Int. J. Dev. Biol. 40: 1065-1080 (1996)

Review

X-chromosome activity: impact of imprinting and chromatin structure

ROBYN V. JAMIESON, PATRICK P.L. TAM* and MARGARET GARDINER-GARDEN

Embryology Unit, Children's Medical Research Institute, Wentworthville, New South Wales, Australia

CONTENTS

Introduction 1066

Features of the inactive X chromosome 1066
Heterochromatic state and asynchronous replication 1066
Hypoacetylation of histone H4 1067
Hypermethylation of CpG islands 1067
Expression of XIST/Xist gene from the inactive X chromosome 1067

Counting and choice of X chromosome to be inactivated 1068

Differential activity of the X chromosome during development 1069
The pre-implantation embryos 1069
The extraembryonic tissues 1069

Developmental consequence of abnormal X-chromosome constitution 1070
Uniparental embryos 1070
Single X chromosome 1071
Uniparental X-chromosome disomy 1072

The possible role of methylation in imprinting of the X chromosome 1072
Methylation of CpG islands 1072
Parent-specific activity and methylation of the Xist gene 1072

Chromatin configuration of the X chromosome 1073
Expression of X-linked genes in the germ cells 1073
Germ cell chromatin and its influence on parent-specific expression in cleavage stage embryo 1074
Histone acetylation 1074
The XIC/Xic in the preimplantation embryo 1074
Influence of chromatin on imprinting in the trophectoderm and the early epiblast 1076

Summary and key words 1076

References 1076

***Address for reprints:** Embryology Unit, Children's Medical Research Institute, Locked Bag 23, Wentworthville, NSW 2145 Australia. Fax: 61.2.96872120. e-mail: patrict@mail.usyd.edu.au

0214-6282/96/$03.00
© UBC Press
Printed in Spain

Introduction

In female mammals, X-chromosome dosage compensation occurs during early embryonic development and is achieved by inactivation of one of the two X chromosomes (Lyon, 1961). The X chromosome that is inherited from the paternal parent (the X^P chromosome) is preferentially inactivated in the somatic cells of a number of marsupials (Cooper *et al.*, 1993) and in the extraembryonic lineages of eutherian mammals, notably rodents (West *et al.*, 1977) and humans (Migeon *et al.*, 1985; Harrison, 1989). Further studies demonstrate that in cleavage stage mouse embryo there is preferential repression of transcription from X^P (Singer-Sam *et al.*, 1992). A novel gene *(XIST/Xist)* which maps to the X inactivation centre (XIC/Xic) is expressed exclusively from the inactive X chromosome (Brockdorff *et al.*, 1991). The activity of the *Xist* gene has been found to be essential in the promulgation of X inactivation (Penny *et al.*, 1996). Preferential paternal *Xist* expression occurs in the extraembryonic lineages and is also seen in cleavage-stage embryos (Kay *et al.*, 1993). At gastrulation, one of the X chromosomes in the precursor cells of the somatic tissues of the embryo is randomly inactivated (Monk and Harper, 1979; Takagi and Sasaki, 1982; Kay *et al.*, 1993). While X-chromosome inactivation has been studied extensively, the mechanisms behind this process, particularly its initiation and the role of genomic imprinting in regulating the activity of the X chromosomes of different parental origins, are not yet fully elucidated.

Genomic imprinting is an epigenetic process which leads to variable behavior of the whole or regions of homologous chromosomes, or the differential expression of alleles of the same genes, according to the parental source from which they were inherited (Surani, 1991; Gold and Pedersen, 1994). An essential feature is the ability to reverse the imprint when the chromosome or the allele is transmitted to the next generation by parents of the opposite sex (Bartolomei *et al.*, 1991; DeChiara *et al.*, 1991). Imprinting is reputed to happen in the germ cells or in the zygotic pronuclei, since this is where the male and female genomes are separate and could be differentially modified. Superimposed on this imprint there can be further somatic regulation so that only one parental allele is differentially expressed in specific tissues (DeChiara *et al.*, 1991). Parental-specific differences in methylation and structural modifications of the chromatin have been postulated as the signature of the imprint (Ferguson-Smith *et al.*, 1993; Stöger *et al.*, 1993; Tremblay *et al.*, 1995; Zuccotti and Monk, 1995; Franklin *et al.*, 1996). It is critical that such an imprint is maintained at each DNA replication for the perpetuation of the parent-specific characteristics as the cells differentiate (Barlow, 1994).

In this review, we examine what is known about the differences between the active and inactive X chromosome, which may have bearing on the imprinting of the X chromosome. The role of the *XIST/Xist* gene and the chromatin structure of the X chromosome will be discussed with respect to differential X-chromosome activity in the preimplantation embryo and in specific tissue lineages.

Features of the inactive X chromosome

Heterochromatic state and asynchronous replication

In adult somatic cells at interphase, the inactive X chromosome (Xi) is visibly distinct from the active counterpart (Xa) and other chromosomes by its formation of a heterochromatic Barr body located near the nuclear membrane (Barr and Carr, 1961; Dyer *et al.*, 1989). At metaphase, when both X chromosomes are condensed, the Xi can be visualised by its intense Giemsa staining reaction (Kanda, 1973) and its resistance to deoxyribonuclease I (DNaseI) digestion (Kerem *et al.*, 1983). When examined after *in situ* hybridization with centromeric and telomeric probes, the Barr body is seen to form an unusual looping structure with closely apposed telomeres attached to the nuclear membrane (Walker *et al.*, 1991). Whether this structural specialization plays a role in initiation or maintenance of X inactivation has yet to be ascertained.

A distinct feature of the Xi is the late replication of the chromosome in S phase (Takagi, 1974). The replication timing of several genes, undergoing inactivation on the Xi, has been shown to lag behind that of their counterparts on the Xa (Schmidt and Migeon, 1990; Boggs and Chinault, 1994; Torchia *et al.*, 1994). There is no direct correlation between the sequence of replication or the extent of asynchrony and the physical location of the genes relative to the *XIST* on the human X chromosome (Boggs and Chinault, 1994). Genes that escape inactivation do not display any asynchrony of replication (Boggs and Chinault, 1994). It has been proposed that this temporal separation of replication is essential for regulating gene expression by maintaining the chromatin disparity between Xa and Xi. Chromatin that is assembled earlier in S phase may be more amenable to activation by transcription factors than chromatin that is assembled later (Riggs and Pfeifer, 1992). Conflicting data has been obtained for the replication timing of the active *Xist* gene on Xi and the inactive *Xist* gene on Xa using different methodologies. 5-bromo-2'-deoxyuridine (BrdU) incorporation, using a probe from the 5' end of the gene, shows the inactive allele replicating first (Hansen *et al.*, 1995). Two studies using fluorescence *in situ* hybridization and DNA probes derived from the body of the gene show the inactive allele replicating last (Boggs and Chinault, 1994; Torchia and Migeon, 1995). The apparent disparity in results could be due to the different probes used. More work is required to determine if the 5' end of the *Xist* gene replicates differently from the body of the gene.

Abbreviations used in this paper: BrdU, 5-bromo-2'-deoxyuridine; DNase I, deoxyribonuclease I; ES, embryonic stem cells; G6PD, glucose-6-phosphate dehydrogenase; HPRT, hypoxanthine phosphoribosyl transferase; *Igf-2*, insulin like growth factor 2; PCR, polymerase chain reaction; *Pdha1*, Pyruvate dehydrogenase a1; PGK1, phosphoglycerate kinase 1; *Prps1*, phosphoribosyl pyrophosphate synthetase 1; RT, reverse transcription; SNuPE, single nucleotide primer extension; Xa, active X chromosome; *Xce*, X-controlling element locus; Xi, inactive X chromosome; XIC/Xic, X inactivation centre; *XIST/Xist*, X inactive specific transcript; X^M, maternal X chromosome; X^P, paternal X chromosome.

Hypoacetylation of histone H4

DNA is wrapped around nucleosomes which comprise two molecules each of the core histones H2A, H2B, H3 and H4. The chromatin of Xa and Xi in adult somatic tissues differs in the level of acetylation of the lysine residues in the amino terminal tail of histone H4 molecules (Jeppesen and Turner, 1993). Xa is associated with hyperacetylated histone H4 whereas Xi chromatin lacks all four acetylated forms of histone H4. This finding is of particular significance since the acetylation of lysine residues in core histones may be associated with enhanced transcriptional activity (Davie and Hendzel, 1994; Brownell and Allis, 1996). The absence of hyperacetylated histone H4 may be a feature of chromatin that is switched off permanently (Worrad *et al.*, 1995). Supporting this theory is the observation that in autosomes, constitutive heterochromatin shows extremely low levels of acetylation of histone H4 (Jeppesen *et al.*, 1992; O'Neill and Turner, 1995). A low level of histone H4 acetylation is present along the entire length of the Xi chromosome, except for three hyperacetylated regions. Two of these regions contain genes known to escape X inactivation in humans and mice (Lyon, 1986; Agulnik *et al.*, 1994).

Interestingly, recent evidence suggests that acetylation of the core histone H4 may not be involved in the regulation of individual genes on the autosomes. Chromatin fractions enriched, or depleted, in hyperacetylated histone H4 were obtained by immunoprecipitation using an antibody specific to acetylated histone H4. Several genes, regardless of their state of activity, hybridized with equal efficiency to both the DNA isolated from hyper- and hypoacetylated chromatin (O'Neill and Turner, 1995). This is in direct contrast to previous studies that have revealed a correlation between acetylation and activity by utilizing a general antibody that detects acetylated core histones in addition to histone H4 (Hebbes *et al.*, 1988; Clayton *et al.*, 1993; Hebbes *et al.*, 1994). Acetylation of other core histones, such as histone H3, may therefore be critical in regulating gene activity. This raises the question of whether differences in histone acetylation between Xi and Xa chromosomes are also found in other histone molecules.

Hypermethylation of CpG islands

In mammals, CpG islands are G+C-rich segments of DNA, in the order of 1kb in length, with a high frequency of CpG dinucleotides. Most mammalian DNA has only about one-fifth the expected frequency of CpG dinucleotides, as computed from the G+C content of the DNA sequence, but CpG islands have close to the expected frequency of CpG dinucleotides. CpG islands encompass the transcription start sites of numerous genes (Gardiner-Garden and Frommer, 1987; Cross and Bird, 1995). The methylation status of CpG dinucleotides within Xi and Xa alleles can be assessed by the susceptibility of sites to digestion by methylation-sensitive restriction enzymes (Grant and Chapman, 1988) and by genomic sequencing techniques that examine all CpG dinucleotides in extended regions of X-linked CpG islands (Pfeifer *et al.*, 1990; Hornstra and Yang, 1994; Park and Chapman, 1994). A consistent difference between Xi and Xa in adult tissues is the extensive methylation of CpG islands on the silenced X chromosome.

The timing of methylation of CpG dinucleotides within CpG islands, with respect to X inactivation, has been found to vary. Using PCR-based restriction enzyme analysis, some sites in CpG islands of the mouse *G6pd* and *Pgk1* genes are found to be methylated at the time of X inactivation (Singer-Sam *et al.*, 1990a; Grant *et al.*, 1992), but by Southern blot analysis, sites in an intron region of the CpG island of the *Hprt* gene are methylated only after X inactivation has occurred (Lock *et al.*, 1987). It is now widely believed that the extensive methylation of CpG islands of Xi is not related to the initiation of inactivation but to the maintenance of the inactive state. The more extensive methylation of the CpG islands in Xi of adult tissues is apparently associated with the more stable inactivated state of the X-chromosome. A less stable inactivation is seen where CpG island methylation is less extensive in cultured extraembryonic tissues and in marsupial tissue (Migeon *et al.*, 1986, 1989).

The use of genomic sequencing techniques has revealed that the CpG islands associated with the inactive alleles of human *PGK-1* and *HPRT* genes are methylated at almost all sites tested. An exception is found with some hypomethylated sites in the *HPRT* promoter, within a 70bp region encompassing four GC boxes which are potential binding sites for transcription factor Sp1 (Pfeifer *et al.*, 1990; Hornstra and Yang, 1994).

The CpG islands associated with the mouse *Pgk1* and *Hprt* inactive alleles are less methylated than their human counterparts (Tommasi *et al.*, 1993; Park and Chapman, 1994). When the CpG island of the inactive *Hprt* gene was analyzed by the bisulphite genomic sequencing technique (Frommer *et al.*, 1991; Clark *et al.*, 1994), the extent of methylation of specific sites differed between individual Xi chromosomes although a consistently high level of methylation was found near the transcription start site (Park and Chapman, 1994). This finding suggests that only a general level of methylation may be required for conferring the inactive status of the *Hprt* gene although specific sites near the transcription start site may be involved. Thus, rather than involving specific methylation-sensitive transcription factors, the establishment of an inactive state of X-linked CpG islands may involve methylated-DNA binding proteins such as MeCP1 (Boyes and Bird, 1992) and MeCP2 (Meehan *et al.*, 1992) which have the ability to bind to and downregulate transcription from methylated DNA but do not require a precise DNA sequence motif. Embryonic stem (ES) cells containing a null mutation of the *Mecp2* gene appear normal whereas chimaeric embryos, formed with a high proportion of these cells, fail to gastrulate (Tate *et al.*, 1996). This might be related to the loss of MeCP2 function which may affect the process of X inactivation that occurs at about the time of gastrulation, but the precise effect of this mutation on X inactivation has not yet been tested.

Hypermethylation of CpG islands is not apparent in X-linked genes which fail to undergo X inactivation (Goodfellow *et al.*, 1988; Luoh *et al.*, 1995; Carrel *et al.*, 1996). Furthermore, the gene coding for human factor IX, which undergoes inactivation but is not associated with a CpG island, does not show differential methylation between the active and inactive X alleles (Ruta Cullen *et al.*, 1986). This suggests that if X inactivation involves a spread of methylation across Xi (Grant *et al.*, 1992), only specific genes become methylated.

Expression of XIST/Xist gene from the inactive X chromosome

Regulation of X-chromosome activity requires the presence of a *cis*-acting X-inactivation centre (Russell, 1963; Cattanach, 1974). The X inactivation specific transcript gene (*XIST* in humans, *Xist* in mice), maps to the XIC(Xic) and the RNA is expressed only by the inactive X chromosome (Borsani *et al.*, 1991; Brockdorff *et al.*,

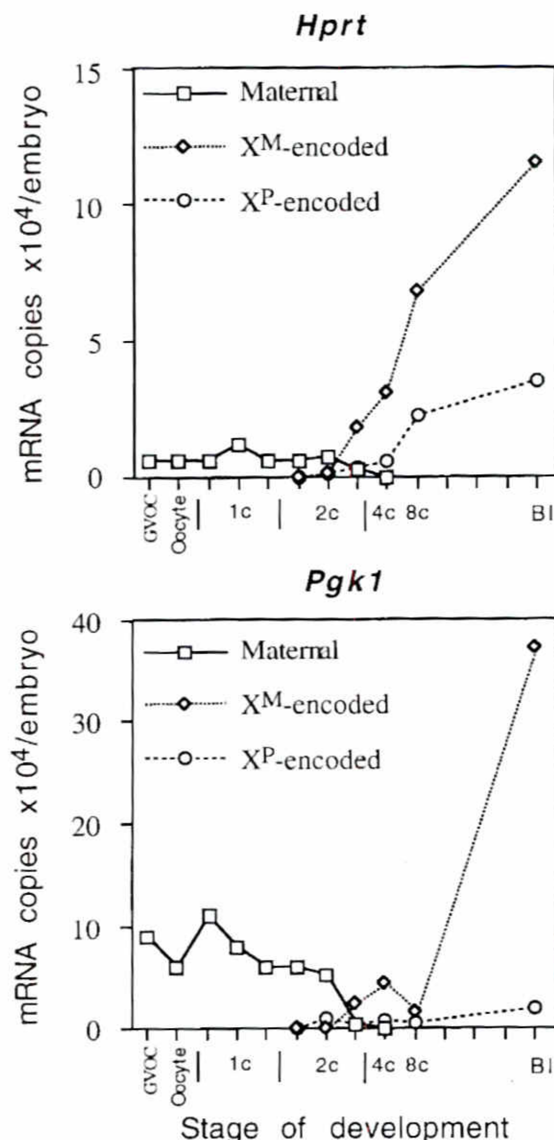

Fig. 1. The changes in the quantity of maternal (oocyte-derived) and zygotic mRNAs during oocyte maturation and pre-implantation embryonic development. *Data is based on the findings of Singer-Sam et al. (1992) and Latham and Rhambhatla (1995). The amount of X-encoded Hprt and Pgk1 mRNAs was determined by the quantitative procedure of reverse transcription and polymerase chain reaction (RT-PCR). The transition from maternal to zygotic transcription was studied by quantifying the Hprt and Pgk1 mRNAs in embryos treated with α-amanitin which inhibits transcription, and in normal untreated embryos (Latham and Rambhatla, 1995). The amount of transcripts encoded by the Hprt and Pgk1 alleles was determined by SNuPE (single nucleotide primer extension) assay (Singer-Sam et al., 1992). For both Hprt and Pgk1 genes, the amount of maternal (oocyte) mRNA declines during the first two embryonic cleavages. Embryonic transcription begins at the late 2-cell stage, with a preponderance of maternally (X^M) over the paternally (X^P) derived transcripts at the 4-cell to blastocyst stages. At the 8-cell stage, the bias for maternal Hprt transcripts is not seen for reciprocal matings (not shown here), which may represent an Xce allelic effect. Stages of development include germinal vesicle stage oocyte (GVOC), ovulated oocyte, one-cell (1c, with three time points of sampling), two-cell (2c, also with three time points of sampling), eight-cell (8c) and blastocyst (Bl).*

1991; Brown *et al.*, 1991). The transcript is large (17kb in the human, 15kb in the mouse) with no conserved open reading frames (Brockdorff *et al.*, 1992; Brown *et al.*, 1992). The absence of a translated protein product suggests that the transcription of the *Xist* locus could lead to a conformational change in the chromatin, which in turn allows the binding of other factors to initiate the X inactivation process (Brockdorff *et al.*, 1992). Alternatively, *Xist* may produce a functional RNA which interacts *in cis* with the chromosome from which it is transcribed (Brockdorff *et al.*, 1992; Brown *et al.*, 1992). In the interphase nucleus, *XIST* RNA has a punctate distribution in the same nuclear territory as the Xi (Clemson *et al.*, 1996). This observation, together with the apparent association of the *XIST* RNA with nuclear matrix material, has led to the idea that these molecules may distribute closely to the Xi chromatin and form junctions with nonchromatin nuclear components. The amount of *XIST* transcript present in cells is not sufficient for it to bind directly along the entire length of the DNA (Buzin *et al.*, 1994). Rather, *XIST* transcripts may interact with chromatin proteins and become involved in the formation of higher levels of packaging (Clemson *et al.*, 1996) which could prevent the entry of transcription factors and polymerases.

The function of the *Xist* gene has been investigated by monitoring the process of X inactivation in ES cells carrying a 7kb deletion including 36 base pairs of the minimal promoter sequence and part of exon I of the *Xist* gene (Penny *et al.*, 1996). In these ES cells, upon differentiation, only the X chromosome bearing the functional *Xist* gene can be inactivated. A *cis*-acting *Xist* gene is therefore essential for X inactivation to proceed.

Counting and choice of X chromosome to be inactivated

A counting mechanism has long been thought to be involved in X-chromosome inactivation since altering the relative dosage of autosomes and X chromosomes, as seen in individuals with abnormal karyotypes, can influence the number of inactive X chromosomes (Jacobs and Migeon, 1989). Normally there is one active X per two sets of autosomes. The choice of which X chromosome is to be inactivated can also be influenced, in certain situations, by the parental origin of the X chromosome, as discussed in this review.

The single allele *Xist* knockout experiment described above (Penny *et al.*, 1996) has shown, that upon differentiation of ES cells, X inactivation occurs in the absence of two intact *Xist* genes. Furthermore, the X chromosome containing the intact *Xist* gene was not always silent suggesting that in a proportion of the ES cells the X chromosome containing the deleted *Xist* gene was chosen for inactivation but failed to inactivate (Penny *et al.*, 1996). It has been speculated that the choice and counting functions might be provided by other elements in the Xic, such as the *Xce* (Simmler *et al.*, 1993). In the mouse, three well-characterised alleles of the X-controlling element locus ($Xce^{a,b,c}$) have been mapped to the Xic (Cattanach, 1975; Simmler *et al.*, 1993). In a mouse which is homozygous for any one of these alleles there is an equal probability that either X will be inactivated. In a heterozygous animal, the presence of different *Xce* alleles may bias the choice of X chromosome for inactivation. Xce^a is the weakest allele and Xce^c is the strongest and in a heterozygous animal the X chromosome with the stronger *Xce* allele tends to remain active (Cattanach, 1975;

Johnston and Cattanach, 1981). A human equivalent of the *Xce* has not been found. An extremely skewed X inactivation pattern has, however, been demonstrated in a family in all seven daughters of one male and the mother of this male. This suggests that in humans there may also be other X-linked gene(s) important in controlling X-inactivation choice (Naumova *et al.*, 1996).

A 450 kb YAC transgene containing the Xic and *Xist* has been incorporated onto autosomes within male ES cells. Upon ES cell differentiation, the expression levels of the endogenous and transgenic *Xist* genes are consistent with individual copies of Xic being counted as separate X chromosomes regardless of their location. This demonstrates that the Xic which includes the *Xce* locus, contains the choice and counting functions for X inactivation (Lee *et al.*, 1996).

Differential activity of the X chromosome during development

The preimplantation embryos

Evidence derived from studies of enzymatic, mRNA and transgene activity suggests that the activity of X^P-linked alleles may be lower than that of the X^M counterparts in preimplantation embryos. Early studies of the activity of X-linked enzymes employed relatively crude methodology and did not always provide a direct assessment of the activity of maternally and paternally derived alleles. These studies indicated that for hypoxanthine phosphoribosyl transferase (HPRT) and α-galactosidase there was a transition from the utilization of maternal enzyme to zygotic enzyme from both X chromosomes at the 4-8 cell stage (Adler *et al.*, 1977; Epstein *et al.*, 1978; Kratzer and Gartler, 1978; Kratzer, 1983). In contrast, only maternally encoded X-linked phosphoglycerate kinase (PGK1) enzyme activity was present in the preimplantation embryo (Kozak and Quinn, 1975). A better delineation of allelic activity has been achieved by examining HPRT activity in mice with the null mutation of the *Hprt* gene *(Hprt^{b-m3})* of known parental origin. In this study, HPRT activity from the paternal X was first detected at the late 2-cell stage. The first indication of differential activity was found at the 4-8 cell stage, where the enzyme activity encoded by the maternal allele was twice that of the paternal allele (Moore and Whittingham, 1992). Pooled embryos at the morula and blastocyst stages of the mouse mutant *Hprt^{b-m3}* continued to show elevated activity of the maternal *Hprt* allele (Moore and Whittingham, 1992).

A more precise picture of the differential activity of the X chromosomes in the cleavage embryo has been obtained by direct analysis of the level of X-linked gene transcripts (Fig. 1) . In the zygote and early cleavage embryos a transition occurs at approximately the late 2-cell stage from activity from inherited maternal (oocyte) mRNAs to zygotic genome activity (Flach *et al.*, 1982; Pikó and Clegg, 1982). This transition has been studied more specifically for X-linked transcripts (Latham and Rambhatla, 1995) in relation to the parental origin of the X chromosomes by the quantitative SNuPE (single nucleotide primer extension) assay (Singer-Sam *et al.*, 1992). Embryonic *Hprt* and *Pgk1* transcripts are first detected at the late 2-cell stage and again there is a general predominance of transcript from the maternally inherited allele at the 4-cell to blastocyst stages (Fig. 1).

Differences in the activity of the X chromosomes in the cleavage-stage embryos are also seen using an X-linked transgene coding for ß-galactosidase. When the transgene is expressed from X^M, *lacZ* expression is evident in fertilized oocytes and up until the 4-cell stage. At the 4-8 cell stage variable blastomere staining patterns are seen, which may reflect the transition from the utilization of maternal transcript to zygotic transcription. Uniform transgene expression is found at the 16 to 32-cell and morula stages. When the transgene is expressed from the X^P, ß-galactosidase activity is not seen until the 16 to 32-cell stage. More significantly, not every cell in the morula shows activity (Tam *et al.*, 1994a), suggesting either that there is a delay in the activation of the X^P-linked transgene or that the X^P may be activated only in a few cells and display a lower activity. This provides further support for the presence of differential activity of the parental alleles at all phases of preimplantation development.

While there is evidence from enzymatic, mRNA and transgene studies that there is suppressed X^P-linked gene activity in the cleavage stages, the first cytogenetic indication of inactivation (revealed by heterochromatic staining of the X chromosome) is not found until the blastocyst stage (Takagi, 1974; Takagi *et al.*, 1978; Sugawara *et al.*, 1985). The early suppressed X^P-linked gene activity may be a prelude to the preferential X^P-inactivation that is found later in the trophectodermal lineage. Some cells in the early embryo may have constantly suppressed X^P activity and be predisposed to the formation of the trophectoderm. If this is so, then the trophectoderm lineage may have been specified as early as the 4-8 cell stage, well before its morphological differentiation in the blastocyst.

The extraembryonic tissues

Asynchronous replication which is indicative of an inactive X chromosome (Yoshida *et al.*, 1993) has been shown to be characteristic of the paternal X in the trophectoderm of the blastocyst (Sugawara *et al.*, 1985), and in the trophectodermal tissues (extraembryonic ectoderm and ectoplacental cone) and the primitive endoderm of postimplantation embryos (Takagi and Sasaki, 1982). In the embryonic ectoderm, asynchronous replication can be found in either X^P or X^M (Takagi and Sasaki, 1982). XP, however, tends to display asynchronous replication earlier than X^M at 5.3-6.3 days (Fig. 2) before a completely random pattern is attained upon completion of inactivation in all embryonic cells (Takagi and Sasaki, 1982). While this may implicate a tendency for X^P to engage earlier in inactivation, the possibility that the higher incidence of inactivated X^P in samples of the postimplantation embryos may have been caused by the contamination of extraembryonic tissues has not been excluded.

Analysis of HPRT activity by comparison of levels in pooled male and female mouse embryos, has indicated down-regulation of X-linked enzyme activity in the females first in the extraembryonic tissues at about 5.5 dpc and later in epiblast tissues at 6.5 dpc (Monk and Harper, 1979). Analysis of electrophoretic variants of the PGK-1 enzyme shows that at 6.5 dpc there is preferential expression of the X^M-encoded enzyme in the extraembryonic ectoderm and the primary endoderm (Harper *et al.*, 1982). Similar analysis of mouse yolk sac endoderm at 12.5 dpc and 13.5 dpc indicates preferential expression of the X^M-encoded enzyme (West *et al.*, 1977; McMahon *et al.*, 1983). Preferential maternal X-chromosome activity in the extraembryonic tissues is also demonstrated using the X-linked ß-galactosidase transgenic mouse line at 3.5 to 6.5 days (Tam *et al.*, 1994a). Interestingly, results from this

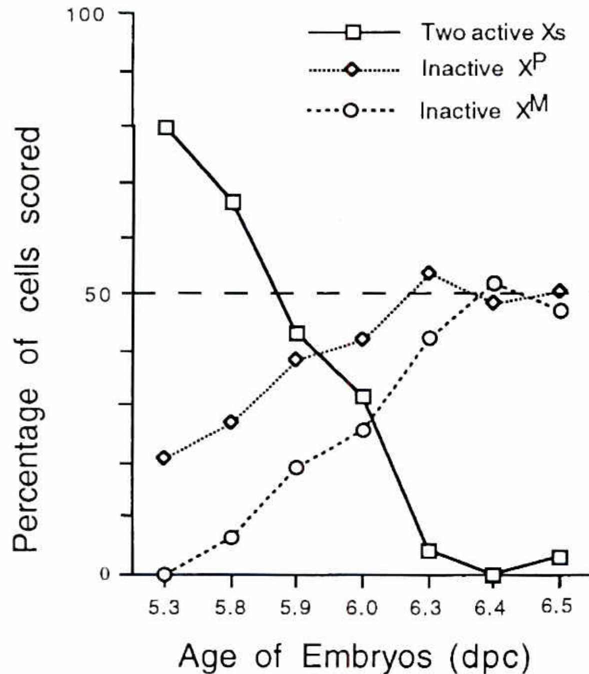

Fig. 2. The progression of X-chromosome inactivation determined by quantifying the relative proportion of embryonic cells that display asynchronous replication of the X chromosome, assayed by 5-bromo-2'-deoxyuridine incorporation. *The parental origin of the X chromosome is deduced from the source of the lengthened chromosome in crosses of wild type mice and mice carrying Cattanach's insertion (Takagi and Sasaki, 1982). The "two active Xs" curve represents the proportion of cells (at metaphase) that do not show asynchrony in X-chromosome replication. This value will decrease as X inactivation proceeds in the cell population. The "inactive" curves show the increases in proportion of cells that display an asynchronously replicating X chromosome that was inherited from the father (X^P) or the mother (X^M). The data show that although the embryonic cells in the gastrulating mouse embryo eventually display random X inactivation (equal proportion of cells with asynchronously replicating X^P or X^M), the X^P chromosome tends to be inactivated sooner than the X^M once the choice of which chromosome to inactivate has been made. Data is based on the findings of Takagi et al. (1982).*

study in the embryonic ectoderm also show that at 5.5-7.5 days the XP may be inactivated earlier than the X^M.

There have been no studies in humans of the X-chromosome behavior in early differentiating extraembryonic tissues. Understanding of these early events relies on extrapolation of observations made on cells of the differentiated placenta. Cultured fetal chorionic villi from first-trimester spontaneous abortuses and one newborn showed preferential expression of one of the two glucose-6-phosphate dehydrogenase *(G6PD)* alleles in some clones but biallelic expression in others (Migeon *et al.*, 1985). In contrast, a study on full-term placental tissues carefully dissected to exclude maternal cell contamination, showed in both fresh and cultured amnion and chorion, as well as cultured villi, preferential maternal allele expression (Harrison and Warburton, 1986). In a pure population of cytotrophoblast cells isolated from the chorionic villi, an excess of maternal allele expression was observed. In contrast, the stromal cells of the villi, which are derived from the mesoderm, exhibited random X-chromosome inactivation (Harrison, 1989).

The results of these studies, particularly those of the mouse, clearly indicate that the X^P and X^M chromosomes behave differently in the extraembryonic tissues of the early embryo. There is also the tantalising suggestion from the mouse studies that, in the embryonic tissues, the X^P chromosome which has been chosen for inactivation may be inactivated ahead of the X^M, even though a random inactivation of either chromosome is ultimately accomplished in the cell population.

Developmental consequences of abnormal X-chromosome constitution

Uniparental embryos

Uniparental embryos which are androgenetic (two paternal genomes) or gynogenetic or parthenogenetic (two maternal genomes) provide an experimental test for parent-specific X-chromosome activity. In parthenogenetic embryos early cytogenetic studies indicated that X-chromosome inactivation may occur in cells of the epiblast and the yolk sac endoderm (Kaufman *et al.*, 1978; Rastan *et al.*, 1980). Inactive X chromosomes are however, found less frequently in parthenogenetic blastocysts than in normal blastocysts (Tada and Takagi, 1992). Using quantitative RT-PCR (Latham and Rambhatla, 1995) (Fig. 3), gynogenetic embryos were shown to contain approximately 1.5 to 2-fold more *Pgk1* mRNA at the morula to late blastocyst stages than was found in normal embryos. Again using quantitative RT-PCR, pyruvate dehydrogenase a1 *(Pdha1)* expression in gynogenones was elevated at the blastocyst stage compared with normal embryos although this was variable, whereas *Hprt* and phosphoribosyl pyrophosphate synthetase 1 *(Prps1)* expression patterns were similar to those in normal embryos (Fig. 3). This indicates that the two maternally derived X chromosomes in the gynogenones are somewhat refractory to inactivation. It has been suggested (Latham and Rambhatla, 1995) that the results may also indicate that genes localized close to the Xic locus, such as *Pgk1*, are less subject to inactivation if they are derived from the maternal source.

The expression of X-linked genes has been studied in pooled preimplantation androgenetic embryos comprising a mixture of X^PX^P, X^PY and possibly YY embryos (Latham and Rambhatla, 1995). By the 8-cell stage, quantitative RT-PCR shows levels of *Hprt, Pdha1* and *Prps1* mRNAs are reduced to about half those of normal embryos, but levels become normal by the late blastocyst stage. *Pgk1* mRNA expression, however, is markedly reduced at all three stages. This suggests the X^P-linked genes are generally less actively transcribed (Fig. 3).

While the results in these two types of uniparental embryos suggest that the X chromosomes of different parental origin behave differently, this may not be entirely specific to the X chromosomes. The impact of the autosomes and the Y chromosome (in X^PY androgenones) which are also derived from the same parent must be taken into consideration. The varying X-linked gene dosage could also be affected by the developmental retardation of the uniparental embryos. Moreover the androgenone experiments were performed on small numbers of embryos where the genotype was not determined. The measurement of transcript level may be biased by the presence of XY embryos with a single dose of X-linked gene activity. Future work needs to address the gene activity in embryos of known genotypes.

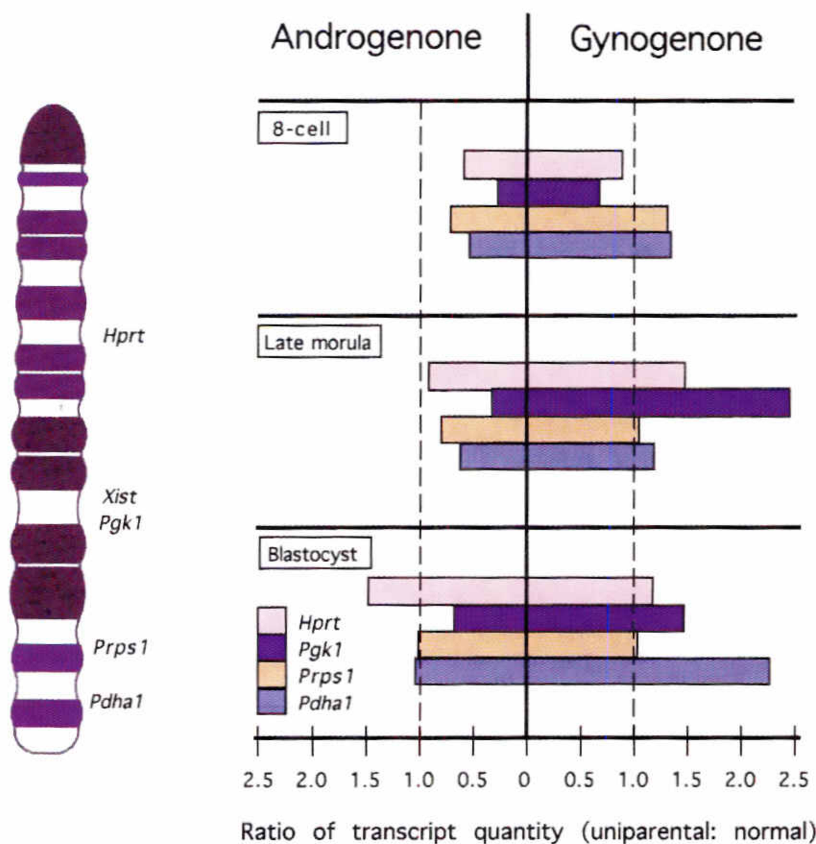

Fig. 3. A comparison of the relative transcriptional activity of the X-linked genes (*Hprt, Pgk1, Prps1* and *Pdha1*) in androgenones (entire genome of paternal origin) and gynogenones (entire genome of maternal origin). *The activity is expressed as a mean ratio of the amount of transcript (assayed by quantitative RT-PCR) in each type of uniparental embryos compared with wild type embryos. A value of 1.0 (marked by the dashed line) indicates that the transcription of the X^P- or X^M-linked gene in the uniparental embryo equals that of the same allele in the normal female embryo. Except for the Hprt activity in the androgenetic blastocyst, the maternally derived allele is generally expressed at a higher level than the paternally derived allele in uniparental embryos. This is most marked for Pgk1 located closest to Xist as seen on the diagram of the X chromosome. Note that values for Pdha1 expression at the blastocyst stage in gynogenones were variable and should be treated with caution. Data are based on the findings of Latham and Rambhatla (1995).*

Single X chromosome

The possible differences in behavior of the X chromosome depending on the parent of origin may be observed in female mice of the 39,XO genotype. The XO mice that have the X of paternal origin (X^PO) are produced in matings of females heterozygous for an inversion of the X chromosome In(X)1H (Evans and Phillips, 1975) with males with a normal X chromosome (Burgoyne *et al.*, 1983a). These XO mice show reduced birthweight, slow postnatal growth, shortened reproductive life span and reduced litter size (Cattanach, 1962; Lyon and Hawker, 1973). X^PO embryos display growth retardation at the preimplantation and immediate postimplantation periods but compensate for the size deficiency by catch-up growth at mid-gestation (Burgoyne *et al.*, 1983b; Banzai *et al.*, 1995). Since the XO embryos studied in the preimplantation period are developing in females also of XO genotype, the possible complicating factor of poor oocyte quality or an abnormal intrauterine environment cannot be ignored (Burgoyne and Biggers, 1976). Indeed, XX and XY embryos in an XO uterus do show growth retardation in the preimplantation period compared with those in an XX uterus, and X^PO embryos in XO mothers show the most delay in development (Banzai *et al.*, 1995). Using a mouse strain with a structurally abnormal Y chromosome, female mice with a normal X^M and a small Y chromosome containing only the centromere and the pseudoautosomal region are produced (XY^{*x}). These mice are functionally X^MO females. Mating of the XY^{*x} females with normal males produced some female embryos thought to be equivalent to X^PO. Results of these matings indicated that none of the X^PO embryos present at preimplantation survives at birth (Hunt, 1991). Again the data may have been influenced by the fact that the mother of the X^PO embryos is functionally XO whereas the mother of the XY^{*x} (=X^MO) embryos is XX. X^PO and X^MO embryos can also be produced by mating mice with an X-linked *Patchy fur (Paf)* mutation (Lane and Davisson, 1990). At about 10.5 days, the X^PO embryos are approximately half the size of their X^MO littermates, but X^MO embryos are similar in size to the XY embryos. Interestingly, both the X^MO and XY are bigger than the XX embryos (Thornhill and Burgoyne, 1993). Again all these embryos are developing in an XO uterine environment. We are currently addressing the question of the developmental potential of X^PO and X^MO embryos at pre- and postimplantation stages using mothers that are XX in both cases.

Overall these studies indicate that X^PO embryos show growth retardation in preimplantation and early postimplantation development with the effect possibly lasting through to birth. This difference between X^PO and X^MO development remains unexplained. If X-chromosome activity in preimplantation development is imprinted, as would seem to be the case, perhaps decreased expression of certain X-linked genes at this time in the X^PO results in growth retardation (Banzai *et al.*, 1995). Alternatively, since X^P is preferentially inactivated in the trophectoderm at 3.5-4.5 dpc, perhaps the obligatory inactivation of the X^P in cells without the backup of X^M may have led to poor extraembryonic development and thus growth retardation. Some cells in the trophectoderm must be able to keep X^P active and override the inactivation effect since the trophectoderm derived tissues of 9.5-12.5 day X^PO embryos do show some X-linked PGK1 activity (Frels and Chapman, 1979; Papaioannou and West, 1981).

Differential X-chromosome activity may play a role in the manifestation of the Turner syndrome phenotype in humans with X chromosome monosomy. In patients with a 45,X karyotype, those with 45,X^M are more likely to express certain features of Turner syndrome (cardiovascular abnormalities and neck webbing) than individuals with 45,X^P (Chu *et al.*, 1994). Several studies have shown that for individuals with 45,X there is a predominance of 45,X^M to 45,X^P both in liveborns and abortuses (Hassold *et al.*, 1988; Jacobs *et al.*, 1990; Mathur *et al.*, 1991; Lorda-Sanchez *et al.*, 1992; Chu *et al.*, 1994). It has been speculated that 45,X which

may arise from errors in meiosis or mitosis would present at a ratio of 2: 1 (45,X^M: 45,X^P) by assuming equal loss at each step and the poor viability of 45,Y (Mathur *et al.*, 1991). The predominance of 45,X^M is more marked than this and has amounted to 2.3: 1 to 4: 1 (45,X^M: 45,X^P) (Lorda-Sanchez *et al.*, 1992; Chu *et al.*, 1994) in both abortuses and liveborns. It is possible that this predominance of 45,X^M in human X monosomy is related to preferential 45,X^P fetal loss before 7.5 weeks gestation due to retarded growth at early development similar to that seen in X^PO mouse embryos.

Uniparental X-chromosome disomy

The activity of the X chromosomes in individuals with uniparental X-chromosome disomy may give insight into the imprinting effects associated with the X chromosome. They are, however, rarely reported in humans and none has been reported in the mouse. A possible imprinting effect was reported in a woman whose two X chromosomes were paternally derived. She had short stature and impaired gonadal function but few other Turner stigmata. The interpretation of the phenotype is, however, confounded by the presence of a low level of 45,X mosaicism (Schinzel *et al.*, 1993; Ledbetter and Engel, 1995). Maternal X-chromosome disomy has been reported in normal fertile women (Avivi *et al.*, 1992). In an effort to examine the impact of two maternal X chromosomes in the mouse, female embryos were examined with an extra maternal X ($X^MX^MX^P$). These embryos showed growth retardation shortly after gastrulation which could be related to excessive X^M-linked gene dosage in the trophectoderm. Some embryos were, however, also monosomic for the distal part of chromosome 4 which by itself is also associated with growth retardation (Shao and Takagi, 1990).

The possible role of methylation in imprinting of the X chromosome

The differential behavior of the X chromosomes in the early embryo is strongly suggestive of a parent-specific modification (imprinting) of the X chromosome that takes place in the germ cells. The effects of the imprint are reflected in first, the delayed expression of certain X-linked alleles in cleavage stage embryos and second, the preferential paternal X inactivation in the extraembryonic tissues together with the precocious inactivation of the paternal X in epiblast tissues. These two phenomena may arise from the same primary imprinting signals. Alternatively since only the latter phenomenon is accompanied by heterochromatinization and asynchronous replication of the X chromosome the phenomena may arise by different mechanisms.

Methylation of CpG islands

In tissues undergoing random X inactivation, CpG islands associated with silenced genes are extensively methylated on Xi and unmethylated on Xa, as described previously. An important question is whether a similar differential methylation exists between X^P and X^M in tissues undergoing paternal X inactivation. Only a limited number of CpG sites have been examined in extraembryonic tissues to date. A single site in the *Pgk-1* gene and another in the *G6pd* gene have been found methylated in mouse extraembryonic tissues, by PCR based restriction enzyme analysis (Singer-Sam et al., 1990a; Grant *et al.*, 1992). The human *HPRT* gene in whole placenta and the human *P3* and *GdX* genes in chorionic villi are, however, unmethylated at the few restriction enzyme sites examined.

One of the earliest experiments, designed to test for differential methylation between *Hprt* alleles, assayed the ability of purified DNA containing the *Hprt* gene to be expressed after its introduction into *Hprt⁻* cell lines. Any difference between the numbers of *Hprt⁺* colonies obtained for different alleles of *Hprt* must be due to a DNA modification, such as DNA methylation, since chromatin proteins are removed from the introduced DNA prior to transfection. To examine the situation in tissues undergoing random X inactivation, DNA was purified from whole X: autosomal translocation T(X;16) fetuses which contained the *Hprt-a* allele on the intact Xi and the *Hprt-b* allele on the translocated Xa. Of the colonies obtained, 100% showed expression of the *Hprt-b* allele, consistent with extensive DNA modification of the *Hprt-a* allele on the Xi. Interestingly, when purified genomic DNA derived from XX yolk sac endoderm, which undergoes preferential paternal X inactivation, was used to transform *Hprt⁻* cells, 70 to 80% of the *Hprt⁺* colonies obtained expressed the maternal rather than paternal allele of the introduced *Hprt* gene (Kratzer *et al.*, 1983). This provides evidence that the level of DNA methylation, or other DNA modification, within the mouse *Hprt* gene is higher for X^P than X^M in yolk sac endoderm. The difference between X^P and X^M alleles in yolk sac endoderm was apparently not as great as that between alleles on the Xa and Xi in fetal tissues (Kratzer *et al.*, 1983). Genomic sequencing of X^P and X^M alleles in extraembryonic tissues is required to determine the exact extent of the methylation difference between alleles.

Two lines of evidence suggest that widespread methylation of CpG islands on the X chromosome is not the elusive signal which marks paternal X chromosomes for inactivation. Firstly, X-linked CpG islands in both sperm and oocyte are found unmethylated at the numerous restriction enzyme sites examined (Yen *et al.*, 1984; Lock *et al.*, 1986; Toniolo *et al.*, 1988; Driscoll and Migeon, 1990). Purified DNA from sperm can efficiently transform an *Hprt⁻* cell line to produce *Hprt⁺* colonies. This finding suggests that the although the *Hprt* gene is silent in sperm, it is not subject to extensive methylation (Venolia and Gartler, 1983). Secondly, the paternal allele of *G6pd* of the marsupial wallaroo *(Macropus robustus robustus)* is completely repressed in all adult tissues (VandeBerg *et al.*, 1987) yet, by genomic sequencing, the associated CpG island is unmethylated in both sperm and somatic tissues (Loebel and Johnston, 1996).

Parent-specific activity and methylation of the Xist gene

Differential inactivation of the X chromosome may be regulated by the imprinting of a controlling gene such as the *XIST/Xist* gene. Preferential paternal expression of the *Xist* gene has been observed in cleavage stage embryos (4 cell, 8 cell and morula) as well as in total blastocyst and yolk sac endoderm derived from 14.5 dpc female embryos (Kay *et al.*, 1993, 1994). This correlates with suppression of paternal X-linked genes. In one study, *Xist* expression in parthenogenetic and gynogenetic embryos is delayed until the morula and blastocyst stages whereas in androgenones expression occurs at the 4 cell stage and decreases at later stages. This study raises the possibility that an imprinted maternally-expressed gene may be required for *Xist* expression after the 4 cell stage (Kay *et al.*, 1994). In another study, however, *Xist* expression is observed in both androgenones and gynogenones at 8 cell, morula and blastocyst stages with the level being much higher in

the androgenones at all stages (Latham and Rambhatla, 1995). The first study examines the total pool of *Xist* transcripts whereas the second study examines polyadenylated transcripts only. The reason for the discrepancy in results is not clear. The preferential paternal expression of the *Xist* gene is consistent with, but does not provide proof that, an imprint resides within the *Xist* gene.

DNA methylation may play a role in parent-specific expression of the *Xist* gene since DNA methylation can be accurately inherited during cell division. In embryos which are severely deficient in DNA methylation due to a homozygous deletion in the DNA methyltransferase gene, there is a loss of imprinted expression pattern of the imprinted genes, *H19*, insulin like growth factor 2 *(Igf-2)* and *Igf-2* receptor (Li *et al.*, 1993). Differences in methylation patterns between parental alleles occur in some imprinted autosomal genes (Neumann and Barlow, 1996).

In several instances, methylation of restriction enzyme sites within the promoter region and exon 1 of the *Xist* gene correlate with suppression of the gene's transcription. Situations where the inactive *Xist* gene has been found to be methylated include X^M in female yolk sac endoderm, Xa in female and male somatic tissues, and Xa in both differentiated and undifferentiated male ES cells (Norris *et al.*, 1994). *Xist* gene activity has been examined in male ES cells and male embryos which are severely deficient in DNA methylation, due to the same homozygous deletion in the DNA methyltransferase gene. In contrast to the normal controls, in the mutant male ES cells after differentiation and in the mutant embryos the *Xist* gene is found hypomethylated and highly active (Beard *et al.*, 1995). A hypomethylated state is not, however, always sufficient for high levels of *Xist* expression since the *Xist* gene is also hypomethylated in mutant male ES which are undifferentiated and show only very low *Xist* expression (Beard *et al.*, 1995). An additional factor, necessary for *Xist* expression, may be absent in these undifferentiated male ES cells.

Two recent studies reported that specific methylation-sensitive restriction enzyme sites in the first exon of the *Xist* gene are unmethylated in sperm and methylated in the oocyte (Ariel *et al.*, 1995; Zuccotti and Monk, 1995) suggesting that a primary imprint may be present in this exon. The two studies examine different sites with the exception of a site which is found, in oocytes, to be methylated in one study (Ariel *et al.*, 1995) and unmethylated in the other study (Zuccotti and Monk, 1995). PCR-based restriction enzyme analyses may be prone to giving false positive methylation signals due to amplification of small amounts of partially digested DNA. Genomic sequencing of this exon may be required to confirm these results.

An important question is the time at which the *Xist* gene first shows differential methylation between alleles in the embryo. Using the PCR-based restriction enzyme method, several sites in the promoter region (Zuccotti and Monk, 1995) and exon 1 of the *Xist* gene (Ariel *et al.*, 1995) appear to be methylated in cleavage stage embryos. These results do not differentiate between X^P and X^M alleles and again may need to be corroborated with genomic sequencing.

It has been reported that the 5' end of the *Xist* gene is about 50% methylated in undifferentiated female embryonic stem (ES) cells (Norris *et al.*, 1994). By presuming that the methylated and unmethylated CpG sites were on separate alleles, the conclusion was reached that methylation of the *Xist* promoter preempts X inactivation in differentiating ES cells. In contrast, further studies have shown that the partial methylation observed in undifferentiated female ES cells is both unstable through cell division and mosaic rather than allele-specific in nature (Mise *et al.*, 1996; Sado *et al.*, 1996). It is known that ES cells do not strictly retain the characteristics of the inner cell mass from which they are derived and that ES cells can lose a genetic imprint. Direct studies of inner cell mass and trophectoderm have not been carried out as yet.

The X-chromosome imprint may reside in other genes or elements within the Xic that may influence *Xist* activity. A precedence for the imprinting of one gene causing the parent-specific expression of another gene is seen in the imprinted genes on mouse chromosome 7. The deletion of the *H19* gene (maternally expressed) has resulted in the loss of imprinting of the *Igf-2* and the insulin 2 genes (both paternally expressed), presumably by removing the competition for common enhancer elements (Leighton *et al.*, 1995). Similarly, an imprinting centre on human chromosome 15 affects both Angelman and Prader-Willi syndromes which are associated with maternal and paternal-specific expression, respectively (Buiting *et al.*, 1995). An *Xist* knockout experiment has shown that while *Xist* is needed for the initiation of X inactivation it is not required for the choice of which X chromosome is to be inactivated (Penny *et al.*, 1996). The *Xce* region which maps distal to *Xist* in the Xic (Simmler *et al.*, 1993) may control the choice of target for inactivation. Different alleles in this region can influence differential X-chromosome activity in somatic tissues of the adult animal (Cattanach, 1975) and in the yolk sac endoderm (Rastan and Cattanach, 1983). The strength of the *Xce* allele has been shown to correlate with methylation of several CpG sites in the DXPas34 locus which lies 15kb distal to *Xist* (Courtier *et al.*, 1995). More extensive studies are required to see if there are other sites within the DXPas34 locus which are preferentially methylated on the paternal allele in extraembryonic tissues. Two newly identified genes, *Cdx4* (Horn and Ashworth, 1995) and *BpX* (Rougeulle and Avner, 1996) are also found within the Xic region, but whether they may be involved with the imprinting of the X chromosome is not yet known.

Chromatin configuration of the X chromosome

Expression of X-linked genes in the germ cells

The presence of isopycnotic X chromosomes and the level of X-encoded enzyme activity have implied that both X chromosomes are active during oogenesis (Ohno *et al.*, 1961; Epstein, 1969; Monk and McLaren, 1981; Tam *et al.*, 1994b). In contrast, the X chromosome of the male germ cell is inactivated during spermatogenesis. The X and Y chromosome form a single mass in the zygotene stage during pairing of the chromosomes at meiosis I (Solari, 1974). In a study assaying incorporation of [^{3}H]uridine, the XY body was found to be transcriptionally inactive compared to autosomes at meiotic prophase (Monesi, 1965). The transcription of several X-linked genes has been examined more precisely by quantitative RT-PCR at different stages of spermatogenesis. The *Pgk1* (Singer-Sam *et al.*, 1990b; Walker *et al.*, 1991; Hendriksen *et al.*, 1995) and the pyruvate dehydrogenase 1 *(Pdha1)* genes (Dahl *et al.*, 1990; Hendriksen *et al.*, 1995) are silent in spermatocytes and spermatids. *Hprt* transcripts diminish in pachytene spermatocytes and in round spermatids (Singer-Sam *et al.*, 1990b) but remain detectable. This suggests either that the *Hprt* gene is transcribed at a low level in spermatocytes and spermatids, or that the *Hprt* transcripts formed in spermato-

gonia are more stable than *Pgk-1* and *Pdha1* transcripts and persist longer in the spermatogenic cells (Singer-Sam *et al.*, 1990b). The *MHR6A* and *Ube1x* genes behave differently during spermatogenesis; expression is reduced in pachytene spermatocytes, but transcripts reappear in round spermatids, indicating the occurrence of post-meiotic transcription (Hendriksen *et al.*, 1995).

The higher level of paternal allele suppression observed for the *Pgk1* gene compared to the *Hprt* gene (Figs. 1 and 3) may relate to the more complete suppression of *Pgk1* transcripts in male germ cells, described above. A plausible hypothesis is that the differential expression of X-linked genes between male and female germ cells may relate to differences in the chromatin structure of specific genes and that these chromatin structures may be maintained as imprinting effects in the zygote. Recently, several groups have demonstrated that viable offspring can be produced by fertilizing eggs with secondary spermatocytes or round spermatids (Aitken and Irvine, 1996). Their observations make it likely that the formation of essential imprinting signals during spermatogenesis is completed before the first meiotic division. Of course, fertilization does not naturally occur with immature sperm so we consider it likely that any imprinting signal formed early, such as at the stage of XY body formation, will be maintained in the mature sperm.

Germ cell chromatin and its influence on parent-specific expression in the cleavage stage embryo

Differences in chromatin configuration of the X chromosome are observed at certain time-points in male germ cells. The XY body, forms a very condensed structure which remains distinct with a close relationship to the nuclear envelope during pachytene and early diplotene (Solari, 1974). Two DNase I hypersensitive sites at the 5' end of the mouse *Pgk-1* gene which are present in spermatogonia and early meiotic prophase are only faintly detectable in pachytene spermatocytes. These hypersensitive sites reappear in round spermatids and disappear again in sperm (Kumari *et al.*, 1996). The imprinting of the X-chromosome activity could involve the binding of testis-specific proteins (particularly at the prophase of meiosis) which set up an inheritable signal. Several testis-specific chromosomal proteins have been identified though their involvement in the imprinting process is not known. Testis-specific proteins may be required in the formation of the XY body since XY germ cells ectopically located in the adrenal gland enter meiosis but fail to form an XY body (Hogg and McLaren, 1985). Two spermatogenesis-specific proteins found to be associated with the XY body in rat may be involved in the inactivation process; the first is specifically associated with the axial structures of both sex chromosomes (Smith and Benavente, 1992) and the second is located along the full length of both sex chromosomes as well as in heterochromatic centromeric regions of the autosomes (Smith and Benavente, 1995). Furthermore, histones such as TH2A, TH2B and H1t are present in spermatocytes but not oocytes (Meistrich and Brock, 1987).

In the post-meiotic stages of spermatogenesis, but not oogenesis, both autosomes and sex chromosomes are subject to extreme chromatin condensation as the vast majority of somatic and testis-specific histones are replaced first by transition proteins at the mid-spermatid stage and then by protamines at the late spermatid stage (Balhorn *et al.*, 1984). A minor subset of histones, estimated at less than 2% in mice (O'Brien and Bellvé, 1980) and about 15% in humans has been shown to be retained in the mature sperm chromatin (Tanphaichitr *et al.*, 1978; Gusse *et al.*, 1986; Gatewood *et al.*, 1987). In humans, these histones are retained in a DNA sequence-specific fashion (Gatewood *et al.*, 1987; Gardiner-Garden *et al.*, unpublished) and they are similar to the histone variants found in active chromatin of somatic cells (Gatewood *et al.*, 1990). The sperm DNA regions which are active or potentially active may retain their histone complement while inactive regions, such as X-linked genes with suppressed activity, may become condensed by protamines.

Histone acetylation

Core histone acetylation may have the potential to impart an X chromosome imprinting signal since hyperacetylated histone H4 is associated with the active X chromosome in female metaphase cells (Jeppesen and Turner, 1993) and histone acetylation patterns can be inherited to some extent through cell division (Wolffe, 1994). It is known that the small subset of histones retained in human sperm, described above, show high levels of acetylation at core histones H3 and H4 compared to somatic cells (Gatewood *et al.*, 1990). The acetylation levels of histones in oocytes have not been studied to date, due to difficulty in procuring analyzable materials. Following fertilization, no difference in overall levels of hyperacetylated histone H4 in maternal and paternal pronuclei is observed (Worrad *et al.*, 1995). Other core histones, such as histone H3, have not been examined. Nevertheless, the two pronuclei clearly create different environments for chromatin remodelling since an inhibitor of histone deacetylase, sodium butyrate, can stimulate promoter activity from a gene construct introduced into the maternal but not the paternal pronucleus (Wiekowski *et al.*, 1993). Furthermore, the unusual peripheral localization of hyperacetylated histone H4, is observed more frequently in the female pronucleus than in the male pronucleus (Worrad *et al.*, 1995).

If histone acetylation levels are critical to imprinted expression of X-linked genes in the cleavage stage embryo, we predict that X-linked genes which are actively transcribed in oocytes will be associated with hyperacetylated histones throughout oogenesis, whereas the same genes which are inactive in spermatocytes will be complexed first with hypoacetylated histones in the XY body and finally with protamines in the mature sperm. When the reorganization of chromatin occurs in the male pronuclei and the protamines are rapidly replaced by new histones (Nonchev and Tsanev, 1990) we envisage that regions of the X chromosome which are complexed with protamine may now become complexed with hypoacetylated histones and a distinction between X chromosomes of paternal and maternal origins can thus be maintained. The determination of whether histone acetylation actually forms an imprint in the case of the X chromosome will require a comparison of acetylation levels of core histones associated with X^M and X^P alleles in male and female gametes and between maternal and paternal pronuclei across the length of the chromosome.

The XIC/Xic in the preimplantation embryo

If a transcriptionally inactive chromatin configuration inherited via the male germline and affecting X^P alleles in the zygote is responsible for the suppression of paternal X-linked gene

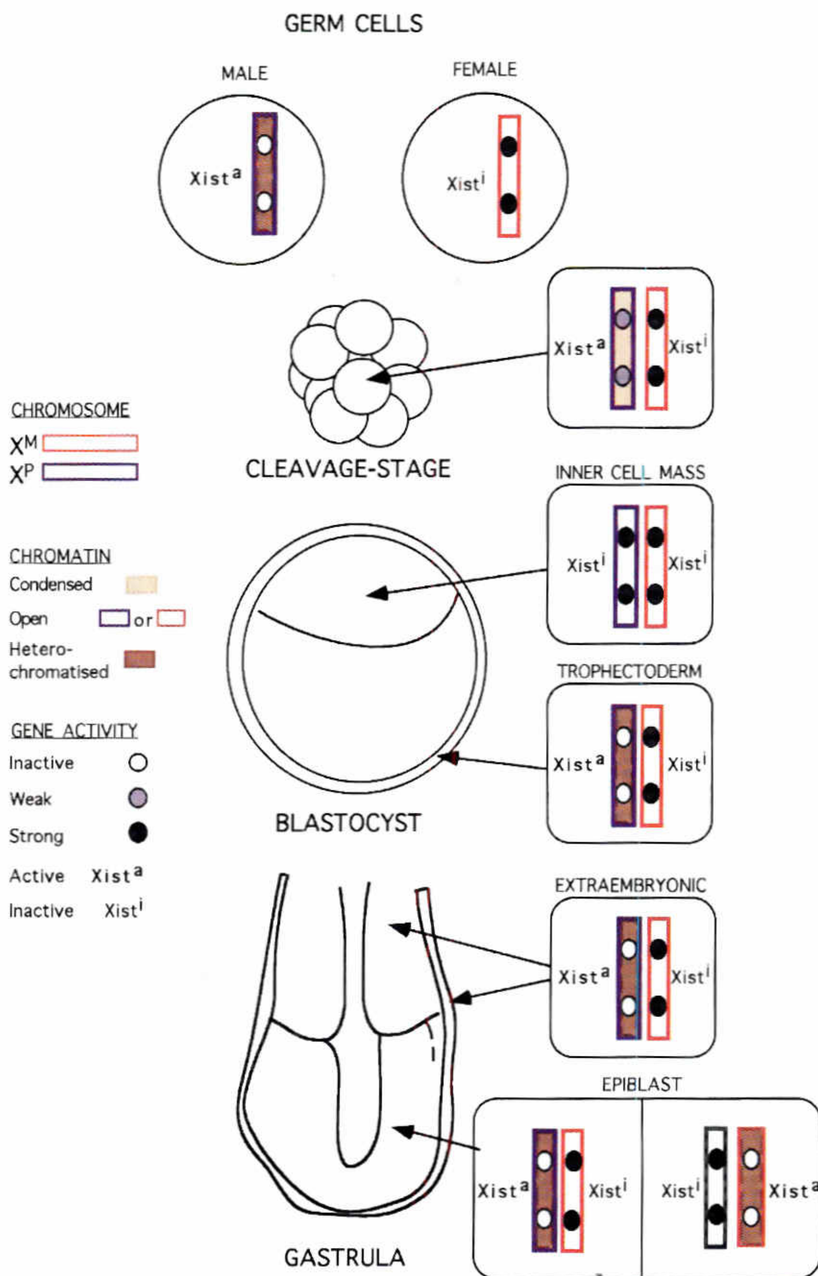

Fig. 4. A model of the developmental profile of X-chromosome activity with respect to chromatin condensation and the differential expression of X-linked genes in the germ cells and early embryos. *The X chromosome of the male gamete is highly condensed and most X-linked genes are transcriptionally silent during spermatogenesis. There is, however, evidence showing a low level of X-linked gene expression (including the Xist gene) in the immature spermatozoa and spermatid. In the female gamete, the X chromosome is not condensed and the X-linked genes remain active probably until the onset of meiosis II. The relatively condensed chromatin structure of the X^P and the open chromatin structure of the X^M may be maintained after syngamy in the female embryos and also persist during pre-implantation development. Studies comparing the transcriptional and enzymatic activity of the X-linked genes reach a consensus that the maternal alleles are generally more active than the paternal alleles, except for the Xist gene. The lower level of expression of X^P-linked genes may be influenced by chromatin configuration established in the germ cells. However, it is also possible that lower transcriptional activity of the X^P-linked genes is associated with the preferential expression of the paternal Xist gene in the cleavage-stage embryo, which reflects the once active state of the gene during spermatogenesis. The condensed chromatin structure of the X^P chromosome may be subject to progressive modification in the undifferentiated embryonic cells. However, when cells are allocated to a defined tissue lineage, the chromatin structure of the X chromosomes becomes fixed in the progenitor cells and their descendants. Cells that are allocated to lineages at an early time-point in development (trophectoderm and primitive endoderm) may therefore contain X chromosomes that have retained evidence of the germ cell-specific chromatin configuration and express a higher paternal Xist activity. This may therefore predispose the preferential (but not exclusive) inactivation of the paternal X chromosome in these two extraembryonic tissue lineages. Other tissue lineages which are specified later are derived from the precursor cells in which the parent-specific differences in chromatin structures of the X chromosomes and the imprints that govern the expression of the Xist genes may have been erased, resulting in the random inactivation of the X chromosomes. There is evidence showing the timing of lineage allocation in the postimplantation embryo may be associated with the setting of lineage-specific schedule of X-inactivation. The X^P chromosome is also inactivated ahead of the X^M chromosome in the epiblast, suggesting the germ-cell chromatin configuration may have a lasting effect on chromosomal behavior.*

activity in the cleavage stage embryo, then the presence of the precocious expression of the paternal *Xist* allele clearly demands an explanation. This *Xist* expression pattern may be related to a more open chromatin configuration endowed by histone hyperacetylation of the Xic on the maternal X chromosome. An autosomal blocking factor has been invoked as part of the counting mechanism of X-chromosome inactivation which is thought to operate when X inactivation becomes random (Lyon, 1996). In the presence of one set of autosomes there is postulated to be enough factor to block one Xic. Perhaps this blocking factor may also operate in the cleavage stage embryo. As a result of an inherited chromatin configuration at the maternal Xic the blocking factor may be able to bind there more easily than to the paternal Xic. As *Xist* starts to be transcribed this may lead to an increased likelihood that the paternal *Xist* will function. Thus the apparent imprinted *Xist* expression may be secondary to this effect. Alternatively, paternal expression of *Xist* may be required for the stable maintenance of a chromatin imprint on the *Xist* gene until X inactivation occurs in the trophectoderm. The *Xist* transcript may not initiate heterochromatinization of the X chromosome in the cleavage stage embryo due to the absence of some crucial factor which is only found later in differentiated cells.

Chromatin may also control the imprinting of the X chromosome by directly influencing the regulation of the *Xist* gene. The *Xist* gene is differentially transcribed in male and female germ cells. A

transcript is not detected in fetal prospermatogonia at 15.5 and 18.5 dpc or in neonatal prospermatogonia at 21.5 dpc whereas it is detected in oogonia at 12.5 dpc. The reverse of this expression pattern is observed in meiotic prophase when *Xist* transcripts are detected in spermatocytes but not in oocytes (McCarrey and Dilworth, 1992). In the sperm, the *Xist* gene or other loci in the Xic may complex with hyperacetylated histones rather than protamines, and in the oocyte these loci may complex with hypoacetylated histones.

Influence of chromatin on imprinting in the trophectoderm and the early epiblast

Differences in the chromatin configuration in the cleavage stage embryo may influence preferential inactivation of X^P in the trophectoderm lineage (West *et al.*, 1977). While it may be possible that widespread chromatin differences might be maintained until the blastocyst stage and be responsible for preferential X^P inactivation in the trophectoderm we consider this unlikely, since this would require the paternal X to undergo increased heterochromatinization in the trophectoderm while undergoing remodelling in the inner cell mass to achieve a similar conformation as the maternal X. We consider it more likely that the differential chromatin configuration may facilitate paternal *Xist* expression or that an imprint on the paternal *Xist* or Xic may be coming into play in the early differentiating lineages of the trophectoderm and the primary endoderm and that the activity of *Xist* is then responsible for X inactivation. It has previously been proposed that X-chromosome differentiation may be linked to cellular differentiation, occurring at different times, in different cell populations, as they depart or terminally differentiate from a pluripotent fetal stem line (Monk and Harper, 1979). The activity of *Xist* and subsequent maintenance mechanisms may then serve to lock imprinted paternal X inactivation into place in the trophectoderm derived lineages (Fig. 4). *Xist* driven X-chromosome inactivation may not occur in the inner cell mass at this time because the cells are not sufficiently differentiated. The imprint affecting X-chromosome activity may be retained to a small extent in the epiblast since the first cells to show X inactivation show preferential X^P inactivation. This is, however, soon lost so that random X inactivation is seen. This loss of imprint may be related to the genome-wide demethylation event reported in the blastocyst (Monk *et al.*, 1987), since DNA methylation can affect chromatin structure (Keshet *et al.*, 1986; Eden and Cedar, 1994). Assessment of the role of chromatin in imprinting of the X chromosome will require study of chromatin differences of the parental alleles in gametogenesis and early embryogenesis.

Acknowledgments

We thank Peter Rowe for reviewing the manuscript. Our work is supported by research grants from the National Health and Medical Research Council (NH&MRC) of Australia and by Mr James Fairfax. RVJ is supported by a NH&MRC Medical Postgraduate Scholarship.

Summary

The analysis of the imprinting of the X chromosome has provided insight into factors that affect the initiation and the choice of the chromosome for inactivation in the early mammalian embryo (Lyon, 1996). There are significant differences in the chromatin configuration, methylation and gene expression between Xi and Xa in somatic cells. Preferential paternal X inactivation that is concomitant with widespread heterochromatinization first occurs in the trophectoderm in the blastocyst. It is now clear that the activity of some paternal X-linked genes are suppressed before this stage. In the epiblast there may be early preferential paternal X inactivation before a random pattern supersedes. These observations suggest that parent-specific modification of the chromosome may determine the choice of which X chromosome is to be inactivated (Lyon, 1996). Differential methylation within the Xist gene or the XIC may lead to imprinted X-chromosome behavior. Alternatively, we postulate that imprinting of the X chromosome may be related to differences in chromatin configuration of the X chromosome in male and female germ cells which may then influence X-linked gene expression in the early embryo (Fig. 4). This may occur with a gene by gene effect leading to suppression of paternal alleles. An overall chromatin difference in the chromosomes may influence imprinted paternal *Xist* expression in early embryos and in the trophectoderm and primary endoderm populations that segregate early from the totipotent progenitors. Alternatively more specific differences in the chromatin architecture of the *Xist* gene or other gene loci in the Xic may constitute the signature of the imprint.

KEY WORDS: *X-chromosome, inactivation, imprinting, Xist, methylation, chromatin structure*

References

ADLER, A., WEST, J.D. and CHAPMAN, V.M. (1977). Expression of alpha-galactosidase in preimplantation mouse embryos. *Nature 267:* 838-839.

AGULNIK, A.I., MITCHELL, M.J., MATTEI, M-G., BORSANI, G., AVNER, P.A., LERNER, J.L. and BISHOP, C.E. (1994). A novel gene with a widely transcribed Y-linked homologue escapes X-inactivation in mouse and human. *Hum. Mol. Genet. 3:* 879-884.

AITKEN, R.J. and IRVINE, D.S. (1996). Fertilization without sperm. *Nature 379:* 493-495.

ARIEL, M., ROBINSON, E., McCARREY, J.R. and CEDAR, H. (1995). Gamete-specific methylation correlates with imprinting of the murine *Xist* gene. *Nature Genet. 9:* 312-315.

AVIVI, L., KORENSTEIN, A., BRAIER-GOLDSTEIN, O., GOLDMAN, B. and RAVIA, Y. (1992). Uniparental disomy of sex chromosomes in man. *Am. J. Hum. Genet. 51:* A11.

BALHORN, R., WESTON, S., THOMAS, C. and WYROBEK, A.J. (1984). DNA packaging in mouse spermatids: synthesis of protamine variants and four transition proteins. *Exp. Cell Res. 150:* 298-308.

BANZAI, M., OMOE, K., ISHIKAWA, H. and ENDO, A. (1995). Viability, development and incidence of chromosome anomalies of preimplantation embryos from XO mice. *Cytogenet. Cell Genet. 70:* 273-277.

BARLOW, D.P. (1994). Imprinting: a gamete's point of view. *Trends Genet. 10:* 194-199.

BARR, M.L. and CARR, D.H. (1961). Correlations between sex chromatin and sex chromosomes. *Acta Cytol. 6:* 34-45.

BARTOLOMEI, M.S., ZEMEL, S. and TILGHMAN, S.M. (1991). Parental imprinting of the mouse H19 gene. *Nature 351:* 153-155.

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.

BOGGS, B.A. and CHINAULT, A.C. (1994). Analysis of replication timing properties of human X-chromosomal loci by fluorescence *in situ* hybridization. *Proc. Natl. Acad. Sci. USA 91:* 6083-6087.

BORSANI, G., TONLORENZI, R., SIMMLER, M.C., DANDOLO, L., ARNAUD, D., CAPRA, V., GROMPE, M., PIZZUTI, A., MUZNY, D., LAWRENCE, C., WILLARD, H.F., AVNER, P. and BALLABIO, A. (1991). Characterization of a

murine gene expressed from the inactive X chromosome. *Nature 351:* 325-329.

BOYES, J. and BIRD, A. (1992). Repression of genes by DNA methylation depends on CpG density and promoter strength: evidence for involvement of a methyl-CpG binding protein. *EMBO J. 11:* 327-333.

BROCKDORFF, N., ASHWORTH, A., KAY, G.F., McCABE, V.M., NORRIS, D.P., COOPER, P.J., SWIFT, S. and RASTAN, S. (1992). The product of the mouse *Xist* gene is a 15kb inactive X-specific transcript containing no conserved ORF and located in the nucleus. *Cell 71:* 515-526.

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.

BROWN, C.J., HENDRICH, B.D., RUPERT, J.L., LAFRENIERE, R.G., XING, Y., LAWRENCE, J. and WILLARD, H.F. (1992). The human *XIST* gene: analysis of a 17kb inactive X-specific RNA that contains conserved repeats and is highly localized within the nucleus. *Cell 71:* 527-542.

BROWNELL, J.E. and ALLIS, C.D. (1996). Special HATs for special occasions: linking histone acetylation to chromatin assembly and gene activation. *Curr. Opin. Genet. Dev. 6:* 176-184.

BUITING, K., SAITOH, S., GROSS, S., DITTRICH, B., SCHWARTZ, S., NICHOLLS, R. and HORSTHEMKE, B. (1995). Inherited microdeletions in the Angelman and Prader-Willi syndromes define an imprinting centre on human chromosome 15. *Nature Genet. 9:* 395-400.

BURGOYNE, P.S. and BIGGERS, J.D. (1976). The consequences of X-dosage deficiency in the germ line: impaired development *in vitro* of preimplantation embryos from XO mice. *Dev. Biol. 51:* 109-117.

BURGOYNE, P., EVANS, E. and HOLLAND, K. (1983a). XO monosomy is associated with reduced birthweight and lowered weight gain in the mouse. *J. Reprod. Fertil. 68:* 381-385.

BURGOYNE, P., TAM, P. and EVANS, E. (1983b). Retarded development of XO conceptuses during early pregnancy in the mouse. *J. Reprod. Fertil. 68:* 387-393.

BUZIN, C.H., MANN, J.R. and SINGER-SAM, J. (1994). Quantitative RT-PCR assays show *Xist* RNA levels are low in mouse female adult tissue, embryos and embryoid bodies. *Development 120:* 3529-3536.

CARREL, L., CLEMSON, C.M., DUNN, J.M., MILLER, A.P., HUNT, P.A., LAWRENCE, J.B. and WILLARD, H.F. (1996). X inactivation analysis and DNA methylation studies of the ubiquitin activating enzyme E1 and PCTAIRE-1 genes in human and mouse. *Hum. Mol. Genet. 5:* 391-401.

CATTANACH, B.M. (1962). XO mice. *Genet. Res. 3:* 487-490.

CATTANACH, B.M. (1974). Position effect variegation in the mouse. *Genet. Res. 23:* 291-306.

CATTANACH, B.M. (1975). Control of chromosome inactivation. *Annu. Rev. Genet. 9:* 1-18.

CHU, C.E., DONALDSON, M.D.C., KELNAR, C.J.H., SMAIL, P.J., GREENE, S.A., PATERSON, W.F. and CONNOR, J.M. (1994). Possible role of imprinting in the Turner phenotype. *J. Med. Genet. 31:* 840-842.

CLARK, S.J., HARRISON, J., PAUL, C.L. and FROMMER, M. (1994). High sensitivity mapping of methylated cytosines. *Nucleic Acids Res. 22:* 2990-2997.

CLAYTON, A.L., HEBBES, T.R., THORNE, A.W. and CRANE-ROBINSON, C. (1993). Histone acetylation and gene induction in human cells. *FEBS Lett. 336:* 23-26.

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.

COOPER, D.W., JOHNSTON, P.G., WATSON, J.M. and GRAVES, J.A.M. (1993). X-inactivation in marsupials and monotremes. *Semin. Dev. Biol. 4:* 117-128.

COURTIER, B., HEARD, E. and AVNER, P. (1995). *Xce* haplotypes show modified methylation in a region of the active X chromosome lying 3' to *Xist*. *Proc. Natl. Acad. Sci. USA 92:* 3531-3535.

CROSS, S.H. and BIRD, A.P. (1995). CpG islands and genes. *Curr. Opin. Genet. Dev. 5:* 309-314.

DAHL, H-H.M., BROWN, R.M., HUTCHISON, W.M., MARAGOS, C. and BROWN, G.K. (1990). A testis-specific form of the human pyruvate dehydrogenase E1a subunit is coded for by an intronless gene on chromosome 4. *Genomics 8:* 225-232.

DAVIE, J.R. and HENDZEL, M.J. (1994). Multiple functions of dynamic histone acetylation. *J. Cell. Biochem. 55:* 98-105.

DECHIARA, T.M., ROBERTSON, E.J. and EFSTRATIADIS, A. (1991). Parental imprinting of the mouse insulin-like growth factor II gene. *Cell 64:* 849-860.

DRISCOLL, D.J. and MIGEON, B.R. (1990). Sex difference in methylation of single-copy genes in human meiotic germ cells: implications for X chromosome inactivation, parental imprinting, and origin of CpG mutations. *Somatic Cell Mol. Genet. 16:* 267-282.

DYER, K.A., CANFIELD, T.K. and GARTLER, S.M. (1989). Molecular cytological differentiation of active from inactive X domains in interphase: implications for X chromosome inactivation. *Cytogenet. Cell Genet. 50:* 116-120.

EDEN, S. and CEDAR, H. (1994). Role of DNA methylation in the regulation of transcription. *Curr. Biol. 4:* 255-259.

EPSTEIN, C.J. (1969). Mammalian oocytes: X chromosome activity. *Science 163:* 1078-1079.

EPSTEIN, C.J., SMITH, S., TRAVIS, B. and TUCKER, G. (1978). Both X chromosome function before visible X-chromosome inactivation in female mouse embryos. *Nature 274:* 500-503.

EVANS, E.P. and PHILLIPS, R.J.S. (1975). Inversion heterozygosity and the origin of XO daughters of *Bpa*/+ female mice. *Nature 256:* 40-41.

FERGUSON-SMITH, A.C., SASAKI, H., CATTANACH, B.M. and SURANI, M.A. (1993). Parental-origin-specific epigenetic modification of the mouse *H19* gene. *Nature 362:* 751-755.

FLACH, G., JOHNSON, M.H., BRAUDE, P.R., TAYLOR, R.A.S. and BOLTON, V.N. (1982). The transition from maternal to embryonic control in the 2-cell mouse embryo. *EMBO J. 1:* 681-686.

FRANKLIN, G.C., ADAM, G.I.R. and OHLSSON, R. (1996). Genomic imprinting and mammalian development. *Placenta 17:* 3-14.

FRELS, W.I. and CHAPMAN, V. (1979). Paternal X chromosome expression in extraembryonic membranes of XO mice. *J. Exp. Zool. 210:* 553-559.

FROMMER, M., McDONALD, L.E., MILLAR, D.S., COLLIS, C.M., WATT, F., GRIGG, G.W., MOLLOY, P.L. and PAUL, C.L. (1991). A genomic sequencing protocol which yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc. Natl. Acad. Sci. USA 89:* 1827-1831.

GARDINER-GARDEN, M. and FROMMER, M. (1987). CpG islands in vertebrate genomes. *J. Mol. Biol. 196:* 261-282.

GATEWOOD, J.M., COOK, G.R., BALHORN, R., BRADBURY, E.M. and SCHMID, C.W. (1987). Sequence-specific packaging of DNA in human sperm chromatin. *Science 236:* 962-964.

GATEWOOD, J.M., COOK, G.R., BALHORN, R., SCHMID, C.W. and BRADBURY, E.M. (1990). Isolation of four core histones from human sperm chromatin representing a minor subset of somatic histones. *J. Biol. Chem. 265:* 20662-20666.

GOLD, J.D. and PEDERSEN, R.A. (1994). Mechanisms of genomic imprinting in mammals. *Curr. Top. Dev. Biol. 29:* 227-280.

GOODFELLOW, P.J., MONDELLO, C., DARLING, S.M., PYM, B., LITTLE, P. and GOODFELLOW, P.N. (1988). Absence of methylation of a CpG-rich region at the 5' end of the *MIC2* gene on the active X, the inactive X, and the Y chromosome. *Proc. Natl. Acad. Sci. USA 85:* 5605-5609.

GRANT, M., ZUCCOTTI, M. and MONK, M. (1992). Methylation of CpG sites of two X-linked genes coincides with X-inactivation in the female mouse embryo but not in the germ line. *Nature Genet. 2:* 161-166.

GRANT, S.G. and CHAPMAN, V.M. (1988). Mechanisms of X-chromosome regulation. *Annu. Rev. Genet. 22:* 199-233.

GUSSE, M., SAUTIÈRE, P., BÉLAICHE, D., MARTINAGE, A., ROUX, C., DADOUNE, J-P. and CHEVAILLIER, P. (1986). Purification and characterization of nuclear basic proteins of human sperm. *Biochim. Biophys. Acta 884:* 124-134.

HANSEN, R.S., CANFIELD, T.K. and GARTLER, S.M. (1995). Reverse replication timing for the *XIST* gene in human fibroblasts. *Hum. Mol. Genet. 4:* 813-820.

HARPER, M.I., FOSTEN, M. and MONK, M. (1982). Preferential paternal X inactivation in extraembryonic tissues of early mouse embryos. *J. Embryol. Exp. Morphol. 67:* 127-135.

HARRISON, K.B. (1989). X-chromosome inactivation in the human cytotrophoblast. *Cytogenet. Cell Genet. 52:* 37-41.

HARRISON, K. B. and WARBURTON, D. (1986). Preferential X-chromosome activity in human female placental tissues. *Cytogenet. Cell Genet. 41:* 163-168.

HASSOLD, T., BENHAM, F. and LEPPERT, M. (1988). Cytogenetic and molecular analysis of sex-chromosome monosomy. *Am. J. Hum. Genet. 42:* 534-541.

HEBBES, T.R., CLAYTON, A.L., THORNE, A.W. and CRANE-ROBINSON, C. (1994). Core histone hyperacetylation co-maps with generalized DNase I sensitivity in the chicken ß globin chromosomal domain. *EMBO J. 13:* 1823-1830.

HEBBES, T.R., THORNE, A.W. and CRANE-ROBINSON, C. (1988). A direct link between core histone acetylation and transcriptionally active chromatin. *EMBO J. 7:* 1395-1402.

HENDRIKSEN, P.J.M., HOOGERBRUGGE, J.W., THEMMEN, A.P.N., KOKEN, M.H.M., HOEIJMAKERS, J.H.J., OOSTRA, B.A., LENDE, T. and GROOTEGOED, J.A. (1995). Postmeiotic transcription of X and Y chromosomal genes during spermatogenesis in the mouse. *Dev. Biol. 170:* 730-733.

HOGG, H. and McLAREN, A. (1985). Absence of a sex vesicle in meiotic foetal germ cells is consistent with an XY sex chromosome constitution. *J. Embryol. Exp. Morphol. 88:* 327-332.

HORN, J.M. and ASHWORTH, A. (1995). A member of the *caudal* family of homeobox genes maps to the X-inactivation centre region of the mouse and human X chromosomes. *Hum. Mol. Genet. 4:* 1041-1047.

HORNSTRA, I.K. and YANG, T.P. (1994). High-resolution methylation analysis of the human hypoxanthine phosphoribosyltransferase gene 5' region on the active and inactive X chromosomes: correlation with binding sites for transcription factors. *Mol. Cell. Biol. 14:* 1419-1430.

HUNT, P.A. (1991). Survival of XO mouse fetuses: effect of parental origin of the X chromosome or uterine environment. *Development 111:* 1137-1141.

JACOBS, P.A. and MIGEON, B.R. (1989). Studies of X-chromosome inactivation in trisomies. *Cytogenet. Cell Genet. 50:* 75-77.

JACOBS, P.A., BETTS, P.R., COCKWELL, A.E., CROLLA, J.A., MACKENZIE, M.J., ROBINSON, D.O. and YOUINGS, S.A. (1990). A cytogenetic and molecular reappraisal of a series of patients with Turner's syndrome. *Ann. Hum. Genet. 54:* 209-223.

JEPPESEN, P. and TURNER, B.M. (1993). The inactive X chromosome in female mammals is distinguished by lack of histone H4 acetylation, a cytogenetic marker for gene expression. *Cell 74:* 281-289.

JEPPESEN, P., MITCHELL, A., TURNER, B. and PERRY, P. (1992). Antibodies to defined histone epitopes reveal variations in chromatin conformation and underacetylation of centric heterochromatin in human metaphase chromosomes. *Chromosoma 101:* 322-332.

JOHNSTON, P.G. and CATTANACH, B.M. (1981). Controlling elements in the mouse IV. Evidence of non-random X-inactivation. *Genet. Res. 37:* 151-160.

KANDA, N. (1973). A new differential technique for staining the heteropycnotic X-chromosome in female mice. *Exp. Cell Res. 80:* 463-467.

KAUFMAN, M.H., GUC-CUBRILO, M. and LYON, M.F. (1978). X-chromosome inactivation in diploid parthenogenetic mouse embryos. *Nature 271:* 547-549.

KAY, G.F., BARTON, S.C., SURANI, M.A. and RASTAN, S. (1994). Imprinting and X chromosome counting mechanisms determine *Xist* expression in early mouse development. *Cell 77:* 639-650.

KÀY, G.F., PENNY, G.D., PATEL, D., ASHWORTH, A., BROCKDORFF, N. and RASTAN, S. (1993). Expression of *Xist* during mouse development suggests a role in the initiation of X chromosome inactivation. *Cell 72:* 171-182.

KEREM, B-S., GOITEIN, R., RICHLER, C., MARCUS, M. and CEDAR, H. (1983). *In situ* nick-translation distinguishes between active and inactive X chromosomes. *Nature 304:* 88-90.

KESHET, I., LIEMAN-HURWITZ, J. and CEDAR, H. (1986). DNA methylation affects the formation of active chromatin. *Cell 44:* 535-543.

KOZAK, L.P. and QUINN, P.J. (1975). Evidence for dosage compensation of an X-linked gene in the 6-day embryo of the mouse. *Dev. Biol. 45:* 65-73.

KRATZER, P.G. (1983). Expression of maternally and embryonically derived hypoxanthine phosphoribosyl transferase (HPRT) activity in mouse eggs and early embryos. *Genetics 104:* 685-589.

KRATZER, P.G. and GARTLER, S.M. (1978). HGPRT activity changes in preimplantation mouse embryos. *Nature 274:* 503-504.

KRATZER, P.G., CHAPMAN, V.M., LAMBERT, H., EVANS, R.E. and LISKAY, R.M. (1983). Differences in the DNA of the inactive X chromosomes of fetal and extraembryonic tissues of mice. *Cell 33:* 37-42.

KUMARI, M., STROUD, J.C., ANJI, A. and McCARREY, J.R. (1996). Differential appearance of DNaseI-hypersensitive sites correlates with differential transcription of *Pgk* genes during spermatogenesis in mouse. *J. Biol. Chem. 271:* 14390-14397.

LANE, P.W. and DAVISSON, M.T. (1990). Patchy fur *(Paf)*, a semidominant X-linked gene associated with a high level of X-Y nondisjunction in male mice. *J. Hered. 81:* 43-50.

LATHAM, K.E. and RAMBHATLA, L. (1995). Expression of X-linked genes in androgenetic, gynogenetic, and normal mouse preimplantation embryos. *Dev. Genet. 17:* 212-222.

LEDBETTER, D.H. and ENGEL, E. (1995). Uniparental disomy in humans: development of an imprinting map and its implications for prenatal diagnosis. *Hum. Mol. Genet. 4:* 1757-1764.

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.

LEIGHTON, P.A., INGRAM, R.S., EGGENSCHWILER, J., EFSTRATIADIS, A. and TILGHMAN, S.M. (1995). Disruption of imprinting caused by deletion of the *H19* gene region in mice. *Nature 375:* 34-39.

LI, E., BEARD, C. and JAENISCH, R. (1993). Role for DNA methylation in genomic imprinting. *Nature 366:* 362-365.

LOCK, L.F., MELTON, D.W., CASKEY, T. and MARTIN, G.R. (1986). Methylation of the mouse HPRT gene differs on the active and inactive X chromosomes. *Mol. Cell. Biol. 6:* 914-924.

LOCK, L.F., TAKAGI, N. and MARTIN, G.R. (1987). Methylation of the *Hprt* gene on the inactive X occurs after chromosome inactivation. *Cell 48:* 39-46.

LOEBEL, D.A.F. and JOHNSTON, P.G. (1996). Methylation analysis of a marsupial X-linked CpG island by bisulfite genomic sequencing. *Genome Res. 6:* 114-123.

LORDA-SANCHEZ, I., BINKERT, F., MAECHLER, M. and SCHINZEL, A. (1992). Molecular study of 45,X conceptuses: correlation with clinical findings. *Am. J. Med. Genet. 42:* 487-490.

LUOH, S-W., JEGALIAN, K., LEE, A., CHEN, E.Y., RIDLEY, A. and PAGE, D.C. (1995). CpG islands in human *ZFX* and *XFY* and Mouse *Zfx* genes: sequence similarities and methylation differences. *Genomics 29:* 353-363.

LYON, M.F. (1961). Gene action in the X chromosome of the mouse *(Mus musculus L.). Nature 190:* 372-373.

LYON, M.F. (1986). X chromosomes and dosage compensation. *Nature 320:* 313.

LYON, M.F. (1996). Pinpointing the centre. *Nature 379:* 116-117.

LYON, M.F. and HAWKER, S.G. (1973). Reproductive lifespan in irradiated and unirradiated chromosomally XO mice. *Genet. Res. 21:* 185-194.

MATHUR, A., STEKOL, L., SCHATZ, D., MacLAREN, N.K., SCOTT, M. and LIPPE, B. (1991). The parental origin of the single X chromosome in Turner syndrome: lack of correlation with parental age or clinical phenotype. *Am. J. Hum. Genet. 48:* 682-686.

McCARREY, J.R. and DILWORTH, D.D. (1992). Expression of *Xist* in mouse germ cells correlates with X-chromosome inactivation. *Nature Genet. 2:* 200-203.

McMAHON, A., FOSTEN, M. and MONK, M. (1983). X-chromosome inactivation mosaicism in the three germ layers and the germ line of the mouse embryo. *J. Embryol. Exp. Morphol. 74:* 207-220.

MEEHAN, R.R., LEWIS, J.D. and BIRD, A.P. (1992). Characterization of MeCP2, a vertebrate DNA binding protein with affinity for methylated DNA. *Nucleic Acids Res. 20:* 5085-5092.

MEISTRICH, M.L. and BROCK, W.A. (1987). Proteins of the meiotic cell nucleus. In *Meiosis* (Ed. P.B. Moens). Academic Press Inc., Orlando, pp.333-353.

MIGEON, B.R., DE BEUR, S.J. and AXELMAN, J. (1989). Frequent derepression of G6PD and HPRT on the marsupial inactive X chromosome associated with cell proliferation *in vitro*. *Exp. Cell Res. 182:* 597-609.

MIGEON, B.R., SCHMIDT, M., AXELMAN, J. and RUTA CULLEN, C. (1986). Complete reactivation of X chromosomes from human chorionic villi with a switch to early DNA replication. *Proc. Natl. Acad. Sci. USA 83:* 2182-2186.

MIGEON, B.R., WOLF, S.F., AXELMAN, J., KASLOW, D.C. and SCHMIDT, M. (1985). Incomplete X chromosome dosage compensation in chorionic villi of human placenta. *Proc. Natl. Acad. Sci. USA 82:* 3390-3394.

MISE, N., SADO, T., TADA, M., TAKADA, S. and TAKAGI, N. (1996). Activation of the inactive X chromosome induced by cell fusion between a murine EC and female somatic cell accompanies reproducible changes in the methylation pattern of the *Xist* gene. *Exp. Cell Res. 223:* 193-202.

MONESI, V. (1965). Synthetic activities during spermatogenesis in the mouse. *Exp. Cell Res. 39:* 197-224.

MONK, M. and HARPER, M.I. (1979). Sequential X chromosome inactivation coupled with cellular differentiation in early mouse embryos. *Nature 281:* 311-313.

MONK, M. and McLAREN, A. (1981). X-chromosome activity in foetal germ cells of the mouse. *J. Embryol. Exp. Morphol. 63:* 75-84.

MONK, M., BOUBELIK, M. and LEHNERT, S. (1987). Temporal and regional changes in DNA methylation in the embryonic, extraembryonic and germ cell lineages during mouse embryo development. *Development 99:* 371-382.

MOORE, T.F. and WHITTINGHAM, D.G. (1992). Imprinting of phosphoribosyltransferases during preimplantation development of the mouse mutant, *Hprt*$^{b-m3}$. *Development 115:* 1011-1016.

NAUMOVA, A.K., PLENGE, R.M., BIRD, L.M., LEPPERT, M., MORGAN, K., WILLARD, H.F. and SAPIENZA, C. (1996). Heritability of X chromosome-inactivation phenotype in a large family. *Am. J. Hum. Genet. 58:* 1111-1119.

NEUMANN, B. and BARLOW, D.P. (1996). Multiple roles for DNA methylation in gametic imprinting. *Curr. Opin. Genet. Dev. 6:* 159-163.

NONCHEV, S. and TSANEV, R. (1990). Protamine-histone replacement and DNA replication in the male mouse pronucleus. *Mol. Reprod. Dev. 25:* 72-76.

NORRIS, D.P., PATEL, D., KAY, G.F., PENNY, G.D., BROCKDORFF, N., SHEARDOWN, S.A. and RASTAN, S. (1994). Evidence that random and imprinted Xist expression is controlled by preemptive methylation. *Cell 77:* 41-51.

O'BRIEN, D.A. and BELLVÉ, A.R. (1980). Protein constituents of the mouse spermatozoon. *Dev. Biol. 75:* 386-404.

O'NEILL, L.P. and TURNER, B.M. (1995). Histone H4 acetylation distinguishes coding regions of the human genome from heterochromatin in a differentiation-dependent but transcription-independent manner. *EMBO J. 14:* 3946-3957.

OHNO, S., KAPLAN, W.D. and KINOSITA, R. (1961). X-chromosome behavior in germ and somatic cells of *rattus norvegicus. Exp. Cell Res. 22:* 535-544.

PAPAIOANNOU, V.E. and WEST, J.D. (1981). Relationship between the parental origin of the X chromosomes, embryonic cell lineage and X chromosome expression in mice. *Genet. Res. 37:* 183-197.

PARK, J-G. and CHAPMAN, V.M. (1994). CpG island promoter region methylation patterns of the inactive-X-chromosome hypoxanthine phosphoribosyltransferase *(Hprt)* gene. *Mol. Cell. Biol. 14:* 7975-7983.

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.

PFEIFER, G.P., STEIGERWALD, S.D., HANSEN, R.S., GARTLER, S.M. and RIGGS, A.D. (1990). Polymerase chain reaction-aided genomic sequencing of an X chromosome-linked CpG island: methylation patterns suggest clonal inheritance, CpG site autonomy, and an explanation of activity state stability. *Proc. Natl. Acad. Sci. USA 87:* 8252-8256.

PIKÓ, L. and CLEGG, K.B. (1982). Quantitative changes in total RNA, total poly(A), and ribosomes in early mouse embryos. *Dev. Biol. 89:* 362-378.

RASTAN, S. and CATTANACH, B.M. (1983). Interaction between the *Xce* locus and imprinting of the paternal X chromosome in mouse yolk-sac endoderm. *Nature 303:* 635-637.

RASTAN, S., KAUFMAN, M.H., HANDYSIDE, A.H. and LYON, M.F. (1980). X-chromosome inactivation in extra-embryonic membranes of diploid parthenogenetic mouse embryos demonstrated by differential staining. *Nature 288:* 172-173.

RIGGS, A.D. and PFEIFER, G.P. (1992). X-chromosome inactivation and cell memory. *Trends Genet. 8:* 169-174.

ROUGEULLE, C. and AVNER, P. (1996). Cloning and characterization of a murine brain specific gene *Bpx* and its human homologue lying within the *Xic* candidate region. *Hum. Mol. Genet. 5:* 41-49.

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

RUTA CULLEN, C., HUBBERMAN, P., KASLOW, D.C. and MIGEON, B.R. (1986). Comparison of factor IX methylation on human active and inactive X chromosomes: implications for X inactivation and transcription of tissue-specific genes. *EMBO J. 5:* 2223-2229.

SADO, T., TADA, T. and TAKAGI, N. (1996). Mosaic methylation of *Xist* gene before chromosome inactivation in undifferentiated female mouse embryonic stem and embryonic germ cells. *Dev. Dynamics 205:* 421-434.

SCHINZEL, A.A., ROBINSON, W.P., BINKERT, F., TORRESANI, T. and WERDER, E.A. (1993). Exclusively paternal X chromosomes in a girl with short stature. *Hum. Genet. 92:* 175-178.

SCHMIDT, M. and MIGEON, B.R. (1990). Asynchronous replication of homologous loci on human active and inactive X chromosomes. *Proc. Natl. Acad. Sci. USA 87:* 3685-3689.

SHAO, C. and TAKAGI, N. (1990). An extra maternally derived X chromosome is deleterious to early mouse development. *Development 110:* 969-975.

SIMMLER, M-C., CATTANACH, B.M., RASBERRY, C., ROUGELLE, C. and AVNER, P. (1993). Mapping the murine *Xce* locus with $(CA)_n$ repeats. *Mammal. Genome 4:* 523-530.

SINGER-SAM, J., CHAPMAN, V., LEBON, J.M. and RIGGS, A.D. (1992). Parental imprinting studied by allele-specific primer extension after PCR: paternal X chromosome-linked genes are transcribed prior to preferential paternal X chromosome inactivation. *Proc. Natl. Acad. Sci. USA 89:* 10469-10473.

SINGER-SAM, J., GRANT, M., LEBON, J.M., OKUYAMA, K., CHAPMAN, V., MONK, M. and RIGGS, A.D. (1990a). Use of a *HpaII*-polymerase chain reaction assay to study DNA methylation in the *Pgk-1* CpG island of mouse embryos at the time of X-chromosome inactivation. *Mol. Cell. Biol. 10:* 4987-4989.

SINGER-SAM, J., ROBINSON, M.O., BELLVÉ, A.R., SIMON, M.I. and RIGGS, A.D. (1990b). Measurement by quantitative PCR of changes in HPRT, PGK-1, PGK-2, APRT, MTase, and Zfy gene transcripts during mouse spermatogenesis. *Nucleic Acids Res. 18:* 1255-1259.

SMITH, A. and BENAVENTE, R. (1992). Meiosis-specific protein selectively associated with sex chromosomes of rat pachytene spermatocytes. *Proc. Natl. Acad. Sci. USA 89:* 6938-6942.

SMITH, A. and BENAVENTE, R. (1995). An Mr 51,000 protein of mammalian spermatogenic cells that is common to the whole XY body and centromeric heterochromatin of autosomes. *Chromosoma 103:* 591-596.

SOLARI, A.J. (1974). The behavior of the XY pair in mammals. *Int. Rev. Cytol. 38:* 273-317.

STÖGER, R., KUBICKA, P., LIU, C-G., KAFRI, T., RAZIN, A., CEDAR, H. and BARLOW, D.P. (1993). Maternal-specific methylation of the imprinted mouse *Igf2r* locus identifies the expressed locus as carrying the imprinting signal. *Cell 73:* 61-71.

SUGAWARA, O., TAKAGI, N. and SASAKI, M. (1985). Correlation between X-chromosome inactivation and cell differentiation in female preimplantation mouse embryos. *Cytogenet. Cell Genet. 39:* 210-219.

SURANI, M.A. (1991). Genomic imprinting: developmental significance and molecular mechanism. *Curr. Opin. Genet. Dev. 1:* 241-146.

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

TAKAGI, N. (1974). Differentiation of X chromosome in early female mouse embryos. *Exp. Cell Res. 86:* 127-135.

TAKAGI, N., WAKE, N. and SASAKI, M. (1978). Cytologic evidence for preferential inactivation of the paternally derived X chromosome in XX mouse blastocysts. *Cytogenet. Cell Genet. 20:* 240-248.

TAKAGI, O.S. and SASAKI, M. (1982). Regional and temporal changes in the pattern of X-chromosome replication during the early post-implantation development of the female mouse. *Chromosoma 85:* 275-286.

TAM, P.P.L., WILLIAMS, E.A. and TAN, S-S. (1994a). Expression of an X-linked *HMG-lacZ* transgene in mouse embryos: implication of chromosomal imprinting and lineage-specific X-chromosome activity. *Dev. Genet. 15:* 491-503.

TAM, P.P.L., ZHOU, S. X. and TAN, S-S. (1994b). X-chromosome activity of the mouse primordial germ cells revealed by the expression of an X-linked *lacZ* transgene. *Development 120:* 2925-2932.

TANPHAICHITR, N., SOBHON, P., TALUPPETH, N. and CHALERMISARACHAI, P. (1978). Basic nuclear proteins in testicular cells and ejaculated spermatozoa in man. *Exp. Cell Res. 117:* 347-356.

TATE, P., SKARNES, W. and BIRD, A. (1996). The methyl-CpG binding protein MeCP2 is essential for embryonic development in the mouse. *Nature Genet. 12:* 205-208.

THORNHILL, A.R. and BURGOYNE, P.S. (1993). A paternally imprinted X chromosome retards development of the early mouse embryo. *Development 118:* 171-174.

TOMMASI, S., LEBON, J.M., RIGGS, A.D. and SINGER-SAM, J. (1993). Methylation analysis by genomic sequencing of 5' region of mouse *Pgk-1* gene and a cautionary note concerning the method. *Somatic Cell Mol. Genet. 19:* 529-541.

TONIOLO, D., MARTINI, G., MIGEON, B.R. and DONO, R. (1988). Expression of the G6PD locus on the human X chromosome is associated with demethylation of three CpG islands within 100 kb of DNA. *EMBO J. 7:* 401-406.

TORCHIA, B.S. and MIGEON, B.R. (1995). The XIST locus replicates late on the active X, and earlier on the inactive X based on FISH DNA replication analysis of somatic cell hybrids. *Somatic Cell Mol. Genet. 21:* 327-333.

TORCHIA, B.S., CALL, L.M. and MIGEON, B.R. (1994). DNA replication analysis of FMR1, XIST and Factor 8C loci by FISH shows nontranscribed X-linked genes replicate late. *Am. J. Hum. Genet. 55:* 96-104.

TREMBLAY, K.D., SAAM, J.R., INGRAM, R.S., TILGHMAN, S.M. and BARTOLOMEI, M.S. (1995). A paternal-specific methylation imprint marks the alleles of the mouse *H19* gene. *Nature Genet. 9:* 407-413.

VANDEBERG, J.L., ROBINSON, E.S., SAMOLLOW, P.B. and JOHNSTON, P.G. (1987). X-linked gene expression and X-chromosome inactivation: marsupials, mouse and man compared. In *Isozymes: Current Topics in Biological and Medical Research*. Alan R. Liss, New York, pp. 225-253.

VENOLIA, L. and GARTLER, S.M. (1983). Transformation of *Hprt* gene with sperm DNA. *Somatic Cell Genet. 9:* 617-627.

WALKER, C.L., CARGILE, C.B., FLOY, K.M., DELANNOY, M. and MIGEON, B.R. (1991). The Barr body is a looped X chromosome formed by telomere association. *Proc. Natl. Acad. Sci. USA 88:* 6191-6195.

WEST, J.D., FRELS, W.I., CHAPMAN, V. and PAPAIOANNOU, V.E. (1977). Preferential expression of the maternally derived X chromosome in the mouse yolk sac. *Cell 12:* 873-882.

WIEKOWSKI, M., MIRANDA, M. and DEPAMPHILIS, M.L. (1993). Requirements for promoter activity in mouse oocytes and embryos distinguish paternal pronuclei from maternal and zygotic nuclei. *Dev. Biol. 159:* 366-378.

WOLFFE, A.P. (1994). Inheritance of chromatin states. *Dev. Genet. 15:* 463-370.

WORRAD, D.M., TURNER, B.M. and SCHULTZ, R.M. (1995). Temporally restricted spatial localization of acetylated isoforms of histone H4 and RNA polymerase II in the 2-cell mouse embryo. *Development 121:* 2949-2959.

YEN, P.H., PATEL, P., CHINAULT, A.C., MOHANDAS, T. and SHAPIRO, L.J. (1984). Differential methylation of hypoxanthine phosphoribosyltransferase genes on active and inactive human X chromosomes. *Proc. Natl. Acad. Sci. USA 81:* 1759-1763.

YOSHIDA, I., KASHIO, N. and TAKAGI, N. (1993). Cell fusion-induced quick change in replication time of the inactive mouse X chromosome: an implication for the maintenance mechanism of late replication. *EMBO J. 12:* 4397-4405.

ZUCCOTTI, M. and MONK, M. (1995). Methylation of the mouse *Xist* gene in sperm and eggs correlates with imprinted *Xist* expression and paternal X-inactivation. *Nature Genet. 9:* 316-320.

Received: September 1996
Accepted for publication: October 1996