Genomic and epigenomic approaches to the study of X chromosome inactivation

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X chromosome inactivation represents a compelling example of chromosome-wide, long-range epigenetic gene-silencing in mammals. The *cis*- and *trans*-acting factors that establish and maintain the patterns and levels of gene expression from the active and inactive X chromosomes remain incompletely understood; however, the availability of the complete genomic sequence of the human X chromosome, together with complementary approaches that explore the computational biology, epigenetic modifications and gene expression-profiling along the chromosome, suggests that the features of the X chromosome that are responsible for its unique forms of gene regulation are increasingly amenable to experimental analysis.

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Introduction

Increasing understanding of the complexities of the structure and expression of our genome has forced refinement of the simple view of a diploid genome as two equivalent copies of the haploid chromosome set. It is now known that there are many hundreds of genes that are expressed from only a single allele or are differentially expressed between alleles in mammalian genomes. Imprinted regions, genes that exhibit allelic exclusion (e.g. those involved in olfaction or immune function), and most genes on the inactive X chromosome all require some coordination by *cis*- and *trans*-acting elements to modify chromatin in order to achieve differential expression of a gene or groups of genes [1–7]. In addition, demonstrable differences in the level of expression between the two alleles of autosomal genes are widespread in the genome [8,9] and focus attention on genomic determinants that can lead to epigenetically distinguishable chromatin states at allelic positions.

The most dramatic example of such epigenetic states in mammalian genomes involves X chromosome inactivation, which results in the modification and transcriptional silencing of one of the two X chromosomes in somatic cells of eutherian females and acts to equalize X-linked gene expression between males and females [10]. Although genes undergoing X inactivation share many epigenetic features with other mono-allelically expressed regions in the genome, dosage compensation of the X chromosome is the only example of an entire chromosome being regulated by a different mechanism to that regulating its homolog. Recent studies investigating expression and chromatin profiles of the X chromosomes, as well as the availability of the recently complete annotation of its sequence, have begun to unravel some of the complexities of X inactivation at the chromosome level. Here, we focus on several complementary genomic and epigenomic approaches to explore the differential states of the active (Xa) and inactive (Xi) chromosomes (Figure 1). Much progress has also been made in understanding the molecular and developmental mechanisms of X inactivation; for this, the reader is referred to several recent reviews on the subject [7,11,12] and to other reviews in this special issue.

Epigenetic marks of Xi heterochromatin

Initiation of X inactivation begins with a series of increasingly well delineated events at the X inactivation center (see Glossary) on the X chromosome, resulting within the first few days of embryogenesis in the stable differentiation of the two X chromosomes in female cells [12]. In somatic cells, the inactive state of the X chromosome is thereafter faithfully maintained with each cell division. At interphase, the heterochromatic Xi territory usually appears next to the nuclear envelope as a dense proteinaceous structure called the Barr body [13]. The Xi is distinguished from the Xa epigenetically by a number of chromosome-wide features, including histone hypoacetylation, methylation of lysines 9 and/or 27 on histone H3, late replication in S-phase, methylation of CpG islands, and enrichment for the histone H2A variant macroH2A [7,14]. In humans, there appear to be two largely distinct heterochromatin complexes that are associated with the Xi (Figure 1) [15•]. One of these complexes, which is marked by trimethylated lysine 27 on histone H3 (H3K27me3), consists of *XIST* RNA (see Glossary), macroH2A and components of the Polycomb group repressor complex. The other complex contains HP1 (heterochromatin protein 1) bound to trimethylated lysine 9 on histone H3 (H3K9me3). These two heterochromatin types occupy spatially distinct, 1015 RNA in

Glossary

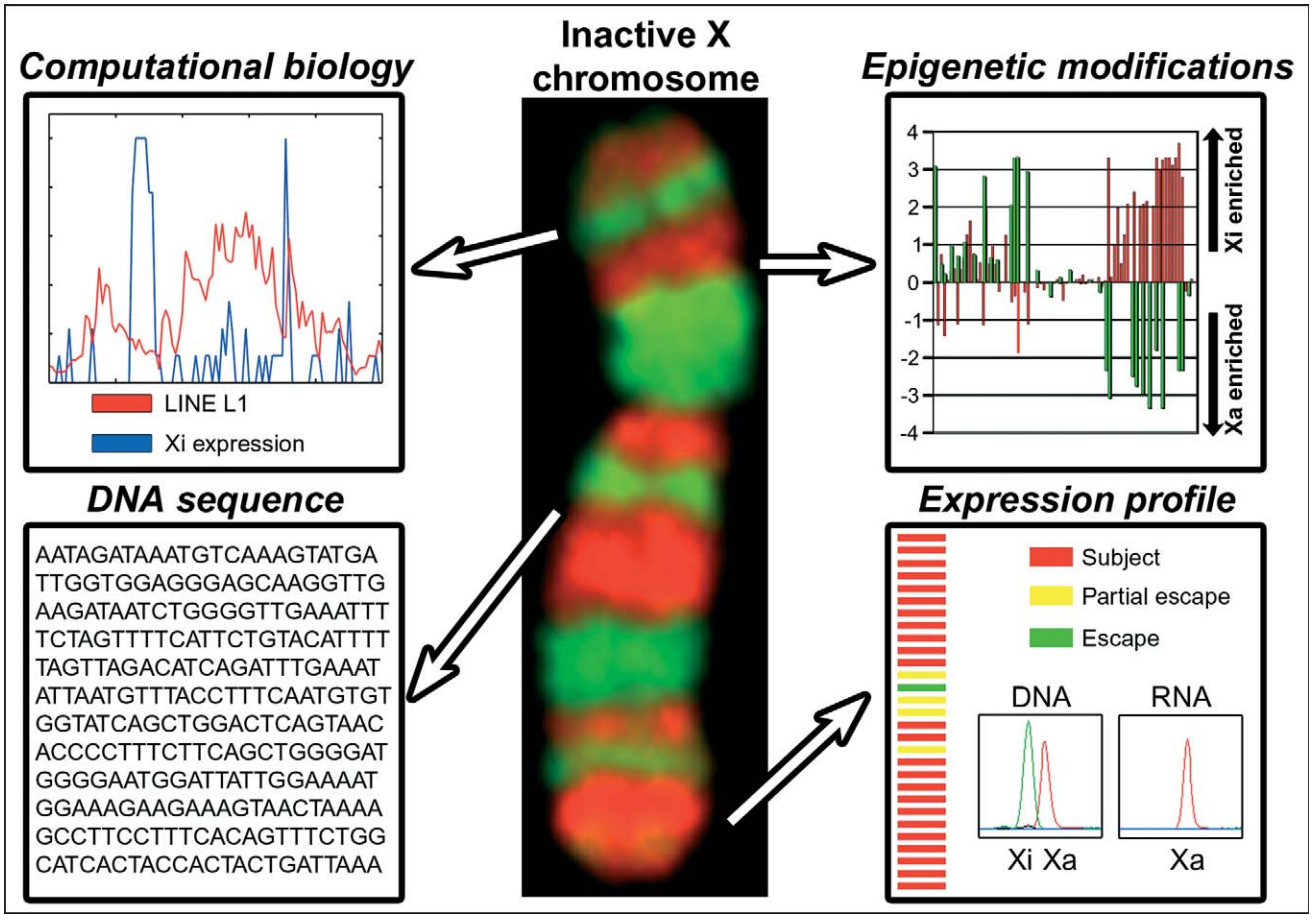
X inactivation center: A complex and specialized locus on the X chromosome, required for the initiation of X inactivation. This region contains the *XIST* gene as well as elements that control expression of *XIST* and that determine which of the two X chromosomes in female cells is chosen to be the active or inactive X.

***XIST*:** The master regulatory gene that is required for X inactivation. *XIST* (Xi-specific transcripts) is expressed only from inactive X chromosomes (or Xs that will become inactive) and is not expressed from active Xs in either males or females. The product of *XIST* is a large non-coding RNA that stays associated with the inactive X and presumably assists in heterochromatin formation on the inactive X.

mouse cells [16], suggesting a genomic basis for the assembly and/or propagation of heterochromatin complexes on the Xi. At the resolution available with immunocytochemistry, it is difficult to discern the boundaries between each domain of heterochromatin; the use of chromatin immunoprecipitation (ChIP) experiments might reveal the precise nature of such transitions and might address the association of specific genomic features with either type of Xi heterochromatin (Figure 1).

To date, a handful of genes have been examined using ChIP and antibodies directed against dimethylated lysine 9 on histone H3 (H3K9me2) and H3K27me3 [17,18]. H3K9me2 is enriched at both the promoter and 3′ regions in female mice, whereas males show only the downstream enrichment. In females, H3K27me3 displays a pattern of deposition similar to that of H3K9me2 but is barely detectable in males at the promoter or downstream. Thus,

Figure 1



Four complementary genomic and epigenomic approaches for the analysis of X inactivation. The center image shows the human inactive X chromosome, stained with antibodies that detect H3K9me3 (red) or H3K27me3 (green) [15•]. Two largely non-overlapping types of facultative heterochromatin are apparent. Lower left panel: the availability of the DNA sequence of the human X chromosome [22••] enables detailed analysis of the sequence composition of different regions of the X containing genes that are subject to or escape from inactivation. Upper left panel: computational analysis reveals different amounts of various sequence features (here, LINE L1 repetitive elements) that correlate with the likelihood of genes being expressed from the Xi. Upper right panel: ChIP experiments demonstrate enrichment of various chromatin features (H3K9me3, red; H3K27me3, green) on the Xi or Xa in different regions of the X. Lower right panel: expression profile of the Xi, determined by allele-specific analysis of gene expression in human female diploid cells [21•]. Genes subject to inactivation (red), partially escaping inactivation (yellow) or escaping inactivation (green) are indicated by the heat map at the left of the panel. Allele-specific analysis is shown for one gene that is subject to inactivation and shows mono-allelic expression from the Xa only.

H3K9me2 and H3K27me3 appear to be located at the same sites on the Xi in the genes tested; surprisingly, however, H3K9me2 does not clearly demarcate the Barr body in mouse cells by immunofluorescence as H3K27me3 does [19]. Whether this reflects mechanistically meaningful differences between human and mouse cells in this regard is uncertain, although there are other instances in which the details of X inactivation appear to differ between these species [14]. This underscores the importance of pursuing, where possible, studies in human cells and highlights the potential merits of an evolutionary and comparative view of X inactivation.

There are several important questions to emerge from recent studies of heterochromatin on the Xi. If *Xist* or *XIST* RNA-based heterochromatin forms at specific regions, whereas H3K9me3- and HP1-based heterochromatin forms in other distinct domains on the Xi, what are the implications for gene expression? Are both types of heterochromatin equally proficient at gene silencing? What are the genomic determinants that influence which type of heterochromatin forms and where? How are H3K9me3 domains established and maintained if they do not include *XIST* RNA and how well conserved are they evolutionarily? It will also be of interest to determine how the boundaries between H3K9me3 and H3K27me3 are established and maintained. DNA sequences that act as heterochromatin barriers or boundaries [20] might prevent the encroachment of one territory on that of another, or they might facilitate the spread of one type of heterochromatin relative to the other.

X chromosome gene expression: Xi dosage compensation

The most notable downstream effect of facultative heterochromatin is to inactive genes on the Xi. Although it has long been recognized, especially in human cells, that not all genes on the Xi are subject to inactivation, [11], the extent of gene expression from the Xi has only become clear as a result of recent genomic studies. An expression profile of an estimated 95% of the assayable genes on the human X chromosome was determined using a combination of allele-specific assays in human cells and species-specific assays in mouse–human somatic cell hybrids (Figure 1) [21•]. Although most of the Xi appears to be heterochromatic at the cytological level, an estimated 15% of genes are nonetheless expressed at significant levels from the Xi and thus escape inactivation in humans. The distribution of genes that escape inactivation is non-random along the chromosome and appears to reflect, at least in part, the history of additions of once autosomal material to the mammalian X chromosome throughout evolution [22••,23•]. The sex chromosomes are thought to have evolved from an autosomal set of chromosomes that diverged approximately 300 million years ago. This resulted in the formation of a series of identifiable evolutionary 'strata' that appear on the current human and mouse X chromosomes and which joined the modern X chromosome at different times [24,25]. Expression profiling of the human Xi [21•] demonstrated that the majority of genes that escape inactivation map to the so-called X-added region (XAR), which corresponds to most of the short arm of the modern human X [23•]. The evolutionary history of the sex chromosomes is explored in greater detail elsewhere in this volume (see reviews by JA Marshall Graves *et al.* [26] and MT Ross *et al.* [27], this issue).

The clustering of genes that escape inactivation into multigene domains raises the question of what genomic features account for the different X inactivation states of adjacent genes. Recent work has promoted the possibility that binding sites for the transcription factor CTCF (CCTC-binding factor) might mark the transition between genes that escape or are subject to inactivation [28•]. Disteche and colleagues [28•] found CTCF-binding sites at the 5′ end of three genes that escape inactivation, each of which was adjacent to genes that are subject to inactivation. They suggested that CTCF prevents the spread of heterochromatin — and specifically DNA methylation — into domains of genes that escape inactivation, thus disrupting stable gene silencing. It will be important to assess the generalization of these findings to a larger number of X-linked genes of known X inactivation status.

Two additional points are worth highlighting from these gene expression studies. First, the data from these studies demonstrate substantial heterogeneity among genes in the degree to which they escape inactivation. Although most genes subject to inactivation show undetectable gene expression from the Xi [21•], those that 'escape' inactivation do so to differing extents Some genes are well expressed from the Xi, being expressed at the equivalent of 50–100% of the level seen from the Xa allele; however, other genes are expressed at only ~10% of the Xa level. Even though it is clear that such genes do indeed 'escape' the X inactivation process that efficiently silences most other genes, it is equally clear that they are largely subject to dosage compensation between male and female cells, despite escaping complete inactivation. Thus, there might be two quantitatively different types of dosage compensation on the Xi: one type that fully silences genes and another type that only partially represses them. Whether these types of dosage compensation are also mechanistically different remains to be explored in depth. However, it is tempting to posit that the two types of heterochromatin noted earlier might be correlated to some extent with the X inactivation status of genes along the chromosome. An important goal of future research will be to assess the role of *cis*-acting genomic sequences, as well as *trans*-acting factors implicated in chromatin remodeling and perpetuation, in determining the X inactivation profile.

The incomplete nature of escape from inactivation for most genes on the human X might explain why previous

studies using X chromosome cDNA arrays to compare gene expression in cell lines that had differing numbers of X chromosomes estimated that less than 5% of genes assayed escaped inactivation [29,30]. This result suggests that allele-specific assays to distinguish Xi and Xa gene expression, rather than male–female gene dosage, might be more suitable for such studies.

The second key observation to come from gene expression studies has been the high degree of heterogeneity between different Xi chromosomes. At least 10% of the genes tested demonstrate significant variation among different females, either in the level of escape from inactivation or in the likelihood that a given gene will either escape or be subject to inactivation [21•]. Given that the number of female cell lines tested was limited (<50), this estimate presumably represents a lower limit on the percentage of genes that might show such variation among females in the general population. Whether such variation has a genetic or epigenetic basis — or both — is not known but is certainly an important question for future work.

The observed variability in escape from inactivation might have important clinical consequences as well. There is an unusual concentration of genes involved in cognition on the X chromosome, and, not surprisingly, there are many more mutations that lead to mental retardation associated with the X than with other chromosomes [31]. There is recent evidence for differences in gene expression in brain tissues between the sexes in both mice and humans [32,33], as well as for the presence of imprinted genes on the X [34,35]. In brain tissues, the level of expression of X-linked genes is more than twofold that of autosomal genes [36••]. The question of exactly how the identity and variation in the level of transcription of genes expressed in females, as well as potential differences in X-linked gene expression between the sexes, might contribute to pathologies of the brain remains open to investigation.

X chromosome gene expression: Xa dosage compensation?

Dosage compensation might not just be involved in determining the levels of gene expression from the Xi. A recent study suggests that the mechanisms of dosage compensation in mammals and *Drosophila* might have more in common than previously thought and adds even greater complexity to the story of gene expression on the sex chromosomes [36••]. A comparison of microarray data for X-linked genes and autosomal genes revealed that in both males and females, and in a number of mammalian species, global transcription from X chromosome genes is double that of global transcription from autosomal genes. Such a mechanism would compensate for gene dosage differences between autosomal genes — these usually exist in two active copies — and X-linked genes that are subject to inactivation — with one active copy in both males and females. Although the mechanism of mammalian Xa dosage compensation is unknown, the similarity with the upregulation of genes on the X chromosome in male flies is notable [37]. How such a process evolved and how the Xa is distinguished from autosomes remain unknown. As in flies, both genomic and epigenetic signals might play a role in humans.

Genomic approaches to X inactivation

A major challenge for X inactivation studies is to determine the basis for a gene being subject to or escaping from inactivation and whether such a decision is entirely epigenetic or whether it is determined, at least in part, by genomic sequence context. Consistent with the latter possibility, Lyon proposed that long interspersed repetitive elements (LINEs) [38] act as boosters for the propagation of the X inactivation signal [39]. In support of this hypothesis, computational analyses have shown that the X chromosome has substantially more LINEs than do any autosomes [40], and, more importantly, that LINEs are over-represented on the ancestral portion of the X (i.e. the X conserved region [XCR]) compared with on the XAR [22••]. Furthermore, the presence of LINEs correlates with the likelihood that a gene is subject to inactivation (Figure 1) [21•,40]. An intriguing possibility is that the distribution of LINEs — and/or other elements — is crucial for determining the transcriptional competence of a gene on the Xi. Some genomic approaches have already hinted at this connection. A study of mono-allelically expressed genes in mouse and in human found that the majority were characterized by a higher density of LINE L1 elements than was found in of bi-allelically expressed genes [41]. Likewise, a comparison of a domain in Xp11.2 containing multiple genes that all escape inactivation to the syntenic region in the mouse where only one gene escaped showed a reduced density of long terminal repeats in the human relative to the mouse, offering a further correlation between repeat content and X inactivation status [42].

Given the recent availability of the complete annotated X chromosome sequence [22••,43], a computational genomic approach to understanding the distribution of sequence elements might provide some insight into sequence features that correlate with epigenetic features of X inactivation. In conceptually similar studies, a machine-learning method has been used to identify imprinted regions in the mouse genome [44•]. Although this method suggests sequence motifs that might be associated with mono-allelic expression, it will require experimental validation to determine which sequence elements are important functionally.

While exploration of the genome sequence will probably provide insights, such computational studies can be complemented by genome-scale study of epigenetic modifications of the X chromosome. Microarray-based experiments have examined the composition of chromatin on a

genome-wide scale in a number of organisms [45–49]. To date, however, the X chromosome, especially in humans, has been primarily analyzed using cytochemical methods. Although immunocytochemistry can reveal gross distributions of chromatin types [15•], it does not provide sufficient resolution to define specific DNA features of the X chromosome. A genomic approach such as ChIP on microarrays ('ChIP-on-chip') [50] will probably be necessary to ask if there are specific sequences with which macroH2A or HP1 heterochromatin associates or how various histone modifications spread in *cis* along specific domains on the X.

To date, efforts to examine histone modifications on the Xi have relied largely on somatic cell hybrids containing the Xa or Xi, or they have compared males with females, considering the male to be representative of the Xa [17,18,51,52]. However, these comparisons might not provide a complete account of how chromatin on the X is organized in a female cell, because they do not directly compare both X chromosomes in the same cell or sample. With the advent of allele-specific arrays and the wealth of single nucleotide polymorphisms in various databases, it should now be feasible to distinguish the Xa and the Xi alleles. For example, such an approach has been effectively used to examine the distribution of macroH2A at imprinted autosomal loci in mouse [53]. An allele-specific approach would facilitate the examination of euchromatin on the Xi, to characterize escape regions and to determine if there are locus-specific elements that actively recruit euchromatic complexes or repel heterochromatin.

Conclusions

Although an increasing number of components involved in X inactivation have been revealed since the discovery of the *XIST* gene 15 years ago, it is still unclear how they work together to silence specific regions of the Xi. With the completion of the X chromosome sequence, and knowledge of the Xi gene expression profile in combination with comprehensive genomic analyses, it should be possible in the coming years to clearly demarcate features of the X chromosome and to ask questions about the importance of specific genomic DNA elements or the epigenetic influence of specific protein or RNA elements.

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