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Higher order chromatin structure at the X-inactivation center via looping DNA

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ABSTRACT

In mammals, the silencing step of the X-chromosome inactivation (XCI) process is initiated by the non-coding Xist RNA. *Xist* is known to be controlled by the non-coding *Xite* and *Tsix* loci, but the mechanisms by which *Tsix* and *Xite* regulate *Xist* are yet to be fully elucidated. Here, we examine the role of higher order chromatin structure across the 100-kb region of the mouse X-inactivation center (*Xic*) and map domains of specialized chromatin *in vivo*. By hypersensitive site mapping and chromosome conformation capture (3C), we identify two domains of higher order chromatin structure. *Xite* makes looping interactions with *Tsix*, while *Xist* makes contacts with *Jpx/Enox*, another non-coding gene not previously implicated in XCI. These regions interact in a developmentally-specific and sex-specific manner that is consistent with a regulatory role in XCI. We propose that dynamic changes in three-dimensional architecture leads to formation of separate chromatin hubs in *Tsix* and *Xist* that together regulate the initiation of X-chromosome inactivation.

Introduction

X-chromosome inactivation (XCI) evolved to compensate for gene dosage in sexually dimorphic species. In mammals, XX females and XY males achieve dosage compensation by the transcriptional silencing of one X chromosome in each diploid female cell (Lyon, 1961). In the embryo proper, the choice of which X chromosome is to be silenced occurs randomly, with both the maternal and paternal X chromosomes having an equal chance of being inactivated. However, in the extra-embryonic tissues, a parent-of-origin effect is observed where the paternal X chromosome is preferentially silenced. Both mechanisms result in one active X chromosome (Xa) and one inactive X chromosome (Xi), which are then stably maintained through subsequent cell divisions.

The molecular players in XCI have been defined over recent years and many are located within a 100-kb region on the X chromosome, known as the X-inactivation center (*Xic*) (Lee et al., 1999b). Non-coding elements within the *Xic* are responsible for the three steps of random XCI, including counting of the X-to-autosome ratio, choice of which X chromosome to inactivate, and silencing of the Xi (reviewed by Plath et al., 2002). The *Xist* gene encodes a large, non-translated RNA that is transcribed from the inactive X chromosome and 'coats' the Xi in *cis*, inducing chromosome-wide gene silencing (Brockdorff et al., 1992; Clemson et al., 1996; Penny et al., 1996). *Xist* is negatively regulated by its antisense partner, *Tsix* (Lee, 2000; Lee et al., 1999a; Sado et al., 2001), also a non-coding, nuclear RNA. *Tsix* is in turn regulated by *Xite*, an upstream locus that produces several non-coding transcripts and harbors several strong DNase I hypersensitive sites (DHS) (Ogawa and Lee, 2003).

Murine embryonic stem (ES) cells have provided an excellent model system for the investigation of XCI and the roles of *Xite*, *Tsix* and *Xist*. In this system, Xist and Tsix RNAs are transcribed at low levels from both X chromosomes in undifferentiated ES cells. Upon differentiation, *Tsix* expression is extinguished on the future Xi (Lee et al., 1999a). Conversely, *Xist* expression is up-regulated in *cis* during the same developmental phase, suggesting that *Tsix* antagonism is responsible for *Xist* regulation. *Tsix* expression persists on the Xa until slightly later in development and *Xist* expression is prevented in *cis*. However, the exact mode of action of *Tsix* on *Xist* is currently still unclear. A number of possibilities exist whereby the process of antisense transcription through the *Xist* locus may prevent *Xist* expression in undifferentiated cells, including promoter blocking, transcriptional interference or alterations in chromatin structure. Recent evidence argues for a role of Tsix RNA-directed chromatin change (Navarro et al., 2005; Sado et al., 2005; Sun et al., 2006), in which Tsix RNA, coupled to anti-parallel transcription through the *Xist* promoter, results in recruitment of specific regulatory factors and chromatin modifications to the *Xist* promoter.

Thus, while long-range interactions between *Tsix* and *Xist* clearly exist, the three-dimensional (3-D) nature of such interactions has yet

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to be addressed. Higher order chromatin interplay has been implicated in other epigenetic systems, such as β-*globin* (Patrinos et al., 2004; Tolhuis et al., 2002), T_H2 and interleukin genes (Lee et al., 2005), and *H19/Igf2* loci (Kurukuti et al., 2006; Murrell et al., 2004), where looping interactions among locus control regions (LCR), enhancers, and co-clustered genes determine the pattern of gene expression. At the *Xic*, only two enhancers have been identified so far. DNase I hypersensitive site (DHS) mapping and transient transfection enhancer assays showed that one such enhancer resides at *Xite* (Ogawa and Lee, 2003; Stavropoulos et al., 2005). Current models posit that the *Xite* enhancer enables *Tsix* to persist allele-specifically on the future Xa. A second enhancer lies within the 5′ end of *Tsix* itself and is essential for high level expression of *Tsix* in ES cells (Stavropoulos et al., 2005). Although *Xist* is also developmentally regulated, transcriptional enhancers have not yet been described for this gene. How these regulatory elements interact with *Tsix* and *Xist* in 3-D space is not known.

The mode of action of enhancers or LCR elements on gene promoters located at large distances is the subject of intense debate. The 'tracking' model postulates that proteins bound to LCRs and enhancers track linearly along the DNA helix and scans surrounding sequences for promoter elements (Li et al., 1999). By contrast, the 'linking' model proposes that enhancer-bound proteins recruit additional protein factors, which then sequentially extend towards the promoter. Finally, the 'looping' model posits that random collisions between regions of the flexible chromatin fiber enable an enhancer to come into direct contact with a gene promoter in three-dimensional space, thereby looping out all intervening DNA sequences (Bulger and Groudine, 1999). The 'chromosome conformation capture' (3C) technique (Dekker et al., 2002) has lend strong support to the concept of looping chromatin in *cis* (Kurukuti et al., 2006; Lee et al., 2005; Murrell et al., 2004; Patrinos et al., 2004; Tolhuis et al., 2002), as well as to the idea of interaction *in trans* between chromosomes (Ling et al., 2006; Lomvardas et al., 2006; Spilianakis et al., 2005; Xu et al., 2006). In principle, an enhancer or an LCR element at the *Xic* might employ one or several of these mechanisms.

Here, to determine how critical elements interact during the process of XCI, we combine DHS identification and the 3C technique to map higher order chromatin structures at the *Xic*. We find several domains of interactions between *Xist*, *Tsix* and *Xite* that are regulated in a developmentally-specific manner. We thus provide a first glimpse of dynamic three-dimensional architectural changes that accompany the onset of XCI.

Materials and methods

Cell lines and culture

Mouse embryonic stem cell lines J1 (40XY, 129Sv/J)(Li et al., 1992) and 16.7 (40XX, 129 × [*M.castaneus* × 129] (Lee and Lu, 1999)) were maintained on gelatin-treated tissue culture flasks with a feeder layer of γ-irradiated mouse embryonic fibroblasts (obtained from d13.5 embryos) in Dulbecco modified Eagle medium plus 15% fetal bovine serum (heat inactivated) and 500 U/ml Leukemia Inhibitory Factor (LIF). Embryoid bodies were differentiated by suspension culture for 4 days without LIF and maintained thereafter under adherent conditions for 6 days. Primary fibroblasts were obtained from d13.5 embryos and maintained in DMEM plus 10% FBS.

DNase I Hypersensitive Site (DHS) mapping

DHS mapping was performed essentially as previously described (Ogawa and Lee, 2003). Briefly, 1×10^8 trypsinized cells were washed with PBS, pelleted, and resuspended in 10 ml ice-cold Nuclear Isolation Buffer (NIB) (0.32 M sucrose, 3 mM $CaCl_2$, 2 mM MgOAc, 10 mM Tris–HCl (pH7.5), 0.1 mM EDTA, 1 mM DTT). One-twentieth volume of NIB+0.3% NP-40 was added and the suspension was subsequently dounced 15 times with pestle B. Nuclei were centrifuged at 400 ×*g* and resuspended in ice-cold NIB at 0.2 mg/ml as determined by total nucleic acid content. For ES cells, DNase I (Worthington) was added to aliquots at a final concentration of 0–10 μg/ml, and samples were subsequently incubated at 37 °C for 2 min. For fibroblasts, final concentration of DNase I was 0–80 μg/ml and samples were incubated for 5 min. Digestions were terminated by adding an equal volume of 2× stop solution (1% SDS, 0.6 M NaCl, 20 mM Tris–HCl (pH7.5), 10 mM EDTA, 0.2 mg/ml Proteinase K) and incubated overnight at 37 °C. DNA was precipitated with 1.25 M NH_40Ac, resuspended, and digested for Southern analysis.

For Southern blotting, panels A, C and D were digested with EcoRI; panels B and D with BamHI. Probes used were positions a) 108150–108354, b) 113059–113266, c) 114507–114718, d) 124506–124723 and e) 91198–91433 from GenBank AJ421279.

Chromosome conformation capture

Chromosome conformation capture experiments were carried out as described in Splinter et al (Splinter et al., 2004) with minor modifications and appropriate controls (Dekker, 2006). Briefly, 10^7 cells were cross-linked with 2% formaldehyde at room temp for 10 min. Cross-linking reactions were terminated by addition of glycine to the final concentration of 125 mM. Nuclei of crossed linked cells were purified by incubating the cells with lysis buffer (10 mM Tris 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP-40 with protease inhibitor cocktail) twice on ice for 4 min, then washed with 5 ml of ice-cold PBS. The purified nuclei were resuspended in 760 μl restriction buffer consisting of 80 μl of 10× restriction buffer H (GE Healthcare) and 680 μl H_2O, and incubated with mild agitation at 37 °C for 1 h with 12 μl 20% SDS. Reactions were treated with 18 μl of Triton X-100, incubated at 37 °C for 1 h and subjected to restriction enzyme digestion with 240 U of BamHI and 200 U of BglII at 37 °C for 2 h. An additional 120U of BamHI and 100 U of BglII was carried out at 37 °C overnight. The digested samples were treated with 40 μl of 20% SDS at 65 °C for 20 min to inactivate restriction enzymes. Samples were transferred to 9.2 ml 1× ligase buffer containing 1% Triton X-100 and incubated at room temperature for 1 h and then incubated with 1600 U of T4 DNA ligase at 16 °C for 4.5 h and additional 0.5 h at room temperature. Ligated samples were treated with 3 μl of proteinase K (20 mg/ml) at 65 °C overnight to reverse cross-linking, and then treated with 2 μl RNaseA (10 mg/ml) at 37 °C for 1 h. Genomic DNA was extracted with phenol/chloroform and precipitated with NaOAc and isopropanol. Precipitated DNA was washed with 1 ml 75% ethanol and resuspended with 600 μl H_2O. To estimate genomic DNA concentration, 5 μl of each sample were analyzed on a 0.8% gel and visualized by EtBr staining. About 40 ng of genomic DNA was used for each 25 μl PCR reaction.

Positive control templates were made by digesting pπJL2 with BamHI and BglII and then random re-ligation of the restriction fragments. To mimic the complexity of the genomic DNA obtained from 3C experiments, the controls PCR reactions were performed with the mixture of 2.0 pg of positive control template (see below) and 40 ng of control genomic DNA that was processed in parallel with other 3C samples, except that the formaldehyde cross-linking step was omitted. Amplification of specific PCR products was strictly formaldehyde-cross-linking and ligation-dependent (data not shown). All PCR reactions were performed with hot-start Taq DNA polymerase (AmpliTaq, Applied Bioscience) and with 34–36 cycles of amplification, where all the amplifications were in the exponential range. PCR reactions amplifying un-rearranged genomic DNA were also carried out as described above but with only 26 cycles of amplification. To obtain quantitative information, amplifications were performed in the exponential phase as confirmed by decreases in PCR product levels as the concentrations of templates decreased by successive 3-fold dilutions (Figs. 2B, 3B, and data not shown). The relative cross-linking efficiency was calculated at least from duplicate PCR analyses of 2 independent 3C preparations (average of at least 4 sets of PCR analyses). PCR primer sequences used for 3C analysis are available on request.

To create a pool of all possible interacting products as control, we digested an 80-kb P1 construct, πJL2 (Lee et al., 1999b), with BamHI and BglII and randomly ligated the resulting fragments. To mimic the complexity of the mammalian genome during the PCR step, we digested female fibroblast genomic DNA with BamHI and BglII, mixed it with the pool of πJL2 control products at 2 plasmid copies per genomic equivalent. This control was included in all 3C experiments. As a control for cross-linking efficiency, we carried out PCR using primers that amplify the rearranged *GAPDH* locus generated by the 3C procedure using primers GAPDH-a (5′-ACACAGG-CAAAATACCAATG-3′) and GAPDH-b (5′-GAATGCTTGGATGTACAACC-3′) and found that the cross-linking efficiency of various samples was similar (Fig. 2A). Positive controls for the rearranged GAPDH locus and inter-chromosomal (Ch7:ChX) ligation products were prepared by mixing the PCR fragments of *GAPDH* locus and X chromosome, digesting with BglII and BamHI, and then randomly ligating the restriction fragments. No detectable inter-chromosome cross-linking was detected after 40 cycles of PCR amplification.

The relative cross-linking frequency between the anchor fragment and the fragment of interest ($\boldsymbol{f}(x)$) was normalized to the cross-linking frequency between the anchor fragment and its adjacent 5′ fragment. The relative cross-linking frequency can be expressed as

$$(\boldsymbol{f}(x)) = [IS(X:A)/IC(X:A)] \,/\, [IS(Y:A)/IC(Y:A)]$$

Where IS(X:A) is the intensity of PCR products amplified from ligation products between the anchor fragment A and any given fragment X in the rearranged genomic DNA of ES cells; IC(X:A) is the intensity of PCR products amplified from ligation products between the anchor fragment A and any given fragment X in the positive control template; [IS(Y:A) is the intensity of PCR products amplified from ligation products between the anchor fragment A and the fragment (fragment Y) that yield the fewest ligation products in the rearranged genomic DNA of ES cells; IC(Y:A) is the intensity of PCR products amplified from ligation products between the anchor fragment A and the fragment Y in the positive control template.