.2% (vol/vol) Triton X-100 and finally, 200 μl of 0.1 M PMSF. The nuclei were mixed and permeabilized on ice for 1–3 min before pelleting at 1,000 × *g* (10 min, 4°C). The pellet was then washed in 20 ml of buffer S followed by a 5-min spin to give a clean white pellet. Finally the nuclei were resuspended in 3 ml of buffer E (buffer A containing 0.5 mM EGTA/0.5 mM 2-mercaptoethanol).

DNase I digestions were carried out on ice with 1.2 × 10⁷ nuclei and a range of enzyme concentrations. Buffer E and MgCl₂ (to 5 mM) was added to each digest to give a volume of 480 μl. Digestion was initiated on addition of nuclei, and after 10 min was stopped by the addition of 20 μl of 0.5 M EDTA (pH 8). Each digest was then transferred to tubes containing 4.5 ml of lysis solution (20 mM EDTA/20 mM Tris-HCl, pH 8/1.1% SDS/proteinase K at 200 μg/ml) and incubated at 50°C overnight. The next day, the DNA was extracted with phenol/chloroform, ethanol precipitated, and resuspended in 0.5 ml of TE (10 mM Tris-HCl, pH 8/1 mM EDTA, pH 8).

Single-Nucleotide Primer Extension (SNuPE) Assay. SNuPE assays were performed as described (13, 29). Novel polymorphisms were identified by sequencing of PCR products and were subsequently checked in trial SNuPE reactions. Optimal conditions were established as follows; U2af1-rs1-flanking primers GAT CAG ACA TAC TCG GAT A and TGT GGT ACG GCC AGC CTA TG (270-bp product), SNuPE primer TAA CTG CAC AGG CCA GCT GT at 94°C 1 min; 65°C 1 min and 72°C 1 min (polymorphism; C57BL6, G; *Mus spretus*, A), *Xist* exon I-flanking primers CAA GGT GGA TTG ACT GTG A and GAG TTA CTT GAA CAT CCT CC (900-bp product), SNuPE primer TCT GTG GAA GAT CAG TGC A, 94°C 1 min; 56°C 1 min and 72°C 1 min (polymorphism; *Mus domesticus*, A; PGK, G). *Xist* exon V-flanking primers ACG ATC CCT AGG TGG AGA TG and GCA TGA GTA GGG TAC AGT (400-bp product), SNuPE primer GGT TCT CTC AGA AGC TAG GA, 94°C 1 min; 56°C 1 min and 72°C (polymorphism *M. domesticus*, A; PGK, G). *Xist* promoter P₁-flanking primers CAT GGC TGG AGC AAG and TAT GGA GTC ACC AGG TTC CCA G (400-bp product), SNuPE primer GGT CCA ATA GAT GTC AGA 94°C 1 min; 62°C 1 min and 72°C 1 min (polymorphism *M. domesticus*, A; PGK, C). *Xist* 10-kb upstream-flanking primers GTA GAC CAG ACT GGG AAT CAG AAA and CAA GTA GGC CAA

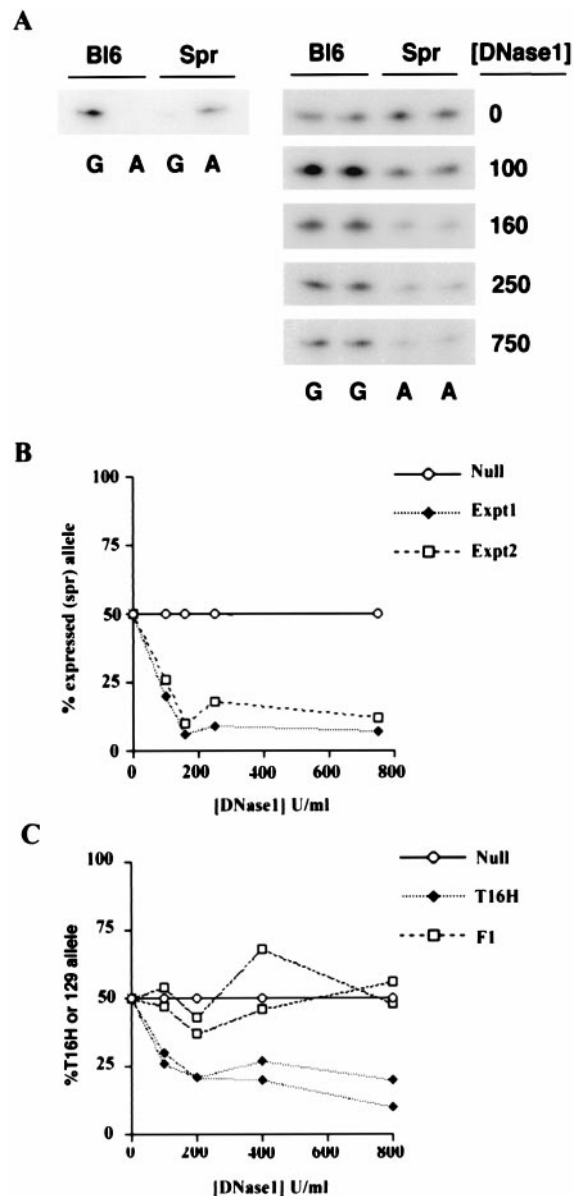


Fig. 1. Allele-specific DNase I sensitivity assay. (A) Left, autoradiograph illustrating SNuPE reactions detecting polymorphism between Bl6 and *M. spretus* (Spr) U2af-rs1 alleles (G and A, respectively). Right, autoradiographs illustrating relative levels of alleles in (C57BL6 × *M. spretus*) F₁ DNA prepared from nuclei treated with various concentrations of DNase I. SNuPE reactions were loaded in duplicate. (B) Quantitation of data shown above (Expt1) and an independent experiment (Expt2) showing expressed allele as % of total signal. The result expected if both alleles exhibit equal DNase I sensitivity (Null) is included for illustrative purposes. (C) Quantitation of two independent allele-specific DNase I sensitivity assays for the *Pgk-1* locus. Data is shown for (PGK × T16H) F₁ female (T16H), and for control (PGK × 129) F₁ female (F₁). Data points in B and C are mean values of duplicate loadings.

TCA ATA CC (400-bp product), SNuPE primer GTA AGT TCC AGA TCA GCC, 94°C 1 min; 50°C 1 min and 72°C 1 min (polymorphism *M. domesticus*, A; PGK, G). *Xist* 20-kb upstream-flanking primers TTC GGA TCT TCC TCT CCT ATA CAG and GTC CCT CAT CCT GCT GGT TT (300-bp product), SNuPE primer AAT GGA CAG AAG GGG TTA, 94°C 1 min; 58°C 1 min and 72°C 1 min (polymorphism *M. domesticus*, A; PGK, G). *Xist* 70-kb upstream-flanking primers TCG GAT CAG CCT GAC TGG TTG and GAG TGG GTT CTC TTA CAG (300-bp product), SNuPE primer TAC AAT

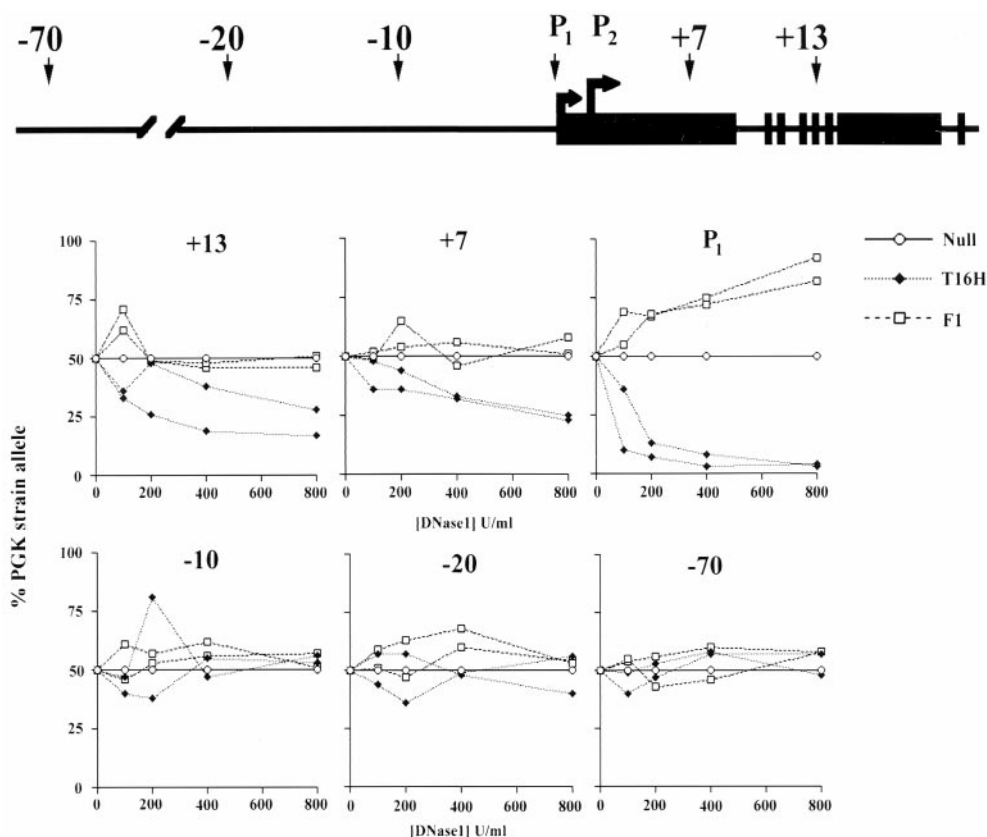


FIG. 2. Allele-specific sensitivity of expressed and silent *Xist* loci. The location of polymorphisms within the transcribed locus and at various sites upstream are shown on the diagram above. DNaseI-sensitivity assay results from two independent experiments are shown for each polymorphism. Both (PGK × T16H) F₁ female (T16H) and (PGK × 129) F₁ female controls (F₁) were analyzed. Data points are mean values calculated from duplicate loadings. Null represents expected result if allelic sensitivities are equivalent.

ACA TGC TTC CTG G, 94°C 1 min; 58°C 1 min and 72°C 1 min (polymorphism *M. domesticus*, A; PGK, C). P_{gk}-1 SNUPE was carried out by using the previously described SNUPE primer and conditions (29) on a 700-bp PCR product generated from genomic DNA by using the flanking primers TTA AAG CTG AGC CCG GCC AAA A and GTC AGT TCC ATA CCA CTA AAC.

SNUPE reactions were quantitated on a PhosphorImager. Data was calculated as mean values for duplicate loadings after normalization for differences in specific activity of nucleotides by using F₁ genomic DNA as a control. For newly described polymorphisms, we tested artificial mixes of genomic DNA (ranging from 1:9 to 9:1) to ensure linearity.

RESULTS

Allele-Specific DNaseI Sensitivity Assay. Active chromatin domains are defined as regions encompassing genes that exhibit general sensitivity to nucleases relative to flanking sequences (30). To assess the extent of the active domain of the expressed *Xist* locus, we established an assay based on single-nucleotide primer extension (SNUPE) (29). The rationale behind the assay is to PCR-amplify regions spanning single-base polymorphisms from genomic DNA isolated from DNaseI-treated nuclei and to then use SNUPE to quantitate allelic ratios. If a given allele is more DNaseI-sensitive within the region defined by flanking primers, then it should be proportionately underrepresented in the resultant PCR products. This approach provides distinct advantages compared with methods that are based on measuring the relative rate of disappearance of polymorphic restriction-length fragments (31–34). Specifically, the SNUPE assay requires only single-base allelic differences, which can be identified more readily

than polymorphic restriction-length fragments. In addition, fragment length differences do not need to be accounted for in assessing relative nuclease sensitivity.

To test the assay system, we first analyzed a region of the imprinted *U2af1-rs1* locus for which general DNaseI sensitivity assays based on digestion of polymorphic restriction fragments demonstrated ≈10-fold greater sensitivity of the paternal (expressed) allele relative to the maternal (silent) allele (34). SNUPE analysis was carried out by using a polymorphism between C57BL/6 (Bl6) and *M. spretus* located within the 5'-untranslated region of *U2af1-rs1*. A representative experiment analyzing DNaseI-treated nuclei from (Bl6 × *M. spretus*) F₁ mice is illustrated in Fig. 1A. Relative levels of the expressed allele (*M. spretus*) are seen to decrease rapidly with increasing concentrations of DNaseI. Quantitation of the above data and a second independent experiment is illustrated in Fig. 1B. In the concentration range of 160–750 units/ml DNaseI, there is an ≈10-fold difference in the levels of the two alleles, consistent with previously reported data (34).

We went on to assess relative DNaseI sensitivity of expressed and silent alleles of the X chromosome-linked *Pgk-1* gene on Xa and Xi. To do this, we analyzed DNaseI-treated nuclei from XX female T(X;16)16H (T16H) × C3H.p_{gk}1a (PGK) F₁ females. T16H causes complete nonrandom X inactivation of the PGK strain X chromosome. As a control, we analyzed nuclei from (129 × PGK) F₁ female animals, which undergo normal random X inactivation. For SNUPE analysis, we used a defined polymorphism between PGK and standard laboratory strains (29). The results obtained from independent experiments by using separate nuclear preparations are illustrated in Fig. 1C. As anticipated, the expressed (T16H) allele exhibited greater DNaseI sensitivity compared with the silent (PGK) allele, and this difference was not detectable in the

control ($129 \times$ PGK) F_1 nuclei. The allelic sensitivity difference was ≈ 2 – 3 fold, less than that seen for *U2af1-rs1*. Importantly, the result was highly reproducible in independent determinations.

DNaseI Sensitivity of the *Xist* Domain. We went on to identify SNUPE polymorphisms between PGK and T16H within the *Xist* locus and flanking regions. Results from two independent determinations are illustrated in Fig. 2. Polymorphisms located in exon V (+13) and exon I (+7) of the gene revealed greater sensitivity of the expressed (PGK strain) allele. In control ($129 \times$ PGK) F_1 female animals, both alleles exhibit similar levels of DNaseI sensitivity. The difference in sensitivity of expressed and silent alleles was similar to that seen at the *Pgk-1* locus, i.e., ≈ 2 – 3 fold and again was reproducible in independent experiments.

We also analyzed a polymorphism close to promoter P_1 . In this case, we observed a very strong difference in sensitivity (Fig. 2C). This likely reflects the fact that the polymorphism is in close proximity to a DNaseI hypersensitive site located over promoter P_1 (35). In control ($129 \times$ PGK) F_1 animals we observed the opposite effect (i.e., greater sensitivity of the 129 allele), albeit to a lesser extent. The reason for this may be that the large differences in allelic sensitivity amplify skewed X inactivation patterns attributable to different *Xce* alleles on 129 and PGK-derived X chromosomes. Theoretically, this effect should be more apparent where there are large differences in allelic sensitivity, e.g., DNaseI hypersensitive sites, compared with regions with relatively mild general DNaseI sensitivity differences.

In contrast to the above, polymorphisms upstream of P_1 (located at approximately -10 , -20 , and -70 kb) showed no significant difference in relative allelic sensitivity. Thus, analysis of general DNaseI sensitivity suggests that the domain of the expressed *Xist* locus on the inactive X chromosome extends no farther than 10 kb upstream. We were unable to define usable SNUPE polymorphisms in the region downstream of *Xist* and therefore cannot determine the 3' boundary of the *Xist* domain by using this approach (but see Discussion).

Acetylation Analysis of the *Xist* Locus. We went on to analyze patterns of histone H4 acetylation at the *Xist* locus by using chromatin immunoprecipitation as described by O'Neill and Turner (22). To distinguish the contribution of the two alleles in XX cells, we compared results for XX and XY cells. As males have only an active X (silent *Xist*), the acetylation status of the expressed allele in XX cells can be indirectly inferred.

Control experiments were carried out by using probes for heterochromatic and genic regions (Fig. 3A). The R198 probe, derived from a minor satellite repeat within *M. musculus* centromeres (27), hybridizes predominantly to the antibody unbound (hypoacetylated) chromatin fraction. This result is consistent with previously published cytogenetic and immunoprecipitation analyses (22, 36). The *DXSmh141* probe, corresponding to a long complex repeat unit sequence island located in the Giemsa-positive A3 band on the mouse X chromosome (28, 37), also hybridizes predominantly to the antibody unbound (hypoacetylated) chromatin fraction. This observation concurs with cytogenetic analysis of histone H4 acetylation levels that suggest that late-replicating Giemsa-negative bands are hypoacetylated (36). In contrast to heterochromatin probes, genic probes for the *Actin* gene and the X-linked *Rps4* gene both hybridize predominantly to the antibody-bound (acetylated) chromatin fraction. Fig. 3B illustrates quantified data from three independent determinations expressed as the ratio of bound to unbound signal. A ratio of <1 was obtained for the heterochromatin probes whereas genic probes give a ratio of >1.5 .

We next analyzed a series of probes derived from across the *Xist* locus and in flanking 3' and 5' regions (Fig. 4A). Probes were hybridized to immunoprecipitated chromatin from XX

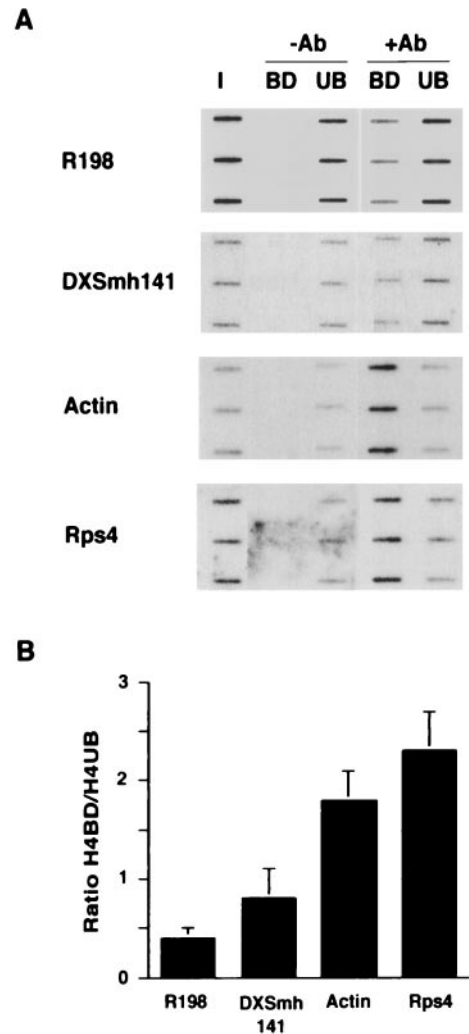


FIG. 3. Histone acetylation assay. (A) Hybridization of heterochromatin and genic probes to slot blots with triplicate loading of ≈ 100 ng of input DNA (I) or DNA from antibody-bound (BD) and -unbound (UB) chromatin fractions without (-Ab) or with (+Ab) antibody to hyperacetylated histone H4 isoforms. (B) Ratio of signal for H4-bound/H4-unbound fractions calculated from three independent determinations. Slot blot data was quantified by using PhosphorImager analysis.

female and XY male animals. Quantified results obtained from at least two independent determinations are shown in Fig. 4B. Selected examples are illustrated in Fig. 4C.

In XX female cells, probes within the *Xist* domain detected similar levels of acetylation compared with the control *Actin* and *Rps4* genic probes shown in Fig. 3B. However, much of the domain was also acetylated to this level in XY somatic cells where *Xist* is never expressed (Fig. 4B, and see for example, w5i in Fig. 4C). Thus, acetylated chromatin is not specifically associated with the domain of the expressed locus. Acetylation patterns in flanking regions were variable but generally similar in male and female cells. Probes HP, HH1.5, 55E(16), and 55E(13) hybridize to hypoacetylated chromatin, most notably in XX cells (Fig. 4B and for example HH1.5 in Fig. 4C). The low levels of acetylation are similar to constitutive heterochromatin (Fig. 3B). Probes NM18B, 19E(2), 2.1(2)P, *Tsx*, and *Cdx4*, on the other hand, detect acetylated chromatin both in XY and XX cells (Fig. 4B). The NM18B probe corresponds to a CpG-rich island (23), but an associated gene has not been identified to date. Both *Tsx* and *Cdx4* probes correspond to genes that are not expressed in the cell type analyzed (24, 25). There are no known genes associated with the other loci. Thus,

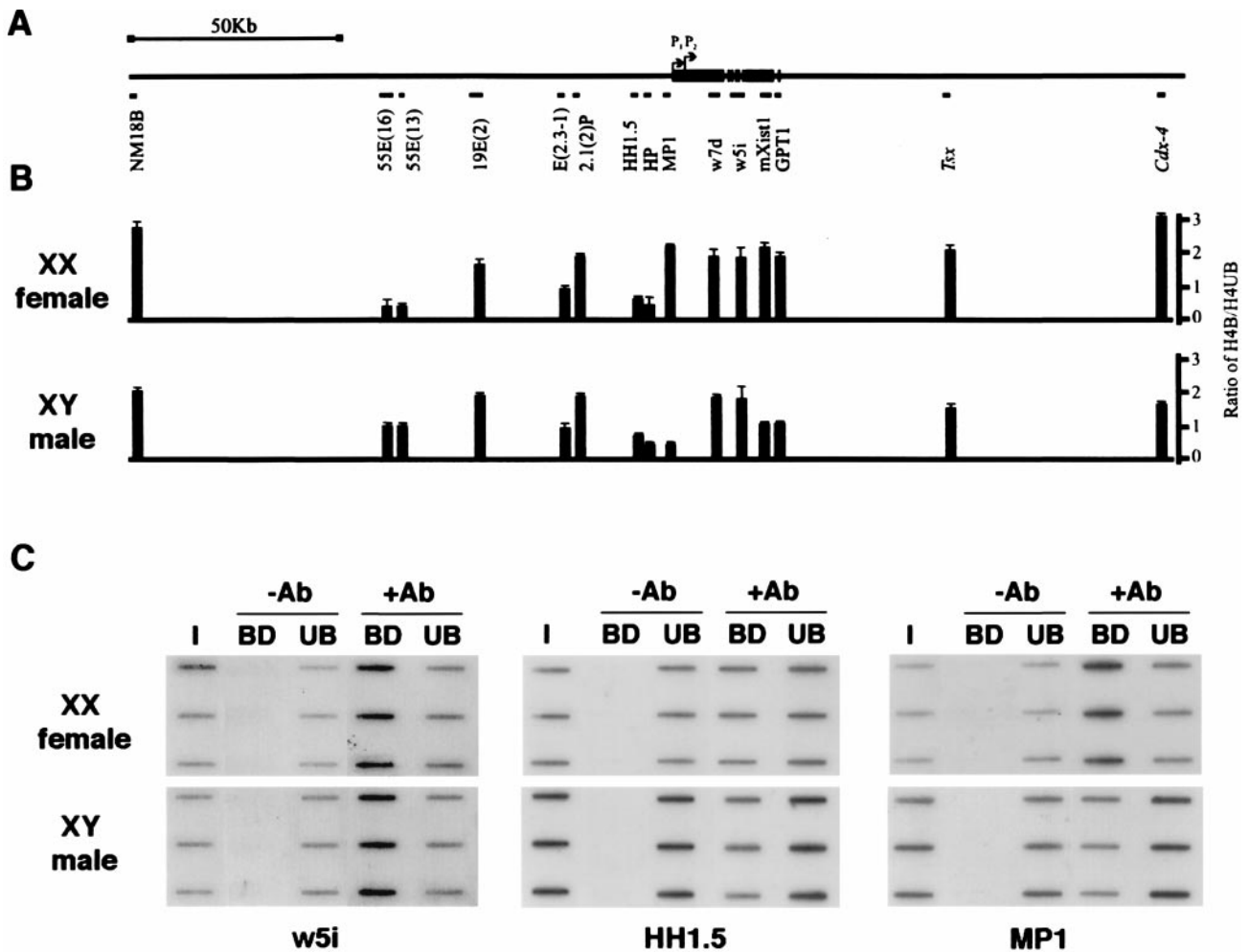


FIG. 4. Chromatin acetylation profile of the *Xist* locus. (A) Diagram illustrating the location of single-copy probes relative to the *Xist* gene. (B) Ratio of H4-bound/H4-unbound chromatin in XY male and XX female cells. Each value represents the mean of at least two independent experiments. (C) Examples of immunoprecipitations of XX female and XY male chromatin probed with w5i, MP1, and HH1.5 showing triplicate loadings of input DNA (I), and antibody-bound (BD) and -unbound (UB) fractions either in the absence (-) or presence (+) of specific antibody (Ab).

as is the case for the *Xist* domain, acetylation patterns do not appear to correlate with the domains of active transcription units.

Importantly, and in contrast to the aforementioned results, the *Xist* promoter probe MP1 hybridized to acetylated chromatin in XX cells but to hypoacetylated chromatin in XY cells (see Fig. 4 B and C). Thus, in this localized region, there appears to be a good correlation between H4 acetylation and expression. Presumably, the acetylated chromatin in XX cells derives specifically from the expressed allele, although this has not been formally demonstrated.

DISCUSSION

One aim of this study was to identify putative boundaries between the expressed *Xist* locus and inactive X chromatin. Our analysis indicates that the upstream boundary lies within 10 kb of promoter P₁. Based on this observation, we suggest that regulatory elements involved in maintaining expression from somatic cell promoters P₁ (and also the recently identified promoter P₂; ref. 21), lie within or downstream of this region. This conclusion is consistent with transgenic studies, which indicate that sequences within 9 kb upstream and 5 kb downstream of *Xist* are sufficient to establish appropriate expression patterns (18).

We were unable to extend our analysis to define a boundary downstream of *Xist*. Previous studies have shown that the *Brx* locus, located 65 kb downstream, is subject to normal X inactivation (38). On this basis, we would anticipate a transition from the expressed *Xist* domain and the silent *Brx* domain somewhere within this span. Interestingly, deletion of the 65-kb span in ES cells does not affect *cis*-inactivation of *Brx* (15). This result suggests that at least in the distal region, a defined boundary with insulator properties is not required to separate the expressed *Xist* locus and *cis*-inactivated material (15).

Our analysis revealed heterogeneity in general nuclease sensitivity differences ranging from 2- to 3-fold for the *Xist* and *Pgk-1* loci to \approx 10-fold for *U2af1-rs1*. The significance of this is not clear, although it may reflect a unique chromatin configuration for the inactive X chromosome. Previous studies have also revealed relatively mild nuclease-sensitivity differences for active and inactive X chromosome alleles (31–33).

Based on both cytogenetic evidence (36) and immunoprecipitation analysis (22), it has been suggested that histone acetylation acts as a marker by which the genome is partitioned into coding and noncoding regions. Our data demonstrating hypoacetylation of the *DXSmh141* repeat sequence island located in a G dark interstitial chromosome band lends further support to this view. Nevertheless, it remains unclear how acetylation levels vary at the level of individual genes. Analysis

of chromatin acetylation at the chicken β -globin locus demonstrated hyperacetylation comapping with the domain of the active locus as defined by general DNase I sensitivity (39). In contrast to this result, O'Neill and Turner (22) have demonstrated that acetylation levels are similar at a number of loci regardless of expression status. Our results indicate that for *Xist*, and also at flanking loci, acetylation levels do not correlate with active chromatin domains and are therefore in agreement with findings in the latter study. However, it should be taken into account that some of the flanking probes used are not fully characterized in terms of being genic or nongenic and also that the probes used do not provide universal coverage of the entire region of interest.

Although we did not find a general correlation between acetylation levels and expression status, we did observe a correlation specifically within the promoter region of *Xist*. This result is consistent with a number of biochemical studies that have highlighted the likely importance of histone acetylation in the initiation of transcription (reviewed in ref. 40). A recent study of Gcn5p-related histone hyperacetylation of target genes in yeast has provided clear *in vivo* evidence that promoter-specific acetylation is linked to transcription initiation (41). Conversely, it is possible that hypoacetylation of the silent P₁ in XY cells is functionally more relevant. In this respect, it is interesting to note that the P₁ region is known to be methylated on the silent *Xist* allele (42) and that recent evidence has linked methylation with the recruitment of histone deacetylase complexes (43, 44).

Results presented here indicate that the *Xist* promoters could provide a useful *in vivo* model to study promoter acetylation. An analysis of acetylation levels of *Xist* in ES cells is the subject of an independent study (45). In further studies, it will be of interest to determine acetylation levels of individual alleles in XX somatic cells, and to this end it should be possible to adapt the SNUPE assay to assess the allelic contribution in immunoprecipitated H4 acetylated chromatin. In addition, it will be interesting to assess acetylation levels at the recently described promoter P₂.

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