Preferential X-chromosome inactivation, DNA methylation and imprinting

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Summary

Non-random X-chromosome inactivation in mammals was one of the first observed examples of differential expression dependent on the gamete of origin of the genetic material. The paternally-inherited X chromosome is preferentially inactive in all cells of female marsupials and in the extra-embryonic tissues of developing female rodents. Some form of parental imprinting during male and female gametogenesis must provide a recognition signal that determines the non-randomness of X-inactivation but its nature is thus far unknown. In the mouse, the imprint distinguishing the X chromosomes in the extra-embryonic tissues must be erased early in development since X-inactivation is random in the embryonic cells. Random X-chromosome inactivation leads to cellular mosaicism in expression and differential methylation of active and inactive X-linked genes. Transgene imprinting shares many features with X-inactivation, including differential DNA methylation.

In this paper we consider when methylation differences in early development affecting X-chromosome activity and imprinting are established. There are processes of methylation and demethylation occurring in early development, as well as changes in the activity of the DNA methylase itself. Methylation of a specific CpG site associated with activity of the X-linked PGK-1 gene has been studied. This site is already methylated on the inactive X chromosome by 6.5 days' gestation, close to the time of X-inactivation. However, differential methylation of this site is not the primary imprint marking the paternal X chromosome for preferential inactivation in the extra-embryonic membranes.

A consideration of factors influencing both X-chromosome inactivation and imprinting suggests that a process of communication and comparison between non-identical alleles might by the basis for the differential modification and expression patterns observed.

Key words: X chromosome, DNA, methylation, imprinting.

Introduction

In all female mammals one or other of the two X chromosomes is inactivated in all somatic cells (Lyon, 1961; reviewed in Grant and Chapman, 1988). This means that females are equivalent to males with respect to X-linked gene dosage. Once established, X-inactivation in somatic cells is clonally inherited and extremely stable. The inactive X chromosome may be distinguished from the active X chromosome by a number of criteria. The inactive X chromosome is: (1) heterochromatic: in certain interphase cells it can be seen as a dark staining body, the sex chromatin or Barr body, in the nucleus; (2) late replicating in the cell cycle (Takagi and Oshimura, 1973); (3) transcriptionally inactive (except for a short region at one end which is homologous to the Y chromosome, and where pairing and recombination between the X and the Y occurs (Burgoyne, 1982); (4) differentially methylated (reviewed in Monk, 1986).

One of the first examples of imprinting observed in mammals was the preferential inactivation of the paternally-inherited X chromosome in female marsupials (Cooper et al. 1971; Richardson et al. 1971; VandeBurgh et al. 1987) and in the extra-embryonic tissues of rodents (Takagi and Sasaki, 1975; West et al. 1977; Harper et al. 1982). In this paper we will review changing patterns of X-chromosome activity and methylation in development and consider the role of methylation in the initiation and specificity of inactivation. This specificity (or choice) of which X chromosome will be active or inactive is affected by a number of factors. These influencing factors may also result in differential allele expression in general, i.e. whether the region concerned is on the X chromosome or is on an autosome (see Monk, 1990b). In order to clarify these influences, we will start by defining what we mean by gamete-specific imprinting in the context of differential gene expression in general.

Classes of imprinting

We define imprinting in a general sense as the differential modification and/or expression in the offspring of the homologous alleles or chromosome regions inherited from each parent.
Gamete-specific imprinting

Gamete-specific imprinting applies to differential modification or expression of an allele depending on whether its inheritance is via the sperm or the egg. The term ‘imprinting’ originally applied to gamete-specific imprinting (Crouse, 1960), and is most commonly used in this context.

Gamete-specific imprinting may be seen only in a particular tissue. For example, in rodents the preferential inactivation of the paternal X chromosome, dependent on gamete of origin, is only seen in the extra-embryonic tissues. Similarly, certain imprinted transgenes showing differential methylation of transgene sequences which depend on the gamete of origin of the transgene may show tissue differences, e.g. the methylation imprint may not occur in certain tissues of the developing conceptus (Reik et al., 1987). In insects, the behaviour of the paternal set of chromosomes may be different in germ line and soma (see Chandra and Nanjundiah, this volume). In maize, only the endosperm shows imprinting (Kermicle and Alleman, this volume).

Strain- and species-specific imprinting

Apart from classical imprinting dependent on the gamete of origin of the X chromosome, strain (Cattanach, 1975) and species (Zakien et al., 1987) differences between the parents also influence the randomness of X-chromosome inactivation. There may be a bias towards inactivation of a particular X chromosome depending upon its strain of origin in the mouse cross (Cattanach, 1975; this volume). There are also similar effects, depending on the combination of mouse strains in a given cross, on the degree of methylation and expression of a transgene in the progeny (Sapienza et al., 1989; Allen et al., 1990; Reik et al., this volume).

Species differences may have even more pronounced effects: there may be preferential inactivation of a particular X chromosome from one of the species in an interspecific cross (e.g. in interspecific hybrid voles, Zakian et al., 1987). However, this is not a rule of interspecific crosses – mules and hybrids between foxes do not show preferential X-inactivation (Serov et al. 1978a,b).

In these latter examples of strain- and species-specific X chromosome imprinting, non-random X chromosome expression is not concerned with whether the X chromosome comes from the mother or the father but with a ‘dominance’ of one X over the other. Nevertheless, interspecific crosses can show parental source effects, i.e. phenotypic differences depending on gamete of origin. The classical example given is the horse/donkey hybrid (Fig. 1; Chandley, 1989) although it has not been clearly demonstrated that the differences are due to gamete of origin of either the horse or donkey genome or to different uterine environments when the horse or the donkey is the mother.

Allele-specific imprinting

Mention should also be made of allele-specific imprinting. Silva and White (1988) demonstrated that in the human a number of loci, distinguished by differences in size of tandem repeat sequences (VNTR), may be differently methylated. The methylation pattern specific for a particular allele is heritable through several generations. The allele appears to be irreversibly modified and imprinted. If such a modification were to result in the silencing of expression of an allele, it would appear to be a mutation. Such a heritable modification of gene expression has been termed an epimutation by Holliday (1987).

X-chromosome activity in development

X-chromosome inactivation occurs in early development. Therefore to understand the mechanisms which
X chromosome, methylation and imprinting

PGK-1A father. Male embryos inherit only PGK-1B on the X chromosome from the mother. Female embryos show random inactivation in epiblast (both PGK-1A and PGK-1B are expressed) and non-random inactivation in the extra-embryonic lineages (only PGK-1B of the maternal X chromosome is expressed). A, PGK-1A control; T, testis control from a PGK-1A male, showing the position of the testis-specific autosomal-coded PGK-2. Data from Harper et al. (1982).

Changes in X-chromosome activity may be monitored by the use of highly sensitive microassays for X-coded enzymes. For example, the two X chromosome from either parent can be marked with different alleles for the electrophoretically distinguishable forms of phosphoglycerate kinase (PGK-1, EC 2.7.2.3). In this way we can see the proportions of cells in a particular tissue with one or the other X chromosome active. Fig. 2 shows evidence for preferential inactivation of the paternal X chromosome in the extra-embryonic tissues of 6.5 day embryos.

Using such highly sensitive assays, we and others (Epstein et al. 1978; Kratzer and Gartler, 1978; Monk and Harper, 1979; Kratzer and Chapman, 1981; Monk and McLaren, 1981) have established the timing and tissue specific patterns of X-activation, X-inactivation and X-reactivation in female mouse embryo development. Fig. 3 summarises these changes. Two X chromosomes are active during oocyte growth and maturation, whilst in the later stages of spermatogenesis the single X chromosome is inactive, heterochromatic and sequestered away from the events of meiosis in the sex vesicle. After fertilisation, the paternally-inherited X chromosome becomes active but then it is inactivated again in the trophectoderm and in the primary endoderm as these extra-embryonic tissues are formed. Clearly, there is some memory mechanism (imprint) distinguishing the paternal from the maternal X chromosome when inactivation occurs in these tissues. However, in the fetal precursor cells inactivation is random. Either the imprint has been erased by this stage or it is not seen in these cells. Reactivation of the inactive X chromosome occurs in the germ line around the time of meiosis.

We are interested in the possible role of methylation in these changes – activation, inactivation, reactivation – and in particular whether methylation is associated with the gamete-specific imprinting of the X chromosome observed in extra-embryonic membranes. We know that differential methylation is correlated with the active and inactive state of X chromosomes in somatic tissues (reviewed in Monk, 1986) and that it is also observed for imprinted transgenes (reviewed by Surani et al. 1988).

Changes in X-chromosome activity and methylation

Several lines of evidence suggest that DNA methylation plays a role in the maintenance of dosage compensation...
of X-linked genes. The observed methylation differences may be summarised as follows (reviewed in Monk, 1986): (1) CpG islands at the 5' end of X-linked genes are methylated on the inactive X chromosome. In contrast, other CpG sequences in the body of X-linked genes may be more methylated on the active X chromosome (Wolf et al. 1984; Yen et al. 1984; Toniolo et al. 1984; Keith et al. 1986; Lindsay et al. 1985); (2) certain tissues are hypomethylated on the inactive X chromosome suggesting that there are other modifying mechanisms apart from methylation which maintain the inactive state (Monk, 1986). For instance, CpG sites on the inactive X chromosome are undermethylated in extra-embryonic tissues in mouse and man and in somatic tissues of marsupials. This undermethylation may explain a greater instability of the inactive state in these tissues (Migeon et al. 1985; Kaslow and Migeon, 1987); (3) MspI and HpaII restriction digest studies only look at a proportion of CpG sites and, in addition, it has been shown recently that not all of these sites show methylation differences which are critically correlated with active and inactive status (Yen et al. 1986; Hansen et al. 1988).

The important questions are when do these methylation changes occur in development and how do they relate to changes in X-chromosome activity and imprinting? Do the methylation differences imprint the X chromosomes in the gametes? Do they precede or follow inactivation or reactivation of the X-linked genes? Similar questions could be asked about the role of methylation in imprinted transgenes, i.e. are certain transgenes differentially methylated in the sperm and egg and does the observed pattern correspond to the methylation imprinting seen in the offspring?

Methylation in early development

Very little is known about methylation in early development at the time when methylation may be playing a role in imprinting, i.e. determining preferential modification and/or expression of the X chromosome, transgenes and endogenous genes. Until recently it has been impossible to look at CpG methylation of specific single copy genes in development because of the shortage of biological material. However, attempts have been made to look at total methylation (Monk et al. 1987) as well as methylation of repetitive and low copy number sequences (Sanford et al. 1987; Monk et al. 1987) at various stages of development. The data is summarised in Table 1 and gives the following picture.

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<th>Table 1. Methylation in development</th>
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<td>Overall</td>
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<tr>
<td>Sperm</td>
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<td>Oocyte</td>
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<td>Blastocyst (ICM)</td>
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<td>Post implantation 7.5d</td>
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<td>Primordial germ cells</td>
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<td>Extra-embryonic cells</td>
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The sperm genome is more methylated than the egg genome, although both are globally undermethylated compared to somatic tissue (Monk et al. 1987). Dispersed repetitive L1 sequences are methylated in sperm but undermethylated in fetal oocytes (Sanford et al. 1984). In the preimplantation embryo, there is a loss of overall methylation by the blastocyst stage. occurring between the 8-cell and blastocyst stages, but this could be due to a lack of methylation in the trophectoderm cells (which are increasing in number) rather than an absolute decrease (Monk et al. 1987). In the rabbit blastocyst the trophectoderm is also markedly undermethylated compared with the ICM (Manes and Menzel, 1981).

In the preimplantation stages there is not much sign of de novo methylation, either overall or for L1 sequences and satellite sequences (Sanford et al. 1987). Detectable de novo methylation commences around the time of implantation in the ICM of the late blastocyst and increases throughout gastrulation. (Since it is occurring independently in different lineages, it is potentially occurring differently in these lineages and may therefore be implicated in differential programming.) We, and others (Lock et al. 1987; Kaslow and Migeon, 1987), proposed that methylation may serve to lock in, or reinforce, patterns of potential gene transcription established by other means.

It is clear that demethylation is also occurring in early development. Dispersed repetitive sequences inherited from the sperm are demethylated in primordial germ cells of the progeny at some time before 11.5 days gestation (Monk et al. 1987). Sperm-derived L1 sequences are also demethylated either before, or soon after, the delineation of extra-embryonic tissues. Either demethylation occurs in the preimplantation embryo and the extra-embryonic and germ line lineages segregate from demethylated precursor cells, or demethylation may occur independently in the extra-embryonic tissues and germ line after their segregation. Methylation is certainly very low in the germ line: this may be a prerequisite for reprogramming of the germ line to developmental totipotency.

Although the data for methylation changes in development is fairly meagre because of the tiny amounts of tissue available, there are clearly processes of both demethylation and de novo methylation occurring in early development.

Are we able to obtain any clues as to what is happening to DNA methylation by looking at the activity of methylase, DNA(cytosine-5)methyltransferase, EC 2.1.1.37? Establishment and maintenance of methylation patterns in development will depend upon...
the availability and activity of the methylase. This in turn will depend on the amount of enzyme inherited in the egg cytoplasm, the stability of the maternally-inherited enzyme and the time of onset of activity of the embryonic gene coding for the methylase. In the egg the low level of methylation observed suggests that methylase activity might be low. The opposite turns out to be true.

Using a highly sensitive microassay for the methylase developed by Roger Adams (Department of Biochemistry, University of Glasgow, U.K.), we showed that the level of maternally-inherited enzyme is extremely high in the egg, and that the activity is stable for the first three cleavage divisions but then is degraded between the 8-cell and the blastocyst stage (M. Monk, R. Adams and A. Rinaldi, unpublished data). There is a marked absolute decrease (10-fold) of methylase activity per embryo. On a per-cell basis, the fall in methylase activity between the egg and the blastocyst is 1000-fold. However, the activity in the egg is so high that, despite the absolute decrease and dilution of enzyme, the final level of enzyme activity at the blastocyst stage reaches a similar level to that observed in cultured cells. The reason for the high level of methylase activity in the egg is open to speculation at this stage. One possibility is that de novo methylation occurs at the onset of development of the fertilised egg although its efficiency may be low.

**Methylation of specific CpG sites in development**

Is differential methylation the primary signal which distinguishes the sperm and egg X chromosomes for preferential paternal X inactivation? If not, when does differential methylation occur – at the time of inactivation of the expression of the X chromosome or later? When is the differential methylation erased – at the time of X-reactivation or earlier, or later? Alternatively, do the X chromosomes in the female germ line escape methylation modification? To attempt to answer some of these questions we need to look at specific CpG sites associated with X-linked genes.

The development of the polymerase chain reaction (PCR) for amplification of specific DNA sequences allows us to look at methylation in development more closely. This procedure is so sensitive that we can now look at changes in methylation of informative single CpG sites whose methylation is correlated with activity or inactivity of a gene when it is on the active or inactive X chromosome (Singer-Sam et al. 1989; 1990a,b).

**Methylation of CpG of X-linked PGK**

This work is being done in collaboration with Judy Singer-Sam, Jeanne LeBon and Art Riggs (Beckman Research Institute, LA, U.S.A.), and Kazuhika Okuyama and Verne Chapman (Roswell Park, Buffalo, U.S.A.) (Singer-Sam et al. 1990a). The methylation of a specific CpG site in the 5' region of PGK is followed by PCR amplification of a sequence containing that site, before and after HpaII digestion. When the site becomes methylated on the inactive X in the female, at some point in development, amplification will be resistant to HpaII digestion. When the site is unmethylated, amplification is not possible after digestion with HpaII. Thus, amplification cannot occur after HpaII digestion of male DNA (single active X chromosome) or female DNA at a time when both X chromosomes are active. However, when one X becomes methylated at this site in the female, amplification will be 50 per cent that observed in control undigested female DNA (as half the X chromosomes will be methylated and resistant to cutting by HpaII). The amplification procedure must be quantitative and this is made possible by incorporating an internal standard in the amplification reaction (Singer-Sam et al. 1989; 1990a,b).

Eggs, sperm, preimplantation embryonic stages, germ cells, and dissected tissues of early post-implantation stages are examined in this way. A small sample of DNA from single embryo samples allows determination of embryo sex by PCR using Y-specific primers (Nagamine et al. 1989; Mardon and Page, 1989). Samples are then cut with either HpaII or MspI and the PGK region containing the diagnostic HpaII site is amplified. The results (see Table 2 and Fig. 4), allow the following conclusions: (1) since the CpG site is not methylated in sperm and eggs, at least this site is not serving as the primary imprint to distinguish the maternal and paternal X chromosomes when inactivation of expression occurs in the extra-embryonic tissues; (2) the CpG site on the inactive X is already methylated by 6.5 days' gestation whether X-inactivation is random, (as it is in embryonic tissue) or preferential paternal X-inactivation (in extra-embryonic tissues). We still do not know precisely if methylation precedes or follows transcriptional silenc-
quantitation of experimental (E) compared to standard (S) amplification. The lanes show amplification of the sequence in DNA from dissected regions of 6.5 day embryos, extra-embryonic ectoplacental cone (epc), extra-embryonic ectoderm (e.e.e.) and embryonic (emb) tissues. DNA was extracted, digested with \textit{Hpa} II (+) or mock digested (–). The standard DNA added and the PCR reactions carried out as described in Singer-Sam et al. (1989; 1990a,b).

**Methylation of CpG sites in an imprinted transgene**

CAT17 transgenic mice are now being studied by Wolf Reik. Sarah Howlett and ourselves, to determine whether a methylation difference exists in eggs and sperm and, if not, to determine the timing of the establishment of the female transmitted methylation imprint in development. Also, using the PCR approach, we can ask whether the methylation imprint is present in the germ line of the progeny, and, if so, when it is erased. Preliminary results suggest that, like the X-inactivation picture, methylation is not the primary imprint in the sperm and egg, and in addition, the methylation imprint does not appear to be present in the primordial germ cells.

**Concluding remarks**

We have considered the choice of which X chromosome is to be active and the role of methylation in the imprinting mechanism determining paternal X-inactivation in extra-embryonic tissues of the mouse. It is clear that the primary imprint in the gametes is not methylation of a key site relating to PGK inactivation in somatic tissues. The methylation difference for the PGK gene on the active and inactive X chromosomes is due to \textit{de novo} methylation in early development and it is in place in both embryonic (random inactivation) and extra-embryonic (paternal X-inactivation) lineages by 6.5 days' gestation, close to the time of X-inactivation itself. Preliminary experiments looking at methylation imprinting in transgenes suggest that there may be parallels between the X-inactivation phenomenon and imprinting.

If we consider the factors operating in a more general context which result in differential transgene and X-chromosome modification and expression (including gamete-specific imprinting), it appears that the difference in origin of the two parental alleles or chromosome regions is the key factor. The differences in ancestry may be simply from the previous generation in the case of gamete-specific imprinting, or may extend further into the past in the case of parental strain and species differences. In interspecific crosses between fish, Whitt et al. (1977) have observed that the more distantly related the parents in the cross the greater the imprinting effects (non-reciprocal lethality, developmental abnormalities, preferential allele expression).

We conclude with a working hypothesis incorporating all these seemingly diverse phenomena — the cross-talk hypothesis (see Monk, 1990) which proposes the following: (1) communication between homologous regions of each chromosome pair allows a comparison of degree of similarity or difference; (2) response to differences detected by modification of one or both (or neither) homologous regions, resulting in "repair" by recombination or gene conversion, duplication, or differential expression (which may be partial or absolute). Irregularities between homologous chromosome regions may be modifications themselves, e.g. due to insertion (transgenes or repeat sequences), rearrangement or deletion, perhaps resulting in cis-position-effect variegation; they could be accumulated epigenetic modifications transmitted through the germ line (ancestral differences), or DNA sequence differences (e.g. in interspecific crosses). In the case of gamete-specific imprinting, the differences may be memories of differential packaging and programming of gene expression in the gametes. Just how homologous chromosomes (X chromosomes or autosomes) communicate and perceive these differences is open to speculation at this stage.

We thank Judy Singer-Sam, Jeanne Le Bon and Art Rigs for the invaluable contribution of the quantitative single site methylation technique and their collaboration in the study of CpG site methylation in the early embryo.

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