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X chromosome inactivation and the *Xist* gene

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Abstract. Recent years have seen rapid progress towards understanding the molecular mechanisms involved in X chromosome inactivation (X inactivation). This progress has largely revolved around the discovery of the X inactive specific transcript (*Xist*) gene, which is known now to represent the master switch locus regulating X inactivation. In adult cells *Xist* is transcribed exclusively from the inactive X chromosome. The transcript has no apparent protein-coding potential and is retained in the nucleus in close association with the domain occupied by the inactive X chromosome. It is thus thought to represent a functional RNA molecule which acts as the primary signal responsible for the propagation of X inactivation. Developmental regulation of *Xist* correlates with the developmental timing of X inactivation. Recent results have demonstrated that *Xist* is both necessary and sufficient for X inactivation. Goals for the future are to understand the mechanism of *Xist* regulation which underlies the establishment of appropriate X inactivation patterns and to determine how *Xist* RNA participates in the process of propagating inactivation *in cis*.

Key words. X chromosome inactivation; X inactivation centre; *Xist*; imprinting; chromatin structure.

The master switch locus

X inactivation represents the mechanism of sex chromosome dosage compensation in mammals (recently reviewed in ref. 1). Transcriptional silencing of a single X chromosome in females (XX) equalizes X-linked gene dosage relative to males (XY). X inactivation initiates in early embryogenesis, and the inactive state is then stably maintained through subsequent cell generations. As the process normally initiates at random, female mammals are comprised of clonal cell populations in which either the maternally (X^m) or paternally (X^p) derived X is inactive. There are also instances where X inactivation initiates nonrandomly. A notable example is the preferential X inactivation of X^p which occurs in marsupial species and in certain extraembryonic lineages of (some?) eutherian species.

In 1965 Russell proposed that X inactivation is mediated by a single *cis*-acting master switch locus which she termed the 'X inactivation centre' (Xic) ([2] and reviewed in ref. 3). The hypothesis arose from observations on balanced X-autosome (X;A) translocations in mouse. Russell noted that inactivation occurs only on one of the two translocation products. By examining a series of different translocations, she deduced that a critical region on the X chromosome is required *in cis* for inactivation to occur. The location of the critical Xic region was refined in subsequent studies, both on mouse and human X chromosomes [4–6]. Thus the Xic was initially defined as a locus from which an inactivation signal is propagated *in cis* along the length of the X chromosome.

Subsequent studies have shown that the Xic regulates the initiation as well as the propagation of X inactivation. Initiation refers to the process by which a cell determines how many (counting) and which (choice) X chromosomes will be inactivated (or remain active). Briefly, it has been shown that cells must register the presence of at least two Xics for X inactivation to occur at all [4, 5]. Together with studies on X chromosome aneuploids and on tetraploid

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embryos [7, 8], these observations led to the formulation of the blocking factor model, in which it is proposed that initiation of X inactivation occurs by blocking a single Xic in all diploid cells. Unblocked Xics then induce X inactivation *in cis*. Importantly, this model suggests that initiation is controlled by marking the single X chromosome that will remain active [4]. The role of the Xic in initiation of X inactivation is further supported by studies on the mouse X controlling element (*Xce*) alleles. Briefly, *Xce* alleles are thought to represent alleles of the Xic which affect the probability of a given X chromosome being elected as the active X chromosome (X^a) in heterozygous animals (reviewed in ref. 3). In the context of the blocking factor model this may translate to *Xce* alleles having different relative affinities for the blocking factor.

In the 1980s the advent of positional cloning technologies opened up the possibility of isolating the Xic. The key breakthrough came in 1991 when Willard's team identified the X inactive specific transcript (*XIST*) gene within the critical interval on the human X chromosome [9]. *XIST* transcription was found to occur exclusively from the inactive X chromosome (X^i) in XX somatic cells. The mouse homologue, *Xist*, was subsequently shown to exhibit the same properties [10, 11]. Characterization of the full-length transcript revealed that the gene produces a large RNA with no apparent protein-coding capacity [12, 13]. Consistent with this, *XIST* RNA was found to be localized in the nucleus [12, 13], and using RNA fluorescence in situ hybridization (FISH), was shown to colocalise with the inactive X chromosome [13]. These findings suggested that binding of *Xist* RNA *in cis* may be the primary signal resulting in propagation of the inactive state along the chromosome.

Characterization of the mouse *Xist* gene afforded the opportunity to examine *Xist* expression at the onset of X inactivation. Thus Kay et al. demonstrated that the first *Xist* expression is detectable at the four- to eight-cell stage and that *Xist* expression occurs predominantly from X^p in preimplantation embryos [14]. These observations were consistent with *Xist* playing a role in initiating imprinted X inactivation in the trophectoderm and primitive endoderm lineages of the blastocyst. Significant expression from X^m was not seen until 5.5–6.5 dpc, the time of initiation of random X inactivation in the embryo proper. Other studies examined the basis of imprinted *Xist* expression [15–18]; low-level *Xist* expression was found to occur in the male germ line [14–17], and regionalized demethylation of the *Xist* promoter was shown to occur in parallel [18]. It was proposed that expression in the male germ line could signal an involvement for *Xist* in silencing of the XY bivalent at the pachytene stage of meiosis [15]. It was further proposed that demethylation of *Xist* in the male germ line could underlie preferential X^p expression and hence imprinted X inactivation in early extraembryonic lineages [14]. Consistent with this idea, oocytes were shown not to express *Xist* (the X chromosome becomes reactivated in the course of female germ cell maturation), and *Xist* alleles in oocytes were found to be methylated [15, 19, 20]. Studies on androgenetic and gynogenetic embryos demonstrated that *Xist* expression in early embryos is governed entirely by parental imprinting [21].

Necessary and sufficient

The first direct evidence that *Xist* represents the Xic came from gene targeting experiments in XX embryonic stem (ES) cells [22]. A large deletion was introduced into a single allele in an XX ES cell line, and it was demonstrated that the X chromosome bearing the targeted allele could not undergo X inactivation. The X chromosome bearing an intact allele, on the other hand, was inactivated. The latter point suggests that Xic sequences required for correct initiation of X inactivation remain intact (deletion of a single complete Xic in XX ES cells would be expected to result in failure to inactivate either X chromosome, as the intact allele would be the only one available to be marked as X^a). Indeed, in the aforementioned experiment there was evidence that initiation of X inactivation occurs normally, i.e. that some cells elect to inactivate the X chromosome bearing the targeted allele but fail to inactivate *in cis* and are then selected against as a result of failed dosage compensation.

A subsequent gene targeting experiment gave rise to comparable conclusions [23]; a similar-sized deletion was introduced into the single *Xist* allele in XY ES cells, and targeted cells were then used to produce chimaeric animals that transmit the mutation. The mutation resulted in early lethality in heterozygous XX progeny when inherited on X^p, and nonrandom X inactivation of the X bearing the intact *Xist* allele when inherited on X^m. Further analysis indicated that lethality occurred as a result of failed imprinted X inactivation in extraembryonic lineages (i.e. these cells always attempt to inactivate from the X^p allele, and the deletion of *Xist* causes failure to X inactivate *in cis*). Nonrandom X inactivation following X^m inheritance is presumably equivalent to the effect seen with the targeting in XX ES cells. These experiments also demonstrated that *Xist* is not required for spermatogenesis. Thus, either *Xist* is not involved in silencing of the XY bivalent, or alternatively, silencing is not required for efficient spermatogenesis.

A number of recent studies have addressed the question of whether ectopically integrated *Xist* constructs

can recapitulate Xic functions [24–28]. Initial experiments examined transgenic mice produced using YAC clones covering the *Xist* locus [24, 25]. The majority of lines did not express ectopic *Xist* either when heterozygous or homozygous and either on an XX or XY background. A single line did express ectopic RNA, and the integration was shown to have occurred in a heterochromatic region of the Y chromosome long arm [24]. There was no evidence to indicate *in cis* inactivation of genes on the Y chromosome. More promising results were obtained using YAC constructs introduced into XY ES cells [26]. A number of lines expressed ectopic *Xist*, and interestingly, the endogenous *Xist* gene was induced in some cells during in vitro differentiation. These findings indicated that the ectopic *Xist* sequences are being counted, and therefore that the introduced sequences encompass the entire Xic. In a subsequent study [27] it was demonstrated that in one case at least, expression of ectopic *Xist* appears to result in long-range *cis*-inactivation of autosomal genes (based on transcriptional analysis of a number of genes and on cytogenetic criteria).

Similar results have now been reported using much smaller constructs; a cosmid covering the genomic *Xist* locus and 9 kb of upstream sequence is expressed and appears to recapitulate the counting function of the Xic when introduced into XY ES cells [28]. There was some evidence for *cis*-inactivation, although further experiments are needed to confirm this. The results nevertheless suggest that *Xist* is sufficient to recapitulate all functions of the Xic. A reasonable working hypothesis is that upstream regulatory sequences are involved in the initiation functions (determining how many and which *Xist* alleles are upregulated), whereas transcribed *Xist* RNA triggers propagation of inactivation by binding along the X chromosome *in cis*. Determining the molecular mechanisms of initiation and propagation are clearly primary goals for the future.

Initiation

Studies to date suggest that initiation of X inactivation is mediated by the regulatory apparatus controlling *Xist* expression. For this reason a number of groups have begun an analysis of sequences immediately upstream of *Xist* [29–32]. Comparative sequence analysis demonstrates conservation of sequence 100–300 bp upstream of the major transcriptional site. Pillet et al. [29] carried out a preliminary in vitro analysis of the mouse *Xist* minimal promoter. A more detailed in vitro analysis and also in vivo footprinting studies have recently been reported by Sheardown et al. and Komura et al., respectively [30, 31]. These studies have identified binding sites for the transcription factors TFIID, SP1 and CBF. In transfection assays, the minimal promoter exhibits constitutive activity both in undifferentiated ES cells and in somatic cells. Similar findings have recently been reported in an analysis of the human *Xist* minimal promoter [32]. In this study, elements located both upstream and downstream were shown to modulate minimal promoter activity in transient transfection assays. The upstream element is a large polypyrimidine-purine tract which represses the minimal promoter. The downstream element is a tandemly repeated motif that enhances minimal promoter activity in transient assays but only when positioned downstream of the promoter. It is possible therefore, that this element acts post-transcriptionally.

Constitutive activity of the *Xist* minimal promoter both in ES cells and somatic cells suggests that upregulation requires elements either upstream or downstream of *Xist*. Specifically, analysis of the ectopically integrated *Xist* cosmid suggested that sequences required for developmental upregulation of *Xist* are present in a region of approximately 9 kb upstream of *Xist*, or alternatively within transcribed sequence or introns [28]. A number of earlier studies had demonstrated that low levels of *Xist* transcript are detectable prior to the onset of X inactivation in undifferentiated XY and XX ES cells [14, 33–36]. Thus developmental upregulation could formally be attributable to increased transcription of the X^i allele (and concomitant repression of the X^a allele), or to increased stability of X^i transcript [32]. Although a priori many considered the former most likely, recent evidence suggests that increased transcript stability is in fact the primary determinant of developmental upregulation [37, 38]. The rate of transcription of endogenous *Xist* was shown to be similar in ES cells and XX somatic cells. Transcript stability, on the other hand, was shown to be 10- to 20-fold higher in XX somatic cells than in ES cells [37]. Quantitative analysis of steady-state RNA levels suggested that developmental upregulation could be accounted for solely by increased transcript stability [37].

Further interesting insights were gained using RNA FISH. Earlier studies had demonstrated that *Xist* RNA occupies a large nuclear domain corresponding to the X^i territory [9, 26, 39]. In ES cells *Xist* RNA was seen as a small punctate signal [34, 35]. A single punctate signal was observed in XY ES cells and two signals in XX ES cells, demonstrating activity of both alleles [35]. The recent studies [37, 38] demonstrated that, following in vitro differentiation of XX ES cells, unstable transcript continues to be produced from the X^a allele for a substantial period (24–48 h) after stabilization and *in cis* accumulation of X^i transcript. Silencing of the X^a allele follows this intermediate stage. This progression also occurs in early embryos undergoing random X inactivation. The temporal control of X^a *Xist* expression is precisely mirrored in XY cells.

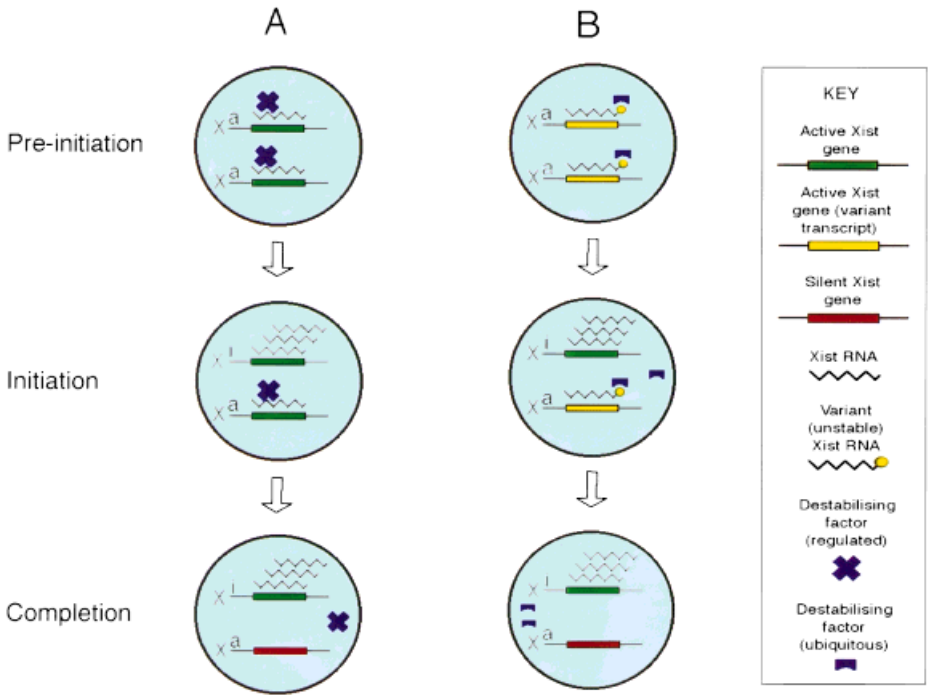

Figure 1. *Xist* RNA stabilization mediates initiation of X inactivation. (*A*) Prior to initiation of X inactivation *Xist* transcript is destabilized by a specific developmentally regulated factor. Reduced levels of the factor at the onset of X inactivation results in transcript from one allele remaining stable (X^a), whereas transcript from the other allele stabilises and accumulates *in cis* (X^i). Transcription from the X^a allele is subsequently repressed (completion). (*B*) An unstable variant is transcribed from both alleles prior to initiation of X inactivation. At the onset of X inactivation production of the unstable variant is retained only on one allele (X^a), whereas the other allele switches to production of stable transcript (X^i). The allele producing unstable transcript is subsequently repressed.

As mentioned previously, establishing appropriate X inactivation patterns is thought to involve marking the X^a by blocking a single Xic. The aforementioned observations have important implications for this model. Silencing of the X^a *Xist* allele clearly occurs after the counting and choice decisions have been made. Thus the mechanism of counting and choice presumably involves selective destabilization of X^a transcript. This could be accounted for either by hypothesizing developmental regulation of the level of specific destabilizing/stabilizing factors, or developmental regulation of transcript processing. The latter would include splice variants with differential stability or the use of different promoters to transcribe unstable versus stable transcript. These models are illustrated in figure 1. Current data favour model A, as no structural differences were detectable between unstable and stable transcripts. A more complete analysis will be required to fully test this possibility.

Regardless of which mechanism operates, it is also necessary to invoke a secondary mechanism which recognizes and silences X^a transcription. There is some evidence that DNA methylation is important in this process. DNA methyltransferase (*dnmt*) mutant XY ES cells produce unstable *Xist* transcript, but following in vitro differentiation, accumulation of stable transcript is observed rather than silencing of the X^a allele. Cell death occurs in differentiated cultures, presumably as a result of silencing the single X chromosome [34, 35]. This also occurs in *dnmt* mutant embryos but only in a small number of cells. Presumably cells which fail to stably repress X^a transcription are being rapidly eliminated by cell selection. Thus methylation appears to be a secondary mechanism important for maintaining, but not for establishing, stable repression of the X^a allele.

The picture is somewhat complicated by observations on imprinted X inactivation [37]. Briefly, RNA FISH analysis indicates that stable X^p transcript accumulates in all cells from the four- to eight-cell stage. Unstable X^m transcript can be seen in some cells. As both X chromosomes are known to be active at this time, accumulation of *Xist* transcript along the X chromosome is presumably insufficient in itself to induce X inactivation. A possible explanation is that factors produced only in differentiated cells are required to respond to the primary signal provided by *cis*-accumulation of *Xist* RNA. Thus in early differen tiating extraembryonic lineages (trophectoderm and

primitive endoderm), prior accumulation of X^p transcript enforces preferential X^p inactivation. Interestingly, inner cell mass (ICM) cells of the blastocyst appear to destabilize X^p transcript and concurrently increase production of unstable X^m transcript. *Xist* expression patterns then resemble those seen in XX ES cells. These events may represent erasure of the imprint and establishment of the preinitiation state required for random X inactivation in the embryo proper [14, 21].

Future studies should be directed toward further elucidation of the basis of differential stability of *Xist* RNA and ultimately toward identifying the *trans*-acting factors mediating this process. Studies to date indicate that this will throw further light on the basis of imprinted *Xist* expression. Also of interest will be studies on the process of X chromosome reactivation, which occurs in developing female germ cells or when XX somatic cells are fused with ES cells. In both cases reactivation is accompanied by a reduction in steady-state *Xist* RNA levels. Application of RNA FISH methods should determine whether reactivation involves resetting to an XX ES cell-like pattern (destabilization of X^i transcript and derepression of unstable X^a transcript) or whether both alleles become fully repressed.

Propagation and maintenance

Spreading of the X inactivation signal was first reported as an example of position-effect variegation in 1959 [40]. Inheritance of a balanced translocation between the X chromosome and chromosome 4, in addition to the normal X {T(X;4)37H}, resulted in a variegated coat colour in female mice. Russell correctly interpreted this phenomenon as the spreading of X inactivation *in cis* into the translocated, autosomal *brown* locus [41]. Until recently, the cytogenetic analysis of several X-autosome rearrangements, using late replication and heterochromatinization as indicators of inactivation, provided the only clues as to the nature of the spreading signal (reviewed in ref. 42). Using these criteria, inactivation was seen to spread bidirectionally into autosomal material. This observation led to the proposal that inactivation must spread from a nonterminal region of the X, named the X inactivation centre or Xic [2, 43–45]. Inactivation of autosomal genes was seen to be attenuated, with less frequent inactivation of those loci furthest from the breakpoint.

Attenuated spread of X inactivation into autosomal material [43] indicates either that X material has evolved to respond more efficiently to the inactivation signal or, alternatively, that cell selection operates to exclude cells in which spread into autosomal material results in haploinsufficiency for critical genes. There is good evidence to support the former idea, that autosomal material is inherently less susceptible to the inactivating signal. Is(InX;7)1Ct (abbreviated to Is1Ct), an insertion of chromosome 7 material into the X chromosome, has been examined in its unbalanced form in which cells are effectively trisomic for the inserted region. Although inactivation of the insertion would restore normal genetic balance, analysis of autosomal coat-colour genes, together with both late replication and heterochromatinization assays, suggested inefficient autosomal inactivation [46–49]. Additionally, in some instances the presence of the insertion appears to block spread of inactivation into X material distal to the insertion [49, 50].

Further evidence comes from the observation that near-random X inactivation occurs with a number of balanced X;A translocations [43]. Cell selection would predict exclusion of the majority of cells in which the translocation product is inactivated. Cell selection clearly does occur with some of the translocations studied, but this can be largely accounted for by selection against functional disomy of X-linked genes that have been separated from the Xic, rather than haploinsufficiency of autosomal genes. An example of this is provided by the T(X:16)16H (abbreviated to T16H) translocation. T16H results in complete nonrandom X inactivation of the normal X chromosome as a result of cell selection [4, 51]. However, monosomy of the region of chromosome 16 translocated *in cis* with the Xic has been shown to have relatively mild effects on normal development [52]. In general, the severity of cell selection appears to correlate well with the extent of functional disomy of X material in X;A translocations (reviewed in ref. 43).

Although selection against cells monosomic for autosomal genes is unlikely to be the primary reason for inefficient spread of inactivation in X;A translocations, there is some evidence that this type of event does occur (reviewed in ref. 53). This observation may be explained by instability of the limited inactivation of autosomal material. Consistent with this, Cattanach reported that as Is1Ct mice age, the *albino* gene on the inserted chromosome 7 material reactivated, producing a coat-colour change [54, 55]. Reactivation with age, however, has also been reported for true X-linked genes [56, 57].

The combination of the aforementioned classical data, and a failure to find X-specific elements in the promoters of several X-linked genes [58], led to models of X inactivation involving 'way-stations'. Way-stations were described as elements regularly spaced along the X chromosome that facilitate spread and are either absent or less frequent on autosomes, thus limiting the spread of inactivation into translocated autosomal material [58]. Genes in the XY pairing pseudoautosomal region, and also genes elsewhere on the X chromosome, escape X inactivation (reviewed in refs 59 and 60). In the context of Rigg's way-station hypothesis, this could be accounted for by proposing that there are X chromosome domains which lack way-stations.

The discovery that the *Xist* gene encodes an apparently 'functional RNA' expressed only from X^i in the nucleus was a major breakthrough, providing a candidate molecule for involvement in the spreading mechanism [12–14]. The results of gene-targeting experiments support a primary role for *Xist* RNA in the propagation of X inactivation *in cis* [22, 23]. FISH techniques have been used to show that *Xist* RNA colocalizes with X^i in female human interphase nuclei [39] and in both interphase nuclei and mitotic cells in female mice [26, 35, our unpublished results]. The RNA does not appear to bind directly to X^i DNA, however, since digestion with DNAse 1 does not disrupt the localization of *Xist* RNA in the nucleus [39]. It has been proposed that *Xist* RNA associates with the nuclear matrix and is involved in higher-order chromatin packaging [39].

Modulation of higher-order chromatin packaging is supported by observations on steady-state levels of *Xist* RNA in mouse XX somatic cells [10, 33]. Levels have been estimated to be approximately 200–1000 molecules/cell, there being variation between different strains and an apparent inverse correlation with the strength of *Xce* allele. The significance of this latter observation is unclear. However, as X inactivation occurs with equal efficiency in the different mouse strains studied, the lowest estimates of *Xist* RNA levels must be sufficient to propagate X inactivation. These values convert to approximately one RNA molecule/1000 kb of X chromosome DNA. Thus *Xist* RNA would appear to regulate large chromatin domains rather than individual genes.

Recent studies have examined the effects of *Xist* transgenes integrated at ectopic autosomal locations [24–28]. In one instance it has been demonstrated that a 450-kb *Xist* YAC transgene is capable of inactivating, *in cis*, specific autosomal genes located at some considerable distance from the transgene [26, 27]. *Xist* RNA was seen to cover the entire autosome (chromosome 12), and at metaphase the autosome was shown to be late replicating and underacetylated at histones H3 and H4, properties normally associated with the inactive X chromosome. These effects could not be seen with two other transgenic integrants. The authors concluded that propagation of inactivation by *Xist* does not require X-specific way-station sequences. They invoked cell selection events to explain these failure to detect autosomal inactivation with the other lines and in classical studies of X;A translocations. However, as has been argued previously, evidence for the relative insensitivity of autosomes to the inactivating signal is compelling.

An alternative explanation for the results of Lee and Jaenisch [26, 27] is that the transgene on chromosome 12, being present in approximately 20 copies, produces artificially high levels of *Xist* RNA that facilitate spread of inactivation via 'pseudo-way-stations'. Classical studies on X;A translocations indicate that different autosomes vary in their relative sensitivity to the inactivating signal, consistent with the concept of varying frequency of low affinity pseudo-way-stations (reviewed in ref. 43). It should also be noted that the results of Lee and Jaenisch, particularly the acetylation of H3/H4 data, indicate that inactivation of the autosome is not as efficient as inactivation of an X chromosome [27]. Our own observations indicate that *Xist* RNA localizes to discrete foci on the X chromosome, consistent with the way-station hypothesis. Further, we have observed that *Xist* RNA does not spread efficiently into autosomal material *in cis* with the Xic in different translocations (S.D., in preparation).

There is still no indication as to how binding of *Xist* transcript is restricted to chromatin *in cis*. One possible model invokes a passive mechanism in which *Xist* RNA associates with proteins localized to high affinity binding sites (way-stations) *in cis*. Co-operative interactions could facilitate *cis* rather than *trans* binding. Thus, for example, binding of RNA to sites close to the Xic could induce chromosome compaction, which in turn draws more distant sites into close proximity with the local concentration of *Xist* RNA (fig. 2A). A second model suggests that the *Xist* transcript must associate first with *trans*-acting factors, which then actively transport it to binding sites *in cis* (fig. 2B). In either scenario, limited spread into autosomes, or escape from X inactivation, could be attributable to low frequency and/or low affinity *cis* binding sites. Since much of the current evidence concerning the involvement of *Xist* RNA in propagation is either indirect, or open to alternative interpretation, careful and direct testing of the RNA hypothesis remains an important goal.

Although it seems highly probable that *Xist* RNA is indeed essential for propagating the spread of the inactivation signal, evidence to date indicates that *Xist* is not required to maintain the inactive state. Two studies [61, 62] demonstrated that in somatic cells that had already undergone X inactivation, subsequent loss of the Xic did not result in reactivation. A likely explanation for these findings is that *Xist* RNA binding *in cis* provides the primary signal for establishment of the inactive state and that, subsequently, additional epigenetic signals serve to maintain the inactive state in successive cell divisions.

Candidates for additional epigenetic signals include replication timing control [63], histone H3/H4 deacetylation [64] and CpG island methylation [65–68]. Replication timing is implicated as an early signal in long-range silencing in the yeast *Saccharomyces cerevisiae*, since the origin of replication complex (ORC) plays a direct role in recruiting silencing proteins to the silent mating type loci [69]. A recent study has shown that the transition to late replication is a relatively early event in the X inactivation process, occurring soon after *cis*-spreading of *Xist* RNA in differentiating XX ES

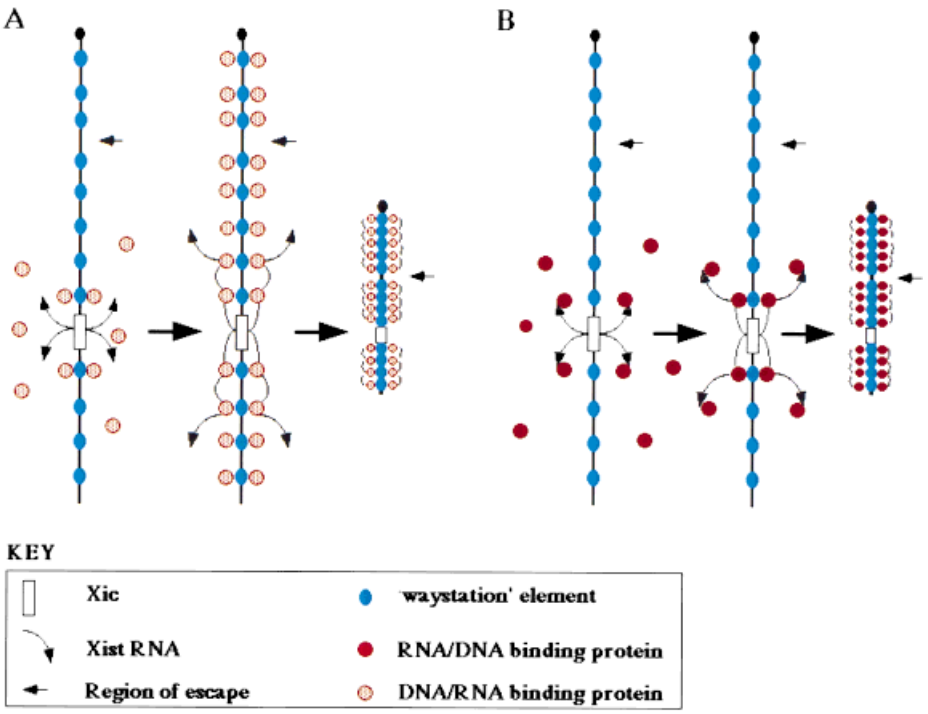

Figure 2. Way-station model for the spread of X inactivation. (*A*) *Xist* RNA associates *in cis* with proteins localized to high affinity binding sites (way-station elements) on the X chromosome. (*B*) *Xist* RNA is actively transported by RNA-binding proteins to way-stations on the X chromosome. In *A*, the way-station binding proteins recognize both X chromosomes, but *Xist* RNA will only bind *in cis* on X^i. In both models, formation of an RNA/DNA/protein complex close to the Xic will induce local conformational changes in chromatin, thus facilitating the spread of inactivation. Regions that escape inactivation either lack way-stations or are protected from the spreading signal by boundary elements.

cells and prior to H3/H4 deacetylation [36]. Methylation of CpG island sequences does not become apparent until some considerable time later [70, 71]. Late appearance of CpG island methylation has also been noted during normal development. This, together with the fact that methylation does not appear to occur on CpG islands of the marsupial inactive X chromosome [72, 73], has led to speculation that methylation is a secondary mechanism which serves to 'lock-in' or stabilize the inactive state.

Reactivation of an inactive X chromosome occurs during the normal development of the female germ line and can also be induced by fusing XX somatic cells with either ES cells or embryonal carcinoma (EC) cell lines. In both instances reactivation has been shown to correlate with downregulation of X^i *Xist* RNA levels. However, a more recent paper [74] reported that a human X chromosome, reactivated by fusion with mouse EC cells, continues to express high levels of *Xist* RNA, even though analysis of human X-linked genes indicated X inactivation had been reversed. Taken together these findings indicate, first, that the human XIC cannot respond to the reactivation signal in mouse EC cells that normally downregulates *Xist* RNA expression and, second, that reactivation requires changes other than, or in addition to, *Xist* downregulation.

The reason for continued X^i *Xist* expression in somatic cells is unclear in view of the various observations above. One possibility is that there is a 'window' following the onset of X inactivation in which *Xist* is required to maintain inactivation. It should be possible to test this using the new Cre-lox technologies that allow conditional gene knockouts to be induced in specific tissues or at specific developmental time points.

It is clear that further study of the *Xist* gene will reveal many of the mysteries that have shrouded our understanding of the process of X inactivation. It will be interesting to see whether *Xist* action in X inactivation turns out to be an evolutionary peculiarity or whether it will provide a paradigm for better understanding other epigenetic silencing phenomena such as genomic imprinting.

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