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XIST RNA AND THE MECHANISM OF X CHROMOSOME INACTIVATION

Kathrin Plath, Susanna Mlynarczyk-Evans, Dmitri A. Nusinow, and Barbara Panning

Department of Biochemistry & Biophysics, University of California San Francisco, San Francisco, California 94143; e-mail: kathrin@itsa.ucsf.edu, smlynar@itsa.ucsf.edu, meter@itsa.ucsf.edu, bpanning@biochem.ucsf.edu

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■ **Abstract** Dosage compensation in mammals is achieved by the transcriptional inactivation of one X chromosome in female cells. From the time X chromosome inactivation was initially described, it was clear that several mechanisms must be precisely integrated to achieve correct regulation of this complex process. X-inactivation appears to be triggered upon differentiation, suggesting its regulation by developmental cues. Whereas any number of X chromosomes greater than one is silenced, only one X chromosome remains active. Silencing on the inactive X chromosome coincides with the acquisition of a multitude of chromatin modifications, resulting in the formation of extraordinarily stable facultative heterochromatin that is faithfully propagated through subsequent cell divisions. The integration of all these processes requires a region of the X chromosome known as the *X-inactivation center*, which contains the *Xist* gene and its *cis*-regulatory elements. *Xist* encodes an RNA molecule that plays critical roles in the choice of which X chromosome remains active, and in the initial spread and establishment of silencing on the inactive X chromosome. We are now on the threshold of discovering the factors that regulate and interact with *Xist* to control X-inactivation, and closer to an understanding of the molecular mechanisms that underlie this complex process.

CONTENTS

INTRODUCTION

Over 40 years ago, Mary Lyon postulated that equalization of X-linked gene dosage between male and female mammals occurs by the transcriptional silencing of one X chromosome in female cells (86). Early observations established that this process occurs at random, such that the X chromosome inherited from either parent is silenced in 50% of cells. The silencing of one X chromosome occurs early in development and roughly coincides with differentiation of pluripotent cells to restricted lineages (105). Analysis of X chromosome aneuploidies showed that one X chromosome remains active in a diploid cell, while all additional X chromosomes are silenced (53). Genetic studies indicated that the silencing of the inactive X chromosome (Xi) is initiated at one location on the X chromosome, termed the *X-inactivation center* (*Xic* in mouse and *XIC* in human). Silencing spreads in *cis* from the *Xic*/*XIC* (137, 155), indicating that this *cis*-regulatory element contains sequences that initiate a chromosome-wide alteration in chromatin structure. Cytological analysis revealed that, once established, the Xi is clonally propagated, such that females are functionally mosaic for X-linked traits (86, 136). Thus, random X-inactivation has often been described as a multistep process involving choice of the active X chromosome (Xa), initiation and spread of silencing on the Xi, and subsequent maintenance of the Xi's silent state. Mouse embryonic stem (ES) cells have become a valuable tool in the study of X-inactivation because they undergo random X-inactivation upon induction to differentiate in culture (115, 150). ES cells can be genetically manipulated and subsequently transmitted through the germline, allowing study of X-inactivation both in culture and in the embryo. As a result, the molecular mechanisms involved in regulation of X-inactivation are best understood in the mouse.

Xist/XIST RNA IS CRITICAL FOR X-INACTIVATION

The Xist/XIST Gene Is Located in the *Xic*/XIC

The location of the *Xic*/*XIC* was determined by analyzing X chromosome translocations that resulted in inactivation of the autosome to which the X chromosome fragment had fused (56). The minimal *Xic*/*XIC* regions are syntenic between mouse and human, although multiple inversions have resulted in shuffled gene order between the species, as depicted in Figure 1 (42, 56). The discovery in 1991 of one gene located within the *Xic*/*XIC*, the *Xi-specific transcript* (*Xist* in mouse, and

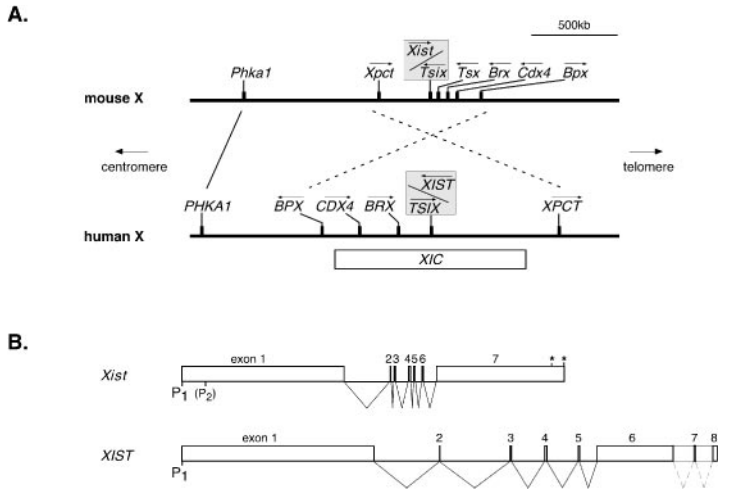

Figure 1 Mouse and human *Xic/XIC* and *Xist/XIST*. *A.* Schematic diagram of the area surrounding the *XIST/Xist* gene on the human and mouse X-chromosomes. The *XIC/Xic* and the surrounding regions of the human and mouse X-chromosomes are depicted; both include the *XIST/Xist* gene. In human, the candidate region for the *XIC* encompassing 700–1200 kb is located within band Xq13 on the proximal long arm of the X chromosome (56). In mouse, the *Xic* region identified by X chromosome rearrangements is considerably larger than that in human (56). However, the regions required to recapitulate all functions of the *Xic*, as defined by transgenic analysis in mouse, contain only the *Xist* gene and minimal surrounding sequences (57, 62, 79, 81, 82, 92). The segment of the X chromosome containing the *Xic/XIC* locus is syntenic but inverted between mouse and human (*dashed lines*) (42, 56). Small black boxes depict the location of genes in the *Xic/XIC* area, and the arrow above the gene name gives the orientation of transcription. Linkage of the two orthologous regions shows some discontinuity as local inversions of transcription units have occurred (42). The existence of a human TSX homolog is unclear (100, 108). *B.* Comparison of the human and mouse *Xist/XIST* genes. P1 indicates the major transcriptional start site for *Xist/XIST*. In mouse, two RNA isoforms of 17 and 17.9 kb may be produced by usage of alternative polyadenylation sites denoted by stars. In mouse, a second promoter, P2, has been described (69) but is not conserved in other *Xist/XIST* genes analyzed to date (108). Extensive alternative splicing of the human gene has been described, yielding *XIST* RNA isoforms lacking, for example, exon 4, half of exon 6, or exon 7 (18, 20). Only the linear splicing events are shown here. As indicated by the broken lines, the two 3′ introns may not always be excised from the processed RNA, yielding an RNA molecule of up to 19.3 kb in length (64). Short, colinear stretches of high homology have been identified throughout the genes including the very 3′ ends (64, 108).

XIST in human), has revolutionized our understanding of X-inactivation (13–15, 18, 20). *Xist/XIST* is the only gene transcribed from the Xi and not from Xa in somatic cells. No significant open reading frame has been identified, suggesting that *Xist/XIST* does not encode a protein. *Xist* has subsequently been shown to be the pivotal player in choice of which X chromosome remains active (89), and in the spread of silencing on the future Xi (90, 117). In simplest terms, the *Xic* can be thought of as the *Xist* gene and *cis*-elements that ensure its correct developmental regulation on both the Xa and Xi.

Xist/XIST Gene Structure Is Conserved, But Sequence Varies

The genomic lengths of mouse *Xist* and human *XIST* are approximately 23 kb and 35 kb, respectively. Transcripts of both genes are spliced and polyadenylated. Mouse *Xist* is comprised of unusually large initial and terminal exons flanking five smaller exons (15, 63, 95). Transcription initiating from the promoter designated P1 may give rise to *Xist* transcripts of up to 17.9 kb depending on polyadenylation site usage (63, 95). The overall structure of the gene, including the transcription start site P1 and exon/intron boundaries, is similar in the human counterpart (17, 18). The longest human *XIST* transcript may be 19.3 kb in length (64). In human, many different *XIST* RNA isoforms are produced by extensive alternative splicing occurring within the 3′ half of the gene (17, 18, 64). So far, the significance of the heterogeneity of *Xist*/*XIST* transcripts is unknown.

On the sequence level, all *Xist*/*XIST* genes sequenced to date display a relatively low degree of conservation (60, 61, 108). Mouse and human *Xist/XIST* show 49% sequence identity overall, with exons being slightly more conserved than introns (60, 108). The homology between rodent and human *Xist*/*XIST* exons is considerably lower than the average identity for protein-coding regions (85%) and even lower than identity between 5′ or 3′ untranslated regions of rodent and human mRNA (∼65–80%) (108). Although several short stretches of unique sequence show relatively high homology between the species (64, 108), the most salient feature of the known *Xist*/*XIST* sequences is the conservation of six repeated elements, designated A through F (15, 18, 108).

Xist/XIST RNA Is Localized to the Xi in Female Somatic Cells

From initial sequence analysis of the *Xist*/*XIST* gene, it was unknown whether its function in X-inactivation, if any, would be mediated by the DNA sequence of the gene or its untranslated RNA product. Analysis of *Xist*/*XIST* RNA distribution quickly led investigators to search for a role for the RNA. *Xist*/*XIST* transcripts are retained in the nucleus of female somatic cells (15, 18). When analyzed by fluorescence in situ hybridization (FISH), *Xist*/*XIST* RNA appears highly particulate and is located to a large nuclear domain that corresponds to the space occupied by the Xi (18, 31, 114). *Xist/XIST* RNA particles are released from the Xi during mitosis (31, 44). A three-dimensional analysis showed that *XIST* RNA is not just bound to the surface of the Xi, but seems to reside within the entire space delineated

by the Xi (31). Such an extensive and intimate association of an RNA with an entire chromosome was unprecedented at the time, but now appears to be shared by the *roX1* and *roX2* RNAs that coat the dosage-compensated X chromosome in male *Drosophila* (71). The close association of *Xist*/*XIST* RNA with the Xi supported a model in which the RNA acts as a functional molecule that regulates X-inactivation.

Cis-Spread of *Xist* RNA Correlates with X-Inactivation During Mouse Development

In male and female mouse embryos, *Xist* is transcribed in the pluripotent epiblast lineage prior to its differentiation into the embryonic tissues. By FISH, *Xist* RNA is detected as a pinpoint signal at the site of its transcription on all X chromosomes, which are active at this point (113, 143). Mouse ES cells, which are derived from pluripotent cells of the early embryo, show this same pinpoint *Xist* expression (114). Consistent with a role in X-inactivation, *Xist* levels increase dramatically in female but not male cells in the developmental window in which silencing of the X chromosome occurs (70). When epiblast cells differentiate into the embryonic germ layers during gastrulation, and when ES cells are differentiated in vitro, both cell types undergo X-inactivation (115, 150, 153) and exhibit similar dynamic *Xist* expression patterns (113, 143). *Xist* RNA coats the presumptive Xi, while the Xa retains a low-level pinpoint of *Xist* RNA that is subsequently extinguished (113, 143). FISH studies on ES cells indicated that genes are silenced soon after *Xist* transcripts coat the Xi (114, 143). That initial coating of the X chromosome by *Xist* RNA coincides with the onset of silencing suggested a causative role for *Xist* in X-inactivation.

Xist RNA Is Necessary and Sufficient for Chromosome-Wide Gene Silencing

To investigate *Xist* function, two groups engineered large deletions, removing the promoter and first exon of the *Xist* gene (117) or the majority of exon 1 through exon 5 (90). The X chromosome bearing the loss-of-function allele could never be inactivated in ES cells or in the embryonic tissues of mice heterozygous for the deletion. Instead, only the wild-type X chromosome ever became the Xi (89, 90, 117), indicating that *Xist* is required in *cis* for silencing of the X chromosome. In addition, use of antisense oligonucleotide analogs to block *Xist* RNA's ability to coat the presumptive Xi during differentiation prevented the formation of a silenced X chromosome (7). These results demonstrate that expression of *Xist* RNA and coating of the chromosome are required to silence the Xi during X-inactivation.

An elegant series of studies from Wutz and colleagues has demonstrated unequivocally that expression of *Xist* RNA is sufficient to induce chromosome-wide silencing (164, 165). A transgene was generated containing a 15-kb *Xist* cDNA, lacking approximately 3 kb at the end of the terminal exon, under control of a tetracycline-inducible promoter. Induction of *Xist* transcription from this transgene

in male ES cells results in *cis*-spread of *Xist* RNA into linked autosomal sequences and silencing of genes (164). This result indicates that high-level expression of *Xist* RNA is sufficient for chromosome coating and silencing, even in male cells. It has been suggested that *Xist* RNA mediates these functions as part of a ribonucleoprotein complex (162, 165). Although *Xist* RNA can induce chromosome-wide silencing in ES cells (164), activation of *XIST*/*Xist* expression and subsequent coating of the Xa in differentiated cells is insufficient to cause silencing (30, 49, 156, 164). Thus, *Xist* transcripts can always coat the chromosome in *cis*, but the RNA can only mediate silencing in a narrow window during differentiation (164). Proper spatial and developmental regulation of *Xist* expression is therefore critical, so that a female cell can reliably keep one X chromosome active, and inactivate the other.

CHOOSING THE ACTIVE X CHROMOSOME

Xa Choice Is Regulated by Several Classes of *cis*-Elements

In contrast to the dosage compensation mechanisms of *Drosophila* and *Caenorhabditis elegans*, where all X chromosomes in the dosage-compensated sex are treated equivalently (33), the two X chromosomes in the cells of female mammals take on radically different fates. In random X-inactivation, mammalian cells designate a single Xa, and then carry out X-inactivation on any remaining X chromosome(s). Cells must choose between two equivalent X chromosomes and randomly make a differentiating mark on only one of them, the future Xa. The simplest model to explain the marking of one X chromosome as Xa-elect hypothesizes that a blocking factor, so named since it functions to block X-inactivation, is responsible for making this mark (Figure 2) (128). Since Xa number increases with the number of autosome complements (21, 46, 161), the amount of blocking factor activity must be determined by autosomal ploidy such that each diploid set of autosomes produces sufficient blocking factor to choose only one Xa. The region of the *Xic* required to mediate blocking factor activity is referred to as the counting element. Modification of the counting element by the blocking factor represents the first molecular difference between the Xa-elect and the presumptive Xi. The consequence of blocking factor's interaction with the counting element on the Xa-elect is the interference with *Xist* RNA's silencing function on this chromosome. The counting element can therefore be seen as the fundamental *cis*-element required for choosing the Xa.

When two X chromosomes bearing identical *Xic* regions are present in a female cell, Xa choice occurs randomly because of the equal probability that blocking factor will interact with either counting element (99). Genetic studies of the *Xic* region have shown the existence of additional *cis*-elements, termed choice elements, that can skew the Xa choice event such that one X chromosome becomes Xa more frequently than the other (103). Skewing of random X-inactivation in female cells heterozygous for a choice element is thought to be the outcome of better or worse competition for the blocking factor interaction compared to the wild-type X. A

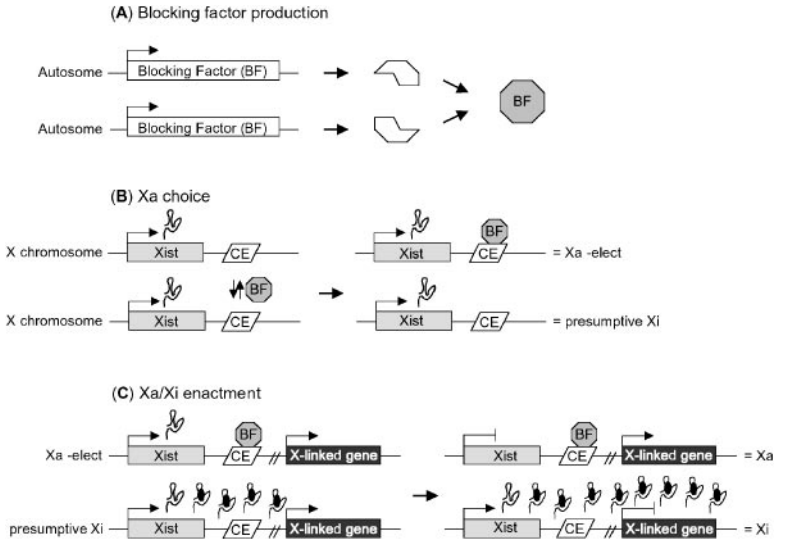

Figure 2 Xa choice by blocking factor. (*A*) An autosomally encoded blocking factor (BF) is produced in sufficient quantity to choose one Xa. (*B*) During the Xa choice process, both X chromosomes compete for the limiting blocking factor at the *cis*-acting counting element (CE). Choice of the Xa is achieved when blocking factor interacts with the counting element on one X chromosome. In this time period, *Xist* RNA molecules (indicated by *black squiggles*) are produced but restricted to the site of transcription. (*C*) During the enactment period, the activity of *Xist* RNA is repressed on the Xa-elect as a consequence of its modification by blocking factor, and *Xist* expression is eventually shut off, resulting in commitment to the Xa fate. On the presumptive Xi, *Xist* RNA, most likely in a ribonucleoprotein complex (indicated by the addition of the *black oval*), coats the chromosome in *cis*, leading to inactivation of X-linked genes, and committing the chromosome to the Xi fate. The location of the counting element and the implication of the drawing that blocking factor is bound at the counting element are speculative.

choice element must therefore affect the affinity of the *cis*-linked counting element for blocking factor, such that there is an increase or decrease in the likelihood that the blocking factor will interact with it. Thus, a combination of *cis*-elements within the *Xic* are employed to allow cells to differentiate an Xa from an Xi during random X-inactivation.

The Minimal Counting Element Is Closely Linked to *Xist*

The definitive test of counting element function is the ability of a sequence to induce inactivation of the single X chromosome in a male cell when integrated as an autosomal transgene. The presence of the counting element in the transgene titers blocking factor away from the endogenous *Xic* such that the single

X becomes the presumptive Xi in a fraction of cells. Several *Xist*-containing transgenes have been reported to induce silencing of the endogenous X chromosome in male cells (57, 62, 79, 81, 82, 92). The minimal region sufficient for counting element function is defined by a 35-kb transgene containing genomic *Xist* flanked by 9 kb of upstream and 3 kb of downstream sequences [(62) based on the recent 3′ end mapping by Hong and colleagues (63)], demonstrating that the counting element is very closely linked to *Xist* (Figure 3). The counting element is unlikely to reside within the transcribed region of the *Xist* gene sufficient for silencing as the *Xist* cDNA transgene is unable to induce ectopic X-inactivation in male cells (164). Like all functional *Xic* transgenes examined so far, the 35-kb transgene containing counting element activity is present in multiple tandem copies (62). No single-copy transgene has been shown to faithfully recapitulate *Xic* activity (57). These

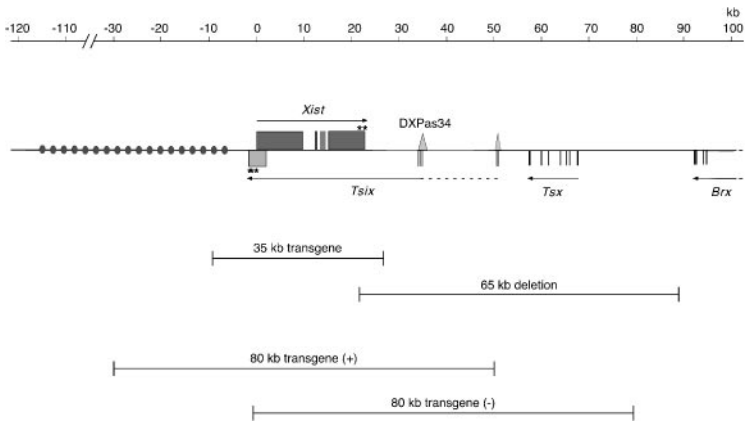

Figure 3 *Xist* and *Tsix* region on the mouse X chromosome. The figure displays the 200-kb region surrounding the mouse *Xist* gene in detail with the zero point of the scale set at *Xist* promoter P1. Transcripts derived from the sense and antisense strands are drawn above and below the line, respectively. Exons are depicted as solid blocks. Major and minor *Tsix* transcription start sites are each associated with CpG-rich region (indicated by the *triangles*). The CpG-rich region at the major promoter is known as DXPas34 (37). Both *Tsix* transcription start sites are the 5′ ends of short ∼200-bp exons that are spliced to an acceptor site located in the antisense region complementary to the 5′ end of *Xist* exon 1. The final exon of *Tsix* is from 2 to 4 kb in length, depending on polyadenylation site usage (indicated by *stars*) (139). The chromatin domain immediately upstream of the *Xist* transcriptional start site P1 is characterized by methylation of histone H3 on lysine 9 (indicated by *ovals*) (59). A 35-kb transgene containing minimal *Xic* activity and sufficient for counting element function (62), and a 65-kb deletion thought to remove counting element function (32) are shown. Two 80-kb *Xic* transgenes, one able (+) and one unable (−) to enact the Xi fate upon differentiation (81), are shown.

results suggest either that position effects, to which single-copy transgenes are often subject, interfere with counting element function, or that the counting element is multipartite, perhaps requiring a certain threshold number of minor elements to function. Even in the cases when multicopy transgenes induce X-inactivation of the endogenous X chromosome, they do not confer truly random choice, because the endogenous X chromosome is chosen as Xa more frequently than expected (81, 82). In contrast, X: A translocations containing the *Xic* compete equally with a normal X chromosome for choice as Xa (128a). Thus, although the transgene contains the counting element, additional sequences regulating the choice process are missing from these transgenes.

Given its pivotal role in receiving the blocking factor-mediated Xa choice signal, the counting element should be absolutely required for an X chromosome to be chosen as Xa. Therefore, deletion of the counting element is predicted to result in ectopic X-inactivation of the single X chromosome in male cells. A 65-kb deletion implicates the region 3′ of *Xist*, beginning in *Xist* exon 7 and continuing downstream, as containing the counting element (Figure 3) (32). Nearly all cells carrying this deletion on their only X chromosome died upon differentiation due to ectopic *Xist* RNA coating and silencing. Although this deletion may have removed the counting element such that the single X chromosome could not be chosen as the Xa, it is also possible that the counting element is unaffected and that a second critical element required for downregulation of *Xist* activity on the Xa, acting downstream of Xa choice, may have been perturbed. Three smaller regions contained within the 65-kb region have been deleted independently in male ES cells without resulting in ectopic X-inactivation upon differentiation (80, 130, 139). Identification of the counting element may provide important clues about the mechanism of Xa choice and may allow isolation of the blocking factor hypothesized to interact with this sequence.

Xist Acts in *cis* to Influence Xa Choice

Like a counting element deletion, deletion of a choice element causes skewed X-inactivation in female cells. In contrast to a counting element deletion, however, deletion of a choice element does not result in aberrant X-inactivation in males, since choice elements affect the relative affinity of the *cis*-linked counting element for blocking factor. Thus, mutations that skew X-inactivation in females but do not cause ectopic X-inactivation in males define choice elements.

Xist has been implicated as a choice element. Upon differentiation of female cells heterozygous for *Xist* deletions, only the wild-type X chromosome was ever inactivated (90, 117). There were two possible explanations for this result. Choice could have occurred normally, but the 50% of cells choosing the wild-type X chromosome as the Xa may have died owing to the inability to silence the *Xist* deletion-bearing presumptive Xi. Alternatively, choice may have been completely skewed such that the deletion-bearing X chromosome was always chosen as Xa and thus the wild-type X chromosome would always be inactivated. By distinguishing

between the wild type and deleted *Xist* alleles using FISH probes, it was determined that the wild-type X chromosome was never selected as the Xa in differentiating embryonic cells (89). The effect of this deletion implicates the transcribed region of *Xist* or *Xist* RNA itself as a choice element that reduces the affinity of the *cis*-linked counting element for blocking factor.

Although loss of *Xist* function increased the likelihood of choosing the mutant X chromosome as the Xa, a chromosome with greater *Xist* expression showed a decreased chance of becoming the Xa. In male ES cells, replacement of 2.5 kb of *Xist* upstream sequences with a selectable marker driven by a constitutive *Pgk-1* promoter resulted in an increase in Xist RNA levels (109). The somatic cells of female mice heterozygous for this replacement exhibited skewed X-inactivation with the targeted X chromosome becoming the Xa in only 10–20% of cells. When the *Pgk-1* promoter and selectable marker were removed, random X-inactivation was restored. These results suggest that increased transcription of *Xist* has a negative influence on Xa choice, consistent with the hypothesis that *Xist* RNA decreases the affinity of a *cis*-linked counting element for blocking factor. Since all the factors required for *Xist* RNA-mediated coating and silencing are present in ES cells (164), one mechanism by which *Xist* RNA could negatively influence Xa choice is by causing local coating and silencing, and regulating accessibility of the closely linked counting element for the blocking factor.

Tsix, an Antisense *Xist* Transcript, Positively Influences Xa Choice

In 1999, several groups simultaneously reported the identification of antisense transcription through the *Xist* locus in ES cells (41, 78, 102). The transcript, named *Tsix* in recognition of it being antisense to *Xist,* was found to initiate at a major transcription start site 13 kb downstream of the *Xist* 3′ end, and to extend across *Xist* into its promoter region (Figure 3) (78). Subsequently, a minor *Tsix* promoter has been identified, and mature *Tsix* transcripts of up to 4 kb have been shown to be produced by splicing (139). Like *Xist*, *Tsix* has no significant open reading frame and is not thought to encode a protein.

The expression pattern of *Tsix* yielded some exciting clues about its function. *Tsix* RNA is detected in ES cells and differentiating cells, and not in somatic cells (41, 78, 102), suggesting that its role might be in regulating initiation of X-inactivation. Whenever low-level *Xist* pinpoint expression is observed in male and female ES cells, antisense *Tsix* RNA is detected as an overlapping pinpoint signal (78). Upon differentiation, *Tsix* expression is extinguished from the presumptive Xi concomitant with *Xist* spreading to coat the chromosome (41, 78). Pinpoint expression from the Xa continues for several days and persists longer than pinpoint *Xist* expression (78, 139). *Tsix* shows the same dynamic expression patterns during random X-inactivation in developing embryonic tissues (77, 139). The correlation between *Tsix* expression and unstable *Xist* expression in *cis* led to speculation that *Tsix* might be a negative regulator of *Xist*.

Deletion of the major *Tsix* promoter in female cells dramatically reduced antisense transcription and skewed Xa choice away from the targeted *Tsix* allele (80, 139). In female cells, the X chromosome bearing the deletion was chosen as Xa in at most 4% of cells, but male cells correctly chose a deletion-bearing X chromosome as Xa, demonstrating that *Tsix* is not the counting element. Insertion of a transcriptional stop signal between the *Tsix* promoter and the 3′ end of *Xist*, with (139) or without (85) deleting the major *Tsix* promoter, abolished antisense transcription and caused a similar dramatic skewing phenotype in female cells. Ectopic X-inactivation occurred in a small proportion of male cells (85, 139). Thus, when in competition with a wild-type *Xic*, the chromosome carrying the *Tsix* loss-of-function allele became the Xi in nearly every cell (41, 80, 85, 139). These results suggest that the normal role of *Tsix* RNA, or the process of transcription antisense to *Xist*, is to promote Xa choice by increasing the affinity of the *cis*-linked counting element for blocking factor. Surprisingly, insertion of the constitutive EF-1α promoter increased *Tsix* RNA levels in undifferentiated cells (146) but did not skew X-inactivation, indicating that higher than normal *Tsix* levels cannot further promote Xa choice.

In addition to skewing Xa choice, *Tsix* transcription affects the abundance of *Xist* RNA in *cis*. In cells with the strongest *Tsix* loss-of-function alleles, *Xist* RNA levels were dramatically higher than in wild-type cells (85, 139). In the presence of the allele that retained transcription from the *Tsix* minor promoter, *Xist* RNA levels rose threefold compared to wild type (78). These results are consistent with *Tsix* transcription normally acting to reduce *Xist* RNA steady-state levels. In contrast, increased *Tsix* transcription from the EF-1α promoter insertion did not affect *Xist* RNA levels (146).

Tsix May Act Through *Xist* to Influence Xa Choice

Both mutations that increase *Xist* RNA levels, such as the introduction of the *Pgk-1* promoter (109), and mutations that decrease *Xist* RNA levels, such as an internal *Xist* deletion (89), skew Xa choice. Interestingly, only those *Tsix* alleles that affect *Xist* RNA levels in *cis* also skew Xa choice, suggesting that *Tsix* could act through *Xist* to regulate Xa choice. Indeed, the levels of *Xist* RNA in cells with different *Tsix* alleles correlate with the degree of skewed Xa choice observed upon differentiation (Figure 4) (80, 85, 139, 146). These results are consistent with a model in which *Tsix* transcription acts to reduce the amount of *Xist* RNA in *cis*, thus reducing *Xist*'s negative influence on the likelihood of blocking factor interacting with the counting element.

How could the process of *Tsix* antisense transcription or the *Tsix* antisense transcript itself affect *Xist* RNA levels? If the process of antisense transcription through the *Xist* locus is important, then this process might prevent efficient transcription of *Xist*, lowering the amount of functional *Xist* molecules that are produced. Alternatively, if the antisense RNA is functional, then *Tsix* RNA may actively destabilize *Xist* transcripts or prevent *Xist* RNA from becoming fully active. One possibility

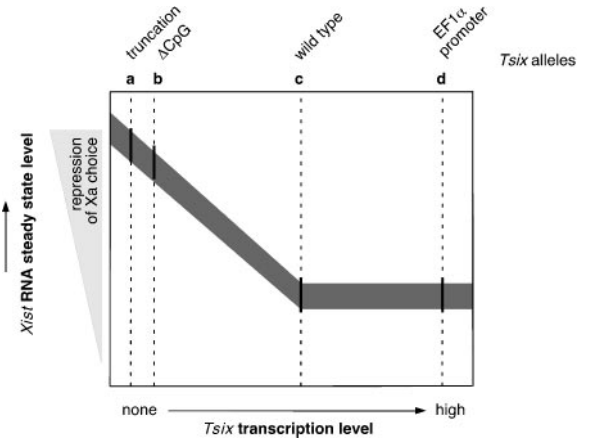

Figure 4 *Tsix* functions through modulation of the *Xist* steady-state level in Xa choice. This figure depicts the effects of *Tsix* transcription on the abundance of *Xist* RNA transcribed in *cis*. The gray band indicates a range of *Xist* RNA steady-state levels achieved by a population of cells bearing the corresponding *Tsix* allele. Four *Tsix* alleles are depicted in order of increasing *Tsix* transcription. *Tsix* alleles are described in the text [allele a (85, 139); allele b (80); allele c, wild type; allele d (146)]. Hypothetically, in a female cell homozygous for a certain *Tsix* allele, each chromosome produces identical steady-state levels of *Xist* RNA in *cis*, and choice occurs randomly. In cells heterozygous for *Tsix* alleles, the allele with lower *Xist* RNA levels will more likely be chosen as Xa; for example, in a cell heterozygous for a wild-type *Tsix* allele (*c*) and a strong *Tsix* loss-of-function allele (*a*), the wild-type *Tsix* allele out-competes the other allele for Xa choice, since the steady-state level of *Xist* is lower on the wild-type allele. This model could be tested by generating female cells heterozygous for these *Tsix* alleles and determining the skewing phenotype.

is that a *Tsix*/*Xist* duplex stimulates a double-stranded RNA-mediated turnover of both transcripts (121). Another possibility is that *Tsix* transcripts interfere with *Xist* RNA folding, *Xist* ribonucleoprotein complex formation, or *cis*-spread along the Xi. Interestingly, the spliced form of *Tsix* RNA contains only 2 kb of overlap with the mature *Xist* transcript (139). This overlap occurs within a domain of *Xist* that is critical for silencing activity (165), suggesting that *Tsix* modulation of Xa choice could involve regulation of *Xist* RNA's silencing activity by affecting its interactions with *trans*-acting factors. ES cells carrying a 65-kb deletion removing both *Tsix* promoters, and possibly the counting element, produced higher levels of *Xist* transcripts that appeared to freely diffuse from the site of transcription (106). Reinsertion of the *Tsix* promoter restored normal transcript levels and re-established pinpoint localization of *Xist* (106). These results are consistent with the possibility that *Tsix* can affect some aspect of *Xist* RNA metabolism that regulates *cis*-spread.

Tsix was the first example of a presumably nonfunctional RNA whose transcription is critical to negative regulation of its functional gene partner. Recently, transcription of another antisense RNA, *Air*, has been shown to be required for regulating imprinted expression of the coding sense gene, *Igf2r*, with which it overlaps (145). As both *Air* and *Tsix* are antisense to the genes they regulate, this may indicate a common antisense transcription or antisense RNA-mediated mechanism for gene regulation in mammals. Antisense transcription appears to be a common phenomenon associated with imprinted genes, and has been detected at the *UBE3A* gene in the Prader-Willi/Angelman syndrome locus (135) and at the *Gnas* locus (83).

Xce Effects May Be Correlated with *Xist* RNA Steady-State Levels

When some divergent mouse strains are crossed, F1 mice exhibit skewing of Xa identity away from the normal 50/50 ratio (56). This skewing is mediated by the X choosing element (*Xce*) locus, which lies downstream of *Xist*, beyond *Tsix* (144). X chromosomes bearing a stronger *Xce* allele are thought to be chosen more frequently as Xa. Interestingly, female somatic tissues from a strain with a strong *Xce* allele showed markedly lower levels of *Xist* RNA than those from a strain with a weak *Xce* allele (14). Indeed, even before differentiation, a female ES cell line heterozygous for *Xce* alleles shows lower levels of *Xist* RNA and somewhat higher levels of *Tsix* transcripts from the stronger *Xce* allele (146). This observation raises the possibility that, like *Tsix*, the *Xce* acts by modulating *Xist* RNA levels in *cis*, either directly or perhaps indirectly through *Tsix*. One study correlated *Xce* strength with methylation levels at *DXPas34* (37), the CpG island located at the major *Tsix* promoter (Figure 3), though differential methylation appeared to follow X-inactivation (122).

ENACTMENT OF THE ACTIVE AND INACTIVE X FATES

After choosing the number of active X chromosomes appropriate to autosome dosage, the next step in establishing proper dosage compensation is to carry out, or enact, the fate assigned to each X chromosome. In addition to the events occurring on the presumptive Xi to achieve inactivation, a distinctly different series of events occurs on the Xa-elect. To protect the Xa-elect from *Xist*-mediated silencing, this chromosome must be shielded from the factors that are required to enact the Xi fate.

An Increase in *Xist* RNA Levels Correlates with Coating and Silencing

The silencing of the presumptive Xi is mediated by *cis*-spread of *Xist* RNA to coat the chromosome (164). Quantitative RT-PCR and slot blot experiments demonstrated a 10- to 20-fold difference in the amount of *Xist* RNA in ES cells versus

female somatic cells (80, 106, 143, 146). These results suggest that an increase in steady-state levels of *Xist* RNA coincides with spreading of *Xist* transcripts in *cis* to coat the X chromosome and mediate gene silencing. Indeed, increased levels of *Xist* RNA in undifferentiated cells can result in premature enactment of the Xi fate (85, 109, 139, 164, 165). The mature *Xist* transcript in somatic cells has a 10- to 20-fold longer half-life than in embryonic cells (113, 143). Thus, an attractive hypothesis is that an increase in *Xist* RNA half-life on the presumptive Xi is a prerequisite for its inactivation.

Multiple Mechanisms Regulate Increases in *Xist* RNA Abundance

The increase in *Xist* RNA half-life that occurs upon differentiation could be achieved by changes in the *Xist* transcript itself or changes in factors that regulate *Xist* RNA stability. Several groups examined whether the increased amount of *Xist* RNA that correlates with onset of X chromosome silencing is caused by production of an alternative transcript possessing higher stability. When analyzed by RT-PCR, the major splicing pattern appeared to be the same in ES cells and female somatic cells (113, 143), suggesting that there are no major changes in processing occurring during Xi-enactment. With an RNA of this enormous size, however, alternative processing may be difficult to detect. An attractive model proposed that *Xist* transcripts might be produced from different promoters in undifferentiated and differentiated cells (69). Transcripts from the somatic promoter P1 might be inherently stable, whereas an alternative embryonic promoter P0, located 6.6 kb further upstream, was suggested to produce unstable transcripts (69). Upon differentiation, a switch from P0 to P1 promoter usage on the presumptive Xi could trigger production of stable *Xist* transcripts, enacting the Xi fate. However, subsequent studies have been unable to detect *Xist* transcription from P0, and transcripts from P1 have been shown to be unstable in ES cells, effectively ruling out the P0 hypothesis (160). The presence of *Tsix* antisense transcription and a highly conserved ribosomal protein pseudogene immediately downstream of the P0 promoter was found to have complicated transcript analysis in this region (133, 160).

Since *Tsix* expression reduces *Xist* RNA levels in *cis* during Xa choice (80, 85, 139), developmentally regulated shutoff of *Tsix* on the presumptive Xi is an excellent candidate for the mechanism of *Xist* RNA stabilization. *Tsix* is shut off as soon as *Xist* transcripts can be seen coating the presumptive Xi (41, 78). Furthermore, when *Tsix* cannot be shut off on the presumptive Xi due to constitutive high-level expression from the EF-1α promoter, *Xist* cannot coat and silence the presumptive Xi (146). Taken in combination, these results suggest that silencing of *Tsix* on the Xi could result in *Xist* RNA stabilization, facilitating its *cis*-spread and X-inactivation. However, lack of *Tsix* transcription does not result in ectopic X-inactivation in the majority of ES cells prior to differentiation (80, 85, 139). Thus, additional developmentally regulated mechanisms contribute to the increase in *Xist* RNA coating and silencing activity on the presumptive Xi upon differentiation.

The increase in *Xist* RNA half-life could be controlled by the developmentally regulated production of *trans* factors that stabilize the RNA. High-level *Xist* RNA produced from the cDNA transgene in ES cells has a half-life equivalent to that of *Xist* RNA in somatic cells and can coat and silence the chromosome in *cis* (164). These results indicate that *Xist* RNA can recruit the factors required for its stabilization in ES cells. However, since *Xist* was expressed at an extremely high level in these experiments, it is still possible that a developmentally regulated stabilizing factor could increase *Xist* activity upon differentiation.

Developmentally regulated activation of *Xist* transcription is also an attractive explanation for the increase in *Xist* activity seen upon differentiation of female cells, since increased *Xist* transcription can enact the Xi fate (109, 165). *Xist* transcription rates in male ES cells and female somatic cells are roughly comparable (113, 143), suggesting that any transcriptional activation at the time of *Xist* RNA *cis*-spread and coating may be minor or transient. Such a minor or transient boost might be sufficient to account for the observed increase in *Xist* RNA half-life on the presumptive Xi. There may be a threshold level of *Xist* RNA that must reached in order to form stable ribonucleoprotein complexes. In theory, *Xist* transcription rates could be tuned precisely relative to the *Tsix*-mediated destabilization rate to limit the amounts of these complexes that assemble in undifferentiated ES cells or even prevent their formation altogether. A slight increase in *Xist* transcription upon entry into the enactment phase could result in assembly of or increase in abundance of functional *Xist* ribonucleoprotein complexes. Since *Xist* RNA can silence very closely linked genes (42), it is reasonable to propose that it could also shut off *Tsix* expression. *Tsix* shutoff by the *Xist* ribonucleoprotein complex would positively reinforce *Xist* activity, since *Tsix*-mediated destabilization of *Xist* transcripts would be eliminated.

Regions Flanking *Xist* Are Implicated in Xi-Enactment

Though many *Xic* transgenes are capable of pinpoint *Xist* expression in ES cells as analyzed by FISH, no single-copy transgene has been found to produce *Xist* RNA that spreads to coat the chromosome in *cis* and mediates silencing upon differentiation (57). Instead, a few days into differentiation, some cells exhibit a faint, dispersed *Xist* signal emanating from the transgene, and all cells ultimately extinguish *Xist* expression. This result suggests that *cis*-elements required to direct increases in *Xist* RNA levels or that facilitate *Xist* RNA spread are missing from these transgenes, or that single-copy transgenes cannot overcome position effects. In contrast, multicopy transgenes as small as 35 kb in size can recapitulate *Xic* function (62), suggesting that duplicate elements present in neighboring copies of the transgene can substitute for endogenous elements missing in a single-copy transgene, or that multicopy transgenes are not as sensitive to position effects (57).

One 80-kb *Xic* transgene containing less than 1 kb of sequence upstream of the *Xist* promoter P1 is notable in that it does not support X chromosome coating and silencing by *Xist* RNA even when present in more than 10 copies (81). The

functioning of other 80-kb transgenes containing 30 kb of *Xist* upstream sequence argues that a critical Xi-enactment element lies in the approximately 30 kb of additional upstream sequence contained in the functional transgene (Figure 3) (81). The minimal region containing this element may be defined by a functional multicopy 35-kb transgene containing 9 kb of upstream sequence (62). A 2.5-kb region containing DNase I hypersensitive sites lies within this candidate region, but its deletion had no effect on X-inactivation, further delineating the location of the *cis*-element required for enactment of the Xi fate (109).

Xa Enactment Employs Several Mechanisms to Repress *Xist* in *cis*

During the period in which stable *Xist* RNA initiates silencing on the presumptive Xi, the Xa-elect continues to express *Tsix* and unstable *Xist* (78, 113, 143). Although *Tsix* destabilizes *Xist* in *cis*, it is not strictly required to block *Xist* RNA-mediated silencing during differentiation since the vast majority of male cells lacking *Tsix* function still enact the Xa fate on their single X chromosome (80, 85, 139). One possibility is that blocking factor functions to prevent the increase in steady-state levels of *Xist* RNA on the Xa-elect, perhaps by interfering with the hypothesized transcriptional activation of *Xist*.

A role for DNA methylation in controlling *Xist* expression during Xa-enactment was suggested by differential methylation of the *Xist* promoter on the Xa and the Xi (111). The *Xist* promoter is methylated on the silent *Xist* allele on the Xa in somatic cells, and less methylated on the *Xist*-expressing Xi in somatic cells and on both X chromosomes in undifferentiated ES cells (6). The phenotype of the *DNA methyltransferase 1* (*Dnmt1*) mutant demonstrated that proper regulation of DNA methylation is involved in enactment of the Xa fate. The *Xist* promoter showed extremely low levels of DNA methylation in undifferentiated male *Dnmt1* mutant ES cells, and did not acquire methylation upon differentiation (6). Mutant male ES cells exhibited normal pinpoint expression of *Xist* RNA. Upon differentiation, these cells showed *Xist* RNA coating and silencing of X-linked genes on the presumed Xa-elect (114). A low but significant percentage of male and female somatic cells in *Dnmt1* mutant embryos also exhibited ectopic X-inactivation (114). These results indicate that DNA methylation is employed in the Xa-enactment mechanism, most likely in repressing *Xist* transcription from the Xa-elect.

An Integrated View of Choice and Enactment

When the data relating to Xa choice and enactment of the Xa and Xi fates are taken together, a coordinated view of these processes emerges. Prior to differentiation, an embryonic cell is uncommitted in its choice of the Xa. Upon differentiation the choice of the Xa is made. During the choice process, the two X chromosomes in a female cell compete for the limiting quantity of blocking factor. Choice elements act in *cis* to influence the counting element's affinity for blocking factor interaction. *Xist* RNA is a choice element, negatively influencing the likelihood

of blocking factor interaction with the *cis*-linked counting element. This negative choice activity may be a local manifestation of the *Xist* ribonucleoprotein complex's silencing activity, affecting the chromatin structure or accessibility of the counting element. *Tsix* transcription, on the other hand, promotes Xa choice, most likely by destabilizing *Xist* RNA and lowering the abundance of functional *Xist* complexes acting on the counting element in *cis*. The blocking factor interaction with the counting element of the Xa-elect represents the first differentiation between the two X chromosomes, allowing the cell to distinguish the Xa-elect from the presumptive Xi.

The Xa and Xi fates are carried out in the enactment phase. To enact the fate of the presumptive Xi, *Xist* activity increases in *cis*. A *trans*-acting factor modulating *Xist* transcription or stability could be regulated by entry into the enactment phase. Even a small boost in *Xist* steady-state levels may lead to an increase in the abundance of functional *Xist* complexes. An increase in *Xist* activity could lead to local silencing of *Tsix*, thus shutting off *Tsix*-mediated destabilization of *Xist* and further increasing *Xist* activity. *Xist* would then be free to spread along the presumptive Xi, silencing genes. Choice as the Xa protects the Xa-elect from this developmentally regulated boost in *Xist* activity; perhaps modification of the counting element by blocking factor does so directly. *Tsix* continues to be expressed on the Xa, contributing to low *Xist* steady-state levels, and DNA methylation may be responsible for turning off *Xist* expression. When the cell exits the enactment window, the active and silent states of the Xa and Xi become irrevocable.

DESIGNATION OF THE ACTIVE AND INACTIVE X CHROMOSOMES IN IMPRINTED X-INACTIVATION

In contrast to random X-inactivation in embryonic tissues, X-inactivation occurs in an imprinted manner in the extraembryonic tissues that support the developing mouse embryo (152). In imprinted X-inactivation, the maternally inherited X chromosome (Xm) always remains active and the paternal X chromosome (Xp) is silenced. Since normal male embryos lack Xp and normal females have a single Xp, this imprinting system results in appropriate dosage compensation. *Xist* and *Tsix* expression patterns during early embryogenesis suggest that differential regulation of both transcripts may be critical to setting the imprint or carrying out the information contained in it. The dynamics of *Xist* and *Tsix* expression are depicted in Figure 5. *Xist* expression begins from the Xp at the onset of zygotic transcription (166) and transcript levels rise quickly during the early cleavage stages (55). By FISH, *Xist* RNA appears to partially coat the Xp (41, 143), which exhibits partial silencing during the cleavage stages (76, 154), but has not yet committed to the Xi fate. On the Xm, a weak pinpoint of *Xist* RNA can be detected (41). While *Tsix* is not expressed from the Xp, it is strongly expressed as a pinpoint signal from the Xm (41, 77). By the late-blastocyst stage of development, when the pluripotent epiblast has reversed *Xist* RNA coating, the extraembryonic lineages have differentiated

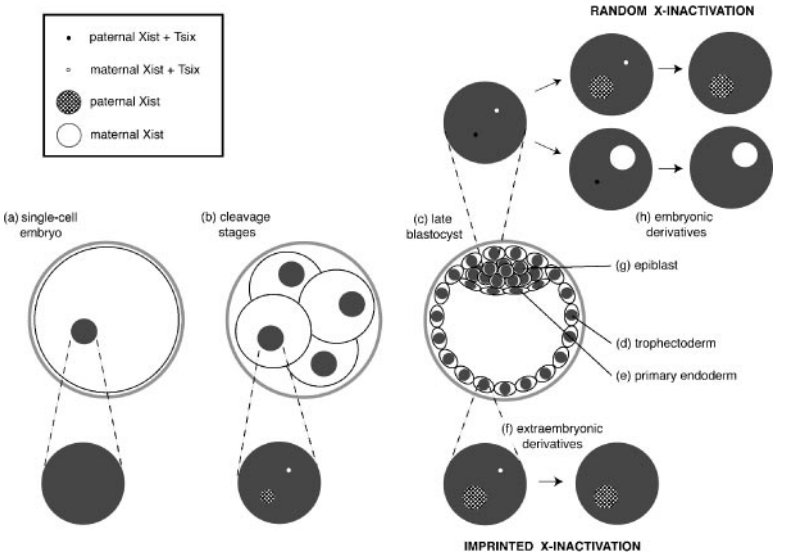

Figure 5 *Xist* and *Tsix* expression during early female mouse development. At the single-cell stage of female mouse embryogenesis (*a*), *Xist* expression is undetectable by RT-PCR or FISH. *Xist* expression commences at the 2-cell stage at the onset of zygotic transcription (166). By FISH, cleavage stage embryos (*b*) exhibit differential biallelic *Xist* expression starting at the 2-cell stage, with *Xist* RNA appearing to coat the Xp at least partially (41, 91, 107, 143), and a weak *Xist* pinpoint signal at the Xm (41). The late blastocyst (*c*) consists of the differentiated extraembryonic lineages, the trophectoderm (*d*) and primary endoderm (*e*), and the pluripotent embryonic lineage precursor, the epiblast (*g*). The trophectoderm and primary endoderm have undergone imprinted X-inactivation by the mid and late blastocyst stages, respectively (151). The Xp (which is now the Xi) has become fully coated by *Xist* RNA (113, 143). After the early embryo implants into the uterus, the extraembryonic tissues derived from the trophectoderm and primary endoderm (*f*) shut off low-level *Xist* expression on the Xm and continue to exhibit *Xist* RNA coating of the Xp throughout subsequent cell divisions. At the late blastocyst stage, the cells of the epiblast have reversed the partial *Xist* RNA coating of the Xp and now exhibit low-level *Xist* RNA pinpoint signals from both the Xm and the Xp (113, 143). Between implantation and completion of gastrulation, epiblast cells differentiate into the embryonic germ layers and undergo random X-inactivation (105, 153, 154). During this time period, *Xist* transcription once again displays differential biallelic expression in the embryonic derivatives (*h*) (113, 143). After completion of gastrulation, embryonic cells cease to express the *Xist* pinpoint signal from the Xa (113, 143). *Tsix* antisense RNA is coexpressed whenever low-level pinpoint *Xist* expression is found (41, 78), and persists from the Xa for a limited period of time after *Xist* RNA shutoff (139).

and the Xp has become completely coated and silenced by *Xist* RNA (113, 143). The Xp in the extraembryonic lineages has now become a committed Xi (151). Thus, high-level *Xist* expression from the Xp and *Tsix* transcription from the Xm correlate with their fates as the Xi and Xa, respectively. *Xist* and *Tsix* expression programmed in the gametes may allow imprinted tissues to bypass the Xa choice mechanism of random X-inactivation.

Xist and *Tsix* Have Opposite, Parent-of-Origin–Specific Effects

Xist deletions are lethal when inherited from the father, but not from the mother. The lethality arising from paternal inheritance of an *Xist* deletion is due to the total lack of X-inactivation in the extraembryonic tissues, indicating that *Xist* is required for imprinted silencing of the Xp (90). As the Xm, which bears a functional *Xist* allele, does not upregulate *Xist* to compensate for the lack of paternal X-inactivation, it appears that the imprint cannot be overridden. *Tsix* promoter deletions have the opposite parent-of-origin–specific effects. *Tsix* deletions are lethal only when inherited from the mother, owing to ectopic *Xist* expression and X-inactivation of the Xm in most extraembryonic cells (77, 139). Therefore, the *Tsix* promoter region, *Tsix* antisense transcription through the *Xist* locus, or the *Tsix* transcript itself is implicated in repression of *Xist* activity on the Xm during imprinted X-inactivation. As expected for genes with opposite effects on the same process, a paternally inherited *Xist* deletion can be rescued by maternal inheritance of a *Tsix* loss-of-function allele (139). The parent-of-origin–specific effects of *Xist* and *Tsix* deletions are limited to the extraembryonic tissues, as X-inactivation occurs normally in the embryonic tissues due to the random choice mechanism. Lethality is due to extraembryonic defects.

A small number of pups inheriting a maternal *Tsix* deletion survive to term, indicating that proper dosage compensation can be achieved in some mutant extraembryonic cells (77, 139). Imprinted X-inactivation is not absolute in extraembryonic tissues, suggesting that the random choice mechanism can function to some extent to establish proper dosage compensation in extraembryonic cells (65). The imprint may normally direct binding of the blocking factor to the Xm in a constitutive manner during imprinted X-inactivation (103). One explanation for occasional maternal X-inactivation in the extraembryonic tissues is that the imprint may be lost at low frequency, allowing the Xm and Xp to compete equally for blocking factor and resulting in random X-inactivation in a subset of cells (65). The ultimate test of the hypothesis that blocking factor is involved in imprinted X-inactivation will require deleting the counting element or knocking out blocking factor activity and testing the effects on imprinted X-inactivation in the mouse.

The Xm Epigenotype Is More Rigid than that of the Xp

Embryos inheriting exclusively paternal X chromosomes or extra maternal X chromosomes have defects in dosage compensation and aberrantly express other

imprinted genes, resulting in embryonic death midgestation. Embryos with exclusively paternal X chromosomes exhibit *Xist* RNA coating of all X chromosomes during the cleavage stages. However, XpXp androgenetic embryos (75, 112) and XpO embryos (91) are progressively able to repress *Xist* expression from the Xp over time and ultimately achieve nearly correct dosage compensation. Correction of dosage compensation in XmXm parthenogenotes by upregulation of *Xist* on at least one X chromosome was highly variable between embryos (75, 91, 107). Whereas XpO embryos had shut off *Xist* expression from the single Xp in nearly 50% of cells by the blastocyst stage, XmXmY embryos activated *Xist* expression from one Xm in at most 10% of cells at this stage (91). The greater lability of the Xp epigenotype compared with the Xm may explain why some embryos survive maternal inheritance of *Tsix* deletions (77, 139), while paternal inheritance of an *Xist* deletion is always lethal (90). *Xist* may be downregulated on the Xp in the former more easily than it may be upregulated from the Xm in the latter. This differential ability to reprogram *Xist* expression patterns on the Xp and the Xm suggests that the imprint controlling X-inactivation is located on the Xm, designating the Xa fate. Thus, as in the choice phase of random X-inactivation, the imprinting mechanism may specifically designate the Xa rather than the Xi. Nuclear transplantation experiments with cytologically marked X chromosomes suggest that the maternal X chromosome's resistance to inactivation is acquired during oocyte growth between prophase of meiosis I and meiosis II, a time when other imprints are placed on the maternal genome (149).

The Xa and Xi Epigenotypes Are Functionally Equivalent to the Xm and Xp

Eggan and colleagues examined X-inactivation patterns in mice cloned by nuclear transfer of female somatic nuclei. Resulting mouse clones exhibited complete nonrandom X-inactivation of the former Xi in the imprinted extraembryonic tissues, whereas X-inactivation was random in embryonic lineages (45). This result indicates that the epigenetic states of the *Xist* alleles on the Xa and Xi in somatic cells are functionally equivalent to the epigenetic states of the Xm and the Xp, respectively. It would be an elegant solution to the imprinting problem if the imprints controlling *Xist* expression in the early embryo were the same as the somatic epigenotypes controlling *Xist* expression from the Xi and the Xa.

Changes in *Xist* Expression During Gametogenesis Correlate with Parental Imprints

Like female somatic cells, the primordial germ cells (PGCs) that will give rise to the germline undergo X-inactivation during gastrulation. However, prior to entry into meiosis midgestation, female PGCs reactivate their Xi (154). This reactivation is accompanied by a loss of *Xist* expression without an apparent restoration of

Tsix expression (107). *Xist* cannot be detected in mature oocytes by RT-PCR, suggesting that transcripts are absent or extremely rare (3, 70). This repression of *Xist* expression during female gametogenesis correlates with extremely low *Xist* expression from the Xm upon fertilization (41, 77).

Male PGCs do not contain an Xi or express *Xist* RNA. During the process of spermatogenesis, however, *Xist* becomes expressed at low levels and is present in mature spermatids (93, 111, 132, 140). By in situ RT-PCR, *Xist* RNA appears to be associated with the heterochromatic sex chromatin body, which consists of the X and Y chromosomes that have been sequestered away from the autosomes during meiosis I of spermatogenesis (4). Even though it is expressed in the male germline, *Xist* is dispensable for spermatogenesis (90), suggesting that *Xist* expression in the male germline may be the result of epigenetic modifications that facilitate *Xist* expression from the Xp early in development.

DNA Methylation Is a Candidate for Establishing or Maintaining the Imprint

Many imprints in mammals require DNA methylation, acquired in the male or female germline, for establishment or maintenance (66). Several groups have searched for differential methylation of the *Xist* gene. In the male germline, CpG dinucleotides at the *Xist* promoter are methylated prior to meiosis, but this methylation is erased during spermatogenesis and is absent in mature spermatids (2, 167) and on the Xp in preimplantation embryos (2, 94, 167). In the female germline, methyl-sensitive restriction analysis suggested that CpGs at the *Xist* promoter and in the first exon become methylated during oogenesis and retain this methylation upon fertilization (2, 167). Using bisulfite sequencing to detect CpG methylation, another group failed to confirm methylation of *Xist* on the Xm in oocytes and early embryos (94). It is therefore unclear whether the *Xist* gene on the Xm is methylated in the early embryo, which would correlate with lack of *Xist* transcription from the Xm. Both male-specific demethylation and female-specific methylation of *Xist* would require reprogramming in germ cells, and suggest that both parental genomes have the potential to acquire epigenetic marks.

Another attractive target for the X-inactivation imprint is the *Tsix* gene. A maternal imprint would direct high maternal *Tsix* expression, whereas a paternal imprint would direct low or absent expression. It has been suggested that the CpG island at the *Tsix* promoter, *DXPas34*, is an imprinting center involved in keeping the *Xist* allele on the Xm silent during early embryogenesis (77). Although *DXPas34* is differentially methylated on the Xa and Xi (37), bisulfite sequencing in oocytes and spermatocytes failed to find any differences in the methylation patterns between the two (122). Binding sites for CCCTC binding factor (CTCF) have been identified in *DXPas34*, the CpG island associated with the major *Tsix* promoter, and CTCF binds to this sequence in vitro (27). Differential methylation of CTCF binding sites has been implicating in establishing the imprint required for expression of *H19/Igf2* gene pair (8, 52, 148). While CpG methylation has

relatively little effect on CTCF binding to *DXPas34* in vitro, non-CpG methylation abolishes CTCF binding in this assay. If CTCF binding occurs in vivo during Xa choice, then searching for an imprint in the form of differential non-CpG methylation at *DXPas34* would be a logical next step.

ACTIVE X CHROMOSOME CHOICE IN HUMANS

Humans and Mice Differ in Developmental Regulation of *XIST/Xist*

In comparison to mouse, little is known about the developmental regulation of X-inactivation in humans. Human *XIST* RNA can be detected by RT-PCR in oocytes and in both male and female preimplantation embryos, increasing in abundance until the blastocyst stage (40, 129). This early *XIST* expression is not exclusively paternal in humans, consistent with a lack of strict paternal X-inactivation in human extraembryonic tissues (54, 134). In addition, both maternally and paternally derived supernumerary X chromosomes are tolerated in humans (88, 123), in contrast to the mouse. These data suggest that X-inactivation does not occur in an imprinted fashion in humans. Thus, the skewing toward paternal X-inactivation detected in some extraembryonic tissue samples (48) could either be a primary effect due to nonrandom Xa choice or a secondary effect due to differential cell survival (99). Given that secondary effects have been demonstrated to be significant in humans (141), this issue can only be addressed by analyzing tissues immediately after X-inactivation.

The Xa Choice Machinery Exhibits Limited Species Conservation

The molecular machinery involved in random choice of a single Xa must be largely conserved between mouse and human. Male mouse ES cells containing 480-kb human *XIC* transgenes exhibited rare cases of *Xist* expression and coating of the single mouse X chromosome upon differentiation (58, 101), suggesting that the human and mouse counting elements can both compete for mouse blocking factor. Surprisingly, human *XIST* is expressed at high levels prior to differentiation of the transgenic ES cells, suggesting that *XIST* is not unstable in undifferentiated mouse cells. Human *TSIX* transcription has been identified in transgenic mouse ES cells as well as in human embryocarcinoma cells and cell lines derived from human PGCs (100). However, the mouse and human *Tsix/TSIX* promoter regions are poorly conserved (78, 100), and the *TSIX* transcript does not appear to traverse the entire *XIST* gene (100). These differences between human *TSIX* and its mouse counterpart could render *TSIX* RNA unable to destabilize *XIST*. RNA FISH for *XIST* and *TSIX* in human preimplantation embryos and ES cells would allow determination of the distribution of these two transcripts in early development and provide insights into their role in Xa choice in humans.

SILENCING AND ALTERATIONS IN CHROMATIN STRUCTURE

Silencing by *Xist* Can Be Divided Into Three Phases

The enactment of the Xi fate requires an increase in the steady-state level of *Xist* RNA on the presumptive Xi. At this time, *Xist* transcripts spread to coat the chromosome in *cis*, and induce gene silencing within 24 hours (164), leading to the formation of extraordinarily stable heterochromatin. Studies employing the inducible *Xist* cDNA in male ES cells suggest that the silencing process can be divided into three steps: initiation, establishment, and maintenance (Figure 6) (164). In the initiation phase, high-level *Xist* expression can cause de novo silencing. Initiation of gene silencing can occur as long as the cells remain undifferentiated and up to 1.5 days after induction of differentiation. In this phase, silencing is reversible, such that *cis*-linked genes can be reactivated if *Xist* expression is extinguished. Extending this result to female development, endogenous *Xist* expression is normally upregulated within one to two days of differentiation in female ES cells (143). The period in which enactment of the Xi fate occurs corresponds to the initiation phase of silencing. A differentiation milestone triggers the establishment phase, which is characterized by the requirement for continued coating by

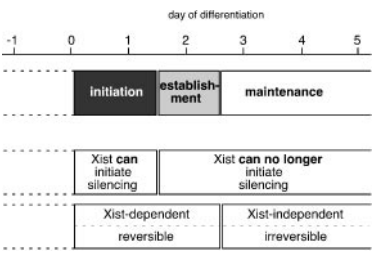

Figure 6 The three steps of the silencing process. The *Xist* RNA-mediated silencing process can be divided into initiation, establishment, and maintenance phases (164). Only during the initiation phase (up to the first 1.5 days of ES cell differentiation), can induction of *Xist* RNA initiate gene silencing. Both the initiation phase and the subsequent establishment phase are characterized by their dependence on coating by *Xist* RNA and the reversibility of the silent state such that continued *Xist* expression is required to prevent loss of transcriptional repression. The maintenance phase is distinguished by the irreversible and *Xist* RNA-independent propagation of silencing, but shares the resistance to *Xist* RNA-mediated initiation of silencing with the establishment phase. The brief 24-hour establishment phase can be envisioned as a window in which the silenced state is locked in. This locking in is tightly linked to ES cell differentiation since it does not occur when silencing by *Xist* RNA takes place in undifferentiated cells. The fact that silencing by *Xist* RNA can be initiated in undifferentiated ES cells is indicated by the extension of the initiation phase to a time before differentiation (−1 days) with dashed lines.

Xist RNA for transcriptional repression and the resistance to de novo silencing by *Xist* RNA. After a defined period of time, which is approximately 24 hours in length, silencing becomes irreversible and *Xist*-independent, demonstrating entry into the maintenance phase. The establishment step may therefore be seen as the process of locking in the silent state, which is then stably maintained.

Silencing Is Accompanied by a Cascade of Chromatin Modifications

The Xi heterochromatin is characterized by a multitude of chromatin modifications that distinguish it from the Xa. The Xi replicates late in S-phase (151), is methylated at promoter CpG islands (163), and hypoacetylated at histones H4, H2A, and H3 (10, 68). In addition, increased histone H3 lysine-9 (H3 Lys-9) methylation and decreased histone H3 lysine-4 (H3 Lys-4) methylation were recently identified as components of the Xi chromatin (12, 59, 97, 119). Each of the above modifications is generally associated with regions of heterochromatin (131), suggesting that X-inactivation employs general mechanisms that are used to regulate gene expression in other contexts. The remodeled Xi chromatin is further characterized by the accumulation of variant histone H2A isoforms termed macroH2A (34). In addition, in human cells, the exclusion of the Barr Body–deficient histone H2A variant (H2ABBD) from the Xi has been reported (26). The function of these H2A variants is unclear.

The appearance of chromatin modifications on the Xi has been catalogued relative to the induction of differentiation, *Xist* RNA coating and gene silencing in female ES cells (Figure 7). *Xist* RNA spread occurs between the first and second days of differentiation and is followed closely by methylation of histone H3 Lys-9,

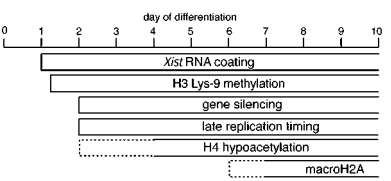

Figure 7 Chromatin modifications on the Xi occur sequentially during the differentiation of female ES cells. The scheme shows the timing of appearance of chromatin modifications that characterize the heterochromatin on the Xi. Solid lines indicate cytological observations (by FISH or immunofluorescence) made when cells were differentiated in embryoid body cultures; the dotted parts of the bars depict results obtained from retinoic acid–induced ES cell differentiation, which appear to place the appearance of modifications at earlier times (59, 73, 96, 127). Once set up, all features of the Xi are stably maintained throughout all somatic cell divisions, as indicated by the open ends of the bars. Although methylation of promoter CpG islands has been observed on the Xi in somatic cells, DNA methylation is not depicted in this figure due to insufficient information on the timing of acquisition of this modification during differentiation.

such that the region of H3 Lys-9 methylation always appears either smaller than or equal in size to the *Xist* RNA domain (59, 97). Concomitant with the appearance of H3 Lys-9 methylation, hypoacetylation of H3 Lys-9 and hypomethylation of H3 Lys-4 on the Xi were observed, indicating that these modifications may be coordinated. These data are consistent with the fact that deacetylation of lysine 9 is a prerequisite for methylation of this residue. The spread of *Xist* RNA and H3 Lys-9 methylation precedes gene silencing and late replication timing, both of which begin on day 2 and are essentially complete around day 4 and day 6, respectively (73). The appearance of H4 hypoacetylation is currently placed either at day 2 or day 4, depending on the differentiation method used; therefore, this modification may occur coincident with or subsequent to gene silencing (59, 73). The accumulation of macroH2A has been detected around day 6 or 7 (96, 127), although one study placed the appearance earlier (126). The timing of the first appearance of promoter CpG methylation has not been analyzed, but has been detected at day 21 post-differentiation (73, 84). When these data are taken together, a sequential progression of chromatin changes appears to underlie the X-inactivation process in female ES cells.

If any one of these modifications is crucial for setting up the silent state of the Xi, then it should be present in the initiation phase of silencing. Using the *Xist* cDNA transgene, the initiation stage can be separated from the differentiation-dependent establishment phase by expressing the *Xist* RNA in undifferentiated ES cells (Figure 6) (164). *Xist* cDNA-induced gene silencing was achieved in undifferentiated ES cells without a shift in the affected chromosomes' replication timing or H4 acetylation status. The relatively early appearance of both modifications during the differentiation of female ES cells suggests, however, that they play a role in the establishment of silencing (73, 164). The co-localization of macroH2A with the Xi takes place only after the transition to the irreversible maintenance phase (127, 164), suggesting that macroH2A does not play a role in establishment. Although it has yet to be shown that modifications on histone H3 occur during the *Xist* RNA-induced initiation phase in undifferentiated ES cells, H3 Lys-9 methylation is the only alteration that appears nearly coincident with *Xist* RNA spread in differentiating female ES cells, implying that modifications of histone H3 are involved in the initiation of silencing (59, 97). Studies to determine whether H3 Lys-9 methylation is necessary for the initation of silencing on the Xi would be of great interest.

A Histone H3 Methylation Hotspot May Be Required for the Initiation of Silencing

A hotspot of H3 Lys-9 methylation, spanning a region of roughly 100 kb 5′ of the *Xist* P1 promoter, was identified by both immunostaining and chromatin immunoprecipitation in undifferentiated female and male ES cells (Figure 3) (59). As ES cells are induced to differentiate, this hotspot gives way to chromosome-wide H3 Lys-9 methylation on the future Xi, while it is retained for up to 5 days on the

Xa-elect. Heard and colleagues proposed that this hotspot of H3 Lys-9 methylation may serve as a nucleation site for the spread of *Xist* RNA along the X chromosome (59). The H3 Lys-9 hotspot is also present on single- and multicopy 480-kb transgenes in undifferentiated male ES cells (59). Upon differentiation, the hotspot disappears from single-copy transgenes more rapidly than associated *Xist*/*Tsix* expression, suggesting that the lack of *Xist* RNA spread from single-copy transgenes may be due to lack of the H3 Lys-9 methylation nucleation center. The absence of a crucial *cis*-element, such as a boundary element that blocks spread of chromatin modifications from sequences flanking the site of transgene insertion, may subject single-copy transgenes to position effects that prevent proper maintenance of the H3 Lys-9 methylation hotspot. Presumably, multicopy transgenes can be shielded from such position effects. Whether or not pinpoint *Xist* expression plays a role in the formation of this hotspot has yet to be tested.

As H3 Lys-9 methylation is a self-propagating modification in other systems (5, 74), it is possible that spread of H3 Lys-9 methylation on the Xi is also self-propagating and does not require *Xist* RNA. Alternatively, *Xist* RNA could simultaneously coat the Xi and propagate H3 Lys-9 methylation along the chromosome by binding nucleosomes that contain H3 Lys-9 methylation, and then inducing this modification on adjacent nucleosomes in a self-reinforcing mechanism. However, H3 Lys-9 methylation per se is not sufficient to mediate *Xist* RNA spread, as *Xist* RNA is absent from regions of constitutive heterochromatin such as centromeres (31, 44), which are characterized by H3 Lys-9 methylation (120). Lys-9 of H3 in constitutive heterochromatin of centromeres is methylated by the histone methyltransferases Suv39h1 and Suv39h2 (120), neither of which is required for H3 methylation on the Xi (119). In addition, the heterochromatin protein HP1, which interacts with H3 methylated on Lys-9 (74), is enriched on centromeric heterochromatin but not on the Xi (119). Thus, facultative heterochromatin of the Xi may employ different histone methyltransferases and methyl-histone binding proteins than constitutive heterochromatin.

MacroH2A Is Recruited to the Xi by *Xist*

The enrichment of macroH2A histone variants on the Xi in female somatic cells revealed the first evidence of differential protein composition between the Xa and Xi (34, 36). MacroH2A1 contains an amino-terminal domain with 64% homology to core histone H2A, which mediates macroH2A's incorporation into nucleosomes as well as its enrichment on the Xi, and a large carboxy-terminal domain of unknown function (24, 116, 118). Two subtypes of macroH2A1 (macroH2A1.1 and 1.2), differing in only a putative leucine zipper within the non-histone domain, are generated by differential splicing of the same gene (116, 125). The third subtype, macroH2A2, is produced from a separate gene and has a histone domain highly similar to that of macroH2A1, but differs slightly throughout the entire non-histone domain (25, 35). Most studies have concentrated on the macroH2A1 subtypes. MacroH2A1 may not only be involved in X-inactivation since it is expressed at

similar levels in male and female ES and somatic cells, is found throughout the entire nucleus, and is located on centrosomes (34, 96, 98, 125, 126). When *Xist* is conditionally deleted from the Xi in female somatic cells (39), or when *Xist* RNA is prevented from coating the Xi (7), macroH2A1 disappears from the Xi. These results suggest that this histone variant is recruited to the Xi in an *Xist* RNA-dependent fashion. A tight association between *Xist* RNA and the Xi chromatin is suggested by the fact that *Xist* RNA co-immunoprecipitates with macroH2A1 and also with core histones (47).

Multiple Mechanisms Contribute to the Maintenance of the Xi

The continued expression and association of *Xist* RNA with the Xi in somatic cells hinted that this RNA plays a role in the maintenance of the Xi (31). However, X-inactivation appears to be stable without continued *Xist/XIST* expression in differentiated cells, suggesting that the RNA is not strictly required to maintain the silent state (19, 38, 39, 124, 164). To investigate a possible role for *Xist* RNA in maintenance, Csankovszki and colleagues generated somatic cells containing an Xi that allowed for the conditional loss of the *Xist* gene (38). The stability of the Xi was analyzed by monitoring the reactivation of two *cis*-linked genes, *Hprt* and a *GFP* transgene. The loss of *Xist* expression on the Xi allowed reactivation of the formerly silent *GFP* and *Hprt* genes in a significant number of cells. Cells generally reactivated only one of the two genes, suggesting that silencing is maintained on a gene-by-gene basis. The approximately 50-fold greater effect of the conditional *Xist* deletion on the endogenous *Hprt* gene compared to the *GFP* transgene suggested that bona fide X-linked genes are more dependent on *Xist*-mediated maintenance of silencing. These data are consistent with the idea that *Xist* RNA coating contributes to the stability of the Xi. When the loss of *Xist* was coupled with treatment by DNA demethylating agents, DNA methyltransferase mutants, and/or deacetylase inhibitors, synergistic reactivation of the *GFP* and *Hprt* genes was observed (38), indicating that multiple redundant mechanisms maintain the silent state of the Xi.

DNA methylation appears to be extremely important for the stability and maintenance of gene silencing on the X chromosome. The use of DNA demethylating agents on human Xi-containing somatic cell hybrids resulted in reactivation of several genes in vitro (104). When similar studies were performed on mouse somatic cell lines, there was a 10- to 20-fold increase in reactivation of X-linked genes (38). A greater effect was seen upon loss of the *Dnmt1* gene from a conditional allele, which resulted in genome-wide hypomethylation. A 1500-fold increase in reactivation of the X-linked *GFP* transgene occurred upon loss of *Dnmt1* activity (38). Similarly, mouse embryos with a mutation in *Dnmt1* cannot maintain the Xi in embryonic tissues, leading to the reactivation of a formerly silenced X-linked *LacZ* transgene in many cells (138).

Inactivation of autosomal genes in cells with X:autosome translocations can be stably maintained without the spread of late replication from the Xi into the translocated autosomal region (142). Therefore, late replication timing is not

absolutely necessary for continued silencing, most likely due to the redundancy in Xi maintenance mechanisms. The importance of late replication timing is only apparent in the absence of CpG island DNA methylation. Humans with ICF syndrome (Immunodeficiency, Centromeric instability, and Facial anomalies syndrome), which is caused by mutations in the de novo DNA methyltransferase *DNMT3b*, can properly establish an Xi even with global hypomethylation at promoter regions of X-linked genes (50, 51). Reactivation of formerly silenced genes in these patients' cells was observed only when regions on the Xi had shifted to an earlier replication timing.

Although delayed appearance of H4 hypoacetylation during differentiation suggests a role in establishment and maintenance of X-inactivation, the use of histone deacetylase inhibitors on female cells did not result in reactivation of the Xi (38, 73). However, when deacetylase inhibitors were combined with treatments that reduce DNA methylation, a twofold increase in reactivation was seen over demethylation treatment alone, indicating that histone hypoacetylation plays some role in the stable maintenance of silencing (38). Considering the importance of proper dosage compensation during development, it is not surprising that multiple mechanisms ensure the stability of the Xi.

Genes that Escape X-Inactivation Lack Chromatin Modifications Characteristic of the Xi

A subset of genes escapes X-inactivation in humans and mice (43). The mechanisms by which X-linked genes can escape *Xist* RNA-mediated silencing remain mysterious. FISH provides insufficient resolution to determine whether escapees are coated by *Xist* RNA. However, it appears that *Xist*/*XIST* RNA does not uniformly coat the X chromosome in mouse and human cells as the RNA is absent from constitutive heterochromatin (31, 44). Chromatin isolated using antibodies directed against histone H3 methylated on Lys-9 contained promoter regions of silenced genes on the human Xi, but not of two X-linked genes escaping silencing (12). Instead, the promoter regions of these escapees could be precipitated using antibodies directed against H3 methylated on Lys-4 (12), consistent with the association of H3 Lys-4 methylation with active regions of transcription and H3 Lys-9 methylation with inactive regions (67). Extending the correlation between escaping genes and modifications associated with actively transcribing regions, the pseudoautosomal region (PAR), which contains a number of genes that escape X-inactivation (43), is methylated on H3 Lys-4 in human cells (12), and acetylated on H4 in both mouse and human cells (68). The human X chromosome region Xp11.2–3, known to harbor escaping genes, and the homologous region A2 in mouse show the same pattern of histone methylation and acetylation as the PAR (11, 68). Furthermore, escapees do not show late replication timing or methylation of CpG islands in their promoter regions (22, 157). Thus, active genes on the Xi are not subject to the same chromatin modifications as silent genes, indicating that there may be *cis*-elements that determine whether *Xist* RNA can mediate silencing on particular regions of the X chromosome.

The genes that escape silencing in humans tend to be found in clusters, suggesting that their expression may be regulated at the level of chromatin domains (23). DNA boundary elements have been demonstrated to separate regions of differentially regulated chromatin (9, 29, 72, 110). Boundary elements could play a similar role in insulating genes that escape X-inactivation from the surrounding heterochromatic environment of the Xi. Another hypothesis is that escaping genes are deficient in sequences promoting *Xist*-mediated silencing. It has recently been suggested that *long interspersed nuclear elements* (*LINEs*) enhance *Xist* RNA spread, since these repetitive elements are enriched on the X chromosome relative to autosomes (87). However, *Xist* cDNA transgenes can coat and silence *LINE*-poor chromosomes (101, 164), indicating that high *LINE* density is not absolutely required for chromosome coating and silencing.

Xi Chromatin Modifications Play Different Roles in the Extraembryonic Tissues

The appearance as well as the importance of Xi chromatin modifications differ in mouse extraembryonic and embryonic tissues, which undergo imprinted and random X-inactivation, respectively. Whereas macroH2A association with the Xi appears to be a late event in random X-inactivation, this histone variant becomes enriched on one X chromosome between the 12-cell stage and blastocyst formation (36). In blastocysts, macroH2A accumulation is mostly restricted to the differentiated extraembryonic cells, suggesting that macroH2A might be involved in the silencing of the Xp in this lineage (36). Aside from *Xist* RNA spread, macroH2A recruitment is the earliest known chromatin modification in extraembryonic tissues. The presence of H3 Lys-9 hypermethylation and hypoacetylation, H3 Lys-4 hypomethylation, and H4 hypoacetylation, chromatin modifications that are associated with the Xi in embryonic lineages, have yet to be examined in preimplantation embryos and the extraembryonic tissues.

From the blastocyst stage until gastrulation, the Xi in the extraembryonic lineages replicates very early in S-phase, before autosomes and the Xa (151). The Xi then shifts to the more familiar late replication timing within a single cell cycle (147, 153). It is unclear why the Xi in the extraembryonic tissues initially replicates very early and subsequently shifts to late replication, although the shift does follow the first appearance of a late replicating Xi in the embryo proper (153).

Whereas DNA methylation is critical for X-inactivation in embryonic tissues, it does not appear to play a major role in extraembryonic tissues. There is less CpG methylation in extraembryonic cells than in those of the embryo proper (28), with genes such as *Hprt* seemingly devoid of CpG methylation compared to their counterparts in the embryonic tissue (84). Consistent with a minor role for DNA methylation in extraembryonic tissues, Xi maintenance in these lineages is unaffected by mutations in *Dnmt1* (138).

Since there is little DNA methylation in extraembryonic tissues, one might expect the maintenance of the extraembryonic Xi to rely more heavily on other

mechanisms. Wang and colleagues showed that mutations in the Polycomb group protein *extraembryonic deficient* (*eed*) cause defects in the development of certain extraembryonic tissues, with females showing a more severe phenotype (159). Further analysis using a paternally inherited X-linked *GFP* transgene showed that *eed* mutants are deficient in maintaining the Xi in the extraembryonic, but not the embryonic tissues (159). Following imprinted X-inactivation, extraembryonic cells were negative for GFP fluorescence. Subsequently, GFP expression reappeared, supporting the idea that these tissues initially underwent X-inactivation, but then failed to maintain it. Since the *eed* protein has been shown to interact with histone deacetylases, and their activity is crucial to *eed*-mediated repression (158), maintenance of the Xi could be more reliant on histone hypoacetylation in extraembryonic than in embryonic lineages. It remains to be determined whether the extraembryonic Xi is hypoacetylated, and if so, whether deacetylation occurs in an *eed*-dependent manner. Even though X-inactivation in the embryo proper was shown to be stable, it would be interesting to challenge embryonic cells from *eed* mutants by compromising an Xi maintenance mechanism to see if *eed* may also play a role in maintaining the Xi in embryonic tissues.

Xist RNA CONSISTS OF FUNCTIONAL MODULES

Xist/XIST RNA Displays a Conserved Repeat Structure

Mouse *Xist* and human *XIST* RNA contain 6 direct repeats, designated *A* through *F*, whose order and sequence are highly conserved (Figure 8). The delineation of these repeats was reinforced and extended by comparison to *Xist* sequences of four common vole species (108). The *Xist/XIST* RNA repeats *A*, *B*, and *F* are highly conserved in copy number, whereas the more complex *C*, *D*, and *E* repeats have been differentially amplified in mouse and human (15, 18, 108). In total, almost half of the *Xist/XIST* transcript is composed of tandem repeats. The strong conservation of repeats contrasts with the overall low homology between mouse *Xist* and human *XIST* RNA, suggesting that the *Xist/XIST* repeats play an important role in the function of the RNA. The differential amplification of *C*, *D*, and *E* repeats in human and mouse suggests that they may be functionally redundant.

Xist Contains Multiple Redundant Domains that Mediate X Chromosome Coating

The inducible *Xist* cDNA transgene approach allowed the first thorough analysis of the requirement of *Xist* RNA sequences for chromosome coating and silencing. In an impressive study, Wutz and colleagues generated 43 *Xist* cDNA constructs containing different deletions, which were individually integrated into the same site on the X chromosome in male ES cells (165). *Xist* expression levels, RNA spread along the X chromosome, and transcriptional inactivation of genes located on the single X chromosome were examined.

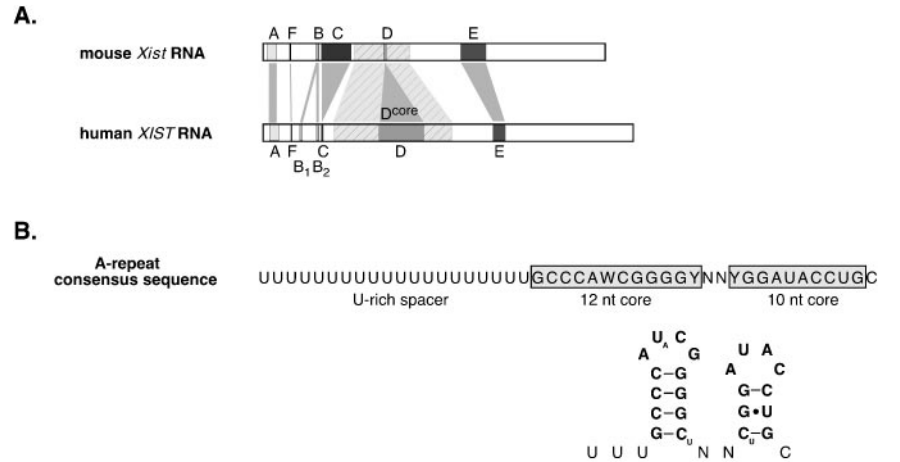

Figure 8 The repeat structure of *Xist*/*XIST* RNA. *A.* Repeats are conserved between human and mouse *Xist*/*XIST* RNA. *XIST*/*Xist* RNA contains six types of repeats, labeled A–F. The E-repeat is located at the 5′ end of exon 6 of *XIST* and at the 5′ end of exon 7 of *Xist*, respectively; all other repeats are contained within the large first exon of *XIST*/*Xist*. The *A*-repeat, located within the first 1000 nt of *XIST*/*Xist* RNA, consists of 8.5 copies of a 43–59 nt unit in the human RNA and of 7.5 copies of a 42–74 nt unit in mouse counterpart [for a more detailed description, see Figure 8*B* (15, 18)]. The short *F*-repeat is located approximately 750 nt downstream of the *A*-repeat (108). It contains two copies of a G/C-rich 10-nt sequence motif (UGGCGGGCUU) separated by 8 nt in mouse *Xist* and 16 nt in human *XIST*. This repeat was identified recently based on its extension to 5 copies in vole *Xist* and may actually function as DNA element (108). Besides the *A*-repeat, the *B*-repeat is the most conserved repeat in *Xist*/*XIST*RNA (15, 18, 108). In the mouse RNA, the low-complexity *B*-repeat is composed of 32 C-rich 4–8-nt units, with 21 of those containing the motif (A/U)GCCCC. In human *XIST* RNA, the B-repeat is split. Twelve C-rich 6–9-nt units, eight of which contain the motif ACCCCCC, are separated by approximately 700 nt of unrelated sequence from 17 C-rich 4–11-nt units, ten of which contain the motif PuPuCCC.

In contrast to the *A*, *B*, and *F* repeats, the three most 3′ *Xist*/*XIST* repeats have been differentially amplified in mouse and human (15, 18). In mouse, the C-repeat contains 14 copies of 120-nt units, which show more than 90% similarity to each other. Only one weakly homologous copy of the *C*-repeat unit can be found in human *XIST* RNA, which, however, is located in the equivalent part of the RNA. In contrast to the *C*-repeat, the *D*-repeat is emphasized in the human RNA (15, 18). The *D*-repeat appears to have the greatest sequence complexity. Recently, the *D*-repeat region of human *XIST* RNA has been extended. The originally described *D*-core region, comprised of seven 300-nt units with more than 75% homology, was shown to be flanked on both sides by truncated copies of the *D*-repeat unit for a total of 19 truncated copies, such that the entire *D*-repeat region encompasses 6000 nt (108). In mouse *Xist* RNA, only 10 partial copies of the *D*-repeat unit were identified within a region of 3000 nt (108). The *E*-repeat encompasses 1350 nt in mouse and 700 nt in human *Xist*/*XIST* RNA, and contains highly variable copies of 20–25-nt units (15, 18, 108).

This analysis allowed designation of functions to certain domains of *Xist* RNA (Figure 9). Large regions, covering roughly the first two thirds of *Xist* RNA, mediate the association of *Xist* transcripts with the Xi, and are functionally redundant such that not all of them have to be present for coating to occur (165). The regions implicated in *Xist* RNA localization contain all of the *Xist* repeats (*A, B, D, E, F,* and at least parts of the *C* repeat; Figure 9). Therefore, it seems likely that all 6 *Xist* repeats are involved in localizing *Xist* transcripts to the Xi, but no single one of them is essential. These observations could be explained if the repeats and surrounding sequences contain low-affinity binding sites for *trans*-acting factors whose occupancy is increased by cooperative interactions, conferring full *Xist* RNA coating function (165). In a different experimental approach, oligonucleotide analogs antisense to *Xist* RNA were used to physically disrupt *Xist* RNA function. By this approach, a highly conserved sequence within the *C*-repeat was found to be important for localizing *Xist* RNA to the Xi, since antisense oligonucleotides to this region induced dissociation of *Xist* transcripts from the Xi in female somatic cells (7) (Figure 9). As this manipulation may have caused a general disruption of *Xist* RNA secondary structure and/or ribonucleoprotein complex formation, these results do not necessarily implicate the *C*-repeat directly in *Xist* RNA localization.

Trans-Acting Factors Required for X Chromosome Coating Are Species-Specific

Interspecies experiments support the idea that *trans*-acting factors are required for the association of *Xist* RNA with the Xi and may give some insights into their nature. In rodent somatic cell hybrids containing a human Xi, *XIST* is highly

←

Figure 8 (*Continued*) *B.* Each *A*-repeat unit forms two conserved stem loops. The consensus sequence of the *A*-repeat unit for mouse and human *XIST*/*Xist* RNA is shown (W = U or A, Y = C or U, (18)). Each *A*-repeat unit contains, within a 25-nt core region, two highly conserved GC-rich motifs of 10 nt and 12 nt, respectively. A variable, primarily U-rich stretch, here depicted as a stretch of 20 uracil residues, serves as spacer between the GC-rich regions of neighboring *A*-repeat units. The length of the spacer (17–49 nt) and its composition vary between individual repeat units, and can be decreased down to 8 residues in every unit without interfering with the function of the *A*-repeat (165). Both the 12-nt and 10-nt GC-rich regions fold into stem loop structures as supported by secondary structure predication and mutational analysis (165). In the secondary structure model of the two GC-rich regions depicted here, nucleotides shown in small font size occur less frequently at the corresponding positions. Mouse *Xist* RNA contains 7.5 copies of the *A*-repeat unit, comprising 8 of the first, larger, and 7 of the second, smaller stem loop; human *XIST* RNA contains 8.5 copies with 9 large stem loops and 8 small ones.

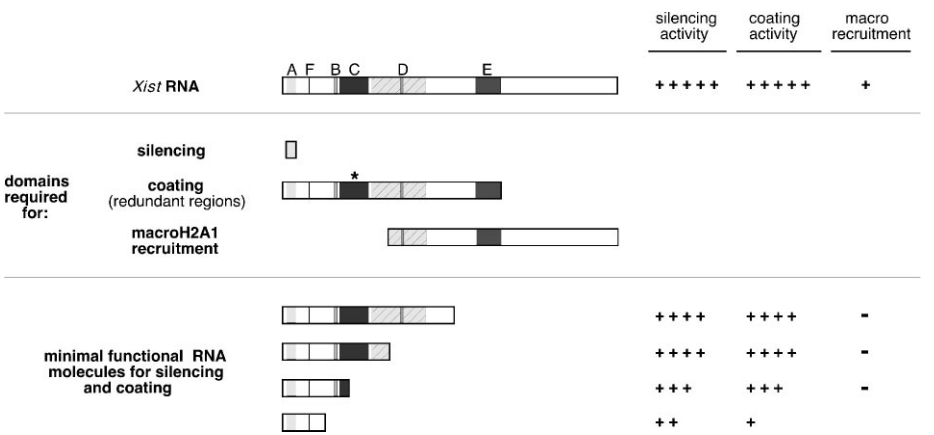

Figure 9 Functional domains of *Xist* RNA. *Xist* RNA is depicted as in Figure 8*A*. Domains required for silencing, chromosome coating, and macroH2A1 recruitment as determined by deletion analysis are shown (165). Deletion of the *A*-repeat region results in an RNA molecule that can coat but not silence the chromosome. The coating function requires multiple partly redundant domains contained within a large sequence block at the 5′ end. A short antisense molecule directed against a highly conserved sequence of the *C*-repeat (indicated by a *star*) completely disrupts localization of *Xist* RNA to the Xi (7).

The deletion study by Wutz et al. (165) defined various large 3′ truncations that for the most part do not interfere with the silencing function of *Xist* RNA. The silencing activity correlates tightly with the coating activity such that on a scale from high (+++++) to no (−) activity, the silencing activity decreases with a reduction of the coating activity. The smaller the minimal *Xist* RNA molecule becomes, the lower the coating activity due to the requirement of multiple domains for coating. MacroH2A1 colocalization is not essential for the initiation of the silencing process, since minimal functional RNA molecules do not recruit macroH2A1 (indicated by + or −).

expressed but not localized tightly to the X chromosome (30). In these cells, some *XIST* RNA is located within the region delineated by the human X chromosome, but it is mostly distributed throughout the rodent nucleus, having a particulate appearance (30, 49). Similar results were obtained when human *XIC* transgenes were analyzed in mouse ES cells. Human *XIST* RNA was found to incompletely coat the mouse autosome carrying the transgene showing a less compact accumulation around the transgenic chromosome (58, 101). Since mouse ES cells contain all the *trans*-acting factors required for chromosome coating by mouse *Xist* RNA (164, 165), it seems that these factors are unable to confer the ability of human *XIST* RNA to coat the chromosome. Thus, factors that mediate localization of mouse *Xist* and human *XIST* transcripts may be species-specific (30). The low degree of primary sequence conservation between mouse and human *Xist/XIST* RNA within regions required for chromosome association, and the differential amplification of

the *C*, *D*, and *E* repeats, could explain the requirement for species-specific factors in *Xist/XIST* RNA localization.

Despite the fact that *XIST* RNA is not properly associated with the Xi in rodent cell hybrids or transgenic mouse ES cells, the half-life, and presumably the steady-state level, of *XIST* RNA is similar to that of *XIST* transcripts in human female somatic cells (30, 49, 58, 101). Therefore, in contrast to localization factors, *trans*-acting factors that stabilize the RNA could be conserved between mouse and human. The hybrid cell data suggest *Xist/XIST* RNA can be stable without chromosomal association. This idea is supported by the fact that *Xist* RNA accumulates at a steady-state level similar to that normally seen in female somatic cells when association with the chromosome is blocked by addition of antisense oligonucleotides (7). While all short *Xist* RNA molecules encoded by cDNA fragments that are able to coat the chromosome are also stable, no fragment that is defective in coating has been shown to be stable (165). Thus, a minimal length of *Xist* RNA may be required to achieve stability.

The A-Repeat Mediates *Xist* RNA's Silencing Function

Wutz and coauthors clearly demonstrated that the silencing function of *Xist* RNA can be attributed to the *A*-repeat region (Figure 9) (165). Deletion of the *A*-repeats in inducible *Xist* cDNA transgenes does not disrupt Xi localization of *Xist* RNA, but abolishes silencing activity. Thus, chromosome coating alone is not sufficient for initiation of X-inactivation (165). When the *A*-repeat region is present, however, coating of the Xi by *Xist* RNA is a prerequisite for silencing (7, 165). Since the *A*-repeat region functions properly even when moved to the 3′ end of *Xist*, it can be viewed as a position-independent silencing module (165). It seems most likely that the *A*-repeat functions by recruiting proteins that mediate silencing.

Wutz and colleagues provide data that support a model in which the highly conserved 25-nucleotide core region of each *A*-repeat RNA unit forms 2 stem loops that are necessary for silencing (Figure 8*B*) (165). Mutations that should disrupt a base pair in the first stem results in the loss of silencing activity. Mutational analysis further showed that the first stem loop, but not the sequence of either the stem or the loop, is essential (165). Detailed analysis of the second stem loop has not been reported. Inversion of the *A*-repeat region in the *Xist* cDNA transgene resulted in production of an RNA containing antisense *A*-repeats that did not confer silencing function (165), indicating some requirement for sequence-specificity of the two stem loops for the silencing function. The length and sequence of the spacer separating the core regions is less important (165). At least 5.5 copies of the *A*-repeat unit, replacing the endogenous *A*-repeat region, are necessary to confer silencing function (165). Higher *A*-repeat copy number improved the efficiency of silencing only slightly. Since no silencing at all is observed with 4 copies, it is very likely that proteins mediating the silencing activity of *Xist*

RNA interact with the stem loops of different *A*-repeat units in a cooperative fashion.

Based on the high degree of conservation of the *A*-repeat sequence between *Xist* and *XIST* RNA, proteins interacting with the *A*-repeat may be conserved between mouse and human. The ability of mouse *A*-repeat-interacting proteins to bind the human *A*-repeat and mediate silencing is suggested by the fact that *XIST* RNA expressed from human *XIC* transgenes in mouse cells can mediate silencing of linked autosomal genes (58, 101). Two groups have identified proteins that interact with the *A*-repeat in vitro. Using an entire *A*-repeat region, hnRNP C1/2 were isolated from nuclear extracts (16). It is perhaps not surprising that two proteins with affinity for polyU stretches, which are highly enriched in the *A*-repeat spacer regions, were isolated. In a second approach, a synthetic RNA oligonucleotide comprising just the first stem loop bound a 120-kD protein of unknown identity (1). Given that the secondary structure rather than the primary sequence of first stem loop is crucial for *A*-repeat function, it is unclear whether specificity will lie within this region of the *A*-repeat. Clearly, identification of the hypothesized *A*-repeat-interacting proteins would provide insight into the mechanism of *A*-repeat-mediated silencing.

Developmental regulation of *A*-repeat-interacting proteins could explain how *Xist* RNA can induce silencing only during the initiation phase of X-inactivation (164). Alternatively, hypothetical *A*-repeat-interacting proteins may be present throughout development, but changes in chromatin structure that occur upon differentiation could interfere with their silencing activity. Of the *Xist* RNA regions required for Xi association, the *A*-repeat region could contribute indirectly to localization, since *A*-repeat-interacting proteins may bind the Xi-chromatin to mediate their silencing function. Thus, the *Xist* RNA domains required for coating and silencing could be non-overlapping. It will also be of interest to determine whether the *A*-repeat is required for *Xist* RNA's role in Xa choice or maintenance of the Xi, which would implicate the silencing activity of *Xist* RNA as crucial in these processes.

Wutz and colleagues found two large deletions in the 3′ half of *Xist* RNA that still allow spreading along the chromosome but are deficient in macroH2A recruitment (165) (Figure 9). The deletion of the *A*-repeat region has no effect on macroH2A localization, indicating that the *Xist* RNA-mediated accumulation of macroH2A on the Xi occurs independently of *Xist* RNA's silencing function. This finding further supported the idea that macroH2A is not involved in the initiation or establishment of silencing but could play a role in the maintenance of the silent state of the Xi (96, 126, 127). It remains possible that the macroH2A histone variants could mediate *Xist* RNA's role in the maintenance of the Xi heterochromatin. *Xist* cDNA transgenes lacking domains required for macroH2A colocalization should be tested for their ability to confer *Xist* RNA's Xi maintenance function.

Taken together, initial analysis of the sequence requirements for *Xist* RNA function indicates that the RNA can be divided into separate domains conferring the activities of chromosome coating in *cis*, gene silencing, and macroH2A

recruitment. The modular nature of *Xist* RNA suggests that the RNA may act as a scaffold to coordinate multiple functions.

CONCLUSION

The different fates taken on by the two X chromosomes in female mammals have long been attributed to the action of a single chromosomal locus, the *X-inactivation center*, which responds to developmental cues orchestrating the X-inactivation process. *Xist* RNA, encoded within the *Xic*, has turned out to be the pivotal player in X-inactivation, as it is both necessary and sufficient for initiation and spread of silencing on the Xi. *Cis*-regulatory elements of the *Xist* gene direct its expression and control the RNA's activity during the period in which the Xa chromosome is chosen. The existence of a critical sequence required to choose an Xa, the counting element, has been inferred, but this sequence remains to be identified. Antisense transcription of *Xist* has recently been identified, generating *Tsix* RNA. Both *Xist* RNA and *Tsix* transcription have been shown to regulate the random choice of the Xa. *Cis*-limited control of *Xist* activity ensures that the Xa will be protected from *Xist* RNA-mediated silencing during the period in which *Xist* activity becomes upregulated on the future Xa. In imprinted X-inactivation, gametic imprints control *Tsix* and *Xist* expression patterns, which direct the maternal Xa fate and the paternal Xi fate. Most advances have been made through the study of X-inactivation in the mouse. Early evidence suggests that the process of Xa choice during human X-inactivation may exhibit some differences.

Silencing of the X chromosome by *Xist* RNA is a multistep process that can only be initiated during a brief developmental window. *Xist* RNA coordinates multiple chromosome-wide chromatin modifications, possibly by recruiting modifiers of chromatin structure. These modifications, characteristic of heterochromatin, stably maintain the silent state of the Xi. Out of all known modifications, methylation on histone H3 Lys-9 spreads on the Xi concomitant with coating by *Xist* RNA, immediately before gene silencing. The spread of methylation on H3 Lys-9, which is involved in transcriptional repression in other contexts, suggests that *Xist* RNA may initiate silencing through recruitment of a histone methyltransferase. Determination of the composition and function of the presumed *Xist* ribonucleoprotein complex will ultimately allow an understanding of how the mammalian dosage compensation system initiates, establishes, and maintains silencing on the Xi.

No *Xic* transgene analyzed to date directs truly random X-inactivation, suggesting that researchers must look beyond the known *Xic* elements, and outside the largest transgene, for sequences that control random choice of the active X and ensure enactment of the inactive X fate. Despite significant advances in the genetics and molecular biology of X-inactivation, many challenges remain before a comprehensive view of the molecular mechanisms directing the two divergent fates of the mammalian X chromosome will emerge.

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CONTENTS