A Functional Chromatin Domain Does Not Resist X Chromosome Inactivation: Silencing of *cLys* Correlates with Methylation of a Dual Promoter-Replication Origin

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To investigate the molecular mechanism(s) involved in the propagation and maintenance of X chromosome inactivation (XCI), the 21.4-kb chicken lysozyme (*cLys*) chromatin domain was inserted into the *Hprt* locus on the mouse X chromosome. The inserted fragment includes flanking matrix attachment regions (MARs), an origin of bidirectional replication (OBR), and all the *cis*-regulatory elements required for correct tissue-specific expression of *cLys*. It also contains a recently identified and widely expressed second gene, *cGas41*. The *cLys* domain is known to function as an autonomous unit resistant to chromosomal position effects, as evidenced by numerous transgenic mouse lines showing copy-number-dependent and development-specific expression of *cLys* in the myeloid lineage. We asked the questions whether this functional chromatin domain was resistant to XCI and whether the X inactivation signal could spread across an extended region of avian DNA. A generally useful method was devised to generate pure populations of macrophages with the transgene either on the active (Xa) or the inactive (Xi) chromosome. We found that (i) *cLys* and *cGas41* are expressed normally from the Xa; (ii) the *cLys* chromatin domain, even when bracketed by MARs, is not resistant to XCI; (iii) transcription factors are excluded from lysozyme enhancers on the Xi; and (iv) inactivation correlates with methylation of a CpG island that is both an OBR and a promoter of the *cGas41* gene.

Functional chromatin domains are extended regions of chromatin containing a gene or gene cluster with all *cis* elements necessary for their appropriate expression. These domains are flanked by domain boundary elements, which may act to segregate the domain and protect against chromosomal position effects (5, 67). Chromosomal position effects are thought to be due to the influence of neighboring repressive chromatin, such as heterochromatin, a type of chromatin that appears more condensed during interphase, is late replicating, and is transcriptionally silent (73). Some of the proteins that affect the spreading and persistence of chromatin-mediated transcriptional repression are known for *Drosophila melanogaster* and *Saccharomyces cerevisiae*; these include heterochromatin protein 1 (HP1), members of the polycomb group, histone deacetylases, and histone methyltransferases (19, 36, 52, 54). Much less is known for mammals, although it has been established elsewhere that methylation of histone H3 is correlated with an inactive chromatin state (7, 38, 46), as are hypoacetylation (39) and DNA methylation (25, 64). A remaining general question is whether a vertebrate functional chromatin domain can be resistant to the spreading effect of nearby heterochromatin.

A special type of heterochromatin is the facultative heterochromatin comprising most of the mammalian inactive X chromosome (Xi). X chromosome inactivation (XCI) is a mammal-specific mechanism for equalizing the expression of X-linked genes between XX females and XY males. Early in embryo-

genesis one of the two X chromosomes in female somatic cells is rendered transcriptionally silent, heterochromatic, and late replicating (for reviews see references 2, 26, 48, 61, and 64). Once this epigenetic change occurs, the Xi is stably maintained for the life of the organism, which can include up to 10¹⁴ cell divisions (61). One of the most difficult challenges in the field of epigenetics is to explain how heritable changes in the chromatin state can be propagated over long distances, in the case of XCI over most of an entire chromosome. Beyond this, XCI is even more difficult to explain because inactivation is exclusively *cis*; that is, the active X chromosome (Xa) remains euchromatic with many active genes, while the Xi in the same nucleus takes on an inactive state indistinguishable from heterochromatin. The silent state persists in the face of all transcription factors necessary for full expression: e.g., ubiquitously expressed, X-linked genes such as *Hprt* and *Pgk-1* are fully active on the Xa yet totally silent on the Xi.

The molecular mechanisms of XCI have been divided into several categories, which include counting, choice, initiation, propagation (or spreading), and maintenance. The counting, choice, and initiation mechanisms all involve the X inactivation center (*Xic*) and associated genes, of which *Xist*, a noncoding mRNA that coats the Xi, is the most well studied (2, 51). *Xist* is necessary for propagation of the inactivating signal along the X chromosome but seems not to be necessary for maintenance (12). Faithful maintenance is, however, dependent on DNA methylation (reviewed in reference 25). Also, the Xi is hypoacetylated (39), hypermethylated at K9 of histone H3 (7, 31), and enriched in the histone variant macro-H2A1 (15, 18) and an as-yet-unknown antigen (32). Little else is known about propagation and maintenance of facultative heterochromatin.

To help explain the spread of heterochromatinization from

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the *Xic* to cover the entire X chromosome, Riggs et al. proposed (62) special "way station" elements that would favor the spread of inactivation. This idea has received support from recent studies showing an especially high abundance of LINE-1 (L1) elements on the mammalian X chromosome (3, 49). The involvement of a common repetitive element is consistent with the observation that XCI can sometimes spread considerable distances into X-translocated autosomal segments (72). A few endogenous X-linked genes escape XCI (70), and in translocations its spread into autosomal regions is usually limited, suggesting that some autosomal segments either are resistant to inactivation or lack the elements required to stabilize and/or propagate the inactivating signal (29). However, X-autosome translocation studies have been mechanistically inconclusive because they represent only situations where XCI spread is not detrimental to the cell and thus selected against. The fact that some normal X-linked genes escape inactivation suggests that control mechanisms act at the level of individual gene loci or chromosomal domains (70). Goldman et al. (29) and Riggs (60) postulated that looped chromatin domains may be key elements of the X inactivation process (29) and that inactivation may not spread continuously along the DNA; rather, the key elements modified by XCI could be in or near the base of loops that are continuously or periodically in close contact (60).

Most autosomal or noneukaryotic transgenes randomly integrated into the X chromosome are subject to XCI. Examples include a mouse alpha-fetoprotein minigene (42), a metallothionein-vasopressin fusion transgene (28), a neomycin resistance gene (20), and a protamine-simian virus 40 transgene (4). But the very nature of these transgenes, their differing copy number in multicopy gene clusters, which by themselves are subject to gene silencing (63), and integration into ill-defined locations on the X chromosome, makes it difficult to draw general conclusions about molecular mechanisms.

Only three transgenes are known to escape XCI, and for two of these escape is only in extraembryonic tissues (42) or partial (3%) (74). The only example of complete escape of a transgene from XCI involves a chicken gene. Goldman et al. (29) examined mice carrying 11 copies of a 17-kb chicken transferrin gene inserted into the D/C region of the X chromosome and found expression on the Xi to be the same as that on the Xa. It was suggested elsewhere that escape from X inactivation may be due to the large size (187 kb) of the transgene and/or the absence of special sequences (29). We wondered if the resistance to inactivation was due to the lack of L1 elements or other way station sequences in chicken DNA and whether all large chicken regions would be resistant to XCI. An alternative model is that the chicken transferrin locus may contain barriers to inactivation. Both models are consistent with avian species not inactivating one sex chromosome (23, 43, 50) and thus not having evolved special sequences relevant to mammalian-type XCI. None of these prior studies included a complete, functional chromatin domain, with boundary elements and all known control elements.

To provide information relevant to models for the propagation and maintenance of XCI, we targeted a functional chromatin domain (the *cLys* domain) from a species that does not undergo XCI into the *Hprt* gene on the mouse X chromosome. The well-studied chicken lysozyme (*cLys*) locus is bounded by

TABLE 1. Summary of female animals used for *cLys* expression analysis on Xi versus Xa

Targeting vector	Transgenic line	LPS treatment	No. of female mice
Lys1	TgH(clysneo)1	—	2
Lys2	TgH(clysneo)2	—	23
		+	6
Lys2	TgH(clys)3	—	6
		+	6

matrix attachment regions (MARs) and contains a number of well-characterized *cis* elements as well as an origin of bidirectional replication (OBR), all within a 21.4-kb region of general nuclease sensitivity (reviewed in reference 9). The *cLys* locus seems to be an autonomous unit resistant to chromosomal position effects, since numerous transgenic mice with randomly inserted copies of this locus have demonstrated correct, macrophage-specific expression dependent on copy number (10). We find that, despite being of avian origin and despite being a complete, autonomous functional unit, the 21.4-kb *cLys* domain is not resistant to silencing by XCI. We also find that the transcriptional silencing of *cLys* is correlated with the absence of transcription factors at a lysozyme enhancer and with extensive methylation of a replication origin located within the *cLys* domain and colocalized with the promoter of the recently discovered *cGas41* gene (16). These results are discussed in the context of present models for the spreading and maintenance of XCI.

MATERIALS AND METHODS

Targeting of the chicken lysozyme domain to the X-linked *Hprt* locus. A bacterial artificial chromosome (BAC) cloning vector, pBeloBAC11 (71), was used for the construction of targeting vectors. pBeloBAC11 was manipulated by using unique restriction endonuclease sites and protocols obtained from the California Institute of Technology's Genome Research Laboratory (<http://www.tree.caltech.edu/>). Recombinants were identified by colony hybridization (14). An 18.6-kb *SalI* fragment of isogenic (129S3/SvImJ) mouse *Hprt* genomic sequence (including exons 2 and 3) previously used as the basis of pRV18.6-I (21) was inserted into the unique *XhoI* site of pBeloBAC11. Next, a 2-kb *XhoI-SalI* fragment containing a *neo* cassette flanked by *loxP* sites was cloned into the unique *XhoI* site in mouse *Hprt* exon 3 to produce a basic replacement vector with a 5' 12-kb arm and a 3' 6.6-kb arm of sequences homologous to *Hprt*. The direction of *neo* transcription is opposite to that of *Hprt*. A 21.5-kb *XhoI-SalI* fragment isolated from pIIIlys (10) and containing the entire *cLys* domain was inserted between the arms of homology. The resulting two targeting vectors contained *cLys* in either the opposite (Lys1) or same (Lys2) orientation relative to *Hprt* transcription.

W9.5 embryonic stem (ES) cells of the 129S3/SvImJ strain were grown as previously described (69). Lys1 and Lys2 were linearized at unique *SrfI* sites, and three electroporations (25 μ g of DNA/ 10^7 ES cells) of each construct were performed. ES cell clones resistant to 175 μ g of Geneticin sulfate (G418) (GIBCO BRL)/ml for 5 days and 2 μ g of 6-thioguanine (6-TG) (Sigma)/ml for an additional 5 days were expanded and analyzed by Southern hybridization (10). Three correctly targeted ES cell clones per construct were injected into (C57BL/6J $\times$ CBA/CaJ) F_1 $\times$ C57BL/6J blastocysts, and the resulting male chimeras were mated to C57BL/6J females to obtain germ line transmission. Mice that inherited the transgene were backcrossed to 129S3/SvImJ to maintain the lines TgH(clysneo)1 from Lys1 and TgH(clysneo)2 from Lys2 (Table 1). To create and maintain a transgenic line without the *neo* cassette, use was made of the *Zp3-cre* mouse line (45). [TgH(clysneo)2 $\times$ *Zp3-cre*] F_1 females that inherited both *cLys* and *cre* were backcrossed to 129S3/SvImJ to produce the mouse line TgH(clys)3. Cre-mediated excision of the *neo* cassette occurred in 100% of animals tested.

Bone marrow harvest and differentiation into macrophages in vitro. Bone marrow cells were flushed from the femurs of 8- to 12-week-old mice with

ice-cold calcium- and magnesium-free Dulbecco's phosphate-buffered saline (Irvine Scientific) and kept on ice. Cells from one mouse were pelleted, resuspended, and cultured in 150- by 15-mm petri dishes (Falcon) at a density of 10^7 cells per dish in 23 ml of Iscove's modified Dulbecco's medium containing 4 mM L-glutamine (GIBCO BRL) supplemented with 10% heat-inactivated, endotoxin-free fetal calf serum (Omega Scientific, Inc.), 100 U of penicillin/ml and 100 μ g of streptomycin (GIBCO BRL)/ml, 0.15 mM monothioglycerol (Sigma), and 10 ng of recombinant mouse macrophage colony-stimulating factor (R&D Systems, Inc.)/ml. For selection, either 2 μ g of 6-TG/ml or 0.1 mM sodium hypoxanthine–0.4 μ M aminopterin–16 μ M thymidine (HAT; GIBCO BRL) was added for the full culture period. After 1 day of culture, nonadherent cells were transferred to new 150- by 15-mm petri dishes to remove any nondividing, adherent macrophages from the population. On day 3, an extra 7 ml of medium was added per dish, and on day 5 the medium was changed completely to again remove nonadherent cells. Selection was carried out for 10 days; by this time cells committed to the macrophage lineage were fully differentiated. In some experiments, 24 h prior to harvest bacterial lipopolysaccharide (LPS; Sigma, Inc.) was added to a final concentration of 5 μ g/ml.

Genomic DNA preparation and analysis. To identify targeted ES cell clones, 10 μ g of genomic DNA was digested with *Kpn*I, resolved on a 1% SeaKem LE (FMC BioProducts) agarose gel in 0.5 $\times$ TBE buffer (65) by pulsed-field gel electrophoresis (CHEF-DR II system; Bio-Rad), stained with 1 μ g of ethidium bromide/ml and then UV irradiated, transferred to a Hybond- N^+ membrane (Amersham Pharmacia Biotech) with 5 $\times$ SSPE (1 $\times$ SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA [pH 7.7]) (44), and hybridized with PCR-generated probes directed to mouse *Hprt* exons 2 and 3 (22). Contour-clamped homogeneous electric field electrophoresis was carried out at 14°C and 6 V/cm for 18 h with a ramped switch interval (0.1 to 6 s). To show that the *cLys* domain was intact and unrearranged, genomic DNA was similarly digested with *Eco*RI, resolved on a conventional 0.8% agarose gel in 1 $\times$ Tris-acetate-EDTA, transferred under alkaline conditions (59), and then probed with the entire *cLys* domain (10). Methylation analysis of the chicken OBR was performed as described by Phi-van and Stratling (57).

In vivo DMS footprinting. Cells were treated with 0.2% dimethyl sulfate (DMS) in phosphate-buffered saline for 5 min at room temperature, and footprinting analysis by ligation-mediated PCR was carried out as described previously (40), with the following nested primers: D376 (–3716) (5'-dGAGCTAC ACACCTTCAGC) (–3735), D376A (–3732) (5'-dCAGCTGTCTCTCCTTG ATGG) (–3755), and D376B (–3748) (5'-dGATGGCAGCCTGCCCAACA AG) (–3768).

RNA preparation and reverse transcriptase PCR (RT-PCR). For measurement of relative expression levels, the Competimer method (Ambion, Inc.) was used. This assay measures expression relative to 18S rRNA, by using a competing oligomer to adjust the PCR amplification of 18S rRNA so that the same number of rounds of amplification can be used for the 18S and the test RNA, keeping both reactions in the linear range. Total RNA was isolated from cultured macrophages with RNeasy B (Tel-Test, Inc.) according to the manufacturer's instructions. One microgram of intact total RNA was treated with 1 U of RQ1 RNase-free DNase (Promega) in a total volume of 10 μ l for 10 min at 37°C followed by heat inactivation of the enzyme at 65°C for 10 min and cooling on ice. cDNA was prepared by adding 0.09 260-nm-optical-density units of random primers (GIBCO BRL), 0.5 μ M deoxynucleoside triphosphates, and 200 U of Moloney murine leukemia virus reverse transcriptase (GIBCO BRL) in a final volume of 30 μ l under conditions recommended by the manufacturer and incubating the mixture at 37°C for 60 min. All reaction mixtures contained 1 U of recombinant RNasin RNase inhibitor (Promega)/ μ l. PCRs were carried out with 3 μ l (10%) of the cDNA reaction mixture with *cLys* primers and Ambion's QuantumRNA 18S rRNA internal standards (Ambion, Inc.). PCRs were done in a total volume of 30 μ l, and the conditions were identical to those previously described for *cLys* (37), except that the primers used in this study flanked intron 1 (c-lysF1, 5'-CGGCTATGAAGCGTCACGGACT, and c-lysR2, 5'-CGTAGT CCGTACTCCCATCGGT). The number of PCR cycles performed was 24 or 19 (plus LPS), each of which was determined empirically to be well within the linear range of amplification. A 5- μ Ci quantity of 3,000-Ci/mM [α -³²P]dCTP (Amersham Pharmacia Biotech) was added to each PCR mixture, and the products were resolved on a 6% polyacrylamide gel (SequaGel; National Diagnostics, Inc.) and imaged with a phosphorimager (Molecular Dynamics). *cGas41* PCR mixtures containing approximately 5% of the cDNA mixture in a total volume of 30 μ l were amplified for 30 cycles with HotStarTaq DNA polymerase and Q-solution (Qiagen). The *cGas41* PCR primers were Forward primer (5'-ATG TTCAAGAGAATGGCTGAG) and Reverse primer (5'-ACCGTCCACTGAT GCGTGTG). *cGas41* PCR products were resolved on a 2.5% agarose gel. No signal was ever obtained in the reactions without reverse transcriptase.

RESULTS

Targeting of the chicken lysozyme chromatin domain to the X-linked *Hprt* gene in ES cells and production of transgenic mice. Homologous recombination employing a BAC targeting vector was used to introduce the 21.4-kb *cLys* domain into the mouse X-linked *Hprt* locus in mouse ES cells (Fig. 1A). The *cLys* fragment used incorporates all previously identified control elements as well as a replication origin and flanking MARs and has been used previously to make multicopy transgenic mice (10). Insertion of the relatively large lysozyme domain was done with a total of 18.6 kb (12- and 6.6-kb stretches) of DNA that is identical in sequence to exons 2 and 3 of *Hprt*. Targeted ES cells, and then mice, were obtained with the *cLys* domain in two orientations, with *cLys* transcription in either the opposite (Lys1) or same (Lys2) orientation as *Hprt* transcription. Twenty ES cell clones resistant to both G418 and 6-TG selection were obtained, 9 for Lys1 and 11 for Lys2. Thirteen out of the 15 clones analyzed contained the lysozyme domain intact and correctly targeted to *Hprt*. We very roughly estimate a targeting efficiency of 10^{-6} for the *cLys*-containing vector, compared to 10^{-4} for the vector without *cLys*. Only a few insertions of this size have been made by homologous recombination in ES cells (21, 75). Our results confirm that large, correctly targeted insertions can be readily accomplished with BACs.

Following blastocyst injections, identification of chimeras by coat color, and breeding for germ line transmission, two lines of transgenic mice were obtained (Table 1). The transgenic line TgH(clysneo)1 was produced from ES cells transfected with the targeting vector Lys1, while the line TgH(clysneo)2 was similarly derived from Lys2. Both lines carry an X-linked *cLys* domain with a *neo* cassette just outside the domain. A third line, TgH(clys)3, without the *neo* cassette, was subsequently created from TgH(clysneo)2 by mating with *cre*-expressing mice.

Expression of *cLys* and *cGas41* on the active X. Males from all three lines were found to express *cLys* in macrophages (Fig. 2A, lanes 5, 6, 10, and 11) (also data not shown). This expression is from the Xa since male cells have no Xi. To the extent so far studied, expression of *cLys* from the Xa is not different from that seen for random, multicopy integrants (10, 11). Consistent with previous studies, *cLys* expression is stimulated by LPS (Fig. 2A, compare lanes 5 and 6 and lanes 10 and 11). Lysozyme is not expressed in early myeloid precursors and is upregulated during macrophage differentiation (reference 37 and data not shown). Figure 2B illustrates that the newly discovered, ubiquitously expressed *cGas41* gene located just downstream of *cLys* (Fig. 1) is also expressed.

An in vitro method for generating macrophages with the active X being exclusively either paternal or maternal. Cells of female mammals have both an Xi and an Xa in each nucleus. Also, due to the randomness of XCI, each adult female is a cellular mosaic, with approximately 50% of the cells having the paternally derived X chromosome inactive (patXi/matXa) and the remaining cells having the maternally derived X chromosome inactive (matXi/patXa). Thus, for this study it was necessary to establish a system that allowed the isolation of cell populations carrying the *cLys* transgene exclusively on either the Xa or the Xi. One can efficiently select either for or against

FIG. 1. Map of the chicken lysozyme chromatin domain and schematic of the expected gene-targeting event with Lys2. (A) The *cLys* domain (thick line) is shown at top, with a thin line representing vector DNA. *cLys* and *cGas41* exons and introns are black and white boxes, respectively, and the direction of transcription is shown. The inverted triangle indicates a 700-bp deletion in the second intron, which was introduced for construction purposes and has no functional consequences (10). *cis*-regulatory elements are indicated. Crosshatched horizontal bars mark the location of the 5' and 3' MARs. E, enhancer; S, silencer; P, promoter. Below is the *Eco*RI restriction map of the lysozyme domain. (B) Schematic representation of Lys2 targeting endogenous mouse *Hprt*. The Lys2 targeting vector and wild-type mouse *Hprt* allele are shown at top, with the expected targeted allele shown below. Homologous regions are indicated by thick lines, and the shaded box represents the *neo* cassette flanked by *loxP* sites. c-Lys, chicken lysozyme.

Hprt expression in a growing population of cells, and culture systems are available (33) in which macrophage precursors derived from bone marrow are able to proliferate and differentiate under the influence of macrophage colony-stimulating factor (10). As illustrated schematically in Fig. 3, we used this in vitro culture system to apply selection during growth and differentiation. When bone marrow cells from a female mouse hemizygous for the transgene are cultured in 6-TG, only those cells carrying the null *Hprt* allele on the Xa survive. These same cells also have the *cLys* domain on the Xa. Conversely, when the cells are cultured under HAT selection, only those cells carrying the null *Hprt* allele (and the *cLys* transgene) on the Xi survive. It will be seen below that this selection is efficient.

Expression is exclusively from the active X chromosome.

Table 1 summarizes the female animals analyzed for *cLys* expression on the Xa and Xi. At least two males from each transgenic line were also analyzed. The expression results shown in Fig. 2 were obtained with the TgH(clys)3 line but are representative of the results obtained for all transgenic lines analyzed. As shown in Fig. 2A, a 131-bp *cLys*-specific RT-PCR product was obtained from female macrophages subjected to either no selection (NS, lanes 2 and 7) or 6-TG selection (lanes 3 and 8; *cLys* on Xa) but not from female cells subjected to HAT selection (lanes 4 and 9; *cLys* on Xi). Importantly, no expression over background was seen for female macrophages under HAT selection in any of the mouse lines (Fig. 2A and data not shown). LPS treatment is known to stimulate expression of *cLys* (35, 37). Figure 2A shows that LPS treatment

FIG. 2. Expression of *cLys* domain genes in mouse macrophages. (A) *cLys* expression was determined by use of Competimer-normalized RT-PCR, as described in Materials and Methods. 18S mouse rRNA was used as an internal standard. Lanes 2 to 4 show results from female macrophages subjected to no selection (NS) and to 6-TG and HAT selection, respectively. Lanes 5 and 6 show male macrophages subjected to no selection and 6-TG selection, respectively. Lanes 7 to 9 show female cells identical to those in lanes 2 to 4 except for the addition of LPS treatment. Similarly, lanes 10 and 11 are male cells identical to those in lanes 5 and 6 except for the addition of LPS. Lanes 1 and 12 are control reaction mixtures containing a single set of PCR primers for either *cLys* (lane 1) or 18S rRNA (lane 12) only. Lane 13 is a no-template control. (B) *cGas41* expression in mouse macrophages. 18S mouse rRNA was used as an internal standard. Lane M shows size markers.

results in a large increase in expression from the Xa (compare lanes 5 and 6 with lanes 10 and 11). In contrast, no *cLys* expression was detected in HAT-selected cells. Allele-specific single-nucleotide primer-extension (66) analysis for *Pgk-1* markers, and other experiments described below, indicated that the Xi was retained in HAT-selected cells. Since we estimate that 1 to 3% of LPS-stimulated *cLys* expression would have been detectable, two conclusions can be made. First, these results confirm that the selection method is efficient. Second, the *cLys* gene on the Xi is not active.

Figure 2B shows that a similar result is seen for the *cGas41* gene. Also, the results of one RT-PCR experiment indicate that the *neo* gene, which is located just outside of the *cLys*

domain in the constructs studied, is also silenced. Similar studies of the two other *cLys* mouse strains (Table 1) gave the same results. Silencing of *cLys* was observed regardless of *cLys* orientation relative to *Hprt* transcription and was not influenced by the parental origin of the transgene or the presence of the *neo* cassette.

Transcription factors are excluded from the inactive X-linked chicken lysozyme enhancer core elements. Biochemical studies to distinguish between the chromatin structures of the Xa and the Xi have heretofore been hampered by difficulty in distinguishing the two chromosome states. Most studies, including in vivo footprinting studies, have been of hybrid cells, e.g., Chinese hamster-human cells (34, 55), not of normal,

FIG. 3. Selection scheme used to generate macrophage populations with known X inactivation patterns. The expected active (Xa) and inactive (Xi) status of each of the X chromosomes (and associated alleles) in a female cell hemizygous for the *cLys* transgene is indicated for bone marrow progenitor cells at harvest (left) and following the culture, differentiation, and selection in vitro of macrophages (right).

primary cells. The macrophage selection method described here improves on this situation. The *cLys* gene can be studied in hemizygous cells either in the active state (euchromatin on the Xa) or in the inactive state (facultative heterochromatin on the Xi). The chromatin fine structure of the *cLys* domain has been very well studied (40), and so it is of interest to compare the chromatin fine structure of the *cLys* domain on the Xa with that on the Xi. To demonstrate the feasibility of this approach and to confirm that the HAT-selected cells retain the transgene-containing Xi, preliminary *in vivo* footprinting experiments have been done on the -3.9 -kb *cLys* enhancer element (see Fig. 4). Since all bands seen are specific for *cLys*, the general similarity in overall band intensities between lanes means that the concentration of the *cLys* target sequence is similar, confirming that, though silent, the Xi with integrated *cLys* is not lost from HAT-selected macrophages. Previous *in vivo* footprinting analysis of lysozyme *cis*-regulatory elements in chicken cells, and in mouse cells with multicopy transgenes, showed that factor binding could be readily seen as footprints showing protection from modification of the DNA by DMS (40; unpublished data). HAT-selected macrophages have the transcription factors necessary to activate lysozyme; therefore, an interesting question is whether these factors are excluded from the *cLys* -3.9 -kb enhancer on the Xi.

Figure 4 shows an *in vivo* DMS footprinting experiment comparing the -3.9 -kb enhancer core region in the parent ES cells (non-*cLys*-expressing, lane 2), male macrophage cells (expressing *cLys* from the Xa, lane 6), and cells with the *cLys* domain on either the Xa (lane 5) or Xi (lanes 3 and 4). On the Xa (lanes 5 and 6), the NF1 site and the ets site show clear protection from DMS, in contrast to Xi (lanes 3 and 4), which is similar to nonexpressing ES cells and naked DNA (lane 1). Though less obvious, the footprints at the C/EBP region have been reproducibly seen on the Xa but not on the Xi or ES cells. In future experiments it now will be possible to analyze all the control elements of the *cLys* domain for differences in chromatin structure, for example, by using the UV-photofootprinting method as described by Kontaraki et al. (40). The absence of macrophage-specific footprints on the -3.9 -kb enhancer on the Xi indicates that, despite the presence of necessary transcription factors in the nucleus, some of which are reportedly able to remodel chromatin (e.g., C/EBP [41]), none is able to efficiently override XCI.

Partial methylation of a dual promoter-replication origin of the *cLys* domain on the inactive X. Hypermethylation of CpG dinucleotides on the Xi relative to the Xa, especially at CpG islands, is a hallmark feature of XCI (reviewed by Riggs and Pfeifer [61]). The *cLys* domain overall has relatively few methylatable sites (CpG dinucleotides), but there is one cluster of CpGs that meets the criteria of a typical CpG island (24). This same CpG-rich region (see Fig. 5) has been reported by Phi-van and Stratling (57) to be a replication origin (OBR). We recently found that the right-hand side of this CpG island also contains the promoter for *cGas41* (16). To investigate possible differential methylation of the *cLys* CpG island on the Xa and Xi, genomic DNA from each selected population was analyzed for CpG methylation. Figure 5A illustrates the location and features of the CpG island region of the *cLys* domain. An interesting feature of this island, which overlaps *cLys* exons 3 and 4 as well as the 3' untranslated region, is that it is early

FIG. 4. *In vivo* DMS footprinting of the -3.9 -kb lysozyme enhancer in the domain transgene on the Xa and Xi. Genomic DNA was prepared from DMS-treated cells and used for ligation-mediated PCR as described previously (40) to reveal *in vivo* DMS footprints. Lane 2, *cLys* transgenic ES cells. Lanes 3 and 4, two independent preparations of HAT-selected macrophages from hemizygous transgenic female mice. Lane 5, 6-TG-selected macrophages. Lane 6, macrophages from *cLys* transgenic male mice. Lane 1, naked DNA control (G). The position and nature of previously identified transcription factor binding sites are indicated on the right. White circles indicate protection from methylation, and black circles indicate methylation enhancement.

replicating and almost completely unmethylated in all chicken tissues (56, 57).

Our investigation of the methylation status of the CpG island on the Xa versus the Xi followed the same Southern blot strategy used by Phi-van and Stratling (57). Genomic DNA was digested with *SacI* either alone or in combination with either *HpaII* or *MspI*. Figure 5B shows that 6-TG selection yields genomic DNA that is completely digested by both *HpaII* and *MspI*. In contrast, HAT selection yields genomic DNA that is only partially digested by *HpaII*, while no selection gives digestion products that are the sum of those seen for 6-TG and HAT selection individually. A homozygous female gives the same result as the hemizygous female, and male genomic DNA is completely digested by both *HpaII* and *MspI*. In conclusion, the 1.3-kb *SacI* fragment containing the CpG island, OBR, and *cGas41* promoter is completely unmethylated at all *HpaII*/*MspI* sites on the Xa and partially methylated at many such sites on the Xi. Moreover, the presence of a full-length 1.3-kb band in the HAT-selected cells demonstrates complete methylation of all *HpaII*/*MspI* sites in a subset of molecules in the population.

FIG. 5. Map of the CpG island in the *cLys* domain and methylation analysis by Southern blotting. (A) At top, the left box labeled *cLys* depicts the 3' end of the lysozyme gene; the right open box is the *cGas41* gene. The directions of transcription are shown by arrows. The hatched box shows where the 3' MAR begins. Below is a map in which each vertical line represents a CpG dinucleotide, and below this is another map with vertical lines indicating the positions of each *HpaII/MspI* site. Black and white lollipops represent the fully methylated and unmethylated sites, respectively, previously identified by Phi-van and Stratling (57). The large half-filled circle signifies the partial methylation on the Xi that we found. (B) Methylation analysis of the CpG island. Southern blot analysis using methylation-sensitive *HpaII* and methylation-insensitive *MspI* was performed as described in Materials and Methods and in reference 57. Restriction enzyme treatment was as follows: S, *SacI*; H, *HpaII*; M, *MspI*; NS, no selection. Cells hemizygous and homozygous for the lysozyme domain are indicated. wt, wild type.

DISCUSSION

The organization of chromatin into discrete looped domains is believed to contribute structurally to the packaging of DNA in the nucleus and functionally to the regulation of expression and replication of the genome (13, 17, 27, 68). The *cLys* chromatin domain is flanked at each end by MARs that bind to the nuclear matrix (47). Matrix attachment may render the lysozyme domain topologically separate from surrounding chromatin and therefore a self-contained functional unit (47, 67). This idea was supported by the finding that stable transfection of a reporter gene controlled by *cLys cis* elements and flanked at each end by the 5' MAR resulted in elevated and position-

independent expression in chicken cell lines (68). However, deletion of the MARs from the lysozyme domain did not affect its copy-number-dependent expression in multicopy transgenic mice (11), and the idea emerged that several elements acting together were necessary for robust position independence (8). To determine whether the complete *cLys* chromatin domain was capable of independent function on the X chromosome and to find out whether avian genes other than the chicken transferrin gene (29) are resistant to silencing by XCI, we used homologous recombination to insert the entire *cLys* domain into the mouse X-linked *Hprt* gene. We find that this single-copy *cLys* transgene is expressed in macrophages, as was seen

previously for multicopy transgenic mice (10). Employing a novel selection system, we obtained macrophages with the inserted *cLys* domain on either the Xa or Xi and found that both genes in the domain, the *cLys* gene and the *cGas41* gene, are transcriptionally silenced by XCI. Thus, there appear to be no chicken- or domain-specific elements capable of insulating against X inactivation, including the domain-flanking MARs. Birds are evolutionarily distant from mammals and lack sex-specific heterochromatin, and the sex chromosome is not late replicating. If dosage compensation occurs, it appears to be by a mechanism different from that of mammals (23, 43, 50). Thus, specially evolved elements are probably not needed for XCI to spread over a 21-kb region. As outlined in the introduction, young L1 repetitive elements have been considered attractive candidates for way station elements that seed or enhance the spread of XCI (3, 49). Avian DNA does not have these L1 elements, and so it is clear that they are not needed for the inactivation of the *cLys* locus. At present, however, we cannot exclude the possibility that L1 elements are necessary for spreading through larger domains or chromosomal regions.

Several experimental approaches have implicated cytosine methylation as part of XCI; these include the reactivation of inactive X-linked loci in somatic cell hybrids by treatment with 5-azadeoxycytidine or other inhibitors of DNA methylation (reviewed in reference 25). Promoter-associated CpG islands on the Xi are heavily methylated, in contrast to CpG islands on the Xa and autosomes, which are almost completely protected from methylation (30). CpG islands are G+C-rich regions, usually greater than 500 bp, that contain a higher-than-expected number of CpG dinucleotides. These islands are characteristically found at the promoters of constitutively expressed genes (6, 24). It is generally accepted that the Xi-specific methylation of CpG islands is a locking mechanism to ensure maintenance of the inactive state (58). Methylation appears to play little role in the regulation of chicken lysozyme expression in its normal chromosomal context. There are only a few CpGs in the known control elements, and the only CpG-rich region, which is found downstream of *cLys*, is almost completely unmethylated in both expressing and nonexpressing chicken cells (57). In primary macrophage cells, we find that this CpG island is unmethylated at all 18 *HpaII* sites on the Xa. In contrast, partial but heavy methylation at all 18 *HpaII* sites of the CpG island is found on the Xi. It is likely that no specially evolved sequences are needed for susceptibility to CpG island methylation on the Xi.

Differential replication timing between the Xa and the Xi, a feature seen even for primitive egg-laying mammals, has been postulated as the key, ancestral mechanism of XCI (60, 61). Replication timing is an attractive mechanism because it is easy to imagine that the enzymatic environment of early-replicating genes is quite different from that of late-replicating genes, and this can be used to establish chromatin states that will persist through replication and thus be somatically heritable (61). For example, methylation of CpG islands may be prevented in early S phase but not in late S phase. It is therefore of special interest that in chickens the CpG island in the *cLys* domain functions as a replication origin, an OBR. In its natural location, the *cLys* OBR is early replicating and unmethylated, even when the *cLys* gene is inactive (56, 57). The early replication and lack of methylation could be due to the fact that the island

contains the promoter for *cGas41*, a second, ubiquitously expressed gene (16). Antequera and Bird (1) have proposed a model in which CpG islands have dual functions both as promoters and as origins of replication. Their typically unmethylated state could be a footprint resulting from transcription inhibiting methylation during early stages of development when de novo methylation is high. Our results establish that, despite the presence of a ubiquitously expressed gene and a potential early-replicating OBR, inactivation and methylation prevail when the domain is located on the Xi. XCI dominates. It seems that the spreading of a repressive chromatin state by XCI to silence the promoters and methylate the CpG island is aggressive, does not need evolutionarily conserved sequences, and is able to overpower a position-effect-resistant chromatin domain.

Our results reported here, studies of CTCF-containing insulator elements (5), and the recent finding of elements that terminate the spreading of mating-type heterochromatin in yeast (53) suggest that the most relevant question to guide future work is whether there are special elements that prevent the spreading of XCI into those genes that resist XCI. In this context, it is relevant that XCI is presumed to spread unidirectionally from the *Xist* locus and yet the orientation of the *cLys* transgene relative to the *Xist* locus made no difference, suggesting that neither the 5' nor the 3' part of the lysozyme locus contains elements able to resist XCI. Studies with other presumptive barrier sequences and multiple copies of the *cLys* domain in the X chromosome will be the focus of future work. Integration by homologous recombination into the *Hprt* locus provides a good, general system to study the effects of heterochromatin on transgenes and specific control elements such as insulators or barriers, with the unique advantage of having a conditional heterochromatin effect (Xi, heterochromatinization on; Xa, heterochromatinization off). It should also be noted that the system described here for generation of macrophages with the lysozyme domain on either the Xa or the Xi can be used for the comparison of chromatin structure between XCI-silenced chromatin (facultative heterochromatin) and active chromatin in nontransformed, primary cells.

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