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Methylation levels of maternal and paternal genomes during preimplantation development

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Summary

The methylation status of three highly repeated sequences was studied in sperm, eggs and preimplantation embryos with different combinations of parental chromosomes. High levels of methylation of the IAP and MUP sequence families were found in sperm and in eggs, whereas the L1 repeat was found to be highly methylated in sperm but only about 42 % methylated in eggs. To assess how the two parental genomes behaved during preimplantation development, normal, fertilised embryos were compared with parthenogenetic embryos where the chromosomes are exclusively of maternal origin. It was observed that the high levels of methylation at the IAP and MUP sequences were retained through early development, with the first signs of demethylation at the IAP sequences apparent on both parental chromosomes in the blastocyst. Methylation at the sperm-derived L1 sequences dropped to about the same level as that of the egg-derived sequences by the late 2-cell stage, both then remain at this intermediate level until around the time of cavitation when levels fell to about 10 % in the blastocyst. High levels of DNA methylase were detected in germinal vesicle and metaphase II oocytes; these high levels were maintained in fertilised and parthenogenetic embryos through into the morula and then declined to be undetectable in the blastocyst. Our comparison of maternal and paternal genomes suggests that methylation levels at repeat sequences are remarkably similar at the time of fertilisation or, as in the case of the L1 sequences, they become so during the first few cell cycles. Hence, there do not appear to be global methylation differences between the genomes that are retained through preimplantation development. The marked loss of methylation from sperm-derived L1 sequences during the first two cell cycles may be indicative of the re-modelling of paternal chromosomes in the egg.

Key words: methylation, preimplantation embryo, parthenogenesis.

Introduction

Imprinting of each parental genome has been proposed to explain the requirement for the participation of both parental genomes in order to achieve full development in the mouse (Surani *et al.* 1984; McGrath and Solter, 1984). Many of the studies that point to the existence of genomic imprinting have been done in mice although there is increasing evidence for its involvement in a variety of human diseases (reviewed Reik, 1989; Hall, 1990). Genomic imprinting is assumed to occur independently during gametogenesis such that by the time of fertilisation epigenetic differences in the two genomes have been established. In this way the parental chromosomes have become imprinted as being of maternal or paternal origin. Genetic data suggest that only certain chromosomal domains are subject to overt imprinting, such that inheriting both copies of such regions from only one parent results in a recognisable phenotype which are often complementary (Searle and Beechey, 1985; Cattanach, 1986). Two endogenous genes, the insulin-like growth factor II and its receptor, have recently been identified as undergoing imprinting (Barlow *et al.* 1991; De Chiara *et al.* 1991). The means by which parental alleles are distinguishable and functionally non-equivalent are important and unresolved problems.

Since it is difficult to look at the two parental alleles separately, the random introduction of transgenes into the mouse genome has been used as a method for probing imprinted chromosomal regions by serendipity (Surani *et al.* 1988). It is then possible to follow a hemizygous transgenic locus in offspring that is inherited from one parent or the other. Independent observations of several different transgenic mouse strains have suggested that methylation may indeed be involved in the imprinting process, although it is far from clear whether or not it is the primary imprinting mechanism (Reik *et al.* 1987; Sapienza *et al.* 1987; Swain *et al.* 1987). In the majority of documented cases of 'imprinted' transgenes, the transgene was more highly methylated when inherited from the mother than when paternally inherited. This was sometimes associated with differences in expression, with the potential for

expression being conferred by paternal transmission. Overall, sperm shows a lower proportion of methylcytosine than does any other adult somatic tissue (Gama-Sosa *et al.* 1983) and this is reflected in the hypomethylated state of both the major and minor satellite sequences (Sanford *et al.* 1984; Ponzetto-Zimmerman and Wolgemuth, 1984); however, other lower copy repeat sequences (Chapman *et al.* 1984; Sanford *et al.* 1987) and many unique gene sequences (Waalwijk and Flavell, 1978; Mandel and Chambon, 1979; Rahe *et al.* 1983) are highly methylated. Foetal and neonatal oocytes are believed to be drastically undermethylated both globally and at various endogenous repeat sequences (Sanford *et al.* 1984, 1987; Monk *et al.* 1987) and the ovulated oocyte is globally undermethylated (Monk *et al.* 1987). By extrapolation, the belief has been that the mature oocyte genome is still also undermethylated at repeat sequences and perhaps also at single copy sequences. Thus it is an attractive idea that methylation may play a role in establishing differences between parental chromosomes. Intermediate levels of methylation of several different repeat sequences observed in the early embryo were interpreted as reflecting the combination of the undermethylated maternal egg DNA and highly methylated paternal sperm DNA (Sanford *et al.* 1987). Hence, it was suggested that differential methylation is retained through the early cleavage divisions, prior to the large scale *de novo* methylation events at gastrulation (Monk, 1988), and this could contribute to the markedly different development of androgenetic and parthenogenetic embryos at these early stages.

As a means of assessing the methylation levels of the two parental genomes separately, we decided to compare parthenogenetic embryos, which contain only maternal chromosomes, with normal, fertilised ones. Ideally we would have included androgenetic embryos in this analysis, unfortunately they are difficult to make and more importantly develop very poorly (Surani *et al.* 1986). If indeed the oocyte genome was drastically undermethylated and remained so following fertilisation, parthenogenetic embryos would have been predicted to show very low levels of methylation. However, contrary to this prediction we found that the Murine Urinary Protein (MUP) and Intracisternal A Particle (IAP) families were highly methylated and L1 repeat fairly methylated in the early parthenogenetic embryo. This led us to a systematic analysis of the methylation levels in sperm, oocytes, eggs and both normal and parthenogenetic embryos. We report here our results that suggest that methylation of several repeat sequences occurs prior to fertilisation in both parental genomes. In the case of the L1 sequence where sperm shows higher levels of methylation than do eggs, methylation at the paternal sequence is lost during the first two cell cycles down to levels equivalent to that of the maternal sequences. Following this, methylation levels of each of these sequences are maintained until the 8- to 16- cell stage when there begins a gradual demethylation over the next 2–3 cell cycles of the L1 and IAP sequences.

Materials and methods

Oocyte, egg and embryo collection

C57BL/6J×CBA/Ca F_1 mice were used throughout. Female mice 4–6 weeks old were superovulated by intraperitoneal injection of Pregnant Mare's Serum (PMS: Folligon, Intervet). Germinal-vesicle stage oocytes were recovered 40–44 h later from ovaries by puncturing with fine forceps and the oocytes released from the surrounding cumulus by careful pipetting up and down within fine bore pipettes. Oocytes were washed through PBI plus Bovine Serum Albumin (BSA, Sigma; Whittingham and Wales, 1969) and carefully scrutinised for adhering cells before collection. For eggs and embryos injection of PMS was followed 48 h later by injection of human Chorionic Gonadotrophin (hCG: Chorulon, Intervet), and for fertilised eggs females were caged overnight with F_1 males and examined for the presence of vaginal plugs next morning (designated day 1). All unfertilised or fertilised eggs were recovered [illegible]–20 h post-hCG as described previously (Howlett *et al.* [illegible]) and cultured further *in vitro*.

Unfertilised eggs to be collected and analysed directly were dezonaed by brief exposure to warm Acid Tyrode's solution, pH 2.5 (Nicholson *et al.* 1985), washed in PBI plus BSA and carefully scrutinised for adhering cumulus cells. This procedure resulted in metaphase-II-arrested oocytes that were mostly devoid of first polar body.

For parthenogenetic embryos, eggs were activated 18.5–19 h post-hCG by exposure to 7 % ethanol in T6 containing 4 mg ml^{-1} BSA (Howlett *et al.* 1987) for 4.5 min, washed and transferred to T6 plus BSA containing 0.5 mg ml^{-1} Cytochalasin B (Sigma) for 4 h as described previously (Surani *et al.* 1984). Fertilised and diploid parthenogenetic embryos were cultured in T6 plus BSA under paraffin oil (BDH) at 37°C in 5 % CO_2 in air until collection. Note that the concentration of sodium lactate in T6 should be 23.7 mM and not 0.045 mM as printed in error in Howlett *et al.* (1987).

Inner cell masses (ICMs) were isolated from day 5 blastocysts by immunosurgery (Solter and Knowles, 1975) as described previously (Barton *et al.* 1985).

Inhibitors

The DNA polymerase inhibitor Aphidicolin (Sigma) was used at 2 µg ml^{-1} in T6 plus BSA from a stock stored at −20°C at 2 mg ml^{-1} in dimethyl sulphoxide (Howlett, 1986). Cytochalasin D (Sigma) was used at 0.5 µg ml^{-1} in T6 plus BSA to inhibit cell division from a stock stored at 0.5 mg ml^{-1} in water at −20°C.

DNA isolation and analysis

All oocytes, eggs and embryos were collected in 200–400 µl of a buffer containing 38 mM NaCl, 15 mM Tris, pH [illegible] and 5 mM EDTA plus 0.5 % sodium dodecyl sulphate and 100 µg ml^{-1} proteinase K and incubated for 1–3 h at 55°C. To aid precipitation of such small quantities of embryonic DNA 10 µg of *E. coli* tRNA was added as carrier before the mixtures were extracted twice with phenol and once with chloroform, the aqueous phase was then made 250 mM with NaCl and the DNA precipitated by addition of 2 volumes of absolute ethanol. Samples were stored at −70°C. Sperm from F_1 males was squeezed from isolated epididymides, collected in PBS and the DNA prepared as above except that 70 mM β-mercaptoethanol and 0.5 mg ml^{-1} proteinase K was used in the lysis solution. DNA was recovered by spinning at 14 000 revs min^{-1} for 10 min in an Eppendorf centrifuge, washed once with 200 µl of 70 % alcohol, dried and

redissolved in 10 mM Tris, pH 8, 1 mM EDTA. Restriction enzymes *Msp*I and *Hpa*II were used under the conditions recommended by the suppliers (NBL). Restriction digest samples were electrophoresed through 0.8 % agarose gels, alkali-transferred to Gene Screen Plus or Hybond N filters and hybridised with L1-specific fragments labelled with α ^{32}PdCTP by oligo-priming (Feinberg and Vogelstein, 1983). Some blots were washed in 1 % SDS, 0.1 SSC at 90°C for 2 h and rehybridised with labelled IAP- (Lueders and Kuff, 1980) or MUP-specific fragments (Kuhn *et al.* 1984) many blots were washed and rehybridised with a mitochondrial probe to check for complete *Hpa*II digestion (Hecht *et al.* 1984). Blots were scanned on a Joyce Loebel chromoscan to enable methylation levels to be quantified. Methylation levels are given (indicated on relevant lanes of figures) such that 100 % means that all the label is found in the limited mobility band at the top of the gel, 50 % indicates that half of this material has been digested down into smaller fragments.

Methylase protein analysis

Oocyte, egg and embryo samples were collected in 20 μl of sample buffer and run on 7.5 % polyacrylamide gels (Laemmli, 1970). Proteins were electrophoretically transferred to nitrocellulose paper in a buffer containing 20 % methanol at 100 mA overnight at 4°C. Blots were then washed and blocked with 3 % BSA in PBS containing 0.1 % Tween-20 (Sigma; PBS-Tween) for 2 h, washed 3×10 min with PBS-Tween and incubated with a monoclonal methylase antibody (a subclone of M2B10, gift from T. Boehm; Pfeifer *et al.* 1985) for 4–6 h at a concentration of 10–20 μg ml^{-1}. After washing, a second layer peroxidase-conjugated rabbit anti-mouse antibody (Miles) was used at 1:400 dilution in PBS for 45–60 min and visualisation achieved with 4-chloro naphthol as substrate (as described previously; Jones, 1986). Photographs of the blots were scanned on a Joyce Loebel Chromoscan to enable amounts of protein in different embryo samples to be semi-quantitated.

Results

Methylation of repeated sequences in sperm, oocytes and eggs

In experiments leading to the observations reported here, we had observed rather high levels of methylation of L1 sequences in parthenogenetic embryos (see below). This was surprising since, based on the previous findings of Sanford *et al.* (1984, 1987) that these sequences were virtually unmethylated in dictyate-stage foetal and newborn oocytes, we had expected to find very low levels of methylation in parthenogenones, which contain only maternally inherited chromosomes. We thus decided to determine the methylation status of oocytes nearer to the time of fertilisation and so ovulated, metaphase II eggs were collected. To avoid somatic contamination from cumulus cells, ovulated eggs were collected after removal of the zonae pellucidae. Thus, pure populations of eggs could be analysed and compared with sperm. DNA was prepared and restricted with the methylation-sensitive enzyme *Hpa*II and the distribution of the L1 repeat sequences compared. The level of methylation of the L1 sequences in metaphase II eggs was found to be lower than in sperm but not as low as would have been

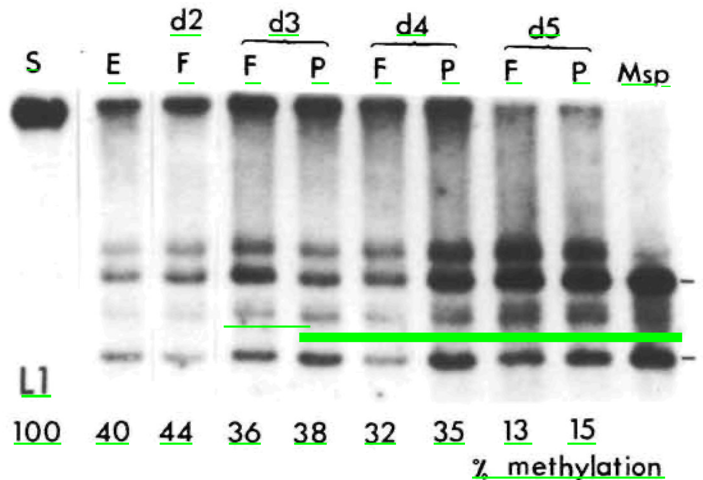

Fig. 1. Methylation of the L1 repeat in sperm, eggs and early fertilised and parthenogenetic embryos. Lanes show the distribution of *Hpa*II-digested DNA from sperm, ovulated eggs (400), day 2 fertilised embryos (2-cells; 180), day 3 fertilised and parthenogenetic embryos, (8-cells; 90), day 4 fertilised and parthenogenetic embryos (cavitating; 30) and day 5 fertilised and parthenogenetic embryos (expanded blastocysts; 10). The last lane is an *Msp*I digest of ten day 5 fertilised embryos as a control, which indicates the two major 5 kb and 3.5 kb fragments generated (marked and referred to as bands c and d respectively in Fig. 8), with two minor ones at about 5.5 and 4.5 kb (see Sanford *et al.* 1987). The percentage methylation (expressed as the amounts of the two major Msp bands as a proportion of the total in each lane) is indicated under each lane.

predicted previously (Fig. 1). From scans of many independent blots, we estimate the average level of methylation in eggs to be 42 %. In previous experiments, the level of methylation of L1 sequences in dictyate-stage oocytes was found to be 5 % (Sanford *et al.* 1987). Using a probe recognising the intracisternal A particle (IAP) and murine urinary protein (MUP) families of repeats, both of which had been found to be undermethylated in dictyate-stage oocytes, we found these sequences to be completely methylated in ovulated eggs and hence indistinguishable from sperm (Figs 2 and 3). To confirm earlier results of undermethylation of these sequences in dictyate-stage oocytes, DNA was extracted from day 15 foetal ovaries (Fig. 4), subjected to methylation analysis using the L1 and IAP probes and compared with foetal testes. From scans we find that about 8 % and 15 % of L1 and IAP sequences are unmethylated in foetal testes and ovaries, respectively (Fig. 4). Assuming that all of the unmethylated fraction is contributed by the germ cells in both testes and ovaries, we must conclude that both germ cell components are completely unmethylated at these sequences (see legend to Fig. 4 and Monk *et al.* 1987; Sanford *et al.* 1987). We also isolated immature oocytes from ovaries three days after birth and found L1 and IAP sequences to be virtually unmethylated (data not shown). These observations suggested therefore that a considerable amount of *de novo* methylation occurs

during the growth and/or maturation phase of oogenesis.

Using a monoclonal antibody against the methylase protein complex for Western blot analysis (Pfeifer *et al.* 1985), we detected extremely high levels of methylase

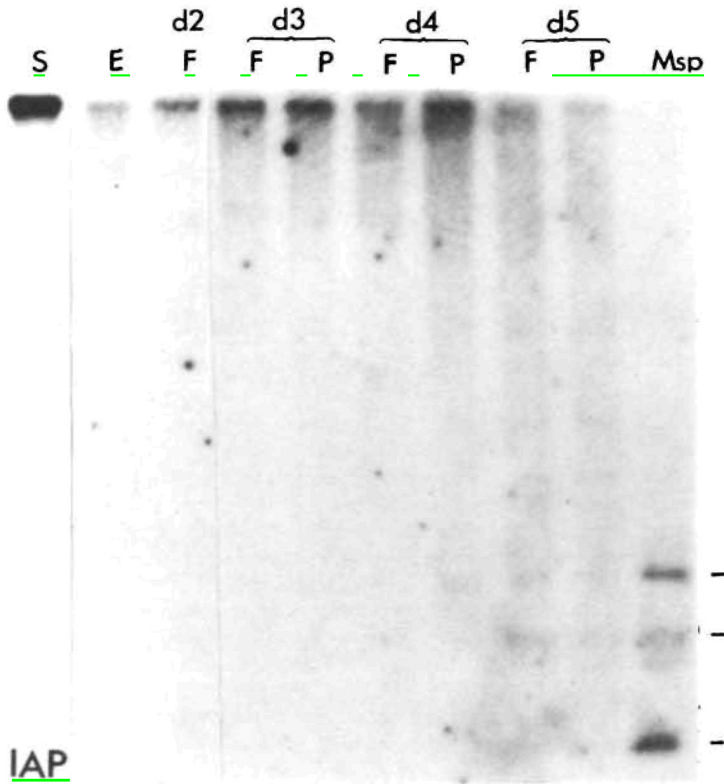

Fig. 2. Methylation of the IAP family in sperm, eggs and early fertilised and parthenogenetic embryos. Lanes show the distribution of *Hpa*II-digested DNA exactly as in Fig. 1, namely from sperm, ovulated eggs, 2-cells (day 2), 8-cells (day 3), cavitating (day 4) and expanded blastocysts (day 5) fertilised and parthenogenetic embryos. The last lane is an *Msp*I digest of blastocyst DNA showing the three major fragments of about 1.7, 0.9 and 0.5 kb (see Sanford *et al.* 1987).

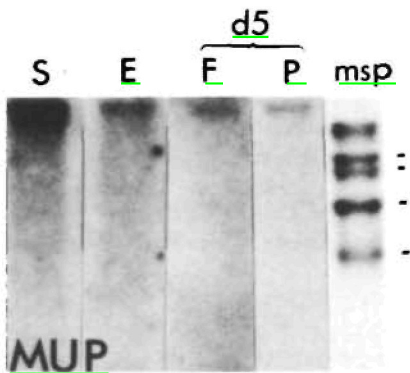

Fig. 3. Methylation of the MUP family in sperm, eggs and early fertilised and parthenogenetic embryos. Lanes show the distribution of *Hpa*II-digested DNA from sperm, ovulated eggs and expanded blastocysts (day 5) fertilised and parthenogenetic embryos. The last lane is an *Msp*I digest of liver DNA showing the four major fragments of about 8.5, 7, 5 and 3 kb (see Sanford *et al.* 1987).

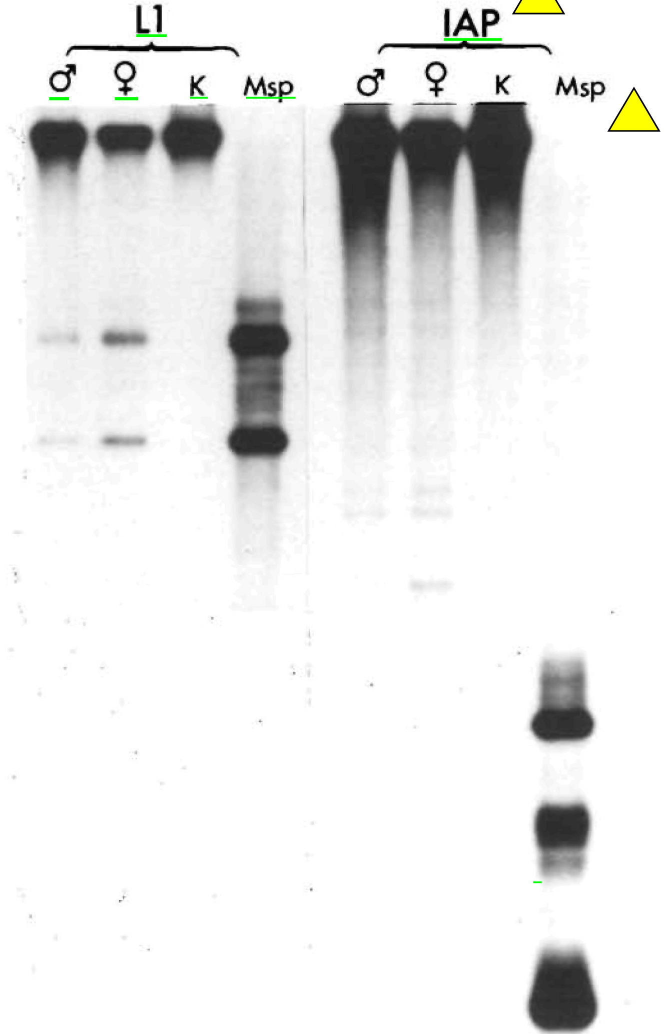

Fig. 4. Methylation of the L1 repeat and IAP family in foetal gonads. Lanes show the distribution of *Hpa*II-digested DNA from day 15 total testes (♂), total ovaries (♀) and kidneys (K), together with an *Msp*I-digested control lane probed with L1 and IAP. Note the complete methylation of both sequences in the kidney samples whereas in foetal gonads of both sexes a sizeable proportion (about 8 % and 15 %, respectively) is unmethylated. On day 15 there are slightly more germ cells in the testis than in the ovary (6×10^3 *versus* 4.7×10^3), however, the testis is twice the size (McCoshen, 1983). Hence for a given amount of DNA the germ cell to somatic cell ratio will be twice that in an ovarian sample compared with that from the testis. If we can assume that the unmethylated fraction is contributed by the germ cells in the testis (i.e. 8 %) then we would expect to find twice this amount in the ovaries if the oocytes are also unmethylated, this is what we see. Therefore, we can conclude that both L1 and IAP sequences are unmethylated in foetal germ cells of both sexes (compare with Monk *et al.* 1987; Sanford *et al.* 1987).

Fig. 5. Methylase protein in eggs and in early embryos. Lanes show the amount of methylase protein detected in germinal vesicle stage oocytes, ovulated eggs, day 4 fertilised and parthenogenetic embryos (cavitating), and day 5 fertilised and parthenogenetic embryos (expanded blastocysts). Each lane contains 200 eggs or embryos. Taking the amount of methylase protein in the ovulated egg as 100 %, the relative amounts in GV oocytes is also 100 %, in day 3 about 50 % (not shown), by day 4 is 30 % in fertilised and 35 % in parthenogenetic embryos, and by day 5 has dropped to about 5 % in fertilised and about 10 % in parthenogenetic embryos. The size of the methylase protein is about 190×10^3 M_r, in accordance with previous findings (Pfeifer and Drahovsky, 1986).

protein in the ovulated egg which were already present by the GV stage (Fig. 5). This is in agreement with the suggestion that the egg possesses high levels of methylase activity, as measured by an *in vitro* assay (Monk *et al.* 1991).

Methylation in the early embryo

We were interested to see how methylation levels on maternal and paternal chromosomes would behave during preimplantation development. In particular, it was of interest to see whether or not the differences detected at the L1 sequences were going to persist for a number of cell divisions following fertilisation. Methylation was therefore compared for the three sequences in normal fertilised and parthenogenetic embryos. For the L1 sequences, methylation in the 1-cell fertilised egg was considerably higher (72 %) than in the ovulated egg (47 %) or parthenogenetic 1-cell (42 %, Fig. 6, compare lane b with lanes a and c). This is consistent with high methylation levels persisting on the paternal chromosomes whereas maternal chromosomes remain at about 40–45 % methylation. However, by day 2 in the late 2-cell fertilised embryo, it appears that most of the sperm-specific methylation has been lost, so that fertilised embryos show very similar levels of methylation to that of eggs and parthenogenetic 1- or 2-cell embryos (Figs 1 and 6). This same intermediate level of methylation remains in fertilised embryos on day 3 and begins to drop on day 4 around the time of cavitation reaching about 13–15 % in the blastocyst on day 5 (Fig. 1). The rate of preimplantation development of parthenogenetic embryos parallels that of fertilised embryos so that cavitation, for example, occurs in both embryos at about 32 cells and at the same time from fertilisation or activation (S.K.H., unpublished observation). Care was taken to analyse the two types of embryos when at the same developmental stages, which was achieved by collecting fertilised embryos 6 h ahead of parthenogenones (6 h being the estimated time between that of *in vivo* fertilisation and experimental ethanol activation). It was, however, often found that

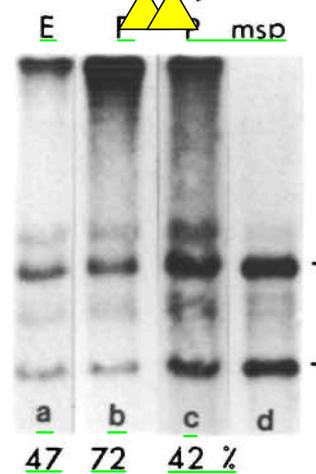

Fig. 6. Methylation of the L1 repeat immediately after fertilisation. Lanes show the distribution of *Hpa*II-digested DNA from 970 ovulated eggs (lane a), 490 fertilised eggs (lane b) and 500 parthenogenetically activated eggs (lane c). Fertilised eggs were collected about 19 h post hCG and the parthenogenetically activated eggs at about 24 h post hCG (about 5 h post activation). Lane d shows the *Msp*I digestion products. Note the similarity of the pattern in eggs before and after parthenogenetic activation (lanes a and c) and the increased amount of undigested DNA in the fertilised egg (lane b) which corresponds to the sperm-derived component. The percentage methylation is indicated under each lane.

demethylation occurred more slowly in parthenogenones than in fertilised embryos, such that methylation appeared to remain slightly higher on days 4 and 5 in parthenogenones (Fig. 1). This observation may well be due to the slight developmental slowing of parthenogenones in culture that is first apparent on day 5 (S.K.H., unpublished observations).

The level of methylation of the two other repeated sequences, the IAP and MUP families, was also analysed comparing normal and parthenogenetic embryos. Both the IAP and MUP families were very highly methylated in the early embryo and there was no difference between normal and parthenogenetic embryos. Some demethylation of the IAP sequences was apparent in the blastocyst (Fig. 2). Unfortunately the signal with the MUP probe was not strong enough to detect any evidence of demethylation in the blastocyst. Whether or not other repeated gene families and indeed single copy sequences become demethylated during preimplantation development remains to be seen, but the overall methylation of the blastocyst genome is clearly very low (Monk *et al.* 1987).

The levels of methylase protein were also determined in the two types of embryos at different stages (Fig. 5). Interestingly, the loss of methylation at the L1 sequences was paralleled by a marked decrease in the levels of methylase in the embryo as has been shown for methylase activity (Monk *et al.* 1991). 8-cell embryos still contained 50–60 % of the levels found in the egg, and this level decreased to about 30 % on day 4 to be virtually undetectable in the day 5 blastocyst (Fig. 5). Thus, on a per embryo basis there is about a 20-fold loss

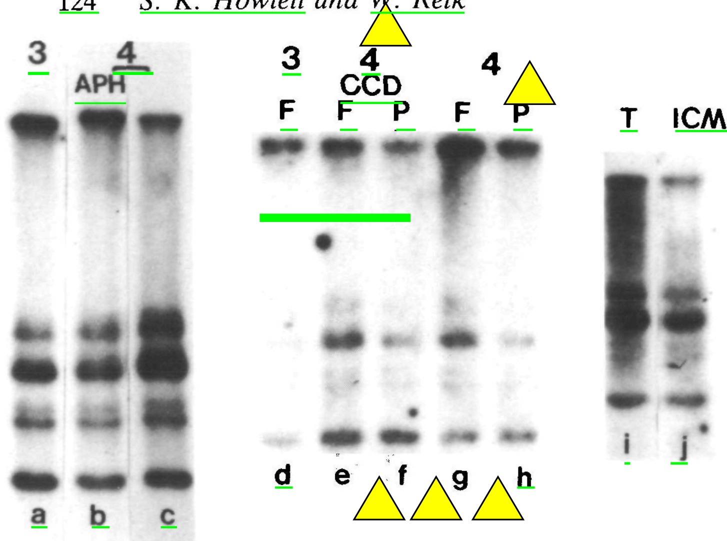

Fig. 7. Demethylation at the L1 repeat. Effects of aphidicolin. Lanes a–c show the distribution of *Hpa*II-digested DNA from 160 day 3 (8-cell) fertilised embryos (lane a), 120 embryos cultured from day 3 (mid 8-cell stage) to day 4 in aphidicolin (lane b) and 68 day 4 fertilised embryos (lane c). The percentage methylation on this gel on day 3 (lane a) is 55 %, on day 4 (lane c) is 36 % and in the aphidicolin-treated embryos (lane b) 51 %. Effects of CCD. Lanes d–h show the distribution of *Hpa*II-digested DNA from 80 fertilised day 3 embryos (lane d), 48 fertilised (lane e) or 46 parthenogenetic (lane f) embryos cultured from day 3 (mid 8-cell stage) to day 4 in CCD and 48 fertilised (lane g) or 46 parthenogenetic (lane h) control day 4 embryos. The percentage methylation on this gel on day 3 (lane d) is 57 %, on day 4 (lanes g and h) 40 % and 38 %, respectively, for fertilised and parthenogenetic embryos and in CCD-treated embryos (lanes e and f) 38 % and 35 %, respectively, for fertilised and parthenogenetic embryos. L1 distribution in the blastocyst. Lanes i and j show the distribution of *Hpa*II-digested DNA from 35 total blastocysts (T; lane i) and 72 isolated inner cell masses (ICM; lane j). The percentage methylation on this gel in total day 5 blastocysts (lane i) is 23 % and in the isolated ICM (lane j) 18 %.

of the protein between the egg and the blastocyst which would correspond to about a 2000-fold decrease per cell. Again we noted that enzyme levels tended to remain slightly higher in the parthenogenetic embryo.

The process of demethylation of the L1 sequence was investigated in more detail by the use of drugs to prevent either DNA replication or cell division. Blocking DNA replication in the 8-cell embryo with aphidicolin allows cleavage to 16 cells but not DNA synthesis during the 4th cell cycle (Smith and Johnson, 1985). Embryos were put into aphidicolin as 8 cells and cultured for a further 24 h when they were arrested at 16 cells; analysis of their DNA showed that demethylation had been almost completely prevented by inhibiting DNA replication (Fig. 7, compare lane b with a and c). 8-cell embryos were also placed into cytochalasin D (CCD) to prevent cell division but not DNA replication. They were cultured for a further 24 h, by which time the nuclear number had increased to 16. The extent of demethylation in such CCD-arrested embryos was similar to that of control day 4 embryos and higher than in aphidicolin-treated embryos (Fig. 7, compare lanes e and f with d, g and h). This suggested that DNA replication but not cell division *per se* is required for demethylation and that it begins during the S phase of the 4th cell cycle.

By the blastocyst stage, the two lineages of trophectoderm cells and inner cell mass (ICM) cells have been established. The trophectodermal and primitive endodermal lineages are known to be markedly undermethylated for both centromeric and dispersed repeat sequences and structural genes while methylation of the same sequences is high in the primitive ectodermal lineages of the embryo itself (Chapman *et al.* 1984; Rossant *et al.* 1986). Therefore, it was of interest to see if the low methylation levels of L1 sequences in the blastocyst solely reflected the trophectodermal contribution. Thus, ICMs were isolated on day 5 and compared with whole blastocysts. Methylation levels of isolated ICMs were also found to be low and appeared very similar to the pattern in the whole embryo (Fig. 7, compare lanes i and j). It appears therefore that there is a fairly uniform low level of methylation of L1 sequences in the blastocyst across both cell lineages. This is at variance with results from rabbit embryos, where high levels of methylation were found in the ICM as compared to the trophectoderm (Manes and Menzel, 1981). In the mouse embryo, global methylation levels seem to increase in the ICM shortly following implantation, and continue to rise during gastrulation (Monk 1988).

Discussion

Previous experiments demonstrated that a number of repeated sequences in the mouse genome are undermethylated in foetal germ cells. Thus, L1, MUP and IAP sequences are undermethylated in female foetal germ cells around days 13–15 (Sanford *et al.* 1987; Monk *et al.* 1987 and Fig. 4), L1 (Monk *et al.* 1987) and IAP (Fig. 4) sequences are also undermethylated in male foetal germ cells. However, in mature spermatozoa as well as in spermatids and pachytene spermatocytes, these sequences are highly methylated, suggesting that there are major *de novo* methylation events in the male germline, perhaps associated with the resumption of mitosis in germ cells after birth (Sanford *et al.* 1987). Our results suggest that there is also *de novo* methylation during oogenesis that most probably occurs during growth of the oocyte such that by the time of ovulation (and probably even by the germinal vesicle stage, our unpublished observations) the female genome has become methylated at several repeat sequences. The extent to which different repeat sequences become methylated in the oocyte varies, with IAP and MUP sequences apparently becoming as methylated as in sperm, whereas the L1 sequence reaches a level where about half of the sequences are methylated. This contrasts with the previously determined level of 5 % in dictyate-stage oocytes (Sanford *et*

al. 1987). As a consequence, the two parental genomes are perhaps more similar in terms of methylation status at the time of fertilisation than might have been predicted. This observation seems to make it unlikely that genome-wide methylation is a means by which imprinting of the maternal and paternal chromosomes during preimplantation development is achieved. In the only other analysis of methylation in oocytes and eggs, it was found that the oocyte genome was globally less methylated than the sperm genome (Monk *et al.* 1987). It is therefore quite possible that there exist other repeat and unique sequences that are differently methylated in egg and sperm. Also, the methylation status of low copy sequences, while thought to be relatively high in sperm (Waalwijk and Flavell, 1978; Mandel and Chambon, 1979; Sturm and Taylor, 1981; Ponzetto-Zimmerman and Wolgemuth, 1984), remains to be determined in eggs. Despite the obvious absence of any genome-wide differences in our experiments, it is still quite possible that certain sex-specific methylation differences exist and hold the key to the parental identity of alleles (see below). So far it is not clear whether imprinted transgenic sequences show any methylation differences between the egg and the sperm copy at the time of fertilisation. However, our observation of *de novo* methylation during oogenesis may be pertinent to transgene imprinting, as the great majority of imprinted transgenes are repressed following maternal inheritance (Surani *et al.* 1988).

We made use of parthenogenetic embryos as a convenient way of looking at the behaviour of the maternal chromosomes in isolation and compared them with normal fertilised embryos where both parental chromosomes are represented. Our results suggest that the high levels of methylation at the MUP and IAP sequences that are found in both egg and sperm are retained through early development and that there begins to be signs of demethylation of both maternal and paternal copies in the blastocyst at least for the IAP family. For the L1 repeat the same levels of about 40 % methylation of the maternal chromosomes are retained through to the morula stage and following cavitation there is a marked decrease down to very low levels in the blastocyst. The highly methylated sperm-derived L1 sequences, however, lose nearly half of their methylation during the first two cell cycles such that by the late 2-cell stage (after 2 rounds of DNA replication) they show an identical level of methylation as do the maternal chromosomes, they then behave in a similar manner to the maternal copies with another marked demethylation beginning after the third cell cycle. This second demethylation at the L1 sequences seems to coincide with the time of onset of cavitation. Indeed observations of differences in timing of this demethylation in different strains of mice whose embryos cavitate with different cell numbers suggest that there may be a link between demethylation and cavitation (S.K.H., unpublished observations). Since our experiments with inhibitors show a dependence of the demethylation on DNA replication and the earliest morphogenetic events in the preimplantation mouse embryo including compaction and cavitation seem to depend on the number of nuclear divisions (Surani *et al.* 1980; Smith and Johnson, 1985), the link may simply reflect the number of replication cycles that the genome experiences.

High global levels of methylation were found in earlier studies in the preimplantation mouse (Singer *et al.* 1979) and also rabbit (Manes and Menzel, 1981) embryo. In the rabbit the overall level of methylation decreased to about half from the 2-cell to the early blastocyst stage (Manes and Menzel, 1981) and a similar decrease in the global level of methylation has been found in the mouse preimplantation embryo (Monk *et al.* 1987). This is accompanied by a drastic reduction in the levels of methylase protein per cell during preimplantation development. However, the activity and specificity of the methylase is unclear since methylation levels of some sequences is maintained but not others – for example, the sperm-derived L1 sequences during the first two cell cycles. It will be interesting to determine the methylation status of unique genes during the first few cycles to see whether the sperm L1 demethylation can be extrapolated to single copy genes; if so, it may explain how paternal genes become expressible by the late 2-cell stage (for example, Sawicki *et al.* 1981). Why the egg should accumulate such abnormally high levels of methylase remains obscure, but it may explain why viral sequences introduced during early preimplantation development become highly methylated (Jahner *et al.* 1982). When analysing the methylation of repeated sequences in the genome, it is impossible to tell whether any intermediate levels of methylation reflect differences between individual copies of the repeats, or between individual cells in the embryo, or both. The apparent requirement for continuing DNA replication for demethylation (and not to a demethylating activity as has been proposed; Gjerset and Martin, 1982) suggests that one component of the demethylation process could involve the loss of activity of maintenance methylase. Loss of maintenance methylation can potentially generate differences in methylation between cells, which may contribute to the mechanism of mosaic expression recently observed in transgenic mice (McGowan *et al.* 1989).

The direct comparison between mature sperm and egg genomes shows that there is a fair degree of similarity in the methylation levels of several repeat sequences at the time of fertilisation, and furthermore it appears that where differences still exist (L1 repeats) they may be removed following the first two rounds of DNA replication such that both paternal and maternal genomes then undergo the same developmental changes in methylation through into the blastocyst. However, there is an indication that subtle but significant differences may persist for longer. The ratio of the two major digestion products of L1, at 3.5 kb and at 5 kb (Fig. 1), was significantly different in normal and parthenogenetic embryos at least until the blastocyst stage (Fig. 8). Since the 3.5 kb and 5 kb *Msp*I bands presumably reflect two subfamilies of the L1 repeat, it is possible that one of these retains methylation slightly longer on paternal and the other one on maternal

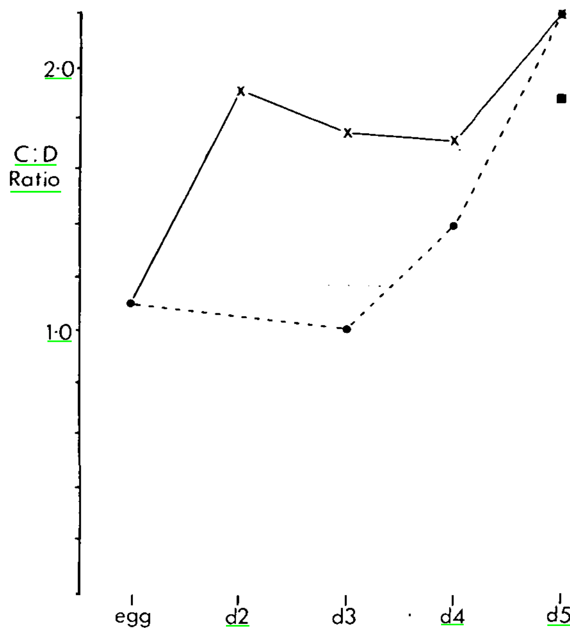

Fig. 8. Ratio of demethylation at the two major L1 subfamilies. The graph shows the ratio of the two major *MspI* digestion products, 5 kb (referred to as C) and 3.5 kb (D), of the L1 repeat in eggs and early embryos. The values given refer to the gel in Fig. 1 for fertilised (×——×) and parthenogenetic (●- - -●) embryos and are representative of many other similar gels. ■ shows the expected *MspI* ratio of the two bands. (Note the greater relative amount of the larger band c fragment in fertilised embryos – compare with Fig. 1).

chromosomes. This indication will, however, require more attention in further experiments.

Our observations highlight the occurrence of major methylation and demethylation events in mouse gametogenesis and early embryogenesis and the apparently tight developmental control. The functional significance of these changes, however, remains largely elusive.

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