Cell, Vol. 92, 657–664, March 6, 1998, Copyright ©1998 by Cell Press

Role of the *Xist* Gene in X Chromosome Choosing

York Marahrens,* Jan Loring,*
and Rudolf Jaenisch*†‡
*Whitehead Institute for Biomedical Research
Cambridge, Massachusetts 02142
†Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts 02142

Summary

In female mammals a "random choice" mechanism decides which of the two X chromosomes will be inactivated. It has been postulated that *Xist* is crucial for heterochromatinization and thus functions downstream of the choice mechanism. Here we report that females heterozygous for an internal deletion in the *Xist* gene, which includes part of exon 1 and extends to exon 5, undergo primary nonrandom inactivation of the wild-type X chromosome. The *Xist* gene, therefore, not only has a role in chromatin remodeling, but also includes an element required for X chromosome choosing. In conflict with the prevailing view of how choosing occurs, the element identified by the deletion plays a positive role in the choice mechanism and forces a reassessment of how X chromosome choosing is thought to occur.

Introduction

Early in the development of female mammals, one of the two X chromosomes is transcriptionally silenced by heterochromatin formation to ensure equal levels of X-linked gene expression in XX females and XY males, a process called X inactivation. Studies of X-autosome translocations led to the discovery of a region called the X inactivation center (Xic), which must be present in *cis* for an X chromosome to be inactivated (Russell, 1963; Russell and Mongomery, 1965; Rastan and Brown, 1990). Furthermore, the cell must contain at least two Xics for X inactivation to occur (Rastan and Robertson, 1985). Consistent with this notion, a YAC transgene containing 450 kb of sequence from the Xic region has been shown to inactivate an autosome in male cells (Lee et al., 1996; Lee and Jaenisch, 1997b), suggesting that the YAC causes a male cell to behave as if it contains more than one Xic. A similar result has been reported with a cosmid-based transgene (Herzing et al., 1997).

A choice mechanism in female somatic cells determines which X chromosome is inactivated and which X chromosome remains active. X chromosome choosing is thought to be performed at the X-controlling element (*Xce*), which has been mapped to the Xic region and is defined by allelic differences in the likelihood of an X chromosome being chosen for inactivation (Cattanach and Papworth, 1981; Simmler et al., 1993). X chromosomes have an equal chance of being chosen if they carry the same *Xce* allele, while one X chromosome is chosen more frequently than the other if they have different alleles. Skewed X inactivation patterns are also seen in some human females and have been correlated with a point mutation in the promoter of the *XIST* gene (Plenge et al., 1997).

It has been proposed that a diploid cell produces a single negative "blocking" factor that randomly associates with one X chromosome and protects it from inactivation while the "unblocked" X chromosome is inactivated (Rastan, 1983; Penny et al., 1996). The *Xce* locus would, therefore, be the likely target for such a factor, and the allelic differences in choosing could be explained by different affinities of two competing copies of the *Xce* for the single blocking factor. This model also provides a simple explanation for the observation that only one X chromosome remains active in diploid cells with more than two X chromosomes (Grumbach et al., 1963).

In contrast to the random X inactivation in somatic tissues, an unidentified mark, called an imprint, always causes the extraembryonic tissues of rodents to inactivate the paternal X chromosome (Takagi and Sasaki, 1975). Imprinting renders the maternal X chromosome resistant to X inactivation as parthenogenetic embryos, which have two maternal X chromosomes, fail to inactivate an X chromosome in extraembryonic cells (Kaufman et al., 1978; Rastan et al., 1980; Endo and Takagi, 1981; Tada and Takagi, 1992). Similarly, mice that inherit an extra X chromosome due to the Robertsonian translocation Rb(X.2)2Ad (Adler et al., 1989) die during embryogenesis if the supernumerary X chromosome is of maternal origin (Tada et al., 1993). It is not known whether imprinted and random X chromosome selection are related.

The *Xist* gene has also been mapped to the Xic region (Borsani et al., 1991; Brockdorff et al., 1991; Brown et al., 1991) but appears to be genetically separable from the *Xce* (Simmler et al., 1993). The *Xist* gene has been shown to be essential for X inactivation in cultured cells (Penny et al., 1996) and in mice (Marahrens et al., 1997). *Xist* encodes a 15 kb untranslated RNA that is stabilized during X inactivation and coats the inactive X chromosome, suggesting a structural role for the RNA in heterochromatin formation (Clemson et al., 1996; Panning et al., 1997; Sheardown et al., 1997). An X chromosome with a large deletion in the *Xist* gene but with an intact *Xce* can be chosen for X inactivation but fails to become inactive, demonstrating that *Xist* is required for heterochromatin formation and functions downstream of the random choice mechanism (Penny et al., 1996). This experiment was performed in a female ES cell line that maintains two active X chromosomes in the appropriate cell culture conditions but undergoes random X inactivation when induced to differentiate.

We previously derived a line of mice that carries a deletion in the *Xist* gene that extends 3′ of the deletion used in the aforementioned cell culture studies (Marahrens et al., 1997). Female pups that inherited the mutation from their fathers were severely growth retarded

‡To whom correspondence should be addressed.

and died during embryogenesis. The lethality was due to a complete failure to undergo X inactivation in the imprinted extraembryonic tissues (Marahrens et al., 1997). The mutant paternal X chromosome was never inactivated, indicating that *Xist* is required for heterochromatin formation. The wild-type maternal X chromosome also remained active in every cell, indicating that the imprinted control of X chromosome choosing in extraembryonic tissues was undisturbed by the mutation. *Xist*, therefore, also functions downstream of the imprinted selection mechanism (Marahrens et al., 1997).

In contrast to paternal inheritance, female pups, heterozygous for a maternally inherited *Xist* mutation, were healthy with the wild-type X chromosome inactive in every cell (Marahrens et al., 1997). In view of the evidence that *Xist* functions downstream of the random choice mechanism (Penny et al., 1996), we considered it likely that the mutant epiblast had generated both balanced cells with an inactive wild-type X chromosome and unbalanced cells with two active X chromosomes at the time of X inactivation, as was seen for the more 5′ deletion (Penny et al., 1996). The unbalanced cells had presumably then been selected against and lost from the mutant embryos.

The loss of cells that express both copies of X-linked genes has been shown to occur in embryos that inherit both products of Searle's X-autosome translocation (T(X;16)16H) from their mothers (McMahon and Monk, 1983). In these embryos, random choice results in either the inactivation of the wild-type X chromosome, resulting in balanced cells, or in the inactivation of the translocation product with the Xic, resulting in unbalanced cells. The cells that inactivated the translocation product are then progressively lost from the embryo (McMahon and Monk, 1983). Despite this massive cell loss, the embryos develop into healthy, but small, newborn pups (Lyon et al., 1964).

If the $Xist^{+/-}$ mice undergo random choice, then one-half of the cells should fail to inactivate an X chromosome, resulting in cell loss and reduced birthweights. In the present study, we report that $Xist^{+/-}$ females undergo primary *non*random inactivation of the wild-type X chromosome in every cell. These surprising findings are incompatible with the leading models for X inactivation and force a reassessment of how X inactivation is thought to occur.

Results

We have previously shown that female mice, heterozygous for a maternally inherited *Xist* mutation, are healthy with the wild-type X chromosome inactive in every cell (Marahrens et al., 1997). Since *Xist* has been shown to function downstream of the random choice mechanism (Penny et al., 1996), we had assumed that the mutant epiblast had undergone random X chromosome choosing and subsequently lost the cells that had chosen, but failed to inactivate, the *Xist*-deficient X chromosome. Loss of cells that express both copies of X-linked genes has been shown to occur in embryos that inherit both products of Searle's X-autosome translocation from their mothers. In these embryos, no significant cell loss has occurred at embryonic day 7 (E7), about one day following X inactivation, but approximately three-quarters of the genetically unbalanced cells are lost between E7 and E8. By E12.5 no unbalanced cells remain (McMahon and Monk, 1983). Despite this massive cell loss, the embryos develop into healthy newborn pups that can be distinguished from their wild-type littermates by their small size (Lyon et al., 1964). Newborn females heterozygous for Searle's translocation are 79% the weight of their wild-type littermates at birth (Figure 1A) (Lyon et al., 1964).

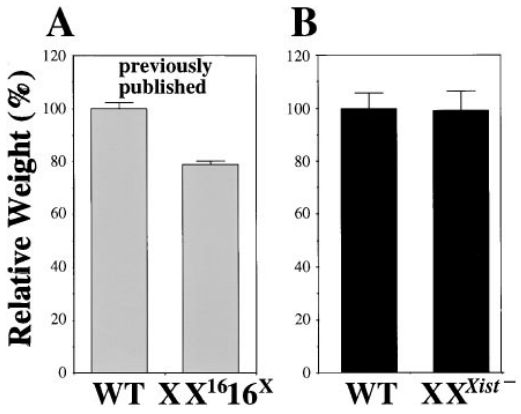

Figure 1. Weights of Newborn Mice

The average weights and standard deviations of (A) 14 newborn females heterozygous for Searle's translocation T(X;16)16H) and 14 normal sibs and (B) 10 newborn $Xist^{+/-}$ females and 31 wild-type littermates are shown. The data in (A) were previously published in a different form by others (adapted from Lyon et al., 1964) and are shown for comparison with the experimental data. Values in each panel are in relation to the average weight of the wild-type littermates, which was assigned the value of 100%. To eliminate the variability in the average weights of the normal pups from different litters, the weight of each pup in a litter was divided by the average weight of all the wild-type pups of that litter and expressed as a percent value. The averages and standard deviations of the percent values assigned to the $Xist^{+/-}$ pups and the wild type (WT) from all the litters are shown. The average weights of newborn $Xist^{+/+}$ females, $Xist^{+}$ males, and $Xist^{-}$ males were virtually identical and all were considered wild-type mice. The T(X;16)16H females were Xce^{a}/Xce^{a}.

In contrast, newborn $Xist^{+/-}$ females were exactly the same weight at birth as their sibs (Figure 1B). This surprising observation suggested that the $Xist^{+/-}$ embryos had retained all of their epiblast because they had chosen to inactivate the wild-type X chromosome in every cell (primary nonrandom X inactivation). The alternative possibility, that the $Xist^{+/-}$ embryos had attempted to undergo random X inactivation and consequently lost one-half of their epiblast, requires the $Xist^{+/-}$ embryos to have experienced compensatory growth that resulted in normal birthweights. This compensatory growth would have to be quite substantial because the *Xist*-deficient X chromosome carries the Xce^{a} allele, which provides the maximum likelihood of being inactivated. Embryos heterozygous for the Searle's translocation rapidly lose three-quarters of their unbalanced cells between E7 and E8 (McMahon and Monk, 1983) and do not balance the reduction in embryo size with compensatory growth during in utero development (Lyon et al., 1964). The loss of

unbalanced epiblast from *Xist*$^{+/-}$ embryos would probably also be rapid and result in *Xist*$^{+/-}$ embryos that are smaller than their wild-type littermates at E8.5 or E9.5. However, a careful comparison of ten *Xist*$^{+/-}$ embryos and their wild-type littermates at E7.5 (2 mutant embryos, 5 wild-type littermates), E8.5 (6 mutants, 19 wild-type littermates), and E9.5 (2 mutants, 7 littermates) led us to conclude that *Xist*$^{+/-}$ embryos were not smaller than the wild-type embryos from the same litter (Figures 2A–2C).

The observation that the *Xist*$^{+/-}$ embryos had maintained the same size as wild-type embryos following X inactivation provided further indication that *Xist*$^{+/-}$ embryos underwent primary nonrandom X inactivation. Nevertheless, it could not be ruled out that X chromosome choosing was random in *Xist*$^{+/-}$ embryos and that the loss of unbalanced epiblast had been gradual, allowing simultaneous compensatory growth to maintain the embryos at a normal size at all times. In Searle's translocation embryos, which rapidly lose the cells expressing a double dose of X-linked genes, unbalanced epiblast cells are abundant at E7 and E8 but undetectable at E12.5 (McMahon and Monk, 1983). A more gradual loss of cells from *Xist*$^{+/-}$ embryos would require the unbalanced cells also to be readily detectable at E7 and E8.

To determine directly whether *Xist*$^{+/-}$ embryos retain cells with two active X chromosomes shortly after X inactivation, we employed fluorescence in situ hybridization (RNA FISH) to assess the X inactivation status of the individual cells of *Xist*$^{+/-}$ embryos. Prior to X inactivation (at about E6), an unstable *Xist* transcript is expressed from both X chromosomes and can be readily visualized as two dot-like RNA signals, one at each *Xist* locus, using FISH (low-level biallelic *Xist* expression pattern, Figure 3B) (Panning et al., 1997; Sheardown et al., 1997). Male cells also express an unstable transcript at this time, which is visible as a single dot (low-level monoallelic). During X inactivation, *Xist* RNA from one allele is stabilized and coats the X chromosome (differential biallelic pattern, Figure 3B) (Panning et al., 1997; Sheardown et al., 1997). The *Xist* gene on the active X chromosome is subsequently silenced (high-level monoallelic pattern, Figure 3B). At E7.5, approximately 15% of wild-type female somatic cells display the differential biallelic *Xist* expression pattern, while 85% of the cells show high-level monoallelic expression (Panning et al., 1997; data not shown). Also, about 15% of wild-type male E7.5 embryos still express an unstable transcript (a single dot-like pattern) (Panning et al., 1997; data not shown). These results indicate that X inactivation is complete at E7.5 but that expression of the unstable transcript has not yet been silenced in all male or female cells.

A probe that recognizes both the wild-type and the truncated *Xist* RNAs (full probe, Figure 3A) revealed that E7.5 *Xist* male embryos, used as controls, displayed a low-level monoallelic expression pattern (Figure 4A) that was indistinguishable from the *Xist* expression seen in wild-type males and is in agreement with previous results (Marahrens et al., 1997). Also consistent with previous reports, X inactivation was complete in wild-type female embryos that displayed high-level monoallelic or

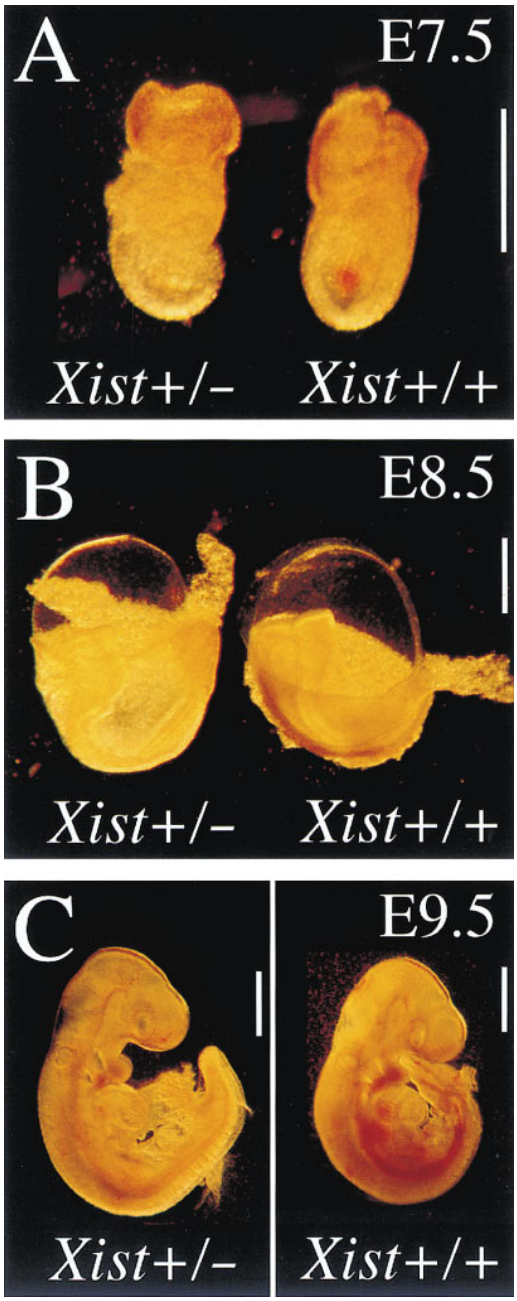

Figure 2. Phenotypic Comparison of *Xist*$^{+/+}$ and *Xist*$^{+/-}$ Female Littermates at 7.5, 8.5, and 9.5 Days of Gestation

(A) E7.5 *Xist*$^{+/+}$ conceptus (right) and a *Xist*$^{+/-}$ littermate; (B) E8.5 *Xist*$^{+/+}$ embryo (right) and a *Xist*$^{+/-}$ embryo with the amnion left intact and the yolk sac partially removed from each embryo; (C) E9.5 wild-type (right) and mutant (left) conceptuses. Bar scale, 0.5 mm.

differential biallelic expression in every cell (Figure 4C). We have previously observed in extraembryonic tissue that the truncated *Xist* RNA cannot coat and inactivate an X chromosome (Marahrens et al., 1997). Consequently, cells that had chosen the mutant X chromosome should *not* display the large signal expression pattern seen in high-level monoallelic– and differential biallelic–expressing cells. High-level monoallelic or differential biallelic (added together: 100%, 283/283 cells)

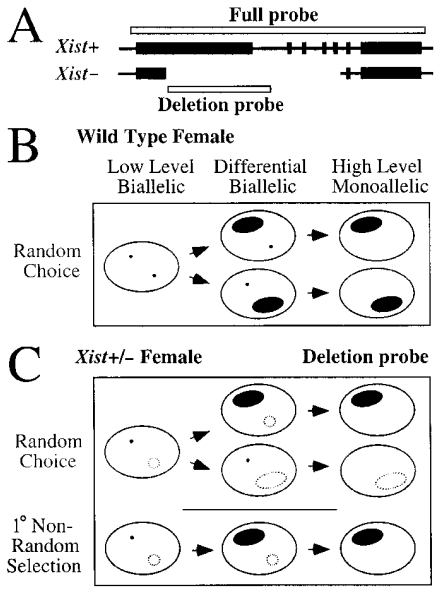

Figure 3. Expected Patterns of *Xist* Expression

(A) Map of wild-type (*Xist*$^{+}$) and mutant (*Xist*$^{-}$) alleles of the *Xist* gene and the probes used.

(B) Pattern of *Xist* expression observed by RNA FISH in female cells during development.

(C) Expected *Xist* expression patterns using the deletion probe in *Xist*$^{+/-}$ embryos if random choosing or primary nonrandom inactivation of the wild-type X chromosome occurred. The dashed circles represent the RNA signals that are absent because one X chromosome is not wild-type.

expression was observed in all cells of *Xist*$^{+/-}$ embryos (Figure 4D), indicating that the full-length *Xist* RNA was coating the wild-type X chromosome and that the mutant X chromosome was never chosen.

To confirm that *Xist*$^{+/-}$ embryos undergo primary nonrandom X inactivation, RNA FISH was also performed on the cells of E7.5 embryos using a second probe that recognizes the wild-type, but not the mutant, *Xist* RNA (deletion probe, Figure 3A). When the deletion probe is hybridized to cells from *Xist*$^{+/-}$ embryos, the pattern predicted by the random choosing of either X chromosome is different from the pattern predicted by the primary nonrandom selection of the wild-type X chromosome (Figure 3C). If the *Xist*-deficient X chromosome is chosen at random in one-half of the cells, then a low-level monoallelic signal should be seen, using the deletion probe, in cells that had selected the mutant X chromosome but had not yet silenced the wild-type *Xist* locus (Figure 3C). Alternatively, if the wild-type X chromosome had undergone primary nonrandom X inactivation in every *Xist*$^{+/-}$ cell from an E7.5 embryo, all cells should display a High Level Monoallelic signal (Figure 3C). As expected, a single dot-like pattern was seen in male wild-type E7.5 embryos (not shown), demonstrating that the deletion probe reliably detects low-level *Xist* expression. Also as expected, no *Xist* signal could be detected in male *Xist*$^{-}$ E7.5 embryos (Figure 4B). A probe recognizing the *Pgk-1* transcript was used as an internal control in these cells to show that the absence of an *Xist* signal was not due to a technical failure (Figure 4B).

Cells from five female wild-type E7.5 embryos produced both the differential biallelic (17%, 47/279 cells) and high-level monoallelic (83%, 232/279 cells) expression patterns at the expected frequencies. The hybridization of the deletion probe to cells from eight *Xist*$^{+/-}$ E7.5 embryos produced exclusively the high-level monoallelic signal (99%, 466/470 cells) and a complete absence of a low-level monoallelic signal (0/470 cells, Figure 4E). We therefore conclude that the *Xist*$^{+/-}$ embryos undergo primary nonrandom inactivation of the wild-type X chromosome.

Discussion

We have examined female embryos and newborn mice carrying a deletion in one of the two copies of the *Xist* gene and determined that every somatic cell had nonrandomly inactivated the wild-type X chromosome. The absence of random choice in the *Xist*$^{+/-}$ cells indicates that the *Xist* gene is required for X chromosome choosing. The observation that this X-linked defect results in the primary nonrandom inactivation of the other, wild-type X chromosome indicates that a positive element that is required by an X chromosome for it to be chosen for inactivation had been deleted. This contradicts the prevailing view of a diploid cell producing a unique negative factor that binds one of the two X chromosomes and blocks its inactivation (Rastan, 1983; Penny et al., 1996). The deletion of the binding site of a negative factor would result in the other, wild-type X chromosome always being blocked and active. Since our deletion has the opposite effect—the wild-type X chromosome is always inactivated—we have located a positive element that is recognized by a factor that initiates heterochromatin formation.

Two Roles for *Xist* in X Inactivation

Our results provide the first demonstration that the *Xist* gene is required for the choice step in X inactivation. Two independent studies had previously shown the *Xist* gene to be required for heterochromatin formation. In one study, female (XX) ES cells, carrying a targeted deletion in one of the two copies of the *Xist* gene, produced two populations of cells when induced to undergo random X inactivation: cells with an inactive wild-type X chromosome and cells with two active X chromosomes (Penny et al., 1996). The relative proportions of the two targeted cell populations accurately reflected the influence of the *Xce* locus, indicating that the random choice mechanism was not disturbed by the mutation (Penny et al., 1996). In another study, we generated mice carrying a different deletion in the *Xist* gene and showed that the deletion abolished heterochromatin formation in the extraembryonic lineage but did not disturb the imprint that controls X chromosome selection in this tissue (Marahrens et al., 1997). Since, in contrast, random choice in the somatic lineage is disrupted by our mutation, our results indicate that random and imprinted X chromosome selection are distinct processes.

The contrast between our results, which indicate that the *Xist* gene is required for random choosing, and the results of Penny et al. (1996), which indicate that random

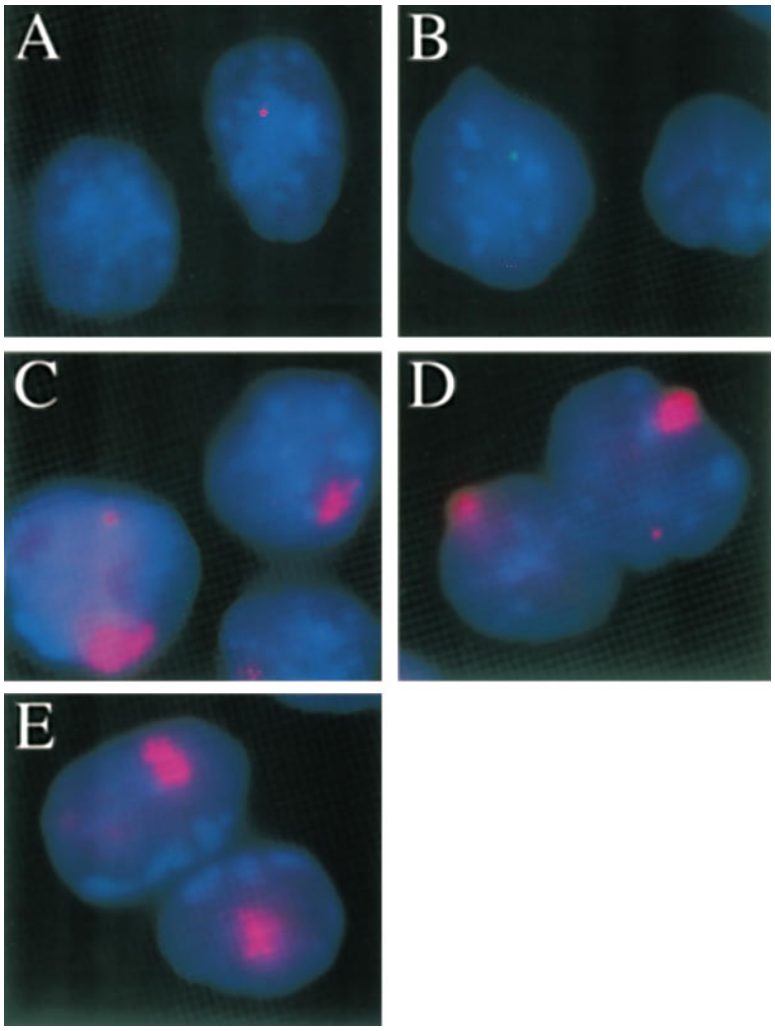

Figure 4. *Xist* Expression in Cells From Female *Xist*$^{+/-}$ Embryos at 7.5 Days of Gestation

Fluorescence in situ hybridization was performed for *Xist* (pink/red) and *Pgk-1* (green) RNA. The full probe was used in (A), (C), and (D); the deletion probe was used in (B) and (E). A probe for *Pgk-1* was also used in (B). (A) *Xist*$^{-}$ male; (B) *Xist*$^{-}$ male; (C) *Xist*$^{+/+}$ female; (D) *Xist*$^{+/-}$ female; (E) *Xist*$^{+/-}$ female.

choosing is unaffected by a large deletion in the *Xist* gene, may lie in the difference in the two deletions. The targeted deletion of Penny et al. extends into the promoter, abolishing transcription, and in addition removes the first 7 kb of exon 1. The deletion used in our study extends from exon 1 to intron 5, leaving all transcription regulatory elements intact. Therefore, the two nonidentical deletions define a 6 kb region that is indispensable for the random choice mechanism. Importantly, the results of Penny et al. indicate that random choosing occurs in the absence of *Xist* transcription, arguing that X chromosome choosing, as defined by our work, does not depend on *Xist* RNA per se but rather on a DNA element that may be located in one of the deleted introns.

Mechanism of Choice

The simplest way to explain the X inactivation patterns seen in wild-type cells and in the two different *Xist* mutants is to postulate that a diploid female cell produces a single initiation factor that binds and inactivates one X chromosome (Figure 5A). According to this model, the unique initiation factor would be unable to recognize a mutant *Xist* allele that is missing the choice element (for example, our deletion) and consequently would always bind and inactivate the wild-type X chromosome. However, a deletion that does not disrupt initiation factor binding but impairs heterochromatin formation (for example, the deletion reported by Penny et al.) would result in both cells with two active chromosomes and cells with an inactive wild-type X chromosome.

A model introducing the concept of a positive initiation factor must explain the observation that diploid female cells carrying supernumerary X chromosomes are capable of inactivating more than one X chromosome. X inactivation in mammals follows the "n-1 rule" (Lee and Jaenisch, 1997a), which dictates that a single X chromosome remains active per diploid set of autosomes: diploid XXX individuals have two inactive X chromosomes and tetraploid (2n) female cells inactivate two X chromosomes and keep two X chromosomes active. Therefore, diploid XXX individuals must produce two initiation factors and XXXX individuals three. A counting mechanism based upon a limiting factor that chooses one X chromosome for inactivation may break down in cells with large numbers of X chromosomes or many copies of an Xic transgene (Lee et al., 1996).

A more complex model for X chromosome choosing,

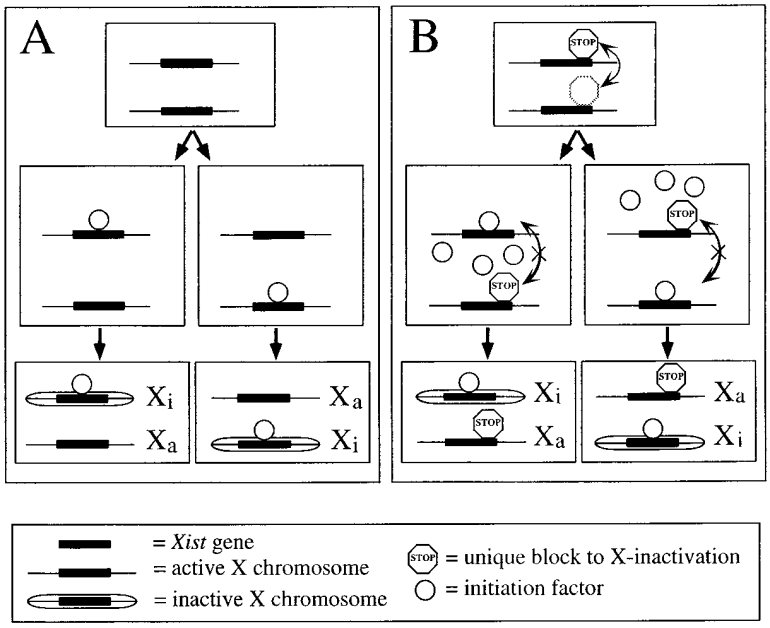

Figure 5. Two Models to Explain X Chromosome Inactivation in Wild-Type and Mutant Female Cells

In both models, the induction of X inactivation is marked by the appearance of a positive initiation factor (circle). The binding of this factor to a "choice element" present on either chromosome determines which chromosome will inactivate and also initiates heterochromatin formation.

(A) In this model, the initiation factor is present in a single copy per cell choosing only one X chromosome for inactivation. When the binding site within the *Xist* transcribed region is deleted from one X chromosome, only the wild-type X chromosome can be selected.

(B) In this model, a unique blocking factor (stop sign) binds reversibly to the X chromosome and switches back and forth between the two X chromosomes. At the time of X inactivation, the initiation factor is expressed and binds all X chromosomes not occupied by the blocking factor. Because binding of the initiation and blocking factors is mutually exclusive, this "locks" the fate of each X chromosome. When the binding site for the initiation factor is deleted from one X chromosome, all cells end up with the blocking factor on the mutant allele.

presented in Figure 5B, would faithfully preserve one active X chromosome in cells with a high number of X chromosomes. According to this model, X chromosome choosing is performed by *two* different factors that select the active and inactive X chromosomes by mutually exclusive binding to the X chromosome. A cell would produce a unique blocking factor that binds the *Xist* gene but switches back and forth between the two X chromosomes. The initiation factor, which is not limiting, would bind all X chromosomes not occupied by the blocking factor. The results seen with the two *Xist* mutations can be easily explained if binding of initiation factor and blocking factor is mutually exclusive. Deletion of the binding site for the initiation factor, as in our mutation, would force the initiation factor to bind the wild-type *Xist* gene, setting the X inactivation pattern. Note that the initiation factor can only bind *Xist* once the blocking factor has switched over to the mutant X chromosome. The deletion in the *Xist* gene 5′ of the choice element would result in cells that have the wild-type X chromosome inactive and the mutant X chromosome active as well as cells with both X chromosomes active. This model predicts that the n-1 rule can be faithfully maintained regardless of X chromosome or Xic transgene number (Lee et al., 1996).

In both of the models presented above, the element we have identified may represent the nucleation site at which heterochromatin formation initiates during X inactivation. The protein(s) that recognizes this element may function by stabilizing the *Xist* transcript (Panning et al., 1997; Sheardown et al., 1997), resulting in heterochromatin formation. In this scenario, the influence of the nearby *Xce* locus, which can skew X inactivation, might be that of an enhancer that influences the binding of the initiator to *Xist*. However, *Xist* has so far only been distinguished from the *Xce* by a single crossover (Simmler et al., 1993), and the two may yet prove to be synonymous. Interestingly, a polymorphism has been identified in the human *XIST* promoter that correlates with the severely skewed X inactivation patterns seen in some families (Plenge et al., 1997). Detailed mutagenesis is necessary to clearly define the functional elements. The identification of the factors that bind these elements should clarify the molecular mechanisms underlying X inactivation.

Experimental Procedures

Characterization of Pups, Isolation of Embryos, Preparation of DNA, and PCR Reactions

Female mice with a maternally inherited mutation in the *Xist* gene (Marahrens et al., 1997) were mated to BALB/c males, and pups were weighed and toe clipped the day they were born. Tail DNA was obtained from each pup, and the gender and presence of the mutant *Xist* allele was determined using the polymerase chain reaction. The PCR primers used to detect the *neo* gene amplify a 477 bp sequence and are neo1 (5′-ATTGA ACAAG ATGGA TTGCA C-3′) and neo2 (5′-TTCGT CCAGA TCATC CTGAT CGAC-3′). Primers used to detect the *Zfy* gene amplify a 618 bp fragment and are Zfy1 (5′-GATAA GCTTA CATAA TCACA TGGA-3′) and Zfy2 (5′-CCTAT GAAAT CCTTT GCTGC-3′). PCR reaction conditions were as previously described (Beard et al., 1995). Embryos were dissected from the uterus as described (Hogan et al., 1994). Color micrographs were taken with a Zeiss DS dissecting microscope, a Zeiss M35 camera, and Kodak Ektachrome Elite 200 film. DNA was isolated from the embryos as described (Beard et al., 1995). PCR primers neo1 and neo2 were used to detect the *neo* gene and primers Zfy1 and Zfy2 were used to detect the *Zfy* gene in the embryonic DNA preparations as previously described (Marahrens et al., 1997).

Slide Preparation and FISH

Embryos were dissected from the uterus and dissociated into individual cells using trypsin, affixed to slides using a cytospin apparatus (Shandon), and then fixed with formaldehyde (Panning et al., 1997). Fixed samples were stored at 4°C in 70% ethanol. Full-length and truncated *Xist* transcripts were detected using λ9–12 and λ3–12,

lambda vectors that, taken together, contain the entire transcribed region of the mouse *Xist* gene (full probe). In *Xist*$^{+/-}$ embryos the full probe also detects transcripts of exon 7 sequence from the *Pgk-1* promoter in a high proportion of cells. This signal, however, is less intense than the low-level monoallelic signal from the *Xist* promoter. Full-length *Xist* transcript was specifically detected using p*Xist*10K, a plasmid that contains all of exon 1 of the *Xist* gene except for the first 912 bp in a XhoI fragment (deletion probe). The XhoI fragment was derived from a P1 clone (Genome Systems) that contains the entire mouse *Xist* gene. *Pgk-1* transcripts were detected using pCAB17, a clone containing 17 kb of mouse genomic *Pgk-1* sequence (Panning and Jaenisch, 1996).

Double-stranded *Xist* sequence was labeled with Cy3 nucleotides by nick translation (Amersham). *Pgk-1* probe was obtained by random priming using biotinylated nucleotides (GIBCO-BRL). After extensive washing, hybridization to *Pgk-1* transcripts was detected using avidin conjugated to fluorescein (Vector Laboratories). DNA was counterstained with DAPI (Molecular Probes), and the slides were coverslipped with Vectashield mounting medium (Vector Laboratories). Fluorescent signals were captured using a Sony Videomax CCD camera mounted on a Nikon Photoskop. Color channels were merged in Adobe Photoshop. Fluorescent signals were also captured using a 35 mm camera mounted on a Zeiss Axioskop using Kodak Ektachrome 1600 slide film. A Kodak Sprintscan 35 slide scanner was used to record the images in digital form so that color channels could be merged in Adobe Photoshop.

Received December 10, 1997; revised January 29, 1998.

Acknowledgments

We would like to thank Barbara Panning and Györgyi Csankovszki for much helpful technical advice. We would also like to thank Ruth Curry for excellent technical assistance, Györgyi Csankovszki for providing plasmids and probes, and Laura Gammill and Mary Ellen Lane for critical reading of the manuscript. This work was supported by a grant from the National Institutes of Health/National Cancer Institute (R35-CA44339) to R. J. During the course of this work, Y. M. was supported by the Cancer Research Fund of the Damon Runyon-Walter Winchell Foundation Fellowship, DRG-1292, and a Postdoctoral Fellowship of the Merck/MIT Collaboration.

References

Adler, I.-D., Johannisson, R., and Winking, H. (1989). The influence of the Robertsonian translocation Rb(X.2)2Ad on anaphase I nondisjunction in male laboratory mice. Genet. Res. *53*, 77–86.

Beard, C., Li, E., and Jaenisch, R. (1995). Loss of methylation activates *Xist* in somatic but not in embryonic cells. Genes Dev. *9*, 2325–2334.

Borsani, G., Tonlorenzi, R., Simmler, M.C., Dandolo, L., Arnaud, D., Capra, V., Grompe, M., Pizzuti, A., Muzny, D., Lawrence, C., et al. (1991). Characterization of a murine gene expressed from the inactive X chromosome. Nature *351*, 325–328.

Brockdorff, N., Ashworth, A., Kay, G.F., Cooper, P., Smith, S., McCabe, V.M., Norris, D.P., Penny, G.D., Patel, D., and Rastan, S. (1991). Conservation of position and exclusive expression of mouse *Xist* from the inactive X chromosome. Nature *351*, 329–331.

Brown, C.J., Ballabio, A., Rupert, J.L., Lafreniere, R.G., Grompe, M., Tonlorenzi, R., and Willard, H.F. (1991). A gene from the region of the human X inactivation centre is expressed exclusively from the inactive X chromosome. Nature *349*, 38–44.

Cattanach, B.M., and Papworth, D. (1981). Controlling elements in the mouse V. Linkage tests with X-linked genes. Genet. Res. *38*, 57–70.

Clemson, C.M., McNeil, J.A., Willard, H.F., and Lawrence, J.B. (1996). *XIST* RNA paints the inactive X chromosome at interphase: evidence for a novel RNA involved in nuclear/chromosome structure. J. Cell Biol. *132*, 259–275.

Endo, S., and Takagi, N. (1981). A preliminary cytogenetic study of X chromosome inactivation in diploid parthenogenetic embryos from LT/Sv mice. Jpn. J. Genet. *56*, 349–356.

Grumbach, M.M., Morishima, A., and Taylor, J.H. (1963). Human sex chromosome abnormalities in relation to DNA replication and heterochromatinization. Proc. Natl. Acad. Sci. USA *49*, 581–589.

Herzing, L.B.K., Romer, J.T., Horn, J.M., and Ashworth, A. (1997). *Xist* has properties of the X-chromosome inactivation centre. Nature *386*, 272–275.

Hogan, B., Beddington, R., Costantini, F., and Lacy, E. (1994). Manipulating the Mouse Embryo: A Laboratory Manual, Second Edition (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Kaufman, M.H., Guc-Cubrilo, M., and Lyon, M.F. (1978). X chromosome inactivation in diploid parthenogenetic mouse embryos. Nature *271*, 547–549.

Lee, J.T., and Jaenisch, R. (1997a). The (epi)genetic control of mammalian X-chromosome inactivation. Curr. Opin. Genet. Dev. *7*, 274–280.

Lee, J.T., and Jaenisch, R. (1997b). Long-range *cis* effects of ectopic X inactivation centres on a mouse autosome. Nature *386*, 275–279.

Lee, J.T., Strauss, W.M., Dausman, J.A., and Jaenisch, R. (1996). A 450 kb transgene displays properties of the mammalian X inactivation center. Cell *86*, 83–94.

Lyon, M.F., Searle, A.G., Ford, C.E., and Ohno, S. (1964). A mouse translocation suppressing sex-linked variegation. Cytogenetics *3*, 306–323.

Marahrens, Y., Panning, B., Dausman, J., Strauss, W., and Jaenisch, R. (1997). *Xist*-deficient mice are defective in dosage compensation but not spermatogenesis. Genes Dev. *11*, 156–166.

McMahon, A., and Monk, M. (1983). X-chromosome activity in female mouse embryos heterozygous for *Pgk-1* and Searle's translocation, T(X;16) 16H. Genet. Res. *41*, 69–83.

Panning, B., and Jaenisch, R. (1996). DNA hypomethylation can activate *Xist* expression and silence X-linked genes. Genes Dev. *10*, 1991–2002.

Panning, B., Dausman, J., and Jaenisch, R. (1997). X chromosome inactivation is mediated by *Xist* RNA stabilization. Cell *90*, 907–916.

Penny, G.D., Kay, G.F., Sheardown, S.A., Rastan, S., and Brockdorff, N. (1996). Requirement for *Xist* in X chromosome inactivation. Nature *379*, 131–137.

Plenge, R.M., Hendrich, B.D., Schwartz, C., Arena, J.F., Naumova, A., Sapienza, C., Winter, R.M., and Willard, H.F. (1997). A promoter mutation in the *XIST* gene in two unrelated families with skewed X-chromosome inactivation. Nature Genet. *17*, 353–356.

Rastan, S. (1983). Non-random X-chromosome inactivation in mouse X-autosome translocation embryos—location of the inactivation centre. J. Embryol. Exp. Morph. *78*, 1–22.

Rastan, S., and Brown, S.D.M. (1990). The search for the mouse X-chromosome inactivation centre. Genet. Res. *56*, 99–106.

Rastan, S., and Robertson, E.J. (1985). X-chromosome deletions in embryo-derived (EK) cell lines associated with lack of X-chromosome inactivation. J. Embryol. Exp. Morph. *90*, 379–388.

Rastan, S., Kaufman, M.H., Handyside, A.H., and Lyon, M.F. (1980). X-chromosome inactivation in extraembryonic membranes of diploid parthenogenetic mouse embryos demonstrated by differential staining. Nature *288*, 172–173.

Russell, L.B. (1963). Mammalian X-chromosome action: inaction limited in spread and in region of origin. Science *140*, 976–978.

Russell, L.B., and Mongomery, C.S. (1965). The use of X-autosome translocations in locating the X-chromosome inactivation center. Genetics *52*, 470–471.

Sheardown, S.A., Duthie, S.M., Johnston, C.M., Newall, A.E.T., Formstone, E.J., Arkell, R.M., Nesterova, T.B., Alghisi, G.-C., Rastan, S., and Brockdorff, N. (1997). Stabilization of *Xist* RNA mediates initiation of X chromosome inactivation. Cell *91*, 99–107.

Simmler, M.-C., Cattanach, B.M., Rasberry, C., Rougeulle, C., and Avner, P. (1993). Mapping the murine *Xce* locus with (CA)n repeats. Mamm. Genome *4*, 523–530.

Tada, T., and Takagi, N. (1992). Early development and X-chromosome inactivation in mouse parthenogenetic embryos. Mol. Reprod. Dev. *31*, 20–27.

Tada, T., Tagaki, N., and Adler, I.-D. (1993). Parental imprinting on the mouse X chromosome: effects on the early development of X0, XXY and XXX embryos. Genet. Res. *62*, 139–148.

Takagi, N., and Sasaki, M. (1975). Preferential inactivation of the paternally derived X chromosome in the extraembryonic membranes of the mouse. Nature *256*, 640–642.