Imprinting by DNA methylation: from transgenes to endogenous gene sequences

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Summary

A number of transgenes in the mouse show variation in methylation and expression phenotypes dependent on parental transmission. It appears that there exist at least two types of transgene imprinting; one is retained on an essentially homozygous background, while the other requires heterozygosity at some modifying loci in the genome and is observed as differences in phenotype in reciprocal crosses. For this type of imprinting to occur, the parental origin of the modifier locus itself is important, and parental asymmetry may involve specific interactions between egg cytoplasm and the chromosomes. Based on the identification of ‘methylation polymorphism’ in the mouse genome, we also show that endogenous gene sequences can undergo imprinting by DNA methylation.

Key words: imprinting, methylation, transgenes, modifiers.

Introduction

The notion of autosomal imprinting in mammals has arisen mainly from three lines of experimentation in the mouse. First, it has been shown that the lethality of parthenogenetic and androgenetic embryos is of nuclear origin (Surani, Barton and Norris, 1984; McGrath and Solter, 1984; Mann and Lovell-Badge, 1984). Second, uniparental disomy of specific chromosomal segments causes particular phenotypes in the offspring; the phenotypes of maternal and paternal disomy are often complementary (Searle and Beechey, 1985; Cattanach and Kirk, 1985). Third, a number of transgene inserts in the mouse show variation in methylation and expression phenotypes dependent on maternal or paternal transmission of the transgene (Reik et al., 1987; Sapienza et al., 1987; Swain, Stewart and Leder, 1987; Hadchouel et al., 1987). Common to these three observations is that the expressivity of genetic traits in the offspring can vary with parental transmission; this may be seen as a violation of Mendel's view of parental reciprocity. This is also the definition of imprinting that we shall adopt in this paper.

In addition to these three lines of experimental evidence, there are many other observations from genetic studies which suggest an involvement of imprinting (reviewed by Solter, 1988; Monk, 1988; Sapienza, 1989; Reik, 1989; Hall, 1990). These range from an association of chromosomal disomy and specific phenotypes in the human, for example in Prader-Willi syndrome, to sex-of-parent-specific phenotypes of monogenic disorders, as in Huntington's chorea where the juvenile onset form is frequently the result of paternal transmission of the mutation. They also include the non-random retention of parental chromosomes in recessive tumour syndromes, for example the preferential loss of maternal chromosomes in Wilms tumour and rhabdomyosarcoma, and the occurrence of non-reciprocal phenotypes in inter- and intra-specific hybrids, like the well known example of the cross between horse and donkey.

It is conceivable that some or all of the particular factors that cause aberrant phenotypes in disomic mice (some of which are early lethalties) are together responsible for the inviability of parthenogenones, gynogenones and androgenones. There is at present no experimental proof that methylation imprinting is a cause of these phenotypes. This is largely due to the difficulty in identifying endogenous gene sequences that undergo imprinting. The study of transgene imprinting has however revealed a number of interesting features, such as its genetic control and potential mechanisms for producing multiple phenotypes at single loci. It should be emphasised that, although methylation is a useful indicator of phenotype, so far there is no evidence for or against methylation being a primary imprinting mechanism. Other epigenetic modifications that are clonally stable must also be considered; these include dosage-dependent chromatin assembly processes that lead to variable heterochromatisation, thought to play a role, for example, in position effect variegation in Drosophila (see Tartof and Bremer, this volume). Indeed differential methylation and heterochromatin assembly, as well as other epigenetic mechanisms, could conceivably interact in the imprinting process.
In this paper we examine some of the aspects of transgene imprinting and ask how useful this model is for understanding an endogenous imprinting process. We also describe our initial attempts at identifying endogenous sequences upon which similar mechanisms may act.

**Imprinting of transgenes**

Some features of transgene imprinting have been commented upon; we summarise these as well as more recent results in Table 1 (Surani, Reik and Allen, 1988; Solter, 1988; Monk, 1988; Sapienza, 1989). The frequency of detecting an imprinted transgene amongst established lines remains high; one expects to find 10–20% of all transgenes to show signs of imprinting. By comparison, imprinted endogenous gene sequences are proving elusive. There are a number of possible explanations for the high frequency of transgene imprinting, including the possibility that insertion into the genome may well be random. If indeed insertion is random, insertion will occur at an appreciable frequency into or near heterochromatic domains and so subject the transgene to variegating position effects analogous to those observed in Drosophila.

Progress in determining chromosomal position of transgene insertions has been slow; it is important to know whether or not the sites of integration coincide with regions of imprinting as defined by uniparental disomy. We have mapped one of our imprinted transgenes OX1-5, as it shows tight linkage to the albino locus on chromosome 7 (Table 2). While this is in principle an imprinted region (Searle and Beechey, 1990), some older experiments by Snell suggest that imprinted loci are actually located distal to the albino locus (Snell, 1946). Hence, it is possible that the imprinted transgene OX1-5 is in a chromosomal region that is not imprinted itself, as judged by the phenotype of disomic mice. The transgene HBsAg has been mapped toChr 13 (Hadchouel et al., 1987), and is therefore in a non-imprinted region (Cattanach and Beechey, this volume). However, its behaviour is slightly anomalous since maternal transmission invariably results in irreversible methylation of the transgene. To our knowledge this has not been tested on genetic backgrounds other than C57BL/6, and so it is not clear whether this property is independent of modifier alleles (see below).

Another imprinted transgene MPA 434, has recently been mapped to Chr 11 band A5 and thus falls into the imprinted region on proximal Chr 11 (H. Sasaki, personal communication). Hence, it appears possible that imprinting of transgenes can occur irrespective of whether or not they lie in imprinted regions as defined by uniparental disomy. Indeed, imprinting could be a widespread genomic phenomenon but only cause visible phenotypes when dosage of the genes involved is of major importance to the development and survival of the animal. In support of this notion, subtle deficiencies in complementation have recently been found with a number of chromosomes not previously thought to be imprinted (see Cattanach and Beechey, this volume).

Very little is known about the nature of the position effect that induces imprinting of transgenes. The cause of imprinting must be sought, at least in part, outside the transgenic DNA itself since not all insertions of any one construct result in imprinting. Only one analysis has recently been completed where sequences flanking the transgene have been cloned and examined for parental-specific methylation in the absence of the transgene, that is, on wild-type chromosomes: in this case no methylation difference was seen at the wild-type locus (H. Sasaki, personal communication). We cannot at present decide whether this is surprising. One might expect, by analogy with the Drosophila situation, that heterochromatisation starts at specific points outside the transgene and is transmitted into the transgenic locus to a greater or lesser extent. However, as far as we are aware, it is not known whether variegation occurs in a wild-type situation in Drosophila, i.e., without the presence of a translocation, or indeed whether a similar parent-specific effect also occurs in Drosophila. Also, the propagation of a heterochromatic domain could conceivably be influenced by the overall sequence arrangement at the transgenic locus. Whilst it is too early to draw any

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**Table 1. Some properties of imprinted transgenes**

<table>
<thead>
<tr>
<th>Designation of locus</th>
<th>Location</th>
<th>Imprinting</th>
<th>Influence of modifier loci</th>
<th>Methylation differences in male germ line</th>
<th>Mosaicism</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT 17</td>
<td></td>
<td>m&gt;p</td>
<td>+</td>
<td>+</td>
<td></td>
<td>Reik et al. (1987)</td>
</tr>
<tr>
<td>TROPONIN 1 139</td>
<td></td>
<td>m&gt;p</td>
<td>+</td>
<td>-</td>
<td></td>
<td>Sapienza et al. (1987; 1989)</td>
</tr>
<tr>
<td>TROPONIN 1</td>
<td></td>
<td>m&gt;p</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TROPONIN 1</td>
<td></td>
<td>p&gt;m</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RSV-Myc</td>
<td></td>
<td>m&gt;p</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBsAg</td>
<td>Chr 13</td>
<td>m&gt;p</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Allen, Norris and Surani (1990)</td>
</tr>
<tr>
<td>TKZ-751</td>
<td></td>
<td>m(B/c)&gt;p(B/c)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>OX1-5</td>
<td>Chr 7</td>
<td>m&gt;p</td>
<td>+</td>
<td>-</td>
<td></td>
<td>H. Sasaki, personal communication</td>
</tr>
<tr>
<td>MPA 434</td>
<td>Chr 11 A5</td>
<td>m&gt;p</td>
<td>+</td>
<td>+</td>
<td></td>
<td>McGowan et al. (1989)</td>
</tr>
<tr>
<td>Tg4</td>
<td>Chr 1</td>
<td>m&gt;p</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

m>p, maternal transmission of the transgene results in higher methylation than does paternal transmission.
m(B/c)>p(B/c), maternal transmission of BALB/c modifier results in higher methylation of the transgene than paternal transmission of the BALB/c modifier.
Table 2. Linkage of the OX1-5 transgene to the albino locus on chromosome 7

<table>
<thead>
<tr>
<th>C/c</th>
<th>c/c</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>71</td>
</tr>
<tr>
<td>+/OX1-5</td>
<td>0</td>
</tr>
</tbody>
</table>

Double heterozygotes (C/c; +/OX1-5) were mated with albino animals (c/c; +/+ ) and their offspring were classified for the albino genotype and the transgene, respectively. Tight linkage is indicated by the absence of any recombinants in 137 offspring analysed.

![OX1-5](image)

Conclusions from this one observation, it is clear that position effects are not necessarily paralleled by methylation changes outside the transgene. It is possible that transgene insertion itself in particular positions can create heterochromatic domains de novo.

In the case of OX1-5, for example, a parent-of-origin-specific methylation change is observed in sequences outside the transgene locus (Fig. 1).

The genetic control of transgene imprinting has received a good deal of attention over the last year (Sapienza et al. 1989; McGowan et al. 1989; Allen, Norris and Surani, 1990). Genes have been identified that influence the methylation and expression phenotypes of transgene loci. These genes are referred to as imprinting (as distinct from imprinted) genes or modifiers. Thus, in the case of the Troponin I 379 transgene, paternal transmission of the transgene will result in two different phenotypes. A high methylation phenotype is observed when the mother contributes a C57BL/6 modifier allele, as compared to a lower methylation phenotype when the mother contributes a DBA modifier allele (Sapienza et al. 1989). In this case, the different degrees of methylation of the paternally derived alleles must be brought about by the action of modifier alleles after fertilization. In contrast to paternal transmission, maternal transmission always results in a high methylation phenotype independent of modifier alleles present. Hence, when this transgene is crossed into the DBA strain of mice, germline-specific imprinting is retained (with maternal transmission leading to high methylation phenotype, whereas paternal leads to low); conversely, when present in a B6 genetic background, imprinting of the transgene is lost because either mode of transmission leads to a high phenotype. Essentially the same is observed with the CAT17 transgene locus for C57BL/6 and BALB/c strains. Breeding this transgene locus in B6 over a number of generations leads to a high methylation phenotype on paternal and maternal transmission, whereas the BALB/c background produces low methylation phenotypes for paternal, and high methylation phenotypes for maternal, transmission. By contrast, imprinting is not retained at the OX1-5 locus when transferred from its original mixed background onto the BALB/c background (Fig. 1).

Imprinting at certain transgene loci can also be observed even on an essentially homozygous background. This is a necessary component of the imprinting process that results in the developmental failure of parthenogenetic and androgenetic embryos. In fact, it has been argued that some loci have to remain imprinted on any homozygous background, and so it is interesting that there is at least one transgene locus where both paternal and maternal transmission have different phenotypic effects regardless of the strains involved (H. Sasaki, personal communication). It has been recently suggested that this invariant component of imprinting is caused by dosage-dependent modifiers on the sex-chromosomes (see Sapienza, this volume).

A seemingly different response to genotype-specific modifiers has been observed for the TKZ 751 locus (Surani et al. 1990; Allen et al. 1990). Although crossing the transgene into the BALB/c strain produced a high methylation type and crossing it into DBA produced a low one, neither phenotype was influenced by parental origin of the transgene itself. However, when a TKZ-DBA (low) male is crossed with a BALB/c female, an increase in methylation is observed in the offspring, whilst in the offspring of the reciprocal cross methylation stays low (Fig. 2). Clearly,
by cytoplasmic factors in the egg interacting differently whether the parental asymmetry in this system is caused segregating population. At present it is not clear BALB/c allele will not. Evidently, this will only result derived BALB/c allele at this locus will increase the phenotype of the transgene is not dependent upon modifier locus controls methylation phenotype at the transgene locus. ("methylation polymorphism"). When reciprocal crosses between the different background strains are carried out, different methylation phenotypes are observed at the transgene locus. This indicates that maternal and paternal transmission of a modifier locus controls methylation phenotype at the transgene locus.

the phenotype of the transgene is not dependent upon its own parental origin, but it is dependent upon the parental origin of its modifying locus. A maternally derived BALB/c allele at this locus will increase methylation in its bearer, while a paternally derived BALB/c allele will not. Evidently, this will only result in parental origin effects in a heterozygous, or segregating population. At present it is not clear whether the parental asymmetry in this system is caused by cytoplasmic factors in the egg interacting differently with BALB/c and DBA chromosomes, or whether the parental origin of a chromosomal factor also plays a role.

If the activity of some modifier loci is influenced by their own parental origin, one could imagine that some of the phenotypes arising from uniparental disomy are caused by different dosage at modifier genes. Particularly provocative are those disomies where antipodal, or opposite, phenotypes are seen (e.g. Chr. 11 and 2), and this is predicted from the dosage-dependent behaviour of modifiers in Drosophila (see Tartof and Bremer, this volume).

We are led to believe that there are at least two types of transgene imprinting, one which is retained on an essentially homozygous background, and the other which is observed in reciprocal crosses between different strains or species. The first type can thus serve as a paradigm for the lethality of parthenogenetic and androgenetic embryos, though its genetic control remains to be unravelled. The second type leads to parental effects in outbred, that is segregating, populations, and can serve as a model for non-reciprocal effects in inter-specific hybrids. Some of the genetic factors are now amenable to analysis through the identification of variant alleles.

**Germline modifications**

Whenever more than two phenotypes are observed in a situation where two different modifier alleles segregate, it must be suspected that the target gene is epigenetically marked in the germline. For example, low somatic methylation phenotype males of the Troponin I 379 strain, when crossed with B6 and DBA females, will give rise to intermediate methylation and low methylation progeny, respectively, whereas high somatic methylation phenotype males will give rise to high and intermediate methylation progeny in the same crosses (Sapienza et al. 1989). Assuming that the same two alleles of a single modifier locus are involved in each case, an epigenetic difference must already exist at the transgene locus between germlines of males with different phenotypes. This epigenetic modification then serves as a template for the modifier alleles to act upon in the next generation. This is most easily explained by the transgene being modified not only in somatic cells, but also in germ cells, for example by an early event prior to the allocation of the germ cell lineage. While no apparent methylation differences were observed in gen cells of low and high methylation phenotype males in the 379 pedigree, such differences have been seen in males of the TKZ 751 (Allen et al. 1990) and of the CAT 17 pedigree (Fig. 3). The overall level of methylation of the CAT 17 locus in male germ cells is very low indeed, as digestion patterns of HpaII and MspI are almost identical (Reik et al. 1987). However, careful comparison of sperm DNA from males with low and high somatic methylation reveals a subtle yet reproducibly higher amount of methylation in sperm DNA from high compared with low somatic males (Fig. 3). Hence, in this case as well as in the case of TKZ 751, the epigenetic modification that persists in the germline to produce different phenotypes in the next generation appears to be methylation itself. In the case of CAT 17 and of Troponin I 379, this can lead to a grandparental effect exclusively on paternal transmission. Interestingly, such a grandparental effect has also been observed in the expression of the parental origin effect in Huntington’s chorea (Ridley et al. 1988).

The observation that some epigenetic information can be transmitted through the germline unchanged raises the interesting possibility that natural selection may act upon alleles that are specifically modified. Normally, if all epigenetic information were lost in the germline, natural selection for specific modified alleles would select for mutations and hence variant alleles of modifier loci. If however some epigenetically modified
alleles are stable over several generations without being changed by modifier variants (Hadjouel et al. 1987; Allen et al. 1990), natural selection would operate on the modified allele itself.

Endogenous imprinting by methylation

Not surprisingly the mouse and the human genome have been scrutinised quite extensively for allele differences in methylation at a variety of loci. Two main approaches have been used: firstly, analysis of the segregation of allelic differences in families using Variable Number of Tandem Repeat (VNTR) markers to distinguish parental alleles (Silva and White, 1988), and secondly, analysis of allelic differences in cell lines or tumours (Chandler et al. 1987; Jones, this volume). The first approach has provided evidence of allelic methylation variants whose inheritance was strictly Mendelian; that is, the same methylation phenotype was invariably associated with the same genotype at the locus in question. The second approach has provided evidence for allelic differences in methylation in cultured cells, but so far analysis of the parental origin of chromosomes has not been made in such cases. Hence the two phenomena could result from the same mechanism, namely different genotypes being associated with specific methylation phenotypes in an invariant fashion. To avoid the need for using restriction enzyme polymorphisms to distinguish between parental alleles, we have in preliminary experiments compared directly methylation patterns at endogenous loci between mice with maternal and paternal disomies for particular chromosomes. A number of probes for chromosome 7 or 11 were tested on DNAs from normal compared with maternal and/or paternal disomies. So far no differences have been detected, suggesting that there are no widespread genomic methylation differences in these imprinted regions (W. Reik, B. Cattanach and T. Searle, unpublished observations).

Another approach has been adopted as a more general strategy to identify imprinted sequences in the mouse genome. Breeding of the transgenic TKZ 751 line had shown that on crossing the transgene onto the BALB/c background it became progressively more methylated, whereas on a DBA background the transgene remained undermethylated (Allen et al. 1990). This constitutes a methylation polymorphism. When reciprocal crosses were performed, the transgene became more methylated when BALB/c was the maternal genotype, but remained undermethylated when DBA was the maternal genotype. Inheritance of the methylation polymorphism was thus non-Mendelian. Accordingly, we screened a number of endogenous gene probes for differences in methylation in different inbred mouse strains. One such methylation polymorphism was identified at the SPARC locus on chromosome 11 (Mason et al. 1986) initially when comparing BALB/c and B6 DNA. The MspI sites at which variable methylation occurred were mapped to the region of the 9th and 10th exon of the SPARC gene (Fig. 4). Whilst mapping these sites we also discovered a structural polymorphism in this region between the BALB/c chromosome and the DBA chromosome (Fig. 4). The BALB/c allele produces shorter MspI fragments because of a deletion in the 9th intron. This provides a means by which to distinguish in a heterozygote the methylation pattern on the BALB/c chromosome from the pattern on the DBA chromosome. This combination was thus used in the reciprocal crosses. A striking difference in methylation on the BALB/c chromosome was found in the reciprocal cross, with one particular HpaII fragment (B) being prominent in the DBAxBALB/c cross, but virtually absent in the BALB/c×DBA cross (Fig. 5). Inspection of the relative intensities of the HpaII fragments produced from the DBA chromosome in the reciprocal cross shows that methylation is also different on that chromosome. We ask then whether the methylation patterns on the parental BALB/c and the DBA chromosomes are different, or whether they are similar. Comparison of the relative intensities of fragments A and B on the BALB/c chromosome and of the equivalent fragments A' and B' on the DBA chromosome shows that the ratio are similar when the two chromosomes are compared, but that they differ in the two types of crosses. This observation is presented schematically in Fig. 6. We conclude that it is not maternal or paternal transmission of the SPARC alleles.
themselves that influence the methylation phenotype, but rather that the parental origin of modified alleles determines methylation phenotype at the SPARC locus. This notion is further supported by the observation that different methylation phenotypes segregate in back crosses (not shown). Again, this type of imprinting would not be expected to occur in an essentially homozygous background, but is limited to segregating populations. It is noteworthy that while the SPARC gene is located on the proximal part of chromosome 11, recent mapping places it distal to the translocation breakpoint in T30H and therefore outside the imprinted region on chromosome 11 (Searle et al. 1989). This reinforces our argument that imprinting, at least by this mechanism, can occur outside the regions defined by clear phenotypic consequences in genetic complementation tests.

This type of imprinting has some interesting implications for the occurrence of different phenotypes in deletion heterozygotes. Consider the expression of a gene to be at different levels in reciprocal crosses; when one copy of this gene is deleted, the remaining one will have a different dosage depending on whether it is maternally or paternally derived. Different phenotypes could thus be produced depending on parental transmission of the deletion. This is the case, for example, in the mouse with the T<sup>bp</sup> deletion (Johnson, 1975), and in the human with the chromosome 15q deletion leading to Prader-Willi or Angelman syndrome (Nicholls et al. 1989). As expected, the severity of maternal transmission of the T<sup>bp</sup> deletion is highly dependent on the genetic background involved.

**How are multiple phenotypes expressed?**

Two types of mechanisms exist that can be used either exclusively or in combination. First, as exemplified by transgenomes with multiple copy insertions, the activity of the locus can be regulated by switching on or off variable numbers of the individual members. This will result in a similar phenotype of all cells that are able to express the transgenic sequences (Allen et al. 1990). It is not clear whether or not this mechanism can operate on single copy gene sequences, but in principle one can imagine a number of methylatable sites to be present, where methylation at specific sites in all cells could go hand in hand with a reduced level of expression in all cells. Second is a type of mechanism whereby phenotype is regulated by the varying composition of cells that themselves have different phenotypes (cellular mosaicism). For example, whenever any two HpaII fragments of the SPARC locus from the BALB/c or the DBA chromosome are present in the same organ, they have to be produced by different cell populations (see Figs 4 and 5). The organ as a whole is therefore composed of cells that carry distinct methylation types and the relative intensity of any one HpaII fragment is proportional to the size of the cell population inheriting that particular methylation type. Hence, variation in phenotype is expressed as variation in composition of a tissue from different phenotypic ‘units’, or, put differently, as methylation or expression mosaicism. This type of phenotype control has also been observed with transgenes (McGowan et al. 1989), and has been inferred from the preferential loss of maternal chromosomes in certain recessive tumour syndromes (Scrable et al. 1989; Reik and Surani, 1989). Of course a well known example of this type of phenotype control is position effect variegation in *Drosophila*, where variegation is usually expressed as mosaicism of mutant and wild-type cells. A recent example of phenotype as a population phenomenon is the behaviour of mating type repression in the yeast *S. cerevisiae* (Pillus and Rine, 1989).

**Conclusions**

It is believed that genomic imprinting is a particular
aspect of phenotype control by modifying genes in the mammalian genome. Because variant alleles at a modifier locus can in principle lead to phenotypic changes of many unlinked loci, rapid adaptive changes are possible with a small number of mutations. The nature of these modifier genes and their mode of action is at present unknown. In particular, it is not yet clear exactly how parental asymmetry is brought about by the action of modifying genes. However, two experimental systems now exist in which their precise action can be investigated: one in which imprinting persists in a homozygous population, and one in which imprinting is observed in a population of individuals that segregate different modifier alleles, and where the parental origin of the modifier genes themselves plays a role. This second situation could be achieved by parent-specific expression at some modifier loci, or by an interaction between cytoplasmic components in the egg and chromosomal genes within the male and female pronuclei, or indeed by a combination of both processes. There is evidence for such nucleo–cytoplasmic interactions introducing parental asymmetry, for example from the DDK mutant in mice (Wakasugi, 1974; Renard and Babinet, 1986). Methylation imprinting is not only observed on transgenes, but also on endogenous gene sequences, as witnessed by methylation differences in reciprocal crosses. Finally, extreme alleles at modifier loci may not only regulate the expressivity and penetrance of mutant genes, but should themselves be regarded as potential candidates for some ‘disease genes’.

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References


