CHROMATIN STRUCTURE OF THE INACTIVE X CHROMOSOME

by

Sandra L. Gilbert

B.A. Biology and B.A. Economics

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Signature of Author .....

Department of Biology

August 19, 1999

Certified by .....

Phillip A. Sharp

Salvador E. Luria Professor of Biology

Thesis Supervisor

Accepted by .....

Terry Orr-Weaver

Professor of Biology

Chairman, Committee for Graduate Students

ABSTRACT

X-inactivation is the unusual mode of gene regulation by which most genes on one of the two X chromosomes in female mammalian cells are transcriptionally silenced. The underlying mechanism for this widespread transcriptional repression is unknown. This thesis investigates two key aspects of the X-inactivation process.

The first aspect is the correlation between chromatin structure and gene expression from the inactive X (Xi). Two features of the Xi chromatin — DNA methylation and late replication timing — have been shown to correlate with silencing of individual genes. This thesis describes a third feature that correlates with silencing of individual genes on the Xi: promoter-specific hypoacetylation of histone H4. Chromatin immunoprecipitation experiments demonstrated that transcriptionally active genes had elevated levels of H4 acetylation at their promoters on both the active and inactive X. In contrast, promoters of X-inactivated genes were markedly deacetylated, which coincided with the methylation of adjacent CG dinucleotides. This suggests that promoter hypoacetylation may be a key component of an X-inactivation machinery that operates at the level of individual genes.

The second focus of this thesis is the nature of the association between *XIST* RNA and the Xi chromatin. Microscopy studies have shown that the noncoding *XIST* RNA colocalizes with the Xi. It is unclear, however, if this colocalization is due to physical association of *XIST* RNA with the Xi chromatin, or if it is a secondary consequence of *XIST* RNA and the Xi being sequestered to the same nuclear territory. This thesis provides evidence from chromatin immunoprecipitation experiments that *XIST* RNA is part of the Xi chromatin. First, *XIST* RNA can be co-precipitated by antisera against macroH2A, a histone H2A variant enriched in the Xi. Second, *XIST* RNA can be co-precipitated by antisera that recognize unacetylated, but not acetylated, isoforms of histones H3 and H4. As demonstrated in this thesis, hypoacetylated histone H4 is enriched at promoters of X-inactivated genes, whereas hyperacetylated histone H4 is found only at promoters of active genes. The preferential association of *XIST* RNA with unacetylated histones therefore suggests that the RNA is not uniformly associated with the Xi chromatin. Further evidence for this conclusion comes from fluorescence *in situ* hybridization in mouse cells, in which *Xist* RNA is shown to localize with the inactivated *Zfx* locus, but not with the *Sts* locus which escapes X-inactivation. These results raise the possibility that association with *XIST* RNA may be a fourth feature that correlates with silencing of individual genes on the Xi. This physical association between *XIST* RNA and the Xi chromatin may facilitate X-inactivation.

Thesis Supervisor: Dr. Phillip A. Sharp,
Title: Salvador E. Luria Professor of Biology

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CHAPTER ONE: INTRODUCTION

SECTION I: OVERVIEW OF DISSERTATION

This dissertation describes an in-depth investigation of the chromatin structure of the mammalian inactive X chromosome (Xi). The primary goal of this investigation was to identify distinct features associated with the Xi chromatin, and to understand how these features may contribute to gene silencing. The dissertation is organized in the following manner:

Chapter One outlines a broad survey of past research in X-inactivation and other relevant fields. Topics include: (1) dosage compensation mechanisms in model organisms; (2) molecular events associated with the X-inactivation process; and (3) the role of chromatin structure in transcriptional regulation.

Chapter Two describes a series of experiments designed to examine the histone acetylation status of individual genes along the Xi. A major emphasis of these experiments was to compare the acetylation status of genes that are subject to X-inactivation with those that escape this process. This study revealed a perfect correlation between the promoter-specific acetylation status of histone H4 and the expression of individual genes on the Xi: genes which escape silencing on the Xi show elevated levels of acetylation at their promoters, while X-inactivated genes are markedly hypoacetylated at their promoters.

Chapter Three describes studies aimed at understanding the molecular nature of the colocalization between *XIST* RNA and the Xi. These studies demonstrated that *XIST* RNA may be a physical component of the Xi chromatin. In addition, results suggested that the distribution of *XIST* RNA along the Xi is perhaps not uniform, but is instead concentrated at hypoacetylated regions of the Xi chromatin.

Chapter Four discusses the significance of the findings reported in this thesis. A model for how *XIST* RNA controls X-inactivation is proposed, in which the RNA serves as a bridging factor that recruits transcriptional silencing machinery to particular genes of the Xi.

One prediction of this model is that genes not subject to X-inactivation should fail to associate with *XIST* RNA. This prediction is partly supported by experiments described in the

Appendix. These experiments demonstrated that *Xist* RNA colocalizes with *Zfx*, an X-inactivated locus in the mouse, but not with *Sts*, a locus that escapes X-inactivation.

SECTION II: DOSAGE COMPENSATION MECHANISMS

One of the fundamental genetic distinctions between males and females of XY-bearing species is the number of X chromosomes that they possess. Dosage compensation is the process by which the sex difference in X chromosome dosage is attenuated through transcriptional regulation of X-linked genes.

The evolution of dosage compensation is thought to be an integral part of sex chromosome evolution. In XY-bearing species, the genetically distinct X and Y chromosomes were once a pair of identical autosomes, but differentiated as most genes on the Y chromosome degenerated. One consequence of this Y chromosome degeneration is the dosage imbalance of X-linked genes between males and females. This imbalance, which is detrimental to proper development, drives the evolution of dosage compensation mechanisms as a countermeasure.

Evolutionary studies have firmly established that sex chromosomes in distantly related lineages evolved on multiple, independent occasions. For each lineage, therefore, the dosage compensation mechanism has also arisen separately. This notion is confirmed by the observation that dosage compensation mechanisms in flies, nematodes and mammals — three lineages with the best characterized sex chromosomes — are disparate in their molecular characteristics. Dosage compensation in *Drosophila* is accomplished by upregulating transcript levels from the single male X to achieve a level comparable to that obtained from the two female X chromosomes. *C. elegans* employs the reverse strategy, in which transcript levels are normalized between males and females by downregulating expression from both X(s) in the hermaphrodite. A third strategy evolved in mammals, in which one of the two female X chromosomes is transcriptionally silenced, almost in its entirety.

Although these three dosage compensation strategies — hypertranscription, hypotranscription, and transcriptional silencing — are evolutionarily unrelated, and appear

mechanistically disparate, each involves alterations in the chromatin structure of the regulated X chromosome. Furthermore, two of the three strategies, hypertranscription in *Drosophila* and transcriptional silencing in mammals, involve noncoding RNA molecules that localize to the regulated X chromosome from which the RNA is transcribed. These commonalities suggest that noncoding RNAs may have the ability to recruit various activities to a chromosome *in cis*.

i) Dosage Compensation in *Drosophila*

Dosage compensation in *Drosophila* is achieved through an approximately two-fold overexpression of transcripts from the single male X. This process requires a number of protein factors, many of which have been identified in genetic screens where their loss-of-function resulted in male-specific lethality. These factors include the male-specific lethal proteins 1, 2, and 3 (MSL-1, -2, and -3) and the maleless protein (MLE) (Belote and Lucchesi, 1980; Fukunaga et al., 1975).

Biochemical and cell biology studies have substantiated the involvement of these genetically identified factors in chromosome-specific regulation of the male X. In these studies, the MSL-1, -2 and -3 proteins were shown to form a heteromeric complex (Copps et al., 1998), which localizes to hundreds of sites along the polytene male X. Although the biochemical mechanism of dosage compensation has not been fully elucidated, potential activities of the MSL and MLE proteins have been characterized to some extent. The MLE protein has RNA/DNA helicase, adenosine triphosphastase (ATPase), and single-stranded (ss)RNA/ssDNA binding activities (Lee et al., 1997). Mutant MLE proteins with defective helicase/ATPase activites still localized to the male X chromosome, but failed to rescue an *mle* phenotype (Lee et al., 1997). This suggests that the helicase/ATPase activities of MLE are needed for transcriptional regulation. Sequence analysis of the MSL proteins revealed several motifs common to transcriptional regulators, suggesting that the dosage compensation machinery indeed regulates transcription (Bashaw and Baker, 1995; Kelley et al., 1995; Palmer et al., 1993).

Binding of the MSL proteins to the upregulated male X is also coincident with the specific acetylation of lysine 16 on histone H4 (Bone et al., 1994; Turner et al., 1992). Males that fail to dosage compensate because they carry mutations in male-specific lethal genes also lack the lysine 16 acetylation on histone H4 (Bone et al., 1994). The reason for this appears to be that the MSL proteins recruit the MOF (*males-absent-on the first*) protein to the male X (Gu et al., 1998), which encodes a putative histone acetyltransferase (Hilfiker et al., 1997). Thus, one way the MSL complex may effect dosage compensation is by alterating chromatin structure of the male X in a manner that enhances transcription of its resident genes.

In addition to protein factors, two noncoding RNA molecules — *roX1* and *roX2* — which originate from the upregulated male X have been implicated in dosage compensation. The *roX1* and *roX2* genes are expressed in many cell types during early development, but become restricted primarily to cells of the central nervous system in adults (Amrein and Axel, 1997; Meller et al., 1997). Both *roX1* and *roX2* produce noncoding RNA molecules which can associate with X-derived chromatin either *in cis* or *in trans* (Amrein and Axel, 1997; Meller et al., 1997). These transcripts bind to the male X in a pattern that coincides with MSL binding (Meller et al., 1997). However, *roX1* is clearly not required for dosage compensation, as its disruption produces no obvious phenotype in males (Meller et al., 1997). It is possible that there are additional, unidentified RNAs that associate with the hypertranscribed male X. In fact, association of the putative RNA helicase, MLE, with the male X is RNase-sensitive (Richter et al., 1996), although it is not affected by disruption of *roX1* (Meller et al., 1997).

ii) Dosage Compensation in Nematodes.

A different dosage compensation strategy has evolved in the nematode *C. elegans*. Here, the active modulation of the dosage imbalance occurs in hermaphrodites (which are XX), rather than in males (which are XO). In hermaphrodite worms, transcription from both X chromosomes is downregulated, such that the steady-state transcript levels of most X-linked genes are

comparable between hermaphrodite and male cells (Donahue et al., 1987; Meyer and Casson, 1986).

Similar to studies of *Drosophila* dosage compensation, genetic screens for sex-specific lethal phenotypes have identified a number of factors in *C. elegans* that contribute to transcriptional downregulation of the hermaphrodite X chromosomes. Functional analysis has led to the identification of a protein complex that includes many of these factors. This protein complex localizes to both of the dosage-regulated X chromosomes of hermaphrodites (Chuang et al., 1994; Chuang et al., 1996). Thus far, the complex has been shown to contain the following proteins: SDC-2, SDC-3, DPY-26, DPY-27, and MIX-1. Some of these proteins, such as DPY-27, appear not to have any function beyond their role in dosage compensation. However, several proteins have other essential cellular functions. For example, the DPY-26 protein initially associates with all mitotic chromosomes in embryos of both sexes. Yet, the initiation of dosage compensation changes this pattern, such that DPY-26 is selectively localized to the hermaphrodite X's in somatic cells and with all meiotic chromosomes in germ cells (Lieb et al., 1996). Similarly, the MIX-1 protein is required not only for downregulation of transcript levels in hermaphrodites, but also for chromosome segregation during mitosis in both sexes (Lieb et al., 1998). SDC-2 and SDC-3 also have several functions, one of which is to target the dosage compensation machinery to X chromosomes (Dawes et al., 1999). The substantial overlap of components between the dosage compensation machinery and the meiotic and mitotic machinery is strong evidence that dosage compensation in nematodes — and conceivably in other species as well — evolved by adapting existing chromosome regulatory mechanisms for a *de novo* task.

iii) Dosage Compensation in Mammals

Mammals employ yet a third strategy for dosage compensation. During the early stages of embryogenesis, one of the two X chromosomes in each female somatic cell is randomly chosen to undergo a widespread transcriptional silencing. Once the silenced state of the chosen X is established, it is clonally inherited through cell divisions and differentiation (Monk and Harper,

1979). A deviation from the random nature of X chromosome choice is observed in marsupials and in the extraembryonic membranes of placental mammals, in which the paternal X is invariably chosen for silencing. The mechanisms of X-inactivation will be described in greater detail in following sections of this chapter.

SECTION III: MECHANISMS OF X-INACTIVATION

The approaches that have been employed to study the mechanisms of X-inactivation are numerous and varied. Most of these approaches can be divided into three broad categories: The traditional approach is a genetic one, whereby regions of the X chromosome that control the X-inactivation process are mapped using mutant chromosomes that are derived from X-autosome translocations. Such an approach led to the original identification of the X-inactivation center (*XIC*) (Brown et al., 1991), and the isolation of the *XIST* gene within the *XIC* (Brown et al., 1991). The genetic approach has been expanded enormously in recent years to include targeted deletions and transgenic insertions which have enabled fine dissections of the chromosomal regions that control various aspects of X-inactivation. A second major approach in the study of X-inactivation is to follow developmental events that occur during the X-inactivation process. In this case, the objective is to chronicle the sequence of events that occur when female cells undergo X-inactivation, either *in vivo* during embryogenesis, or in tissue culture during embryonic stem (ES) cell differentiation. A third approach to study X-inactivation, which has gained greater popularity in recent years, is to identify molecular features that distinguish the inactive X chromosome from its active counterpart. Comparative analysis has identified the following features unique to the Xi chromosome: association with *XIST* RNA, late timing of DNA replication, general histone hypoacetylation and methylation of CG islands associated with the 5' ends of X-inactivated genes.

Intense investigations with combinations of the various approaches have provided detailed descriptions of the X-inactivation process. Although these descriptions are often phenomenological rather than mechanistic, they are beginning to yield clues as to how this intriguing biological process may be regulated at a molecular level.

i) X-Inactivation is Controlled by the X-Inactivation Center (*XIC*)

X-inactivation is a multi-step process which involves: (1) counting the number of X chromosomes and choice of the one that will remain active, (2) initiation of silencing on all remaining X's, and (3) clonal maintenance of the inactive state once it is established. Most of these functions — other than maintenance — are specified by a region on the X termed the X-inactivation center (*XIC*). The *XIC* is located in interval Xq13 in humans, and was originally defined as a region common to all rearranged X's that were capable of being inactivated (Brown et al., 1991). More recently, the transgene-based approach has been used to define the minimum functional *Xic* in the mouse by its ability to induce inactivation at ectopic sites in ES cells (Lee et al., 1996; Herzing et al., 1997; Lee et al., 1999). Such studies have suggested that an 80 kb region surrounding the mouse *Xist* gene may be sufficient to induce many features associated with X-inactivation at ectopic sites, such as hypoacetylation of histone H4, late replication timing, and downregulation of X-linked gene expression (Lee et al., 1999). However, a caveat of the transgene-based approach is that most transgenes are present in multiple copies. One study demonstrated that single copy transgenes were insufficient to induce inactivation, suggesting that some of the elements needed for initiation or spread of X-inactivation are lacking in these transgenes and are artificially supplied by multimerization (Heard et al., 1999).

Another strategy to dissect the function of the *XIC* is to identify and characterize genes located within this critical interval. Several genes have been mapped to the *XIC*, but only one — the *XIST* gene — has been conclusively implicated in X-inactivation. In addition to its physical location within the *XIC*, *XIST* is the only known X-linked gene that is expressed exclusively from the inactive X in differentiated somatic cells (Brown et al., 1991). Furthermore, *XIST* RNA is apparently not transported out of the nucleus as most mRNAs are; rather, it colocalizes with the inactive X chromosome from which it is transcribed (Brown et al., 1992). Human *XIST* encodes a 17 kb spliced and polyadenylated transcript. Initially, evidence for the role of *XIST* in X-inactivation had been circumstantial. More recently, targeted deletion studies provided conclusive

evidence that the *XIST* gene is required for initiation of X-inactivation (Marahrens et al., 1997; Penny et al., 1996).

To date, the mode by which the *XIST* gene functions in X-inactivation is unknown. Several models have been proposed (Brockdorff et al., 1992; Brown et al., 1992), including: (1) the *XIST* gene encodes a critical protein factor that participates in X-inactivation; (2) the *XIST* genomic locus acts as a chromatin organizing center from which X-inactivation is propagated along the chromosome; or (3) *XIST* encodes a structural RNA which is a crucial component of the X-inactivation machinery. The first model is most likely incorrect, as there is no evidence that *XIST* encodes a protein. First, the nuclear localization of *XIST* RNA suggests that it is not accessible to the cytoplasmic translation machinery. Second, *XIST* lacks any significant open reading frames (Brockdorff et al., 1992; Brown et al., 1992). Finally, the few short open reading frames that do exist are not conserved amongst mammals. The other two models for *XIST* function — either that it is a chromatin organizing center or that it encodes a functional RNA — are thus far both consistent with the data. The observation that *XIST* RNA colocalizes with the Xi tends to favor the functional RNA model, although it does not exclude the possibility that the *XIST* locus functions as a chromatin organizing center.

Genetic studies have also identified a sequence element within the *XIC* which controls the likelihood that the chromosome bearing this element will be chosen for X-inactivation. This X controlling element (*Xce*) has at least four alleles in mice — Xce^a (weakest), Xce^b, Xce^c, Xce^d — such that X-inactivation is biased towards the chromosome carrying the weaker allele (Migeon, 1994). Targeted deletion of a large region encompassing this element resulted in constitutive *Xist* expression from the disrupted X (Clerc and Avner, 1998). The only gene identified in this interval to date is *Tsix*. *Tsix* is a 40 kb nuclear transcript that is antisense to *Xist* RNA (hence, its name is "*Xist*" spelled backwards)(Lee et al., 1999). In addition to its antisense orientation relative to *Xist*, the expression pattern of *Tsix* makes it a candidate regulator of *Xist*: Prior to X-inactivation, *Tsix* is expressed biallelically. At the onset of X-inactivation, however, its expression becomes restricted to the future Xa and persists until *Xist* expression is repressed. While the evidence

that *Tsix* is an antisense regulator of *Xist* is tentative at best, it is worth noting that antisense transcripts have also been detected in the vicinity of imprinted genes, which are also subject to allele-specific regulation (Moore et al., 1997; Rougeulle et al., 1998).

In addition to *Xist* and *Tsix*, several other genes have been mapped to the *XIC* in both human and mouse. These include the brain-specific gene *Brx* (Rougeulle and Avner, 1996), the testes-restricted gene *Tsx* (Simmler et al., 1996), the ribosomal protein pseudogene *pS19X* (Rougeulle and Avner, 1996), and a homologue of the caudal gene, *Cdx4* (Horn and Ashworth, 1995). The expression patterns and the putative functions of these genes are not consistent with their having a role in X-inactivation, although this has not been conclusively demonstrated by genetic analysis.

ii) Counting and Choice

In placental mammals, the selection of X chromosomes for inactivation proceeds according to the "n-1 rule", which silences all but one X per diploid genome. The term "counting" reflects the fact that one X is kept active per diploid genome. Increasing amounts of ploidy are able to support additional active X's in a cell, as triploid individuals with a 69, XXY karyotype are able to maintain two active X's (Weaver and Gartler, 1975). However, the term "counting" can be somewhat misleading, since supernumerary X's in a diploid genome do not necessarily need to be counted to become X-inactivated. For instance, it may be that all X chromosomes are normally silenced by default, but that there exists a mechanism that actively prevents just one X from undergoing the default process. It has not been possible to experimentally distinguish whether the "n-1 rule" is implemented through counting the total number of X's, or merely by protecting one X from the inactivation process. Regardless of whether the cell actually counts the number of X chromosomes in the nucleus, it has been hypothesized that "blocking factors" produced in diploid cells prevent one *XIC* from initiating X-inactivation such that it remains active, while all remaining unblocked *Xics* inactivate their host chromosomes (Lyon, 1996).

The element(s) that are required for counting and/or choice have not been fully delineated. The random nature of X-inactivation in the embryonic cells of eutherian mammals can be biased by alleles at the X-controlling element (*Xce*), as described above. The genetic relationship between *Xist* and the *Xce* is unclear. Targeted deletion of a region encompassing the mouse *Xce* and *Tsix* caused constitutive expression of the linked *Xist* gene (Clerc and Avner, 1998). Another element that contributes to choice is present in the *Xist* gene itself. Targeted deletion of sequences corresponding to exon 1 through intron 5 of *Xist* disrupted the ability to choose the mutant X, such that only the intact chromosome became inactivated (Marahrens et al., 1998). Further genetic studies will be needed to better define the sequences and the effector molecules that are critical for choice.

iii) Initiation and Spread of X-inactivation

Time course analysis of X-inactivation has relied primarily on mouse ES cells, which are experimentally more tractable than developing embryos. Female ES cells possess two active X chromosomes, one of which becomes inactivated as cells differentiate in culture. This process of *in vitro* differentiation recapitulates many of the stages of X-inactivation that occur during embryonic development.

Temporal observation of X-inactivation during ES cell differentiation, and in some cases in the developing mouse embryo, has revealed a series of events that occur in a defined sequence over a period of several days (Keohane et al., 1996). The first detectable event in the process of X-inactivation is the increase in levels of *Xist* RNA from the incipient Xi (Kay et al., 1993; Lee et al., 1996; Panning et al., 1997). Prior to this event, *Xist* is transcribed at a low-level from both X chromosomes of undifferentiated ES cells. Yet, this high-level *Xist* expression initiates X-inactivation only if it occurs within a critical developmental period: Reactivation of mouse *Xist* in differentiated cells did not induce silencing of genes *in cis*, despite the fact that the ectopic *Xist* RNA localized to its chromosome-of-origin (Clemson et al., 1998). That *Xist* RNA can initiate X-inactivation during embryogenesis, but not at later stages of development, suggests that a

change occurs either in the composition of available auxiliary factors, or in the RNA itself. It is possible that one contributing factor to this difference is the change in levels and location of *de novo* and maintenance methyltransferases in early embryonic cells relative to terminally differentiated cells (Razin and Shemer, 1995).

Xist upregulation coincides almost immediately with a shift to late S phase in the replication timing of the Xi (Keohane et al., 1996). Within 2 days of *Xist* upregulation, transcriptional downregulation of several genes on the Xi is completed. Although it is commonly held that X-inactivation spreads out from the *Xic* in both directions, RT-PCR analysis of transcript levels has not detected a gradient in the timing of inactivation for X-linked genes (Keohane et al., 1996). Nevertheless, the notion that X-inactivation spreads laterally from the *XIC* is not an invalid one, since studies of X:autosome translocations have shown that X-inactivation can traverse the translocation boundary to affect the proximal portion of the autosomal region. However, the incomplete spreading of X-inactivation on these translocation chromosomes has led to the proposal that "way station" elements evolved on the X chromosome to facilitate the spread of X-inactivation (Gartler and Riggs, 1983). Mary Lyon has proposed that LINE elements, which are disproportionately enriched on the X and which correlate with the X-inactivation potential of autosomes, may facilitate *XIST* RNA association with the Xi and contribute to its X-inactivation (Lyon, 1998).

iv) Maintenance of the Inactive State

Once an X chromosome has been chosen for inactivation, its inactive state is stably and clonally transmitted during subsequent cell divisions and differentiation. The mechanism responsible for the maintenance of the inactive state is apparently separable from that responsible for its initiation. This assessment is based on the observation that maintenance of the inactive state, unlike initiation of X-inactivation, does not require the *XIC*. In cultured human/rodent somatic cell hybrids in which the human X is stably inactivated, induced deletion of the *XIC* — including the

XIST gene — from the Xi does not alter its transcriptionally repressed state (Brown and Willard, 1994; Rack et al., 1994).

Because CG islands associated with the 5' ends of silenced genes on the Xi are hypermethylated, in contrast to their counterparts on the Xa or on most autosomes, methylation is thought to provide "memory" of the inactive state. The greater stability of the Xi in eutherian cells is also attributed to the methylation of CG islands, which is not detectable in marsupials (Kaslow and Migeon, 1987; Loebel and Johnston, 1996) and is believed to have evolved in eutherians as a means to "lock-in" the repressed state (Wakefield et al., 1997). Another molecular feature which distinguishes the Xi from the Xa and from all other chromosomes is a chromosome-wide histone hypoacetylation evident in metaphase microscopy (Belyaev et al., 1996; Boggs et al., 1996; Jeppesen and Turner, 1993). Time course analysis of differentiating ES cells suggested that both methylation and the microscopically observable deacetylation of the Xi may follow, rather than precede, downregulation of gene expression, suggesting that they are important for maintenance of the inactive state (Keohane et al., 1996).

SECTION IV: ESCAPE FROM X-INACTIVATION

Important clues about the mechanism of X-inactivation have come from analyzing exceptions to the rule — namely, genes which are present on the Xi but which are not inactivated. Despite the apparently sweeping nature of X-inactivation, transcription from the inactive X is not uniformly repressed. The first indication that gene expression from the Xi is needed for important cellular functions came from studies of Turner syndrome (TS). Patients with TS have partial or complete sex chromosome monosomy (an XO karyotype), and exhibit a range of defects including short stature, gonadal dysgenesis and multiple anatomical abnormalities. TS individuals can be thought of as either missing an X (when compared to normal XX females), or a Y (when compared to normal XY males). Based on this reasoning, Ferguson-Smith postulated that TS is due to the haploinsufficiency of "TS genes" which should be present on both X and Y, and should escape X-inactivation on the X (Ferguson-Smith, 1965). The immediate implication of

this hypothesis is that X-inactivation is incomplete; that is, there are genes on the X that escape silencing to fulfill the critical requirement of their double dosage during development.

The hypothesis that X-inactivation is incomplete has been validated by experimentation in the ensuing years. Since the 1980's, genes that escape X-inactivation have been continually discovered. Currently, the most powerful method to identify such genes is to assay X-linked gene expression in human/rodent somatic cell hybrids that retain either the active or the inactive human X (Mohandas et al., 1980). Human genes that undergo X-inactivation are expressed only in the Xa-containing hybrid, whereas genes that escape X-inactivation are expressed in both types of hybrids. This and other approaches have led to the identification of nearly two dozen genes that escape X-inactivation in humans (Brown et al., 1997). These genes fall into two categories: those in the pseudoautosomal regions, and those in the X-specific region.

Pseudoautosomal regions (PARs) are located at the distal tips of the sex chromosomes. Within PARs, the X and Y undergo meiotic recombination just as two homologous autosomes do. As a result, PARs on the X are identical to corresponding PARs on the Y. In males, PAR genes are expressed biallelically, from both the X and the Y. For these genes to be expressed at a comparable level in females, they must escape X-inactivation. Indeed, all pseudoautosomal genes examined, other than *SYBL1*, are expressed from both Xi and Xa in females. They constitute approximately one-third of the human genes known to escape X-inactivation. The remaining genes that escape inactivation reside in the X-specific portion of the X — the portion that does not recombine with the Y in meiosis. Consistent with Ferguson-Smith's hypothesis, many of these genes have functional homologs on the Y chromosome.

Interestingly, X-inactivation appears to be much more complete in the mouse, as only four genes have been shown to escape X-inactivation (reviewed in (Disteche, 1995)). The fact that fewer mouse genes should escape X-inactivation is consistent with the observation that the XO karyotype in the mouse produces a much milder phenotype in comparison to TS in humans: XO female mice are fertile, and are often indistinguishable from normal XX females (Banzai et al., 1995).

The mechanism by which some genes escape X-inactivation is largely unappreciated. The fact that genes which escape X-inactivation are dispersed throughout the X suggests that the mechanism by which genes escape X-inactivation operates on a rather localized basis. Occasionally, however, several genes which escape X-inactivation are found clustered together, suggesting the possibility that local control may extend beyond the level of a single gene (Miller and Willard, 1998).

Another way to understand the mechanism by which some genes escape X-inactivation is to perform time course analyses of X-linked gene expression in the course of development. These studies have suggested that the X-inactivation status of a gene can be dynamically altered during development. Several studies have reported that the mouse *Smcx* gene is originally silenced during the initiation of X-inactivation in either differentiating ES cells (Penny et al., 1996) or in early embryos (Lingenfelter et al., 1998). However, *Smcx* expression from the Xi is reestablished later in development such that expression from the Xi and Xa are nearly comparable (Lingenfelter et al., 1998; Sheardown et al., 1996). These results reveal the highly complex nature of both initiation and maintenance of X-inactivation.

SECTION V: FEATURES OF THE INACTIVE X CHROMATIN

Several features distinguish the chromatin of the Xi from that of the Xa. Some of these features, such as hypoacetylation and association with *XIST* RNA, have been described at the level of the entire chromosome. Other features — such as DNA methylation and late replication timing — distinguish genes that are silenced on the Xi from those on the same chromosome that escape inactivation. Studies of these features have provided important clues to the mechanism of gene silencing on the Xi.

i) Colocalization of *XIST* RNA and the Xi

The observation that the RNA product of *XIST* localizes to the inactive X Barr body (Brown et al., 1992; Clemson et al., 1996) is the primary reason to favor the model that *XIST* functions as a

noncoding RNA during the inactivation process. There are at least two possibilities that may account for the observed colocalization: either *XIST* RNA may be sequestered to the same nuclear compartment as the Xi, perhaps by mechanisms that confine the Xi to a transcriptionally repressive environment, or *XIST* RNA may be physically attached to the Xi chromatin.

Several attempts have been made to better characterize the colocalization between *XIST* RNA and the Xi. In one experiment, procedures that extracted 90-95% of DNA and most of the histones from the nucleus hardly diminished the *in situ XIST* RNA signal. This led to the speculation that *XIST* RNA is attached to the nuclear matrix and that it is not part of the chromatin (Clemson et al., 1996). However, the results of this experiment are subject to multiple interpretations, as there exist alternative technical or biological reasons for the inability to extract *XIST* RNA from the nucleus along with chromatin. In addition, it was reported that *XIST* RNA does not hybridize directly to the Xi DNA, as it is not degraded by RNase H which digests RNA/DNA hybrids (Clemson et al., 1996).

ii) Heterochromatinization

A number of observations suggest that the Xi is distinguished from other chromosomes, including the Xa, by higher-order changes in chromatin structure. First, the fact that the majority of genes on the Xi are silenced, more or less simultaneously, suggests that their repression is coordinated at the level of the chromosome. In addition, the Xi appears to be visibly condensed into a heterochromatic Barr body that is usually located at the nuclear periphery (Barr ML, 1961). It is thought that higher-order packaging of chromatin is responsible for an altered conformation of the Xi, including a bend in its Xq arm during metaphase (Flejter et al., 1984), and interactions between the telomeres that produce a looping of the Xi (Walker et al., 1991).

Molecular analyses of individual genes has also revealed differences between the higher-order chromatin structure of the Xi and Xa. Three DNase I-sensitive regions were detected for the X-linked *Pgk* gene that are unique to the Xa allele (Riley et al., 1986). These differences in nuclease accessibility reflect differences in chromatin structure that correlate with gene expression.

Similarly, the promoter region of human *HPRT* contains a DNase I-sensitive region on the Xa, but not on the Xi (Lin and Chinault, 1988). In addition, chromatin fractionation by differential centrifugation showed that several genes on the Xi were enriched in the heterochromatin fraction, whereas their counterparts on the Xa were enriched in the euchromatic fraction (Endo et al., 1998; Endo et al., 1999).

iii) DNA Methylation

Mammalian DNA is methylated at cytosine residues within CG dinucleotides and maintained through cell division by the action of maintenance methylases on hemimethylated templates. C G islands that are associated with 5' ends of X-inactivated genes are heavily methylated on the Xi but completely unmethylated on the homologous Xa alleles (Norris et al., 1991; Tribioli et al., 1992). Furthermore, CG islands associated with genes on the Xi that escape silencing are not methylated, demonstrating a correlation at the level of individual genes.

Although there appears to be a perfect correlation between methylation and X-inactivation status (Jegalian and Page, 1998; Tribioli et al., 1992), the precise role of methylation in the process has not been fully elucidated. Several lines of evidence suggest that methylation contributes to maintenance of the inactive state, once it has been established by other methods. First, treatment of cells with 5-azacytidine results in DNA hypomethylation and consequent reactivation of genes on the Xi (Mohandas et al., 1981). Second, marsupial Xi genes, which are known to lack CG methylation (Loebel and Johnston, 1996), are more easily reactivated in cell culture (Kaslow and Migeon, 1987). The fact that marsupials can achieve X-inactivation without methylation suggests that methylation was not essential for the process in mammalian ancestors, but that it was a later evolutionary invention to help "lock-in" the repressed state (Wakefield et al., 1997) Although methylation is implicated in maintenance of the inactive state in eutherian mammals, it may be dispensible for initiation of X-inactivation. The strongest evidence for this claim is the observation that *Xist*-mediated X-inactivation can be initiated in cells which are deficient for DNA methyltransferase activity (Beard et al., 1995; Panning and Jaenisch, 1996).

iv) Replication Timing

Many heterochromatic regions of the genome replicate late in S phase, after the majority of the genome has already undergone DNA synthesis. Consistent with its heterochromatic state, the Xi also replicates late in S phase with respect to its active counterpart in female cells (Gartler and Riggs, 1983). This feature is one of the earliest detectable changes to the Xi chromatin, immediately following the upregulation of *Xist* RNA during embryonic development (Keohane et al., 1996; Takagi et al., 1982). Replication timing has also been assessed at the level of several individual X-linked genes in human cells. Genes that are subject to X-inactivation replicate earlier on the Xa than do their counterparts on the Xi. In contrast, genes that escape X-inactivation were found to replicate synchronously on Xi and Xa, as measured either by RNA FISH analysis or by chromatin fractionation following BrdU incorporation (Boggs and Chinault, 1994; Hansen et al., 1996; Schmidt and Migeon, 1990). This correlation was further substantiated by the finding that reactivation of several silenced genes on the Xi with the hypomethylating agent 5-azacytidine coincided with a shift to an earlier timing of replication, although there were some exceptions to this rule (Hansen et al., 1996).

Although late replication is a prominent feature of the Xi chromatin which coincides temporally with X-inactivation, some studies suggest that it is neither necessary nor sufficient for the process: Several cell lines have been isolated that contain an early-replicating Xi which is nevertheless transcriptionally repressed (Yoshida et al., 1993). Thus, X-inactivation can be maintained, at least in cell culture, without late replication of the Xi. Furthermore, a shift to a late replication timing, induced by the deacetylase inhibitor Trichostatin A, was not sufficient to induce silencing of X-linked genes in the absence of other features associated with X-inactivation (O'Neill et al., 1999).

v) Histone Acetylation

Immunofluorescence microscopy with antibodies against acetylated isoforms of histones revealed that the metaphase Xi in both human and murine cells is distinguished by a marked reduction in the acetylation of H2A, H3 and H4 (Jeppesen and Turner, 1993; Belyaev et al., 1996; Boggs et al., 1996). Antibodies which could distinguish amongst acetylation of individual lysine residues confirmed that the metaphase Xi was reduced in all acetylated isoforms of histone H4. This is similar to the pattern of reduced H4 acetylation observed on metaphase chromosomes for other regions of markedly reduced transcriptional activity, such as centric heterochromatin and G bands (Jeppesen and Turner, 1993). The role of histone hypoacetylation in gene silencing on the Xi is further corroborated by the finding that the histone deacetylase inhibitor Trichostatin A prevented the silencing of X-linked genes in differentiating ES cells (O'Neill et al., 1999).

Xi chromosomes from mouse metaphase spreads occasionally showed three major bands of H4 acetylation, as did human Xi chromosomes that were exposed to the deacetylase inhibitor sodium butyrate (Jeppesen and Turner, 1993). These three regions of persistent acetylation correspond to homologous regions on the mouse and human Xi, suggesting that the modification has been conserved. Despite the imprecision inherent in cytological localization, it appears that these persistent acetylation signals correspond, at least in part, to regions which contain genes that escape X-inactivation.

While most analyses of the acetylation status of the Xi have been at the level of the whole metaphase chromosome, several recent studies have attempted to examine the acetylation status of individual genes on the Xi. One report showed that there is a promoter-proximal increase in acetylation levels of the *Xist* gene in female cells (McCabe et al., 1999). A more comprehensive analysis of the acetylation status of X-linked genes reported in this thesis (Chapter Two) indicates that promoters of genes that undergo X-inactivation are enriched in hypoacetylated isoforms of H4, whereas promoters of genes that escape transcriptional silencing are acetylated on H4 at elevated levels.

SECTION VI: EVOLUTION OF X-INACTIVATION

Mammals are the only living descendants of synapsid reptiles, from which they first emerged 200 million years ago, as estimated by fossil records. Extant mammals belong to three subclasses — monotremes, marsupials, and eutherian (placental) mammals. All three subclasses are thought to employ X-inactivation as the means of X chromosome dosage compensation. Comparisons of the molecular features associated with X-inactivation amongst these three subclasses, and amongst different species within the same subclass, have provided clues to both the evolution and the mechanism of the X-inactivation process.

Monotremes, the only egg-laying mammals, are the most primitive of the three mammalian subclasses. They diverged from the therian lineage (marsupials and eutherians) approximately 150-170 million years ago (Air et al., 1971). Many of the features associated with eutherian X-inactivation have not been detected in monotremes to date. In fact, the only indication that X-inactivation may occur in monotremes is the asynchronous replication in some tissues of the short arm of the monotreme X, which has no counterpart on the Y chromosome (Wrigley and Graves, 1988). Using asynchronous replication of the Xp arm as a marker, monotreme X-inactivation was judged to be incomplete and tissue-specific, occurring in lymphocytes, but not in fibroblasts (Wrigley and Graves, 1988).

X-inactivation is also observed in marsupials, which diverged from eutherian mammals 120-150 million years ago (Hope et al., 1990). The occurrence of X-inactivation in marsupials has been confirmed by a variety of observations, including late replication of one of the two female X chromosomes in kangaroos, monoallelic expression of kangaroo isozymes (Richardson et al., 1971), and transcriptional downregulation (Wakefield et al., 1997). The fact that no *XIST* homologs have been detected in either monotremes or marsupials to date could indicate either that *XIST* is not present in these primitive mammals, or that its poor conservation has made it difficult to detect.

The primary feature which distinguishes X-inactivation in marsupials from that observed in eutherians is that the paternal X is invariably inactivated (Sharman, 1971). Interestingly, the same

imprinted manner of X-inactivation is also observed in the trophectoderm of placental mammals, in which the paternal X is always silenced (Takagi, 1974). Given that imprinted X-inactivation is employed by both marsupials and the extraembryonic tissues of placental mammals, it is likely that it represents the evolutionarily more primitive state, and that the random nature of X-inactivation observed in the embryo proper of placental mammals is a later invention. In the eutherian lineage, this imprinted manner of X-inactivation persisted in extraembryonic tissues, but was replaced by random X-inactivation in the embryo proper.

The short arm of the marsupial X (Xp) has homology to and replicates synchronously with the Y chromosome. The long arm (Xq), however, is asynchronously replicating and subject to tissue-specific inactivation (Graves and Dawson, 1988). This Xq region also appears underacetylated on histone H4 by metaphase microscopy analysis (Wakefield et al., 1997). The strong evolutionary conservation of histone underacetylation implies that it was a component of X-inactivation in ancestral mammals. The fact that DNA methylation is not as conserved led to the suggestion that DNA methylation was a later evolutionary development which contributes to maintenance of the inactive state in eutherians (Wakefield et al., 1997). This is consistent with the finding that the marsupial Xi, which lacks methylation of CG islands, is more susceptible to reactivation in cell culture (Kaslow and Migeon, 1987).

The evolution of X-inactivation was an integral part of sex chromosome evolution. Mammalian sex chromosomes are believed to have arisen from a pair of autosomes approximately 300 million years ago. This initially homologous pair of chromosomes continually differentiated as the X remained conserved, while the Y lost most of its genes. It is thought that functional degeneration of the Y created the selective pressure that drove the X to become dosage compensated (Adler et al., 1997; Jegalian and Page, 1998). Evolutionary comparisons of sex chromosome genes in diverse eutherian orders have revealed that Y degeneration is an ongoing process, which has advanced to varying degrees in different eutherian orders. The *RPS4Y* gene for example, is conserved only on the Y chromosome of primates, but has become extinct in all other eutherian orders. Similarly, the *ZFY* gene is conserved on the Y of all

eutherians except in rodents. The fact that Y degeneration evolved on a gene-by-gene basis raises the possibility that X-inactivation, which is a counterresponse to Y degeneration, also evolved on a gene-by-gene, or at least a region-by-region basis. This hypothesis is supported by two observations. First, when a particular X gene is examined across diverse eutherian orders, its X-inactivation status is correlated with the presence or absence of a homologous gene on the Y chromosome. That is, in species where the Y homolog is present, the X gene escapes X-inactivation, whereas in species that lack a conserved Y homolog, the X gene is subject to X-inactivation (Jegalian and Page, 1998). Second, when multiple X genes are examined within the same species, the same general correlation holds: most X genes that lack Y homologs undergo X-inactivation, while X genes that have conserved Y homologs escape X-inactivation.

The gene-by-gene or region-by-region evolution of X-inactivation implies that the mechanism responsible for transcriptional repression on the Xi should operate at a local level, and that this mechanism should be sufficiently malleable to allow particular genes to change their X-inactivation status as their Y homologs become degenerate.

SECTION VII: CHROMATIN STRUCTURE IN TRANSCRIPTIONAL REGULATION

i) Nucleosome Structure

In eukaryotes, DNA is packaged by histone and non-histone proteins into a highly ordered and extremely condensed nucleoprotein complex known as chromatin. At the first level of packaging is a nucleoprotein structure called the nucleosome, which contains 146 basepairs of DNA wrapped in 1.75 superhelical turns around a octamer of core histones. There are four types of core histones — H2A, H2B, H3 and H4 — which are present in two copies each in a nucleosome (Kornberg and Thomas, 1974). Repeating arrays of nucleosome cores are further organized into higher-order chromatin structure by the linker histone H1, which brings neighboring nucleosomes into closer proximity. At this level of compaction, chromatin exists as 30-nm nucleosome fibers

(Shen et al., 1995). These fibers are further organized into 50- to 100-kb loop domains by attachment to either the nuclear matrix or the nuclear membrane (Gasser and Laemmli, 1987).

ii) Core Histone Variants

Histones are amongst the most conserved eukaryotic proteins, reflecting their ancient origins and their fundamental importance in many cellular processes. Each of the four core histones is comprised of a globular domain that participates in histone-histone interactions required for nucleosome formation, as well as a highly charged, unstructured tail at its N-terminus that protrudes out of the nucleosome. Histones H3 and H4 are especially conserved, showing very few amino acid substitutions or deletions between human, *Tetrahymena*, and yeast. Histones H2A and H2B are much more divergent between species, especially at their N-termini.

Several variant species of nucleosomal histones have been described. Although their exact functions are not known, they are typically implicated in more specialized chromatin functions. One variant of histone H3 is CENP-A, which is localized to centromeres (Sullivan et al., 1994). Several variants of histones H2A and H2B have been reported in metazoans. One particular variant of H2A which has been conserved from protozoa to humans is H2A.Z. The H2A.Z homolog in *Tetrahymena*, which is known as hv1, is enriched in nucleoli (Allis et al., 1982). Although the functional significance of this enrichment is not yet known, the *Drosophila* homolog, H2A.vD, is essential for early development (van Daal and Elgin, 1992). To date, no variants of H4 have been described.

A histone variant that may be particularly relevant to the understanding of X-inactivation is macroH2A (mH2A), which contains an N-terminal region that is 64% identical to conventional H2A, and a non-histone region which includes a basic region, a leucine zipper, and a region that is homologous to viral RNA-binding proteins. Based on the homology to viral RNA-binding proteins, it has been suggested that mH2A may also have RNA-binding activity (Pehrson and Fuji, 1998). Immunofluorescence analysis indicates that mH2A is enriched in the inactive X chromosome in female cells, similar to the pattern of *Xist* RNA localization (Costanzi and Pehrson,

1998). Although its function is not yet known, the fact that there is greater than 95% identity between the rat and chicken proteins suggests that mH2A plays a critical role in nucleosome structure and that this role is not limited to X-inactivation (which is not present in birds).

iii) Nucleosomes and Gene Repression

The ability to compact a large amount of DNA into the nucleus has enabled eukaryotes to have genomes that are orders of magnitude larger than prokaryotic genomes. This may have facilitated the evolution of enormous genetic complexity in eukaryotes. The packaging of DNA into chromatin also provides an important means of transcriptional regulation. Many studies have shown that chromatinized DNA is significantly more refractory to transcription than is naked DNA, presumably due to reduced accessibility of DNA to the transcriptional machinery. It has been shown by *in vitro* studies that the inhibition of transcription by nucleosomes can occur at the level of activator binding (Owen-Hughes and Workman, 1994), transcriptional initiation, or elongation (Izban and Luse, 1991; Studitsky et al., 1994).

iv) Histone Acetylation

Core and linker histones are subjected to a variety of post-translational modifications at their N-termini, including acetylation, ubiquitination, methylation, ADP-ribosylation, glycosylation, and phosphorylation (Bradbury, 1992). These covalent modifications change the biochemical properties of histones, which then alter the structure of chromatin in ways that have important consequences for gene expression.

Most of the present studies on histone modifications and their role in transcriptional regulation have focused on histone acetylation. Although it was first proposed that acetylation of core histones may correspond to transcriptional activity 35 years ago (Allfrey et al., 1964), it was not until recently that mechanistic insights emerged as to how acetylation might affect chromatin structure and transcriptional competence. Genetic studies of yeast, for example, have shown that mutants which are unable to acetylate the N-terminus of histone H4 have disrupted patterns

of transcription (Durrin et al., 1991). In another study, it was shown that treatment of mammalian cells with histone deacetylase inhibitors, which increased the acetylation level of bulk chromatin, led to a coincident alteration in the expression of many genes (Yoshida et al., 1995). In addition to these studies which attempt to directly address the effect of acetylation in transcription, numerous lines of circumstantial evidence are consistent with the role of histone acetylation in promoting transcription. Chief among them is the recent recognition that many transcriptional regulatory complexes possess histone acetylase or deacetylase activities. This recognition has served as an intellectual bridge to unite the chromatin and transcription fields.

While the precise effects of histone acetylation on transcriptional regulation are still not fully understood, the consensus in the field is that acetylation of nucleosomes results in a more accessible chromatin structure which facilitates transcription. There are several possible mechanisms by which histone acetylation may affect the transcriptional competence of chromatin. The first is that acetylation of lysine residues at the N-termini of histones neutralizes their positive charge, thus reducing the net charge of histone tails. This reduction in charge weakens the histones' interaction with DNA, such that transcription factors are better able to access their binding sites (Cary et al., 1982; Lee et al., 1993). Second, acetylation of histones may facilitate transcription by disrupting inter-nucleosomal contacts that are also inhibitory to transcription factor binding (Luger et al., 1997). The third mechanism by which histone acetylation may enhance transcription is by direct recruitment of transcription factors. Some transcriptional regulators have been shown to have affinity not for DNA, but for histones in a hyperacetylated state. These regulators contain the so-called bromodomain, a motif that specifically binds to acetyl-lysines in the N-termini of H3 and H4 (Winston and Allis, 1999). Thus, histone acetylation may function both directly and indirectly in recruiting transcriptional regulators to chromatin.

v) Histone Acetyltransferases and Deacetylases

Histone acetyltransferases (HATs) are the enzymes that transfer the acetyl moiety from acetyl coenzyme A to the ε-amino group of target lysine residues in the N-terminal tails of histones.

There are two classes of HATs, which are distinguished by their subcellular distribution and their biological function. Cytoplasmic, or B-type HATs acetylate newly synthesized histones H3 and H4 prior to chromatin assembly during DNA replication. Nuclear, or A-type HATs, acetylate histones in the context of the nucleosome, in reactions that are closely associated with transcriptional activation.

Many nuclear proteins which were found to possess histone acetyltransferase activity had previously been identified as transcriptional regulators, although their mechanism of action was initially unappreciated. One histone acetyltransferase — TAF130/250 — is a subunit of the TFIID complex (Mizzen et al., 1996), which is associated with nearly all promoters during transcriptional initiation. Another acetyltransferase, CBP/p300 (Bannister and Kouzarides, 1996; Ogryzko et al., 1996), was initially identified as a transcriptional coactivator capable of interacting with a variety of enhancer-binding proteins. Recently, CBP/p300 has been shown to associate with the RNA Polymerase II holoenzyme, suggesting that it may have more general functions in transcription (Cho et al., 1998; Nakajima et al., 1997). HAT activity is also intrinsic to ACTR (Chen et al., 1997) and SRC-1 (Spencer et al., 1997), two transcriptional activators that associate with nuclear receptors in a hormone-dependent fashion. P/CAF is another HAT (Yang et al., 1996), which associates with CBP/p300 and with ACTR and SRC-1 to form complexes which contain multiple histone acetylases.

In yeast cells, the Gcn5 acetyltransferase is needed for the normal expression of no more than 5% of the genes (Holstege et al., 1998). This suggests that the activity of some HATs may be specific for particular genes. In fact, Gcn5-dependent transcriptional activation of *HIS3* was shown to correlate with preferential promoter acetylation, suggesting that Gcn5 is targeted to the *HIS3* promoter (Kuo et al., 1998). In contrast, some HATs may be targeted to promoters in a manner that is nonspecific for particular genes. The TAF130/250 HAT, for example, is likely to be present at virtually all functional promoters in the cell.

In order for histone acetylation to impact signaling pathways that regulate transcription, the modification must be reversible. Histone deacetylases (HDACs) catalyze removal of the

acetyl group from N-terminal lysines in the core histones. Several of the deacetylases that have been described are members of the HDAC1/Rpd3 family, which has homologs from yeast to human (Rundlett et al., 1996; Taunton et al., 1996). Rpd3p often exerts transcriptional repression in concert with Sin3p (Vidal et al., 1991). Yeast and mammalian Rpd3p/Sin3p are recruited to promoters by their interactions with sequence-specific repressors, including the Mad/Max heterodimer, unliganded nuclear hormone receptors, Ume6p and Ikaros (Alland et al., 1997; Heinzel et al., 1997; Kadosh and Struhl, 1997; Nagy et al., 1997; Yang et al., 1996; Zhang et al., 1997; Koipally et al., 1999). This recruitment of deacetylases to promoters has been demonstrated to repress transcription from adjacent reporter genes.

vi) Site Usage of H4 Acetylation

The N-termini of histone H4 contains four lysines that can each be acetylated. Several lines of evidence suggest that differences in the site usage of H4 acetylation — that is, which of these particular lysines become acetylated — may have different biological consequences. Using antisera that distinguish amongst histones acetylated at different lysine residues, immunofluorescence studies of *Drosophila* polytene chromosomes have shown that the hypertranscribed male X is uniquely acetylated on lysine 16. The same studies showed that centromeric heterochromatin appears to be acetylated on lysine 12 (Turner et al., 1992). Studies of yeast chromatin have also revealed that acetylation of particular lysine residues has specific functional consequences. Substitution of positively charged lysine residues in H4 with residues that resemble either constitutively acetylated or deacetylated lysines has shown that certain patterns of site usage are required for proper silencing of mating type genes (Braunstein et al., 1996; Park and Szostak, 1990). While it is unclear how differences in the site usage of acetylation should translate into different biological consequences, it has been proposed that site-specific acetylation may serve as a "recognition code" for the binding of transcriptional regulators (Hirano, 1999). The selectivity of this recognition may derive in part from the bromodomain motifs found in

some transcriptional regulators, which can bind to acetyl-lysine in a manner that is sensitive to the flanking sequence context (Ornaghi et al., 1999).

How are specific patterns of site usage maintained? Different HATs and HDACs have been shown to have different preferences in their site usage. It is therefore possible that the opposing activities of a number of HATs and HDACs serve to maintain the acetylation of specific lysine residues within a region of chromatin targeted by these enzymes.

vii) Histone Hyperacetylation Correlates with Gene Expression

A large body of correlative evidence demonstrates that histone acetylation coincides with transcriptional activity (Braunstein et al., 1993)(reviewed in (Kuo and Allis, 1998)). Genetic studies have indicated that the N-terminus of histone H4 is essential for proper expression of the yeast mating type loci, whereas the N-terminus of histone H3 contributes to the efficiency of the process (Kayne et al., 1988; Park and Szostak, 1990; Thompson et al., 1994). When examined directly, transcriptional activity of the HM loci in yeast correlated with hyperacetylation of histone H4 (Braunstein et al., 1993). A correlation between induced expression of an integrated serum response factor-controlled reporter gene and rapid acetylation of histone H4 in nucleosomes in reporter gene chromatin was also observed (Alberts et al., 1998). This suggests that H4 acetylation may be a prerequisite for activation of immediate-early gene expression.

Several studies have established a more direct connection between nucleosome hyperacetylation and transcriptional activation. First, mutations in the HAT domain of Gcn5p that failed to acetylate histone H3 *in vitro* also failed to acetylate the promoter and activate transcription from the GCN5-dependent *HIS3* promoter *in vivo* (Kuo et al., 1998). Second, studies with preacetylated nucleosomal templates have shown that acetylation of nucleosomes is sufficient to activate transcription (Lee et al., 1993; Vettese-Dadey et al., 1996; Steger et al., 1998). Third, targeting of HATs to promoters *in vivo* leads to transcriptional stimulation of reporter genes (Georgakopoulos et al., 1995). Finally, regulation of several inducible genes is inhibited by anti-CBP or anti-p300 antibody injection (Arias et al., 1994).

viii) Histone Hyperacetylation Modulates Higher-Order Chromatin Structure

At the level of the nucleosome, histone acetylation is able to relieve some of the inhibitory effects of decreased transcription factor accessibility to the template. Histone acetylation may also function to promote transcription by disrupting higher-order folding of chromatin. Model systems were employed in which 12-mer nucleosomal arrays were reconstituted using three different levels of acetylated histone octamers in the absence of linker histones (Tse et al., 1998). The resulting arrays were incubated in *Xenopus* oocyte extracts, and the degree of compaction was assessed by sedimentation and electrophoretic analysis. High-level acetylation of nucleosomes disrupted folding into higher-order structures, and produced a concomitant enhancement in RNA Polymerase III transcription. This is consistent with evidence that acetylation of nucleosomes may disrupt internucleosomal contacts (Luger et al., 1997). In contrast, high-level acetylation had only a moderate effect on the ability of the arrays to assemble into oligomers, which is thought to provide an *in vitro* representation of the long-range fiber-fiber interactions found in intact chromosomes (Schwarz et al., 1996). These results indicate that histone acetylation has functional consequences for chromatin structure and gene expression at numerous levels, from the individual nucleosome to higher-order chromatin domains.

ix) DNA Methylation Alters Chromatin Structure and Function

Studies performed in *Xenopus* extracts indicate that DNA methylation is inhibitory to transcription only in the context of nucleosomal templates, but not when it is present on naked DNA (Buschhausen et al., 1987; Kass et al., 1997). The methyl-binding protein MeCP2 binds as a monomer to symmetrically methylated DNA in any sequence context (Lewis et al., 1992; Meehan et al., 1992) and stably binds methylated DNA in nucleosomal arrays (Nan et al., 1997). Biochemical studies have shown that MeCP2 copurifies with a complex that contains histone deacetylase activity. This suggests that repression due to methylation is indirect, mediated through hypoacetylation of nucleosomes in target genes (Jones et al., 1998; Nan et al., 1998).

Further indication that deacetylases are a component of methyl-directed repression comes from analysis of transcription from methylated *hsp70* promoters. When injected into *Xenopus* oocyte nuclei, these methylated templates become transcriptionally repressed and lose their DNase I hypersensitivity. Both of these properties can be reversed by addition of the histone deacetylase inhibitor Trichostatin A (Jones et al., 1998). Thus, deacetylase activity is required in many cases for chromatin remodeling and transcriptional silencing on methylated nucleosomal templates.

SECTION VIII: SUMMARY

In model organisms such as *Drosophila* and *C. elegans*, mechanistic insights into the process of dosage compensation have been gained from a combination of genetic, developmental, and biochemical approaches. In contrast, there has been a paucity of molecular information about the mechanisms of mammalian dosage compensation. This thesis is an attempt to identify molecular features which distinguish the inactive X chromosome, with the goal of understanding how these features may contribute to the transcriptional silencing of X-linked genes.

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CHAPTER TWO: PROMOTER-SPECIFIC HYPOACETYLATION OF X-INACTIVATED GENES

Summary

The histone H4 acetylation status of the active X (Xa) and inactive X (Xi) chromosomes was investigated at the level of individual genes. A moderate level of acetylation was observed along the lengths of genes on both the Xi and Xa. However, this moderate level of acetylation was specifically modified in promoter regions. Transcriptionally active genes showed elevated levels of acetylation at their promoters on both the Xi and Xa. In contrast, promoters of X-inactivated genes were markedly hypoacetylated, which coincided with the methylation of adjacent CG dinucleotides. This promoter-specific hypoacetylation may be a key component of an X-inactivation machinery that operates at the level of individual genes.

Introduction

In mammals, X chromosome dosage is equalized between males and females by X-inactivation, the transcriptional silencing of one of the two X chromosomes in female cells. The chromatin of the inactive X (Xi) is distinct from all other chromosomes, including the active X (Xa), in that it is late-replicating (Schmidt and Migeon, 1990; Hansen et al., 1996), methylated on CG dinucleotides (Riggs, 1975; Norris et al., 1991), enriched in the histone H2A variant macroH2A (Costanzi and Pehrson, 1998), and hypoacetylated on histones H2A, H3 and H4 (Jeppesen and Turner, 1993; Belyaev et al., 1996; Boggs et al., 1996). These unique features of the Xi chromatin are thought to contribute to the silencing of its several thousand resident genes.

Although most genes on the Xi are transcriptionally repressed, there are clear exceptions: First, the *XIST* gene which initiates X-inactivation is transcribed only from the Xi (Brown et al., 1991). Second, in both humans and mice, a number of genes escape X-inactivation – that is, they are transcribed from the Xi as well as the Xa (Disteche, 1997). Genes that escape X-inactivation typically have homologs on the Y chromosome, which in theory obviates their need for dosage compensation (Jegalian and Page, 1998).

Among the unique properties of the Xi chromatin, CG methylation and late replication

most closely correlate with gene expression. Where examined, these traits are associated with particular genes that are silenced, but not with those that escape X-inactivation (Mohandas et al., 1981; Goodfellow et al., 1988; Hansen et al., 1996). The correlation between histone acetylation and the activity of individual genes on the Xi has not yet been extensively examined. Immunofluorescence microscopy analysis demonstrated that all chromosomes stained brightly with antisera against acetylated H4 isoforms, with the exception of the Xi in female cells (Jeppesen and Turner, 1993). This led to the prevailing view that the entire Xi chromosome is hypoacetylated on H4.

A chromatin immunoprecipitation (IP) technique has recently been developed which allows the acetylation status of individual nucleosomes to be examined (Braunstein et al., 1993). We have used this method to investigate the acetylation status of histone H4 associated with individual genes on both the Xi and Xa. In contrast to the prevailing view that there is a chromosome-wide H4 hypoacetylation on the Xi, our results indicate that nucleosomes on the Xi are in fact acetylated along the lengths of genes at low levels that are comparable to their counterparts on the Xa. Notable exceptions to this general observation are found at promoter regions. On the Xi, promoters of genes that undergo X-inactivation are enriched in hypoacetylated isoforms of H4, whereas promoters of genes that escape transcriptional silencing are acetylated on H4 at elevated levels. The hypoacetylation of nucleosomes at the promoters of silenced genes correlated with methylation of coincident CG dinucleotides.

Results

Silencing of X-linked Genes Correlates with Hypoacetylation of Histone H4 in Promoter Regions

The chromatin IP assay (Braunstein et al., 1993; Alberts et al., 1998) was used to examine the acetylation status of histone H4 at selected sites on the human X chromosome. To observe a single human X in isolation from its homolog, experiments were performed using human/hamster somatic cell hybrids. These cell lines contain either the active or the inactive human X. Previous

reports indicate that gene expression, methylation status, and replication timing of the human X in these hybrid cells closely resemble those observed in human cells (Hansen and Gartler, 1990; Hornstra and Yang, 1994; Hansen et al., 1996). By RT-PCR analysis, the expected expression status of numerous X-linked genes in these hybrid cell lines was confirmed (data not shown).

To perform chromatin IP assays, cells were fixed with formaldehyde, followed by sonication. This produced chromatin fragments containing 200-1000 bp of DNA, which corresponded to one or several nucleosomes (data not shown). Chromatin IPs were performed using antisera which recognized histone H4 acetylated on any one of its four N-terminal lysine residues (anti-AcH4), but which did not recognize unacetylated H4 (Lin et al., 1989). Immunoprecipitates were subjected to PCR amplification to assay for the presence of particular sequences in the acetylated fraction. The promoter regions of *OCRL* and *PGK1*, two genes known to be silenced on the Xi, were examined first (Figure 1). PCR products corresponding to these regions were not detected in immunoprecipitates recovered from the Xi-containing hybrid, but were present in IPs recovered from the Xa-containing hybrid. In contrast, PCR products from the promoter regions of *ZFX* and *SMCX*, two genes known to be expressed from both Xi and Xa, were recovered by chromatin IPs from both hybrids. Finally, a PCR product corresponding to the promoter region of *XIST*, a gene expressed only from the Xi, was recovered by chromatin IPs only from the Xi-containing hybrid.

The above results strongly suggest a correlation between the acetylation status of H4 in nucleosomes at the promoter and transcriptional activity of the adjacent gene. To further substantiate this correlation, additional genes that either escape or undergo X-inactivation were assayed. As summarized in Table I, hypoacetylation of histone H4 at the promoter indeed correlated with transcriptional silencing for all X-linked genes examined. The expression status of each of these genes in hybrid cells was again confirmed by RT-PCR analysis (data not shown).

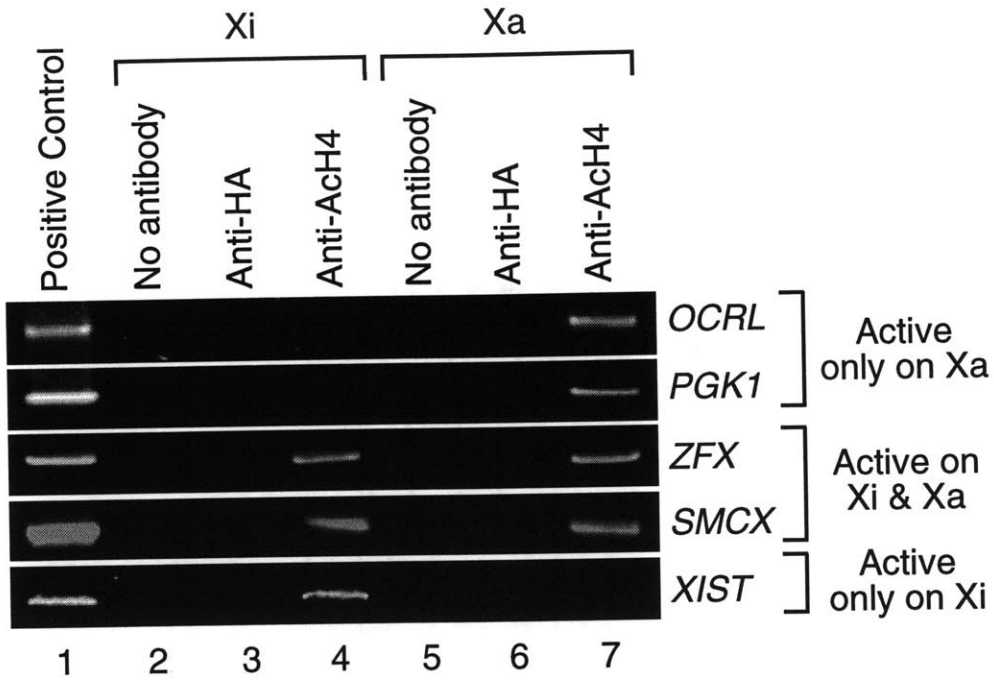

Figure 1. Histone H4 Acetylation at Promoter Regions of X-Linked Genes

Chromatin immunoprecipitations were performed using antisera as designated on human/hamster somatic cell hybrids that contain either the human Xi or Xa. PCR reactions were performed on immunoprecipitates using primers that amplified promoter-proximal regions of genes. The positive control is genomic DNA (lane 1). Negative controls for immunoprecipitations were provided by experiments with either no antibody (lanes 2 and 5) or with anti-HA (Hemaglutinin) antiserum, which does not recognize chromatin (lanes 3 and 6). The AcH4 antisera recognizes histone H4 acetylated at any one of four positions in its N-terminus (lanes 4 and 7).

Table 1. H4 Acetylation Status of 5' Region of X-linked Genes

Gene	X-Inactivation Status	H4 Acetylation	
		Xi	Xa
ZFX	Escape	+	+
SB1.8	Escape	+	+
SMCX	Escape	+	+
OCRL	Subject	—	+
PGK1	Subject	—	+
POLA	Subject	—	+
SOX3	Subject	—	+
IDS	Subject	—	+
XPCT	Subject	—	+
NDP	Subject	—	+
XIST	Active only on Xi	+	—

+ indicates that the promoter region of the gene is present in the H4 acetylated fraction
– indicates that the promoter region of the gene is not detectable in the H4 acetylated fraction

Analysis of Site-Specific Lysine Acetylation Confirms Acetylation Status of Promoter Regions

The amino-terminus of histone H4 contains four highly conserved lysines (Lys-5, -8, -12 and -16) that can each serve as the substrate for acetylation. To test whether the hypoacetylation observed at promoters of silenced genes with antisera against acetylated H4 truly reflected lack of acetylation at all four lysine acceptor sites, chromatin IPs were performed using antisera that could distinguish among H4 isoforms acetylated on either Lys-5, -8, -12 or -16 (Turner and Fellows, 1989). Nucleosomes at the transcriptionally silent *OCRL* and *POLA* promoters on the Xi were indeed deficient in acetylation of all four lysine acceptor sites on the Xi (Figure 2). In contrast, nucleosomes at the transcriptionally active *OCRL* and *POLA* promoters on the Xa, or at the *ZFX* promoter on both the Xi and Xa, were acetylated on all four lysine acceptor sites.

Genes on both Xi and Xa are Acetylated at Similar Levels in Regions Downstream of the Promoter

The histone H4 acetylation status along the entire lengths of several representative genes was determined. *ZFX* was chosen as an example of a gene that is expressed from both the Xi and Xa. To compare the degree of H4 acetylation at various sites along the *ZFX* locus, in both Xi- and Xa-containing hybrid cells, a competitive PCR assay was used. In this assay, chromatin IPs were subjected to PCR using two pairs of primers: one pair which amplified the *ZFX* promoter region was added to all reactions as an internal standard, while the other primer pair was varied to sample different positions along the *ZFX* locus. PCR conditions were optimized such that the total amount of product obtained was linearly dependent on the amount of input DNA (see Materials and Methods). The degree of H4 acetylation at a particular site was then determined by its relative enrichment compared to the *ZFX* promoter in the chromatin immunoprecipitate. Finally, to control for differences in amplification efficiency between each pair of primers, the ratio of the two products from immunoprecipitates was normalized to the ratio obtained from an input DNA control. This input DNA control was derived from cells that had been processed in parallel,

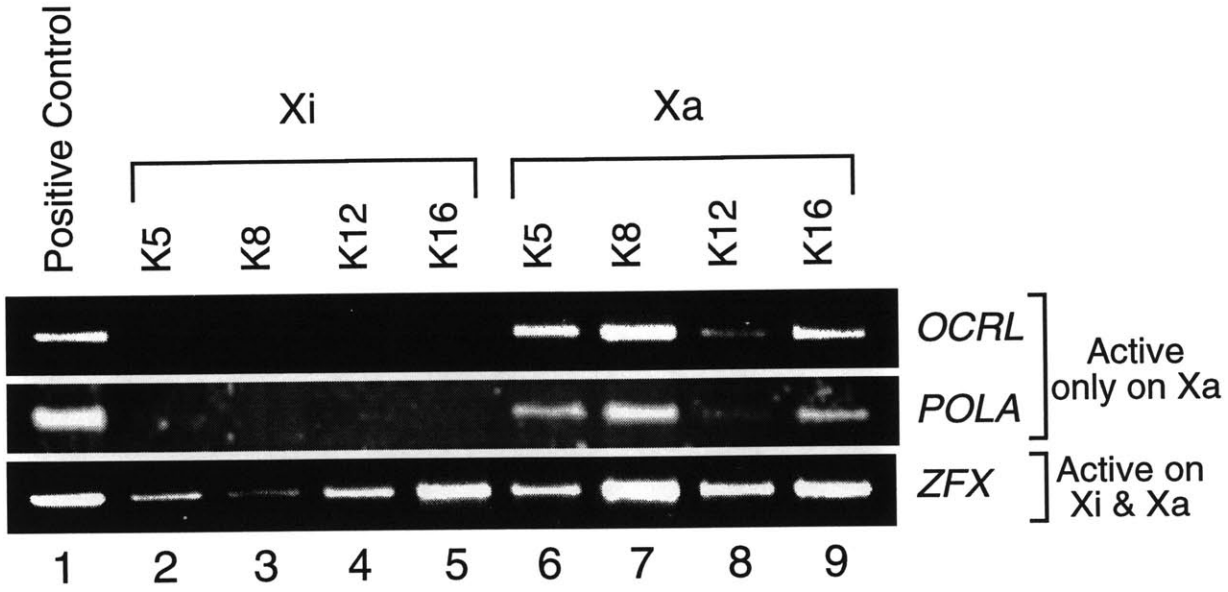

Figure 2. Site-specific Acetylation of Histone H4 at Promoter Regions

Chromatin immunoprecipitations were performed on hybrid cells using antisera that distinguish among H4 isoforms acetylated on particular lysine residues (K5, 8, 12 or 16) as indicated. PCR primers were the same as those used in Table 1 to amplify promoter regions of X-linked genes. Genomic DNA provided a positive control for the PCR assay (lane 1).

but that had not been subjected to chromatin immunoprecipitation.

Figure 3A shows a representative gel-image of the competitive PCR analysis which sampled three sites in the *ZFX* genomic locus, using the *ZFX* promoter-proximal region *ZFX*(0) as the internal standard. The three sites are: *ZFX*(-8), 8 kb upstream of the transcription start site; *ZFX*(50), 50 kb downstream from the transcription start site within the transcribed portion of the gene; and *ZFX*(65), 5 kb downstream from the poly(A) site. Densitometry analysis of PCR products, after correcting for variation in primer amplification from the input DNA control, was used to calculate the ratio of each site relative to the promoter site in the Xi- or Xa-containing hybrids. Figure 3B is a graphical representation of H4 acetylation profiles at several sites along the *ZFX* gene on both Xi and Xa, obtained by averaging results from two independent experiments that varied by less than 10%. Because the same immunoprecipitate was used to assay acetylation levels of various sites along a single X chromosome, comparisons among these sites are internally controlled. Comparisons of the same site between Xi and Xa are also valid because when equal amounts of chromatin from the Xi- and Xa-containing cells were used in immunoprecipitations, comparable amounts of PCR product were obtained for *ZFX*(0) – the site used as the internal standard – from Xi and Xa (data not shown). Two features are evident in the profiles: First, the highest levels of H4 acetylation are found at the *ZFX* promoter on both Xi and Xa. The levels of acetylation decrease both upstream of the promoter and along the transcription unit in each case. Second, the degree and distribution of H4 acetylation along the *ZFX* gene is comparable between Xi and Xa.

Similar assays were also performed for the *OCRL* gene, which represents the many genes expressed only from the Xa. The degree of acetylation along the gene was once again normalized to the *ZFX* promoter on the same chromosome. This experiment was also duplicated, and the results – which varied by less than 10% between the two experiments – were averaged and depicted in graphical form in Figure 3C. The profile obtained for *OCRL* on the Xa was similar to that obtained for *ZFX*: the acetylation levels were greatest at the promoter region, and declined to much lower levels in the downstream portion of the gene. However, on

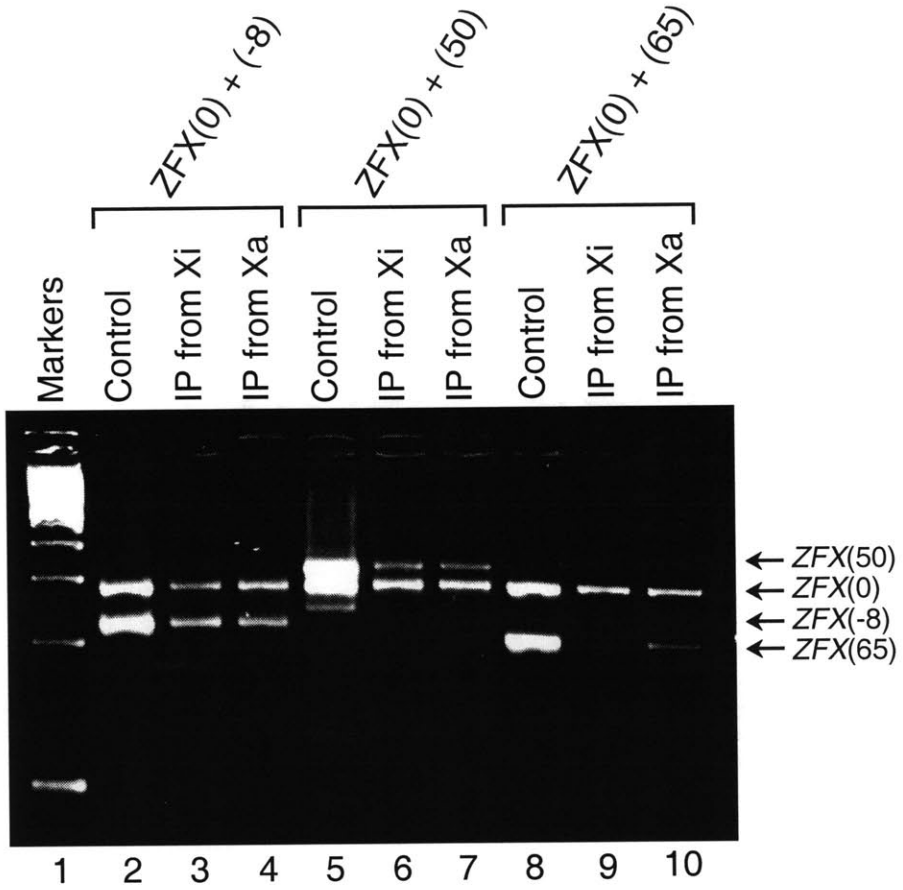

Figure 3. Quantitative PCR Analysis of Histone H4 Acetylation Along X-linked Genes

(A) Sample gel of PCR with internal standard used to quantitate relative enrichment of various *ZFX* sites in chromatin immunoprecipitate. Two sets of primers were used in each PCR reaction: one set, *ZFX*(0), amplified the *ZFX* promoter-proximal region and served as an internal standard of comparison, while the other set sampled positions along the *ZFX* genomic locus: *ZFX*(-8) is 8 kb upstream of the promoter, *ZFX*(50) is 50 kb downstream of the promoter in the transcribed portion of the gene, and *ZFX*(65) is 5 kb downstream of the poly(A) site. The control is genomic DNA isolated from cells that were processed in parallel, but that were not subjected to immunoprecipitations. To eliminate differences that were due to amplification efficiency between primer sets, the amplification ratio from immunoprecipitates was normalized to the ratio obtained with control genomic DNA. After normalization, the relative intensity of the sample PCR product was used to estimate its enrichment relative to *ZFX*(0) in the immunoprecipitate.

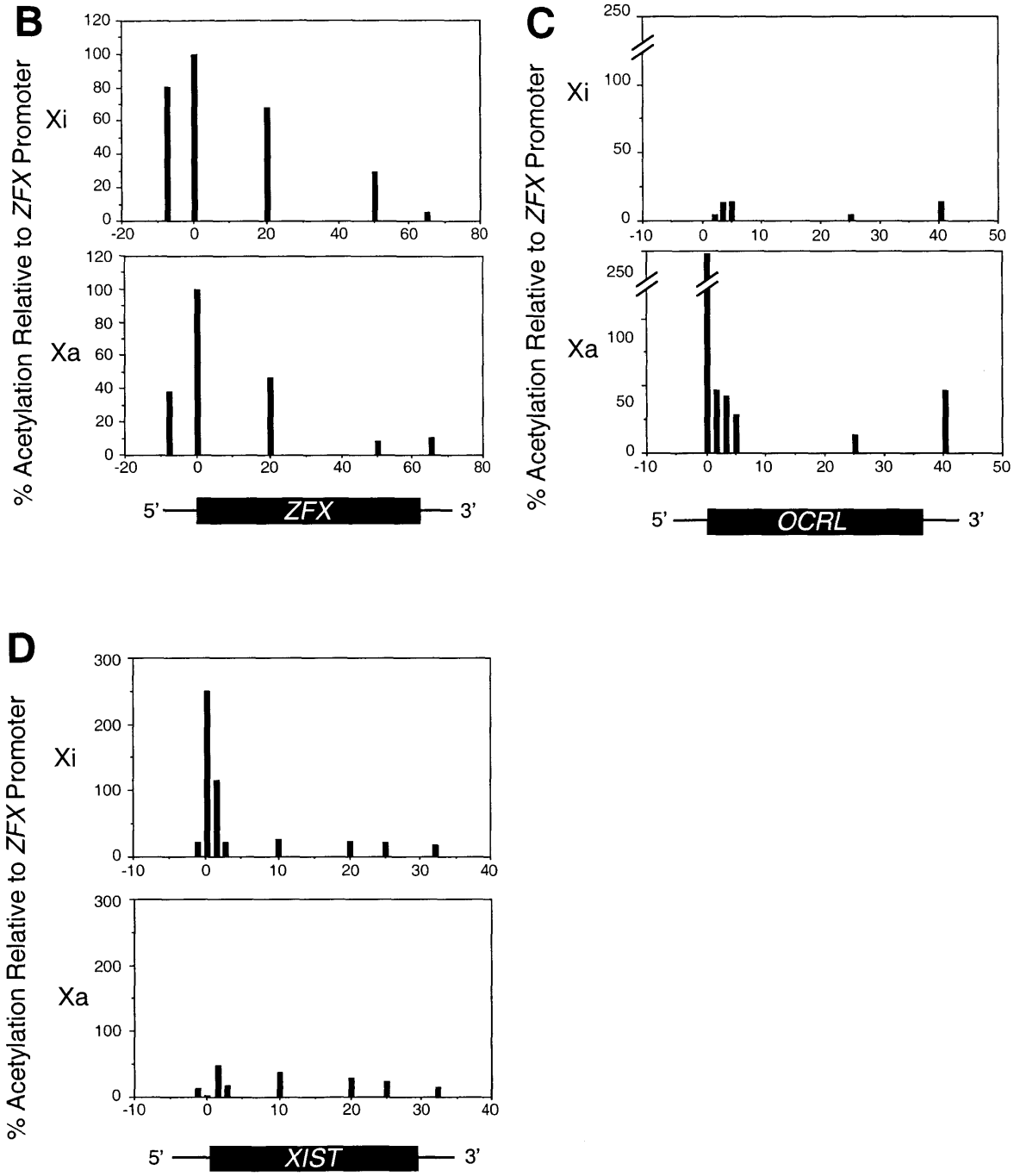

Figure 3. Quantitative PCR Analysis of Histone H4 Acetylation Along X-linked Genes

(B-D) Graphical representations of relative H4 acetylation levels along X-linked genes as assayed by PCR with an internal standard. Three genes were assayed: *ZFX*, which escapes X-inactivation (B); *OCRL*, which undergoes X-inactivation (C); and *XIST* which is transcribed exclusively from Xi (D). The *ZFX*(0) site was arbitrarily set as the unit of comparison (100%), and acetylation levels at all other sites were expressed as a percentage relative to *ZFX*(0).

the Xi, there was an absence of acetylation of *OCRL* in the vicinity of the promoter, not only relative to the promoter on the Xa, but also relative to the remainder of the *OCRL* locus.

Finally, PCR with an internal standard was performed on *XIST*, which is the only gene known to be expressed exclusively from the Xi. By normalizing to the *ZFX* promoter on the same chromosome, as was done before, the correlation between promoter-proximal acetylation and gene expression was again evident: the *XIST* promoter was highly acetylated on the Xi, from which it is transcribed, but was acetylated at a markedly lower level on the Xa, from which it is not transcribed (Figure 3D). The differences between alleles on Xi and Xa were again confined to the promoter region, and the overall acetylation levels were comparable between the two alleles outside of the promoter.

The above results suggest that there is a fairly constant level of histone H4 acetylation extending throughout most of the genetic loci on the Xi. This low-level acetylation does not correlate with transcriptional status. In contrast, the status of H4 acetylation in the vicinity of the promoter strongly correlates with transcriptional activity of the gene.

Methylation of Promoter Regions of X-linked Genes Correlates with H4 Hypoacetylation and Transcriptional Silencing

To explore whether the reduced acetylation observed at the promoters of silenced genes coincided with methylation of CG dinucleotides, genomic DNA samples from female cells, male cells, and human/hamster cell hybrids were digested with methylation-sensitive enzymes. Digestion was monitored using PCR assays with primers that spanned several such restriction sites at promoters of tested genes. If the CG sites were methylated, they would resist digestion and the DNA would then be available to serve as template in the PCR reaction. Figure 4 shows the results of this analysis: Methylation was detected at promoter regions of X-inactivated genes, including *OCRL*, *PGK1*, *POLA*, *SOX3* and *XPCT*, in all cell types which contain an Xi. In contrast, the promoter of the *SMCX* gene, which is acetylated and expressed from the

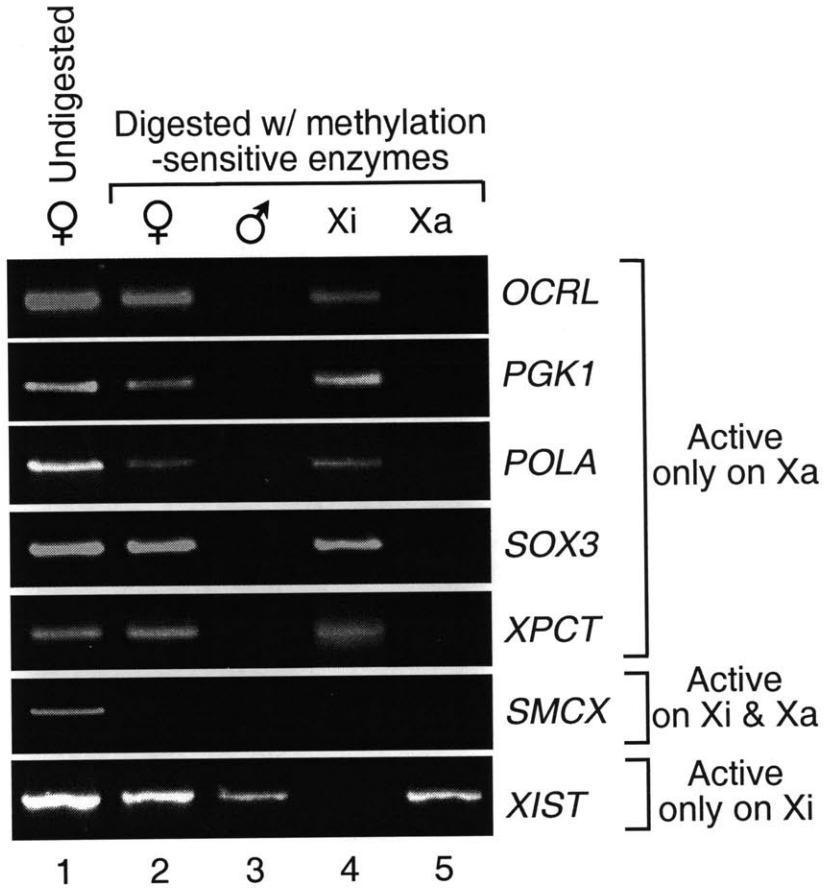

Figure 4. Methylation Analysis of Promoter Regions of X-Linked Genes

Genomic DNA prepared from various sources was digested with methylation-sensitive enzymes. Digestion was monitored by a PCR assay using primers that spanned several restriction sites. Methylated sequences escape digestion and can serve as template in the PCR reaction. Undigested genomic DNA provided a positive control for the PCR assay (lane 1). PCR assays were performed on digested DNA isolated from female (293) cells (lane 2), male (lymphoblastoid) cells (lane 3), and human/hamster cell hybrids containing either the Xi (lane 4) or Xa (lane 5). PCR primers were the same as used to assess the H4 acetylation status in Table 1.

Xi as well as the Xa, was not detectably methylated on either Xi or Xa. Finally, the promoter region of the *XIST* gene was methylated only in cells which contained an Xa chromosome. These results are consistent with previous methylation studies of some of these genes (Tribioli et al., 1992; Hendrich et al., 1993; Jegalian and Page, 1998). The most significant aspect of these findings is that the primers used here were the same as those used to assess H4 acetylation status in Table 1. This indicates that the lack of acetylation corresponds to the presence of methylation at promoters of X-inactivated genes. In addition, these results confirm prior reports that human X chromosomes retain the same methylation status in hybrid cells as in intact human cells (Hansen et al., 1998).

Discussion

Previous analyses of the histone acetylation status of the active and inactive X chromatin have relied primarily on immunofluorescence microscopy of metaphase cells (Jeppesen and Turner, 1993; Belyaev et al., 1996; Boggs et al., 1996). The chromatin immunoprecipitation studies described here provide the first extensive examination of individual nucleosomes associated with numerous X-linked genes. These results revealed complex patterns of acetylation that were not previously detected.

First, a low-level H4 acetylation is present along all examined genes on the Xi, except at the promoters of silenced genes. This low-level acetylation is comparable to that present on the Xa and does not correlate with transcription. Second, promoter regions of expressed genes, regardless of whether they are located on the Xi or Xa, are hyperacetylated relative to their downstream regions. Third and most significantly, promoter regions of X-inactivated genes are dramatically hypoacetylated, even as compared to the low-level acetylation found in downstream regions of the same loci. Thus, promoter hypoacetylation is another feature, along with C G methylation and late replication, that correlates with silencing of individual genes on the Xi. Finally, DNA methylation coincided with promoter-proximal hypoacetylation: methylation of C G dinucleotides was observed at hypoacetylated promoters of silenced genes on the Xi, but not at

hyperacetylated promoters of active genes.

It has been shown in yeast that hyperacetylation of chromatin at the active *HIS3* promoter is the direct result of recruitment of the histone acetyltransferase Gcn5p (Kuo et al., 1998). A similar process may account for the hyperacetylation of active promoters on the Xi observed here. In this case, various combinations of transcription factors and coactivators may recruit histone acetyltransferases to the active promoters.

In the particular case of gene silencing on the Xi, where there is a perfect correlation between methylation of promoter regions and repression (Tribioli et al., 1992; Jegalian and Page, 1998), the promoter-specific hypoacetylation is likely due to methyl-directed recruitment of deacetylase-containing complexes. The ability of methylated CG sequences to bind MeCP2, which can recruit complexes with histone deacetylase activity, has been demonstrated in biochemical analysis (Jones et al., 1998; Nan et al., 1998). Because it is heritable through cell division, DNA methylation provides a mechanism that can contribute to long-term hypoacetylation, which may affect silencing in turn. However, it is also possible that hypoacetylation at promoters of silenced genes results from recruitment of deacetylases by sequence-specific transcriptional corepressors that bind near promoters. Various studies have shown that targeted recruitment of deacetylases to promoters results in transcriptional repression of reporter genes in both yeast and mammalian cells (Alland et al., 1997; Heinzel et al., 1997; Kadosh and Struhl, 1997; Nagy et al., 1997; Yang et al., 1996; Zhang et al., 1997). In the case of Ikaros recruitment to heterologous promoters in yeast, the repression correlates with hypoacetylation at promoter sites and is relieved by histone deacetylase inhibitors (Koipally et al., 1999). The notion that deacetylases play a central role in X-inactivation is supported by a recent study which shows that chemical inhibition of deacetylase activity in differentiating ES cells prevents the appearance of some of the properties associated with X-inactivation (O'Neill et al., 1999).

Although the metaphase Xi, as observed by immunofluorescence microscopy, may be hypoacetylated relative to all other chromosomes (Jeppesen and Turner, 1993; Belyaev et al., 1996; Boggs et al., 1996), gene-containing regions of the Xi are clearly acetylated in asynchronous

cells, as measured by the chromatin IP assay. In fact, promoters of expressed genes are hyperacetylated on the Xi. The apparent differences between immunofluorescence microscopy and the chromatin IP assay in detecting histone acetylation status could reflect a multitude of differences between the two experimental systems, including detection threshold, signal resolution, and cell-cycle-associated variations in histone acetylation. Indeed, it has been shown that bulk histone acetylation is reduced in metaphase cells relative to interphase cells (D'Anna et al., 1983; Turner and Fellows, 1989).

Promoter-specific hypoacetylation may play a critical role in X-inactivation, but is it generalizable to the repression of autosomal genes? There have been conflicting reports regarding the acetylation status of active and inactive genes on autosomes. Since hypoacetylation on the Xi was of a localized nature, promoter-specific acetylation status may have escaped detection in some prior studies if the chromatin fragment size or probe size was not sufficiently small. It is therefore possible that promoter-specific hypoacetylation could have a broad role in gene regulation.

Several features of the Xi chromatin, such as colocalization with *XIST* RNA and chromatin condensation, have been described at the level of the entire chromosome, leading to the view that changes in chromatin structure of the Xi occur on a chromosome-wide basis. Yet, the observation that acetylation differences between expressed and silenced alleles are confined primarily to promoter regions indicates that this model is incomplete. Instead, it suggests that chromatin structure of the inactive X can be regulated at the level of individual genes. Evolutionary studies also support this model. Phylogenetic comparisons have shown that X-inactivation did not evolve in a single, broad sweep; rather, genes acquired X-inactivation throughout evolution on an individual or regional basis (Jegalian and Page, 1998). For each gene, acquisition of X-inactivation was an eventual response to the decay of its homolog on the Y chromosome.

Initiation of X-inactivation during embryonic development involves a cascade of events. The first event is a dramatic increase in the level of *Xist* signal associated with the X that will become inactivated (Beard et al., 1995; Lee et al., 1996; Panning et al., 1997). This may then

trigger downstream events (Lee and Jaenisch, 1997) – such as late replication, H4 hypoacetylation, DNA methylation and down-regulation of gene expression – which appear to proceed in a defined order during differentiation (Keohane et al., 1996). If *Xist* directs these downstream processes, it may do so by targeting repressive factors to promoters of genes that are silenced. Histone deacetylases are likely to be one component of this X-inactivation machinery. The resulting hypoacetylation of promoters could then serve to maintain the repressed state of X-inactivated genes in adult tissues.

Experimental Procedures

Cell Culture

The GMO6318 (NIGMS Human Genetic Mutant Cell Repository) human/hamster somatic cell hybrid containing a single human Xa was maintained under selection in HAT medium. The X8-6T2S1 human/hamster cell hybrid (Hansen et al., 1988) containing a single human Xi was grown in RPMI medium containing 10% fetal calf serum.

Chromatin Immunoprecipitations

10 cm plates of confluent cells were incubated in PBS containing 1% formaldehyde for 10 minutes at 37° C. Chromatin immunoprecipitations were then performed using various polyclonal antisera as previously described (Alberts et al., 1998). Briefly, sonication of crosslinked nuclei was performed in a cup horn (Branson Sonifier 450) under conditions that gave a range in DNA fragments from 200-1000 bps. 5 ul of anti-AcH4 or anti-H4Ac12 antisera (Upstate Biotechnology), 10 ul of anti-H4Ac5 or anti-H4Ac8 antisera (Upstate Biotechnology), 5 ul of anti-H4Ac16 (Serotec), or 3 ul of anti-HA antisera (Boehringer Mannheim) were incubated overnight with precleared nuclear lysates. Immune complexes were then recovered with Protein A Sepharose beads.

PCR

One-tenth of the chromatin immunoprecipitate was added to a 20 ul reaction mix containing 1.5

mM MgCl2, 50 mM KCl, 10 mM Tris pH 9.0, 1% Triton X-100, 0.5 uM each primer, 0.1 mM dNTPs and 1 Unit Taq Polymerase. Competitive PCR reactions contained an additional 1 U of Taq Extender (Stratagene). After denaturation at 95° C for 1.5 minutes, 30 cycles of PCR were performed where each cycle consisted of 1 minute at 95° C, 45 seconds at 60° C, and 45 seconds at 72° C. Under these conditions, PCR product yield was linearly dependent on input genomic template up to a maximum of 50 ng DNA. Typically, less than 10 ng genomic DNA from the chromatin IPs was added to PCR reactions, which is well within the linear range of the assay. Products were then resolved on 4% NuSieve agarose (FMC BioProducts) gels. Quantitation of PCR products was performed using the Eagle Eye II imaging system and EagleSight software (Stratagene).

Methylation Assays

The methylation status of promoter regions of all genes other than *XIST* was measured by PCR using HpaII-digested genomic DNA as template. The methylation status of the *XIST* promoter was assayed by PCR using genomic DNA that had been digested with *Hha*I, *Ava*I and *BstU*I as described (Hendrich et al., 1993). Genomic DNA was prepared from human female (293) cells, human male lymphoblastoid cells, or human/hamster somatic cell hybrids which contained either the human Xi or the Xa. Primers spanned several methylation-sensitive restriction sites in the 5' end of tested genes. 50 ng of template DNA was subjected to 31 cycles of PCR using the conditions described above, supplemented with 2% DMSO.

Primer Sequences

The following primer sets are specific for human genomic DNA (Table 2):

Table 2. Primers for PCR Analysis

Gene	Forward	Reverse
Promoter-specific primers:		
OCRL	refer to *OCRL*(0) below	
PGK1	ACGCGGCTGCTCTGGGC	TTAGGGGCGGAGCAGGAAG
SMCX	CTTGTTCCTCCGCCGTTGCA	CCATCTTGGTTTGTCAGCGT
SB1.8	CAGGGTGCTGTGGAATCTATT	TTGTCATACTCCTGCGCCA
POLA	CTGGGGAAAACGATCCAACC	CTGAAAGCCAATCAGCGGC
SOX3	CGAACCTGTCAATCACGGGT	CCTGATGAGTTCTCTCGAAC
IDS	GCGCAGTCTTCATGGGTTC	CGGGGTGGCGGCATTTCG
XPCT	CTGGCCCGGCTCCTGGC	GCTTTGTTTGCGCCAACCTG
XIST	xst31r (Brown et al., 1992)	xst 29r (Brown et al., 1992)
ZFX	refer to *ZFX*(0) below	
ZFX-specific primers:		
ZFX(-8)	CACATCTCTCCTCTACTTCCT	TGCTTCCCAGGTTTTCACTA
ZFX(0)	GTGCTGTGTTAAAGGATAGC	AGGAGCCCAATTGGGTATGG
ZFX(20)	CTGGTTACCCTTGTGGGAAC	GGTCTATCAAGTGCACACAT
ZFX(50)	AAGACTGTACCGCCTTCACT	TGGGTACAGGTGGTTCTCC
ZFX(65)	GGTCACTGTAATGCCAGATC	GCATATAGGCATAGCATCTG
XIST-specific primers:		
XIST(-0.5)	TCCGTTCTCTTATGGTTGGG	TACCATCTTCAAGGGACTTC
XIST(0)	ACGTGTCAAGAAGACACTAG	xst29r (Brown et al., 1992)
XIST(1.5)	AAGGTCTTGCCGCAGTGTAA	GTTGGGTTATGCAGCAATCC
XIST(3)	CTGTGTTGGCCACCTAAAAC	GCACAGCAAAAAGCGCAGTA
XIST(10)	CTTGTTAAGCAAGCGCCCCA	CATGACACCATGGCTACCTG
XIST(20)	TGAAGACCCATGTCTCTACA	TGGCTCACGTTCTGCTTTTC
XIST(25)	CTTTGCTCTCCTAGATGTGG	GAGAAGGGGAAGGGGTAACA
XIST(32)	CAGCATGGGTGACCACCAGA	GTTAGGGACAGTGAGTTAGAA
OCRL-specific primers		
OCRL(0)	ACAAGTCTAGCTCCCAGCT	CCGATCCGACGACACTGGC
OCRL(1)	GAAAGACTTCCCAGTTTCTG	ATCACCATATTTGGCCTGAC
OCRL(2)	CCAACACCATGCGGAAGCTC	TATCAACAGGCCACTGTCTG
OCRL(5)	AGGCATTTAGCTACCAGAAG	CAGGGACCTATATGACAGGA
OCRL(25)	GCCTATCACTTCCTTGCTTA	AGGCACAGAGACAGTAAATC
OCRL(40)	GCAGCAAAACAGCTGGCTGT	AGGAGAAGGAGCATACAGAA

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CHAPTER THREE: *XIST* RNA IS A COMPONENT OF THE INACTIVE X CHROMATIN

Microscopy studies have shown that *XIST* RNA colocalizes with the inactive X chromosome (Xi)[1, 2]. It is unclear, however, if this colocalization is due to physical association of *XIST* RNA with the Xi chromatin, or if it is a consequence of *XIST* RNA and the Xi being sequestered to the same nuclear compartment. Here we provide evidence from chromatin immunoprecipitation experiments that *XIST* RNA physically associates with the Xi chromatin. First, *XIST* RNA can be co-precipitated by antisera against macroH2A, a histone H2A variant enriched in the Xi[3]. Moreover, *XIST* RNA can be co-precipitated by antisera that recognize unacetylated, but not acetylated, isoforms of histones H3 and H4. Interestingly, unacetylated histones have previously been shown to be enriched at promoters of X-inactivated genes[4]. The physical association between *XIST* RNA and the Xi may facilitate X-inactivation.

X-chromosome inactivation in mammals is controlled by the *cis*-acting *XIST* locus which in humans encodes a 17 kb noncoding RNA expressed only from the inactive X (Xi)[1]. X chromosomes that carry targeted deletions of the *Xist* gene lose the ability to undergo inactivation[5, 6]. In addition, ectopic expression of *Xist* RNA from multicopy transgenes during ES cell differentiation can induce features typically associated with X-inactivation, including late replication, histone H4 hypoacetylation and transcriptional downregulation on autosomes[7, 8]. These studies indicate that the *Xist* locus is necessary for X-inactivation, but they do not distinguish between several possible modes of its action: One model is that the *Xist* genomic locus serves as a chromatin organizing region which initiates changes in chromatin structure that are propagated along the length of the Xi[9]. An alternative model is that the noncoding RNA expressed from the *Xist* gene is functional, and physically participates in the silencing process[1].

One observation suggesting that the *XIST* transcript may physically participate in silencing comes from fluorescence *in situ* hybridization (FISH) experiments which show that *XIST* RNA colocalizes with the Xi[1, 2]. However, the limited resolution of microscopy analysis does not provide any information on the molecular nature of this colocalization.

To investigate whether *XIST* RNA physically interacts with the chromatin of the Xi, we developed the chromatin-IP/RT-PCR assay, which is a variation of the previously developed chromatin immunoprecipitation assay[10]. Sonicated chromatin was first precipitated with antisera

against known components of the Xi chromatin. After DNase digestion, immunoprecipitates were subjected to RT-PCR to assay for the presence of *XIST* RNA. The first chromatin protein used for immunoprecipitation was macroH2A (mH2A), a histone H2A variant. This protein was chosen for two reasons: First, mH2A is highly enriched in the Xi[3]. Second, mH2A has homology to RNA-binding proteins from several RNA viruses. This prompted the speculation that mH2A may also have RNA-binding ability, and that its enrichment in the Xi may be a consequence of its binding to *Xist* RNA, or vice versa[11].

Immunofluorescence microscopy with polyclonal antibodies directed against the major spliced variant of mH2A confirmed the expected enrichment of mH2A in the two Xi chromosomes of human female XXX (293) cells (Fig. 1a). When the same antibodies were used in the chromatin-IP/RT-PCR assay, they were able to co-precipitate *XIST* RNA from 293 cells (Fig. 1b, lane 5). The primers used in this assay distinguish genomic DNA from RNA because they span two alternatively spliced introns, yielding products of 242 bps and 179 bps, respectively (Fig. 1b, lane 3). Several spurious bands were produced in the genomic DNA control. However, these cannot account for the signal in the RT-PCR reaction because they do not correspond to the sizes expected from the reaction. There are several additional reasons why the products obtained in lane 5 cannot be due to contaminating DNA: (1) the immunoprecipitates were DNase-treated, and (2) the products were dependent upon the presence of reverse transcriptase (Fig. 1b, lane 4). Therefore, the signal is derived from RNA rather than DNA. The specificity of the anti-mH2A antibodies in recovering *XIST* RNA, rather than other cellular RNAs, was demonstrated by control RT assays in which the abundantly expressed *FIBRONECTIN* and *ACTIN* mRNAs were not co-precipitated with anti-mH2A antibodies (data not shown).

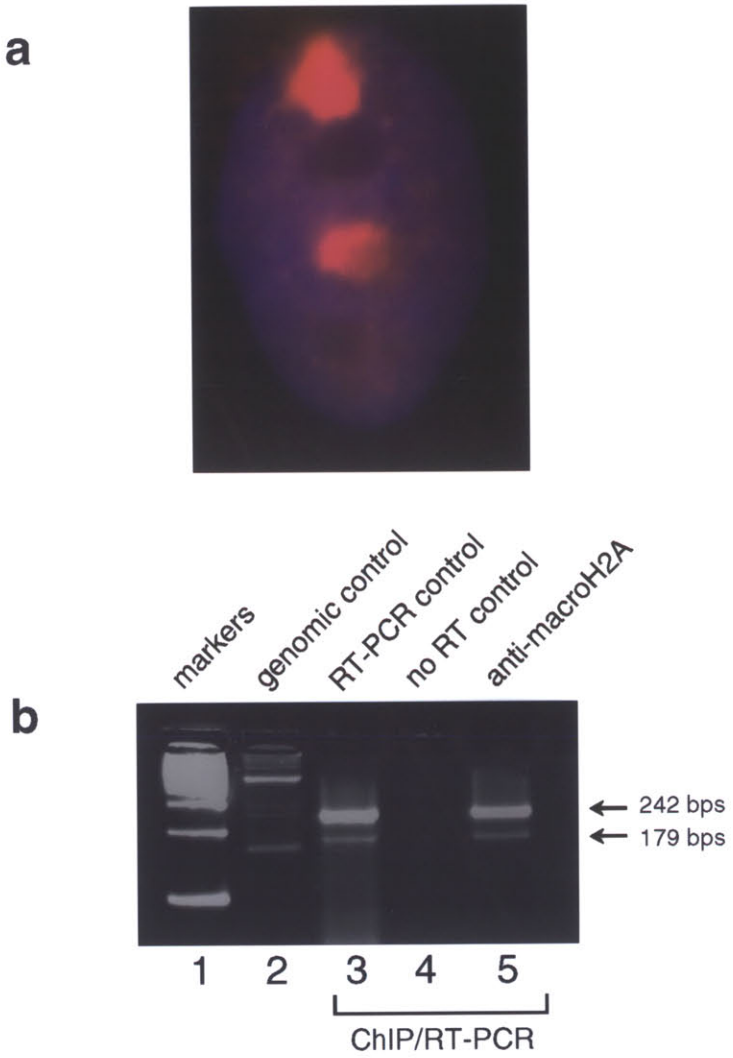

Figure 1 Immunofluorescence and immunoprecipitation analysis of histone mH2A. **a**, Immunofluorescence image showing a female (293) cell which contains 3 X chromosomes, stained for macroH2A1.2 (red) and DNA (blue). **b**, Chromatin IP/RT-PCR analysis of *XIST* RNA recovery with affinity-purified polyclonal antibodies against macroH2A1.2. Immunoprecipitates were DNase-digested, reverse transcribed and subjected to PCR using primers that span two introns in *XIST* RNA. This produces two products, of 242 bps and 179 bps, respectively. 50 ng of sonicated genomic DNA provided a negative control for amplification. The RT-PCR control was performed using total RNA isolated from the identical 293 cell line. Nonspecific antibody controls are provided by anti-acetylated H3 and H4 antibodies in Figure 2.

PCR was performed under conditions such that the amount of product is linearly dependent on the amount of input cDNA (data not shown). Thus, it is possible to make comparisons about the relative abundance of the product bands. The relative proportion of the two alternatively spliced *XIST* products in the anti-mH2A immunoprecipitate (lane 5) is similar to that obtained from cDNA prepared from total cellular RNA (lane 3), although the significance of this result is not clear.

We next examined whether *XIST* RNA could be recovered by antibodies directed against other chromatin proteins. Histone hypoacetylation has been shown to feature prominently in X-inactivation: Metaphase microscopy studies have shown that histones H2A, H3 and H4 are underacetylated in the Xi as compared to other chromosomes[12-14]. Furthermore, our recent work demonstrated that histone H4 is hypoacetylated at promoters of X-inactivated genes, but is moderately acetylated in regions downstream of promoters[4]. Antibodies which can distinguish between the acetylated and unacetylated isoforms of H3 and H4 were used in the chromatin-IP/RT-PCR assay. In this case, *XIST* RNA was co-precipitated by the antibodies against unacetylated H3 and H4 (Fig. 2a, lane 4 and Fig. 2b, lane2). In contrast, *XIST* RNA was not co-precipitated by antibodies against acetylated H3 and H4 (Fig. 2a, lane 5 and Fig. 2b, lane 3). This precipitation profile suggests that *XIST* RNA associates with the Xi chromatin. Unacetylated and acetylated histones H3 and H4 are both present in the Xi chromatin. Thus, the ability of antibodies against unacetylated histones, but not antibodies against acetylated histones, to recover *XIST* RNA provides an internal control. These results demonstrate that the recovery of *XIST* RNA is not a spurious consequence of its being in the same nuclear compartment as the Xi. Rather, it suggests that *XIST* RNA may be specifically concentrated at regions of the Xi chromatin which are deficient in histone H3 and H4 acetylation.

It is worth noting that the immunoprecipitations are enriched for the smaller of the two alternatively spliced *XIST* transcripts. Sequencing analysis confirmed the identity of the 179 b p band in Figure 2b, lane 2 (data not shown). Although the significance of this enrichment is unclear,

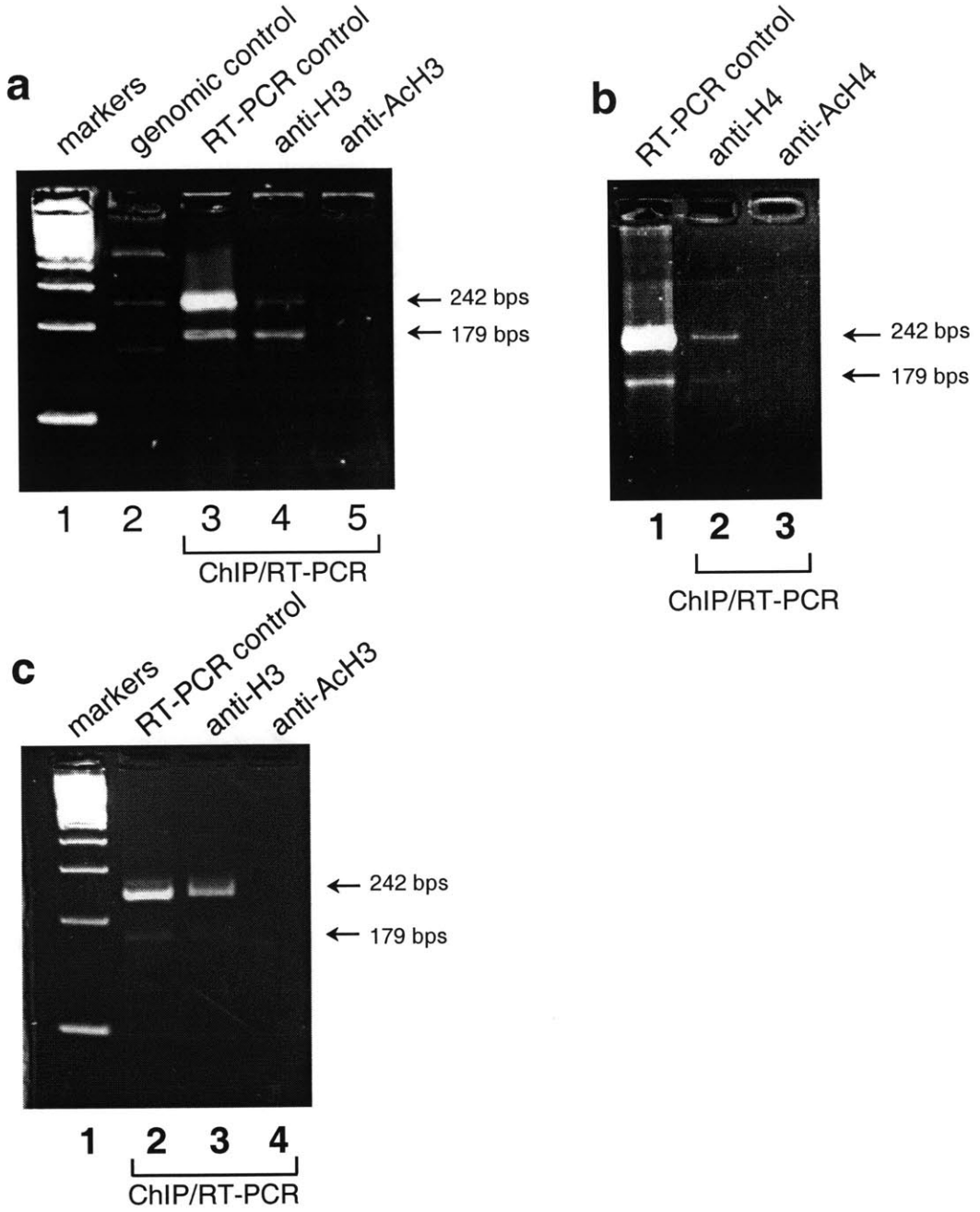

Figure 2 Immunoprecipitation profile of *XIST* RNA with various anti-histone antibodies. **a, b,** Crosslinked, sonicated chromatin was prepared from female cells and subjected to IPs using the designated antibodies. Immunoprecipitates were DNase-digested, reverse transcribed, and subjected to PCR using primers which detect two alternatively spliced *XIST* transcripts. 50 ng genomic DNA provided a negative control for PCR. The positive RT-PCR control was provided by total RNA isolated from 293 cells. **c,** Chromatin fragments were prepared from female cells by MNase digestion without crosslinking, and subjected to chromatin-IP/RT-PCRs as previously described.

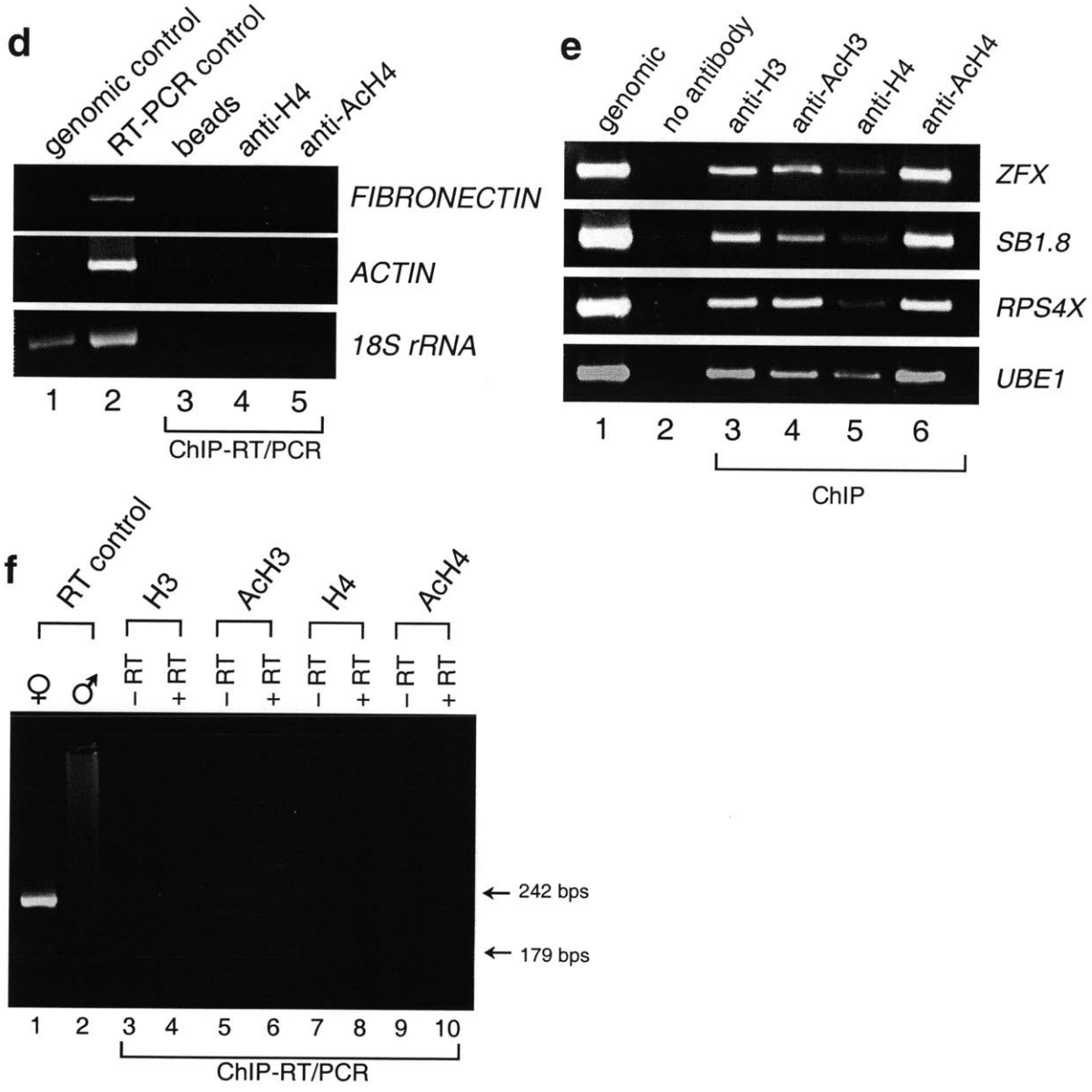

Figure 2 Immunoprecipitation profile of *XIST* RNA with various anti-histone antibodies. **d**, Chromatin IP/RT-PCR analysis to determine whether several control RNAs could be recovered with the chromatin fraction. **e**, PCR analysis of efficiency of various antisera in recovering chromatin associated with X-linked genes. **f**, Crosslinked, sonicated chromatin was prepared from a male cell line (BJEH) and subject to chromatin IP/RT-PCRs using the indicated antibodies. Genomic controls were provided by 50 ng genomic DNA, while RT-PCR controls were provided by total RNA isolated from female or male cells, as indicated.

the fact that the relative abundance of the two alternatively spliced variants in the immunoprecipitate is not the same as that found in total cellular RNA suggests that the recovery is specific.

Until now, the chromatin-IP/RT-PCR experiments had been performed in the presence of formaldehyde crosslinking. To examine whether the association between *XIST* RNA and histone H3 was dependent on crosslinking, an alternative method was used to generate chromatin in which intact nuclei were permeabilized and nucleic acids were digested briefly with micrococcal nuclease (MNase). The resulting native chromatin fragments were then subjected to chromatin IPs. The same results were obtained using this method — that *XIST* RNA was recovered with antisera against unacetylated, but not acetylated H3 — suggesting that *XIST* RNA is stably associated with nucleosomes (Fig. 2c, compare lanes 3 and 4). The only discernable difference between this method and the crosslinking approach is that immunoprecipitations with native chromatin fragments are enriched for the larger *XIST* spliced variant. Again, the significance of the differential recovery of alternatively spliced products is not clear.

The abundantly expressed *FIBRONECTIN* and *ACTIN* mRNAs, as well as 18S rRNA, provided specificity controls for the H3 and H4 immunoprecipitations. None of the anti-histone antibodies recovered any of these transcripts, indicating that the ability of antibodies against unacetylated histones to recover *XIST* RNA is specific (Fig. 2d, lanes 4 and 5).

Chromatin IPs performed in parallel indicated that all anti-histone antibodies used in our assays were efficient in precipitating chromatin, except that the antibodies against unacetylated histone H4 appear to recover chromatin with reduced efficiency (Fig. 2e). Yet, these antibodies against unacetylated histone H4 were able to recover *XIST* RNA, while the more efficient antibodies against acetylated H4 did not. Finally, we showed that, as expected, *XIST* RNA was not recovered from male cells by any of the anti-histone antibodies (Fig. 2f). This control confirms that the signal is not an artifact introduced by the antibodies.

To estimate the size of *XIST*-containing complexes following MNase digestion, native chromatin fragments were applied to 10-30% glycerol gradients. Fig. 3a shows the distribution of

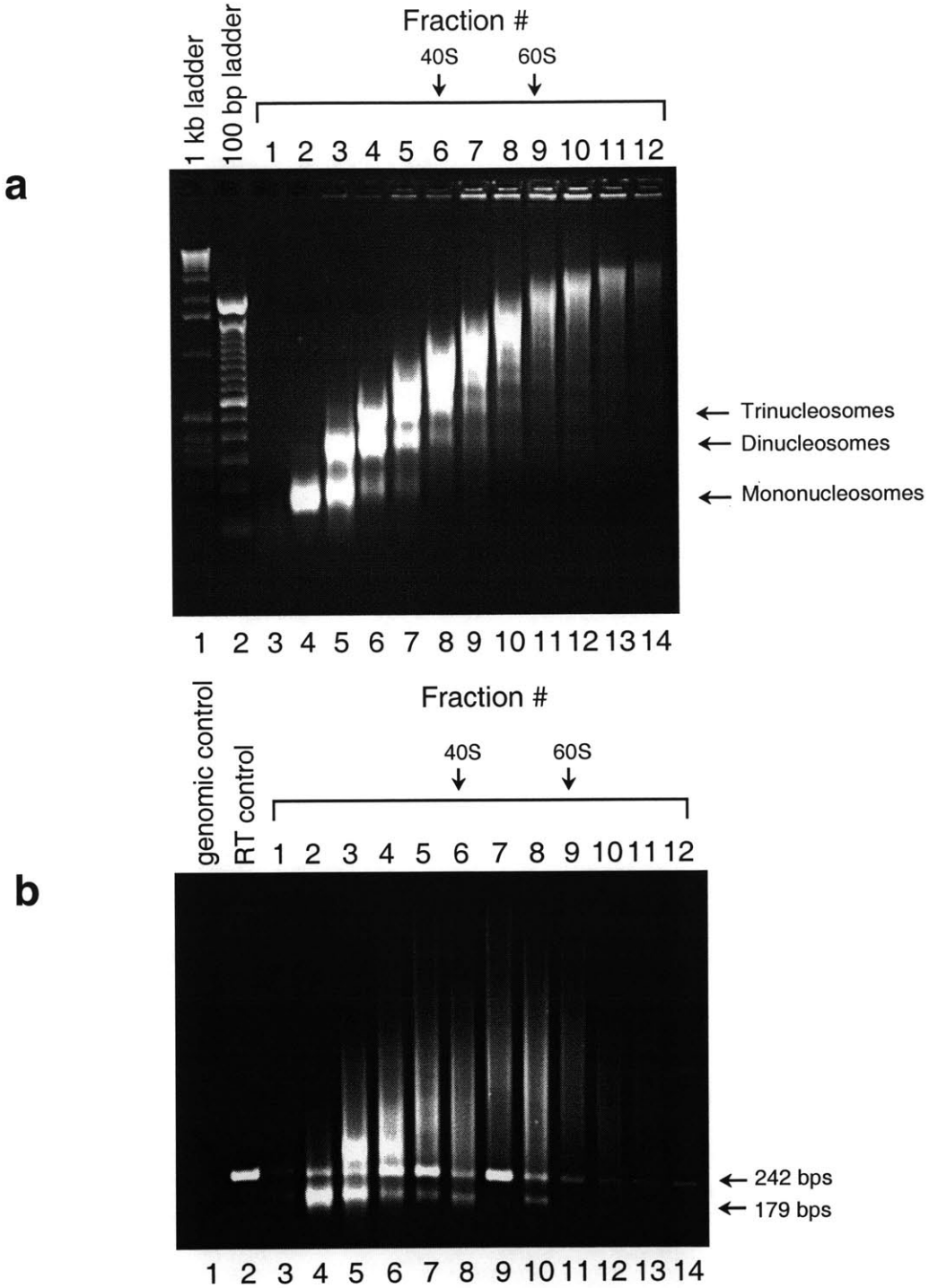

Figure 3 Sedimentation of non-crosslinked, MNase-digested chromatin in 10-30% glycerol gradients. 12 fractions were collected from the gradient, as indicated above the relevant lanes. **a,** Visualization of nucleosomal DNA ladder in gradient fractions. **b,** Gradient fractions were DNase-digested, then subjected to RT-PCR using *XIST*-specific primers. PCR was performed under conditions where the amount of product was linearly dependent on the amount of input cDNA, enabling quantitative comparisons to be made between the products.

nucleosomes throughout the gradient. The migration of *XIST* RNA in the gradient was also monitored by RT-PCR analysis (Fig. 3b). This demonstrates that the majority of *XIST* RNA comigrates with chromatin fragments which contain several nucleosomes. Unexpectedly, the two alternatively spliced *XIST* transcripts migrated to different positions in glycerol gradients. It is unclear whether this difference in migration of the spliced variants is attributable to their 60 nt size difference, or if it is because the variants associate with different protein complexes. Chromatin-IP/RT-PCRs on one gradient fraction again verified that *XIST* RNA could be recovered from complexes which contained unacetylated, but not acetylated, histone H4 (data not shown).

Microscopy studies have shown that, at low resolution, *XIST* RNA colocalizes with the Xi. This apparent colocalization could be the result of at least two processes: First, *XIST* RNA may occupy the same nuclear compartment as the Xi because it is an integral component of the Xi chromatin. Alternatively, the Xi is sequestered to the nuclear periphery, and *XIST* RNA may be similarly localized as a secondary consequence of its expression within the sequestered domain. Our data demonstrates that *XIST* RNA is present in a complex which contains several histone species. As *XIST* RNA can be recovered with particular anti-histone antisera, even in the absence of crosslinking, *XIST* RNA appears to be located in close proximity to the chromatin of the Xi. This suggests that *XIST* RNA is, in fact, a physical component of the Xi chromatin. Furthermore, its association with the Xi appears to be selectively enriched in hypoacetylated regions of the Xi, which may include promoters of silenced genes. This selectivity in *XIST* RNA association with particular regions of the Xi is consistent with FISH analysis of metaphase chromosomes, which showed that *XIST* RNA is excluded from regions which are actively expressed from the Xi[15, 16].

A previous report suggested that *XIST* RNA was a component of the nuclear matrix, as it remained with the insoluble nuclear fraction after removal of chromosomal DNA[2]. The discrepancy between this result and ours may be due to a multitude of factors, including differences in the solubility of *XIST* RNA following sonication or MNase treatment as compared

to detergent extraction of nuclei. Furthermore, it is possible that the *XIST* signal observed here may represent only a fraction of the total nuclear signal.

Three features of the Xi chromatin — promoter-specific histone H4 hypoacetylation[4], DNA methylation[17, 18], and late replication timing[19] — have been shown to correlate with the silencing of individual genes. The results presented here raise the possibility that association with *XIST* RNA may be another important feature that correlates with silencing of individual genes on the Xi. This suggests that *XIST* RNA may function as an adaptor to recruit factors to particular regions of the Xi that have a repressive effect on transcription. These putative factors may be structural, such as histone mH2A, or regulatory, such as histone deacetylases, *de novo* DNA methylases, or chromatin remodeling activities.

Noncoding RNAs have also been implicated in *Drosophila* dosage compensation, in which transcription is upregulated from the single male X chromosome to approach the level obtained from the two female X's. The chromatin structure of the upregulated male X is modified by this process, acquiring a rare acetylation of lysine 16 on histone H4 and a diffuse microscopic appearance[20, 21]. Here again, noncoding RNAs (*roX1* and *roX2*) were shown by microscopy to colocalize with the structurally altered male X chromosome[22, 23]. Perhaps the direct, physical association of noncoding RNAs with chromatin provides a *cis*-acting mechanism of recruiting factors that exert changes in chromatin structure at multiple sites along a chromosome.

Methods

Chromatin Immunoprecipitations

10 cm plates of confluent cells were incubated in PBS containing 1% formaldehyde for 10 minutes at 37° C. Chromatin immunoprecipitations were then performed using various polyclonal antisera as previously described[24]. Briefly, sonication of crosslinked nuclei was performed in a cup horn (Branson Sonifier 450) under conditions that gave a range in DNA fragments from 200-1000 bps. An alternative method was employed to prepare native chromatin: Nuclei were permeabilized in buffer containing 0.4% NP-40, as described [25]. After a 5 minute digestion with MNase, chromatin was subjected to immunoprecipitations as before. The following antibodies were incubated

overnight with precleared nuclear lysates: 10 ul of anti-histone H3 or H4 antibodies, or 5 ul of anti-AcH3 or AcH4 antibodies (Upstate Biotechnology), or 5 ul of affinity-purified, polyclonal anti-mH2A1.2 antibodies.

PCR

One-tenth of the chromatin immunoprecipitate was added to a 20 ul reaction mix containing 1.5 mM MgCl2, 50 mM KCl, 10 mM Tris pH 9.0, 1% Triton X-100, 0.5 uM each primer, 0.1 mM dNTPs and 1 Unit Taq Polymerase. After denaturation at 95° C for 1.5 minutes, 30 cycles of PCR were performed where each cycle consisted of 1 minute at 95° C, 45 seconds at 60° C, and 45 seconds at 72° C. Products were then resolved on 4% NuSieve agarose (FMC BioProducts) gels. PCR primer sequences were: *ZFX* forward (5'-GTGCTGTGTTAAAGGATAGC-3') and reverse (5'-AGGAGCCCAATTGGGTATGG-3'); *SB1.8* 3A and 4A[26]; *RPS4X* forward (5'-TGAGATGGATTGAATGTGGC-3') and reverse (5'-TTAAAGAGGGTGCCCAGGTA-3'); *UBE1* forward (5'-AGTTTGTGGAGCGAACACTG-3') and reverse (5'-TGCCGGATGTTGTTCGAGTA-3').

RT-PCR. Chromatin immunoprecipitates were subjected to first strand cDNA synthesis using random primers and M-MLV RT (GIBCO) according to manufacturer's instructions. One-quarter of the first strand synthesis was added to PCR reactions using the conditions described above. The following primer sets were used: XIST s1 and s2 [1]; *FIBRONECTIN* forward (5'-TGATCATGCTGCTGGGACTT-3') and reverse (5'-TGTGCCTCTCACACTTCCAC-3'); *ACTIN* forward (5'-TGTGAAGCAGCTCCAGCTAT-3') and reverse (5'-CATGTCGTCCCAGTTGGTGA-3'); 18S forward (5'-TGCATGTCTAAGTACGCACG-3') and reverse (5'-CCGGGTTGGTTTTGATCTGAT-3').

Immunofluorescence. Cells were grown on slides and then incubated for 30 seconds in Cytoskeletal Buffer (100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 10 mM PIPES pH 6.8) on ice, followed by 30 seconds in Cytoskeletal Buffer with 0.5% Triton X-100, and another 30 seconds in Cytoskeletal Buffer. Slides were fixed in 4% buffered paraformaldehyde for 10 min at room temperature, quenched in PBS containing 0.2% Tween, and incubated in blocking buffer composed of PBS with 3% BSA and 0.2% Tween. The primary antibody was diluted in blocking buffer and added to cells for 1 hour at 37° C, followed by washes with PBS/Tween and incubation with rhodamine-labelled goat anti-rabbit IgG (Cappel) for another hour at 37° C. Slides were again washed with PBS/Tween, counterstained with DAPI, mounted in Vectashield (Vector Laboratories) and viewed with a Zeiss Axioplan 2 immunofluorescence microscope.

Glycerol Gradients. Chromatin fragments were loaded onto 12 ml gradients containing 10-30% glycerol and sedimented in an SW41 rotor at 40 krpm for 3.5 hours as described [27]. Visualization of the nucleosomal DNA ladder was done on a 1.25% agarose gel after protein extraction. RT-PCR was used to monitor the presence of *XIST* RNA in each fraction. Sedimentation coefficients for 40S and 60S ribosomal subunits were obtained in parallel gradients [28].

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CHAPTER FOUR: DISCUSSION

Nearly four decades ago, Mary Lyon formulated the hypothesis that X-inactivation — the silencing of one of the two X chromosomes in female cells — compensates for dosage differences in X-linked genes between females and males (Lyon, 1961). Since then, biologists have described this intriguing process in ever greater detail. Yet, the underlying mechanism responsible for silencing the majority of genes *in cis* on one of the two X chromosomes has remained elusive. The most important breakthrough in the effort to understand the mechanistic basis of X-inactivation was the discovery of the *XIST* gene (Brown et al., 1991). *XIST* is a noncoding RNA that is transcribed exclusively from the inactive X (Xi) in differentiated cells. The RNA remains in the nucleus, apparently colocalizing with its chromosome-of-origin. Targeted deletion approaches have demonstrated that the *XIST* locus is essential for initiation of X-inactivation (Marahrens et al., 1997; Penny et al., 1996). However, the mechanism by which *XIST* contributes to silencing the majority of X-linked genes is not understood. This thesis provides a detailed description of the changes in chromatin structure that accompany X-inactivation, which offers a preliminary understanding of how this widespread, *cis*-acting gene repression may be effected.

X-inactivation may be Regulated through Hypoacetylation of Individual Promoters

Several observations are consistent with the notion that X-inactivation can be controlled at the level of individual genes. First, genes on the Xi are not all regulated in the same manner. Indeed, despite the sweeping nature of transcriptional silencing on the Xi, a subset of genes remain active and are largely indistinguishable from their counterparts on the active X (Xa) (Disteche, 1997). Second, several features of the Xi chromatin, such as CG methylation and late replication timing, are associated with genes that are silenced, but not with those that escape X-inactivation (Goodfellow et al., 1988; Hansen et al., 1996; Mohandas et al., 1981).

What mechanism(s) of silencing are able to operate at the level of individual genes? Neither of the two features that correlate with individual X-inactivated genes — late replication timing or DNA methylation — have been shown to inhibit transcription directly. Late replication is

perhaps a secondary consequence of changes in the condensation state of chromatin, rather than the primary cause of gene repression (Yoshida et al., 1993). DNA methylation may mark particular genes for silencing, but evidence suggests that its role in transcription is indirect, mediated by other factors (Jones et al., 1998; Nan et al., 1998). In contrast, there is a third feature associated with the Xi, histone hypoacetylation, which has been causally implicated in gene silencing (Lee et al., 1993; Vettese-Dadey et al., 1996; Steger et al., 1998). Yet, histone hypoacetylation had previously been ascribed to the entire Xi, not to individual X-inactivated genes (Jeppesen and Turner, 1993; Belyaev et al., 1996; Boggs et al., 1996). This thesis provides the first conclusive evidence that histone hypoacetylation indeed occurs at the level of individual genes, and that this may be causal for the silencing of most, but not all, Xi genes.

The correlation between histone hyperacetylation and transcriptional activity is well established (Braunstein et al., 1993). Acetylation of lysine residues facilitates transcription by increasing transcription factor access to the DNA through several mechanisms. Some effects are direct, as bromodomain-containing transcriptional regulators have greater affinity for acetylated histones (Lee et al., 1993; Vettese-Dadey et al., 1996; Winston and Allis, 1999). Several other effects are indirect, and make the DNA more accessible to transcription factors (Cary et al., 1982; Lee et al., 1993; Luger et al., 1997; Tse et al., 1998). Conversely, hypoacetylated nucleosomes are refractory to transcription factor binding. Thus, the demonstration of promoter-specific hypoacetylation of silenced genes on the Xi provides a direct rationale for their repression, by interfering with transcriptional initiation. Furthermore, the confined nature of the hypoacetylation to promoters affords a basis for regulation at the level of individual genes.

The restriction of histone hypoacetylation to promoters of inactivated genes suggests that it results from a specific targeting process. This pattern could be produced by binding of transcriptional corepressors near promoters, which then recruit histone deacetylases. Another method of recruiting deacetylases to promoters might be employed as well: Recent reports have shown that methylated CG dinucleotides associate with the methyl-binding protein MeCP2, which can also recruit complexes with histone deacetylase activity (Jones et al., 1998; Nan et al.,

1998). Given that a perfect correlation has been observed between methylation of CG islands at 5' ends of X-linked genes and their silencing (Tribioli et al., 1992; Jegalian and Page, 1998), DNA methylation and the coincident hypoacetylation at promoters of silenced genes may be mechanistically related. In fact, results reported in this thesis provide the first direct demonstration of promoter-proximal histone hypoacetylation that coincides with CG methylation *in vivo*.

The silencing of X-linked genes observed in somatic cells is the result of at least two distinct processes: initiation and maintenance of X-inactivation. Expression of *XIST* RNA appears to be essential for initiation of X-inactivation (Marahrens et al., 1997; Penny et al., 1996). A time-course analysis of X-inactivation in differentiating ES cells revealed that chromosome-wide methylation and H4 hypoacetylation were evident only after genes on the Xi had been downregulated (Keohane et al., 1996). DNA methylation and histone hypoacetylation are thought to contribute to maintenance of the repressed state after it has been established, as their appearance follows, rather than precedes, transcriptional downregulation in differentiating ES cells (Keohane et al., 1996). It is unclear whether histone hypoacetylation also contributes to the initiation of gene silencing on the Xi. One study suggests that it may: Addition of the deacetylase inhibitor Trichostatin A to differentiating ES cells delayed some of the properties associated with X-inactivation (O'Neill et al., 1999). Thus, deacetylase activity may be important both for the initiation and maintenance of X-linked gene silencing.

Given that the majority of X-linked genes are silenced by X-inactivation, and that the Xi acquires a highly condensed state, it has long been assumed that X-inactivation is regulated by events that alter the chromatin structure of the whole chromosome. The data presented in this thesis suggest instead that several, hierarchical levels of control regulate X-linked gene expression. One level of control may in fact operate at the level of the whole chromosome to produce alterations in chromatin structure that are generally inhibitory to transcription. However, the fact that the chromatin structure of individual promoters correlates with activity of the adjacent genes suggests that local sequences may ultimately specify the transcriptional activity at particular

promoters, and may be able to override the inhibitory effects of the global chromatin environment.

The model that X-inactivation is ultimately regulated at the level of individual genes is supported by phylogenetic data. Analysis of X-linked gene expression in diverse mammalian lineages is consistent with a model in which X-inactivation did not initially encompass the entire chromosome. Rather, it appears to have developed locally, on a gene-by-gene or region-by-region basis, in response to decay of homologous genes on the Y chromosome (Jegalian and Page, 1998). The data presented here suggests that the acquisition of X-inactivation potential for a given gene may be specified by sequence evolution in its upstream regulatory region. Comparisons between the regulatory regions of homologous genes with different X-inactivation phenotypes in various mammalian lineages may help to identify such sequences. One such analysis was performed on the CG islands associated with the *Zfx* gene, which is subject to X-inactivation in mouse but which escapes inactivation in human. This study did not find any sequence differences in the remarkably conserved *Zfx* CG islands that might account for the differences in their X-inactivation and methylation phenotypes (Luoh et al., 1995). This indicates either that the key differences in regulatory sequences lie outside CG islands, or that relatively minor sequence changes in CG islands may have drastic effects on its potential for methylation and X-inactivation of the adjacent gene.

XIST RNA is a Component of the Inactive X Chromatin

A second major finding of this thesis is that *XIST* RNA is a component of the inactive X (Xi) chromatin. Fluorescence *in situ* hybridization (FISH) analysis had already demonstrated the colocalization of *XIST* RNA and the Xi at low resolution (Clemson et al., 1996). The results described here extend those analyses to show that a physical association between *XIST* RNA and the Xi chromatin may provide the underlying basis for the colocalization.

XIST RNA can be co-precipitated with particular anti-histone antisera, even in the absence of crosslinking, suggesting that it is stably associated with the Xi chromatin. Furthermore,

the specificity of *XIST* RNA recovery with antibodies against unacetylated, but not acetylated, H3 and H4 isoforms suggests that *XIST* RNA may be preferentially associated only with particular regions of the Xi chromatin. Since unacetylated histones are enriched at promoters of silenced genes (see Chapter Two), *XIST* RNA may also be enriched in chromatin associated with silenced genes. Preliminary dual-label FISH analysis supports the model that *Xist* RNA may be associated with individual genes that are silenced, but not with genes that escape silencing. In interphase mouse embryonic fibroblasts (MEFs), *Xist* RNA colocalizes with the inactivated *Zfx* locus, but not with the *Sts* locus which escapes X-inactivation (see Appendix). Similar conclusions have been drawn from metaphase microscopy analysis, in which *Xist* RNA was not associated with translocated autosomal sequences (Duthie et al., 1999) or with ectopic *Xist* loci (Lee et al., 1999) which were actively transcribed from the Xi. This suggests that the noncoding *XIST* RNA may indeed play a functional role in the silencing process, recruiting factors that have a repressive effect on transcription to particular genes on the Xi. These results raise the possibility that association with *XIST* RNA may be a fourth feature — along with promoter-proximal histone hypoacetylation, CG methylation, and late replication timing — that correlates with individual silenced genes on the Xi.

Model for Regulation by *XIST* RNA

An emerging theme in the dosage compensation literature is that noncoding RNAs may coordinate extensive changes in gene expression in a variety of organisms. The fact that these noncoding RNAs associate with the dosage compensated chromosomes suggests that they play a functional role in the regulation. In fact, expression of a noncoding RNA from a chromosome may provide a convenient method of marking it for extensive, coordinated modulation. This method can even be used to distinguish between two homologous chromosomes by selective transcription of the RNA, as occurs with X-inactivation.

The manner in which *Xist* RNA interacts with the Xi chromatin is unclear. The interaction must be general enough that *Xist* RNA can associate with some autosomal chromosomes *in cis*

when ectopically expressed from multicopy transgenes (Lee et al., 1996; Herzing et al., 1997; Lee et al., 1999). Yet, it must be specific enough such that it can be excluded from some regions which are actively transcribed from the inactivated chromosome (Duthie et al., 1999; Lee et al., 1999). The fact that *XIST* RNA can be recovered with anti-histone antisera suggests that it is indeed a component of the Xi chromatin. It is not yet known whether the association between *Xist* and histones is due to direct binding, or to indirect interactions, however.

The two main findings presented in this thesis — that X-inactivated genes are hypoacetylated on histone H4 at their promoters, and that *XIST* RNA associates with hypoacetylated histones — provide a basis for a model of X-inactivation. First, *XIST* RNA association with the Xi is a product of its selective stabilization upon differentiation (Panning et al., 1997; Sheardown et al., 1997). This association has a physical basis, in that *XIST* RNA is located in close physical proximity to histones that may be simultaneously incorporated into the Xi chromatin. The underlying basis for this physical association is unclear, but it has been proposed that *XIST* RNA may associate with LINE elements, or factors associated with these elements, which are disproportionately enriched on the X (Lyon, 1998). By associating with particular regions of the X chromosome, *XIST* RNA may serve as an adaptor to recruit factors that are repressive to transcription. These putative factors may ultimately produce promoter-specific hypoacetylation and concomitant gene silencing. Factors recruited by *XIST* RNA might be either structural components of chromatin, such as mH2A (Pehrson and Fuji, 1998), or regulatory ones, such as histone deacetylases or *de novo* DNA methylases (Clemson et al., 1998), all of which might repress transcription. According to this scenario, *XIST* RNA association might lead to local modifications of chromatin structure on the Xi. Once initiated, the repressed state of the Xi can then be maintained in an *XIST* RNA-independent manner (Brown and Willard, 1994).

An understanding of the mechanism by which *XIST* RNA association with the Xi chromatin results in repression of target genes will require identification of factors that associate with this RNA. Prior to this dissertation, no factors had been reported to associate with *XIST* RNA. This study shows that *XIST* RNA is present in complexes which include several histone isoforms,

including the histone variant macroH2A (mH2A). It had been proposed, on the basis of its homology to RNA-binding proteins of several viruses, and its colocalization with *XIST* RNA, that mH2A may have *XIST* RNA-binding ability (Pehrson and Fuji, 1998). This thesis presents evidence that *XIST* RNA can in fact be recovered in complexes which contain mH2A. This association may increase the local concentration of mH2A in the vicinity of the Xi, increasing the chance that it may be assembled into nucleosomes on the Xi. Perhaps incorporation of mH2A into chromatin associated with particular genes is important for the initiation of X-inactivation. Although it has not been demonstrated that mH2A incorporation into chromatin alters gene expression, mH2A contains a leucine zipper motif which could potentially bind DNA at specific sites, thereby influencing nucleosome positioning (Pehrson and Fried, 1992).

Why does the presence of *XIST* RNA lead to specific repression of most genes on the Xi? Given that *XIST* RNA is needed for initiation, but not maintenance, of X-inactivation (Brown and Willard, 1994), it may function indirectly in long-term gene silencing. At initiation, it might trigger a cascade of downstream events which are then ultimately responsible for repressing transcription. It may be that *XIST* RNA is able to recruit factors to the Xi which are needed for initiation of silencing, and which have the ability to perpetuate themselves in the Xi chromatin thereafter even in the absence of *XIST* RNA. Although no factors other than histones have been found to associate with *XIST* RNA, several possibilities can be envisioned: For example, *XIST* RNA might recruit a *de novo* DNA methylase to the Xi chromatin which has the unique ability to methylate CG islands of target genes. Once the CG islands have been methylated, they could be perpetuated through future cell divisions by maintenance methylases even if the *de novo* methylase initially recruited by *XIST* RNA were no longer available. Alternatively, *XIST* RNA may recruit histone deacetylases to particular regions of the Xi, as suggested by the fact that *XIST* RNA is preferentially associated with unacetylated histones. This might induce a change in chromatin structure that is refractory to transcription, which initiates X-linked gene silencing. These events themselves, or perhaps subsequent, unidentified events could be responsible for maintenance of the repressed state.

Future Directions

One of the immediate questions raised by this work is whether or not *XIST* RNA can directly bind to mH2A or to other histones. This question could be addressed at a biochemical level by gel shift assays in which exogenous fragments of labeled *XIST* RNA are incubated with nuclear extracts. The specificity of any resulting bandshifts can be assayed by supershift experiments with the same anti-histone antibodies used in the chromatin IP experiments. Any putative interactions could be further analyzed by gel shifts using purified factors, such as free histones and nucleosomes. If gel shifts are not observed with the purified components, it may be that additional factors are needed to mediate the interaction. Biochemical fractionation may be able to identify such factors.

If one function of *XIST* RNA is to recruit mH2A to the Xi chromatin, then mH2A may be enriched along with *XIST* RNA in regions with hypoacetylated histones H3 and H4. Chromatin immunoprecipitations with anti-mH2A antibodies followed by competitive PCR could be used to address whether mH2A is preferentially enriched in X-inactivated genes, and whether it shows any difference in levels along the length of particular genes. If mH2A were indeed enriched in X-inactivated genes, it would support the model that mH2A is recruited to the Xi chromatin by its association with *XIST* RNA. A correlation between mH2A and gene silencing would also be suggestive that the histone variant contributes to gene repression.

X-inactivation results in the acquisition of several distinct features by the Xi chromatin. The data presented in this dissertation has focused on differences in post-translational modifications of histones that correlate with gene activity from the Xi. To identify additional non-histone proteins that correlate with the activity of individual genes on the Xi, the chromatin immunoprecipitation analysis could be expanded to examine other known chromatin proteins. In particular, it may be informative to test antibodies directed against heterochromatin-associated proteins. For instance, antibodies against Heterochromatin-Associated Protein (HP1) (Powers and Eissenberg, 1993) or Polycomb group proteins (PcG) (Strutt and Paro, 1997) might be employed. Chromatin IPs using

these antisera — followed by competitive PCR — might reveal whether these repressive factors are disproportionately enriched in particular genes that are silenced on the Xi, relative to genes that escape silencing or to their Xa counterparts.

Additionally, evidence presented in Chapter III raised the possibility that *XIST* RNA may physically associate with X-inactivated genes. This was based on an indirect line of reasoning — that hypoacetylated histones are enriched in X-inactivated genes, and that *XIST* RNA associates with hypoacetylated histones. To directly examine whether *XIST* RNA is preferentially associated with inactivated genes, antisense affinity selection techniques might be employed to recover *XIST* RNA and its associated factors. If this approach included limited digestion of DNA, it may be possible to recover DNA sequences of X-linked genes along with the noncoding RNA. Competitive PCR could be used to determine the identity and the relative enrichment of these sequences in the *XIST* RNA-containing fraction. This would conclusively determine whether *XIST* RNA was preferentially associated with hypoacetylated promoter regions of X-inactivated genes. It could also be used to test the hypothesis that *XIST* RNA associates with the LINE elements that are disproportionately enriched on the X chromosome (Lyon, 1998). If such an antisense affinity purification were employed, it would also be interesting to examine whether histone deacetylase activity were recovered along with *XIST* RNA.

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APPENDIX: LOCALIZATION OF *XIST* RNA RELATIVE TO X-LINKED GENES

The evidence presented in this thesis suggests that *XIST* RNA is a physical component of the Xi chromatin. The association between *XIST* RNA and the Xi chromatin may not be uniform; rather, it appears that *XIST* RNA may be enriched in hypoacetylated regions of the Xi chromatin. This tentative conclusion is based on the observation that *XIST* RNA was recovered from female cells with antisera against unacetylated, but not acetylated histones (Chapter Three). Chapter Two of this thesis reported that hypoacetylated histones are enriched at promoter regions of X-inactivated genes. This raises the possibility that *XIST* RNA may associate with silenced genes, which are distinguished by unacetylated histones, but not with genes that escape silencing. It was previously suggested that regions of the Xi may escape inactivation by excluding *Xist* RNA binding: first, ectopically expressed *Xist* RNA associated with an autosome except where it "skipped" the multicopy transgenic *Xics* (Lee et al., 1999). Second, it was reported that *Xist* RNA did not associate with autosomal sequences that escaped inactivation despite having been translocated to the Xi (Duthie et al., 1999).

To address whether genes that are normally expressed from the Xi exclude *Xist* RNA, double-label FISH experiments were performed on mouse embryonic fibroblasts (MEFs). Figure 1 shows the results of a dual *in situ* detection of murine *Xist* RNA with the *Zfx* genomic locus. The location of the *Zfx* gene, which is subject to X-inactivation in mouse, was detected by a 250 kb genomic probe. Four patterns of localization between *Xist* RNA and the *Zfx* locus were possible by two-dimensional analysis: (1) complete overlap, in which the *Zfx* signal is completely coincident with the *Xist* signal; (2) partial overlap, in which the signals intersect but are not entirely coincident; (3) no overlap, whereby the signals can be separately resolved; and (4) no signal. Two independent experiments were performed, in which a total of 28 cells were scored. Table I shows the results of this analysis, in which the majority (71%) of cells show complete overlap between *Xist* RNA and the *Zfx* genomic locus.

A different pattern was observed for the localization of *Xist* RNA relative to the transcript derived from the *Sts* locus, which escapes X-inactivation in the mouse (Figure 2). Here, the location of the *Sts* transcript was assumed to approximate the position of the *Sts* genomic locus.

The pattern of overlap – obtained from two independent experiments – was significantly different from that obtained for *Xist* RNA and the inactivated *Zfx* gene. In this case, only a minority of cells showed complete overlap between the *Xist* and *Sts* RNAs in two-dimensional analysis (Table I). Rather, many nuclei showed either a partial overlap, or no overlap between the *Xist* and *Sts* transcripts.

The localization of *Xist* RNA to a genomic locus that is subject to X-inactivation, but not to one that escapes silencing, is consistent with a model in which association of *Xist* RNA with individual loci results in their silencing. This may indicate that *Xist* RNA recruits silencing factors to particular genes on the Xi.

To more thoroughly evaluate the model that *Xist* RNA associates with genes that are silenced, but not with those that escape, additional genes of each type may be examined by dual-label FISH analysis. The *Sts* gene analyzed here is located in the pseudoautosomal region of the X, at the distal tip of the X chromosome, amidst other genes which also escape inactivation. These features may have increased the likelihood of resolving the signals corresponding to the *Xist* and *Sts* transcripts. It would be informative to compare the localization between *Xist* and genes which escape inactivation, but which are located in the X-specific region of the chromosome, amidst genes that are X-inactivated. However, as the resolution of FISH microscopy is approximately 130 kb on chromosomes in interphase nuclei (Lawrence et al., 1988), it may not always be possible to detect exclusion of *Xist* RNA from a locus by this method, even if it does occur. An alternative method to detect the DNA sequences that *Xist* RNA associates with – at high resolution – would be to affinity purify *Xist* RNA from cells and to analyze the enrichment of particular X-linked genes in the pulldown fraction by competitive PCR.

Experimental Procedures

FISH. Cells that were used in RNA FISH experiments were grown and fixed in 4% paraformaldehyde on slides as previously described (Clemson et al., 1996). Briefly,

hybridization was performed without chromosome denaturation such that probes did not detect genomic DNA. For simultaneous DNA/RNA FISH, cells were denatured prior to probe hybridizations, according to the method of Clemson et al. (1996). *Xist* RNA was detected using the plasmid pXist3k, which contains a 3 kb fragment from exon 1. *Xist* probes were directly labeled by nick translation with Cy3-dCTP (Amersham). Random priming from the 8.9 kb *Sts* genomic clone or the 250 kb *Zfx* BAC clone was used to generate biotin-labeled probes (GIBCO BioPrime kit) that were subsequently detected by Fluoroscein Avidin DCS (Vector). Images were collected with a CCD camera mounted on a Zeiss Axioplan 2 microscope and merged with IP Lab Spectrum software.

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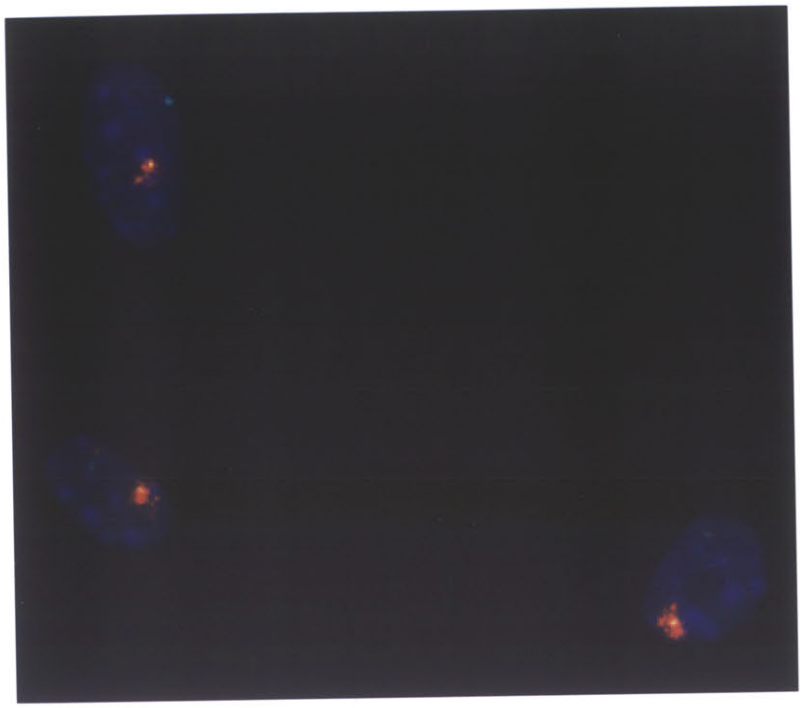

Figure 1. *Xist* RNA Overlaps with the Inactivated *Zfx* Locus

Simultaneous DNA/RNA Fluorescence *in situ* hybridization (FISH) analysis of mouse embryonic fibroblasts (MEFs) grown and fixed on slides. Dual labeling was performed on denatured chromosomes with probes for *Xist* RNA (red) and the *Zfx* genomic locus (green). A single large *Xist* RNA signal was observed in female nuclei which corresponds to the inactive X chromosome. Two *Zfx* signals were observed in each nucleus. Nuclei were visualized by DAPI counterstaining.

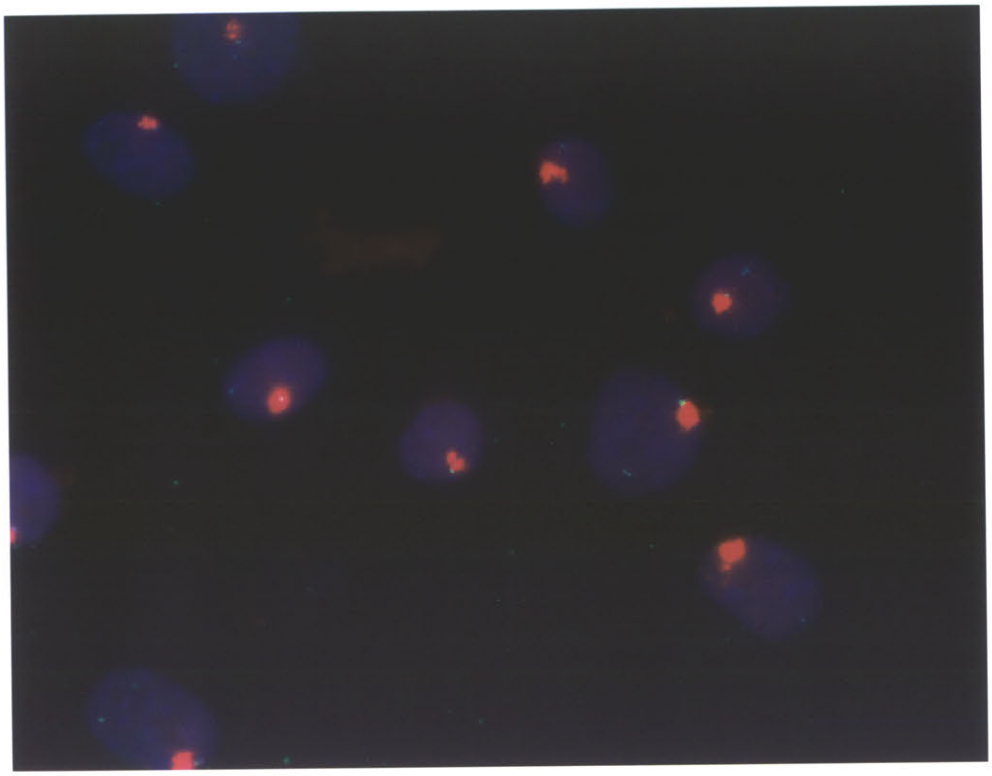

Figure 2. *Xist* RNA Rarely Overlaps with the *Sts* Gene, which Escapes X-inactivation

Dual label RNA FISH analysis of female MEFs using probes against *Xist* RNA (red) and *Sts* RNA (green). The large *Xist* RNA signal can often be resolved from the *Sts* RNA signal which also emanates from the Xi. Nuclei were visualized by DAPI counterstaining.

Table I. Overlap Between *XIST* RNA and X-linked Genes.

Pattern of Overlap	With *Zfx* genomic locus	With *Sts* RNA
(1) Complete Overlap	20/28 = 71%	4/31 = 13%
(2) Partial Overlap	7/28 = 25%	11/31 = 35%
(3) No Overlap	1/28 = 3.5%	14/31 = 45%
(4) No Signal	0/28 = 0%	2/31 = 6%

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curriculum vitae of **Sandra L. Gilbert**

Center for Cancer Research
Building E17-528, 40 Ames St.
Cambridge MA 02138

Tel: 617-253-6457
Fax: 617-253-3867
Email: sgilbert@mit.edu

EDUCATION

1991 - present **Massachusetts Institute of Technology**
Candidate for Ph.D. in Biology

1986 - 1990 **University of Pennsylvania**
B.A. in Biology and B.A. in Economics

RESEARCH EXPERIENCE

1992 - present **Graduate Research Assistant**
Laboratory of Phillip Sharp, MIT
Analysis of chromatin structure of the inactive X chromosome in mammals.

1990 - 1991 **Research Technician**
Laboratory of Margaret Fuller, Stanford University
Identification of genes that interact with microtubules during spermatogenesis in *Drosophila*.

1989 - 1990 **Undergraduate Research Assistant**
Laboratory of Andrew Binns, University of Pennsylvania
Investigation of the role of wounding and nutrient deprivation on gene expression in transformed tobacco cells.

EMPLOYMENT

1998 - present **Science Consultant**
Sensimetrics, Inc.
Designing interactive software for teaching cell biology to middle school students.

TEACHING EXPERIENCE

- Teaching assistant for Microbial Genetics Project Laboratory at MIT.
- Teaching assistant for Introductory Biology at MIT.

HONORS

- 1998 Lorraine DiSano Memorial Prize for outstanding cancer research.
- Graduated *magna cum laude* from University of Pennsylvania with Distinction in Biology.
- Benjamin Franklin Scholar with General Honors Award at University of Pennsylvania.

PUBLICATIONS

Promoter-Specific Hypoacetylation of X-inactivated Genes
Submitted

***XIST* RNA is a Component of the Inactive X Chromatin**
In preparation

The Role of Nutrients in the Modulation of Amidohydrolase Activity in Transformed Tobacco Cells
Undergraduate Thesis