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Adult phenotype in the mouse can be affected by epigenetic events in the early embryo

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SUMMARY

Major epigenetic modifications apparently occur during early development in the mouse. The factors that induce such modifications are complex and may involve the various components of a zygote. We have started to explore whether changes in the nucleocytoplasmic composition brought about by micromanipulation can induce phenotypic effects through epigenetic modifications. Nucleocytoplasmic hybrids were therefore prepared by transplanting a female pronucleus into a recipient egg from a different genotype. As a result, the maternal genome was of a different genetic background as compared with the egg cytoplasm. Specifically, experimental zygotes had cytoplasm from the inbred strain C57BL/6, a maternal genome from DBA/2, and a paternal genome from C57BL/6 (termed BDB hybrids). The mirror-image combination, termed DBD, was also made. The reconstituted zygotes were transferred to recipients and allowed to develop to term. Mice born from manipulated zygotes showed transcriptional repression and DNA methylation of major urinary protein genes in their liver, as well as growth deficiency resulting in reduced adult body weight. No altered phenotype was observed in controls in which the maternal pronucleus was simply transplanted back into another zygote of the same genetic background. These results clearly demonstrate phenotypic as well as molecular effects on DNA methylation and expression of at least one gene. Phenotype was therefore no longer predicted by genotype as a result of epigenetic modifications in experimental embryos. What precisely triggers the phenotypic and epigenetic changes is unknown, but presumably, nucleocytoplasmic interactions in hybrid zygotes may be partly responsible.

Key words: DNA methylation, epigenetic modification, mouse embryo, nuclear transplantation

INTRODUCTION

In the early mouse embryo, major changes in epigenetic modifications of the genome, such as DNA methylation, occur postzygotically before a stable somatic pattern is established at later stages (Monk, 1990). Indeed, many gene sequences become rapidly demethylated at cleavage stages and remethylated at early gastrulation (Monk et al., 1987; Sanford et al., 1987; Howlett and Reik, 1991; Kafri et al., 1992). In addition, a number of transgenes are known to undergo epigenetic modifications following fertilisation (Sapienza et al., 1989; McGowan et al., 1989; Allen et al., 1990; Reik et al., 1990; Chaillet et al., 1991; Engler et al., 1991). Moreover, this epigenetic programming, which can possibly occur at an early stage, can be genotype-specific and presumably involves different alleles of modifier genes. For example, some transgenes when inherited from the father, show different levels of DNA methylation in the offspring depending on the genotype of the mother. More importantly, there are some transgenes that show this response only to different maternal but not to different paternal genotypes (Allen et al., 1990; Surani et al., 1990). These maternal effects could be caused either by products of genotype-specific modifier genes present in the egg cytoplasm or expressed exclusively from the maternal genome after fertilization.

Endogenous genes may also undergo such genotype-specific epigenetic modification, as judged by observations on differences in gene expression in reciprocal crosses of inbred strains of mice (Reed, 1937; Bander et al., 1989; Klose and Reik, 1992; Vogel and Klose, 1992). Certainly, imprinted genes carry epigenetic modifications that uniquely mark their parental origin and have a long-lasting effect on gene expression later in development (Sasaki et al., 1992; Ferguson-Smith et al., 1993; Stöger et al., 1993). In a recent study it was suggested that parental imprinting of certain genes may also depend on genotype-specific modifiers (Forejt and Gregorova, 1992). Clearly, epigenetic modifica-

tions, at least those based on DNA methylation, do have an important role in development since loss-of-function mutations in the methyltransferase gene result in embryonic death at postimplantation stages (Li et al., 1992).

The factors that influence epigenetic modifications after fertilisation are apparently complex. In anisogamous species, the zygote consists of at least three distinct components: the egg cytoplasm, a maternal genome and a paternal genome. Inherited oocyte cytoplasmic RNA and proteins are essential for the initial events following fertilization, namely replication of DNA and activation of transcription from the embryonic genome. In mammals, maternal proteins can persist at least during preimplantation development, but longer lasting influences of cytoplasmic components are not known (except of course those that are brought about by maternally inherited mitochondria).

However, the precise interaction of egg cytoplasm and the genomes is crucial for normal development, as judged for example by observations on the DDK strain of mice in which these interactions are aberrant and result in early embryonic lethality (Babinet et al., 1990). Recent studies suggest that epigenetic genome modifications can be influenced by such interactions (Latham and Solter, 1991). In addition, it is possible that some of the genotype-specific methylation patterns of transgenes are influenced by interactions between cytoplasmic components and the nuclei.

We have initiated studies to perturb the various components in the zygote likely to affect epigenetic modifications. Here we introduce a maternal genome into recipient egg cytoplasm of a different genotype. This situation of course never occurs in nature. In order to assay gene expression on a broad basis, we make use of high resolution 2-dimensional electrophoresis of polypeptides in adult animals resulting from these reconstituted zygotes. We show that in animals derived from experimental zygotes, there is a striking repression of a major group of liver polypeptides. We identify the genes that encode these proteins and present a molecular analysis of their repression.

MATERIALS AND METHODS

Recovery, micromanipulation and transfer of fertilised eggs

Immature female mice (C57BL/6 and DBA/2 from Charles River) of about 5 weeks of age were superovulated by intraperitoneal injection of 7.5 i.u. pregnant mare's serum (Folligon, Intervet) followed 48 hours later by 7.5 i.u. human chorionic gonadotrophin (hCG; Chorulon, Intervet) and caged with stud males (C57BL/6 or DBA/2) overnight. The females were checked for vaginal plugs the following morning and this was counted as day 1 of development. Fertilised eggs were recovered from oviducts between 12 and 2 p.m. on day 1 as previously described (Howlett et al., 1987). Mature (C57BL/6×CBA) F_1 mice that had mated with vasectomised males were used as pseudo-pregnant recipients on the day of their plug (i.e. day 1) for experimental and control eggs.

For control experiments, eggs were recovered and placed in culture immediately in T6 + BSA (Howlett et al., 1987). Control and experimental eggs were either transferred later on day 1 or after overnight culture as 2-cell embryos on day 2, but always into recipients on day 1 of their pseudopregnancy.

Pronuclear transfer procedures were as described previously (Barton et al., 1987). Briefly, eggs were placed into PNC medium, which contains cytochalasin B (5 μg/ml; Sigma) and nocodazole (1.5 μg/ml; Sigma) in PB1 + BSA. Pronuclear transfers were performed in drops of PNC under oil on a Leitz micromanipulator and using inactivated Sendai virus as fusogen of the transferred karyoplasts. Following manipulation, eggs were washed thoroughly and then cultured in T6 + BSA. Only eggs were used where unequivocal identification of male and female pronuclei was possible.

Two-dimensional protein electrophoresis and microsequencing

All animals were analysed when they were older than 12 weeks. A piece of perfused mouse liver was pulverized in liquid nitrogen and suspended in one part (w/v) of 50 mM Tris/HCl buffer pH 7.4 containing protease inhibitors (2 mM EDTA, 2 mM benzamidin, 4.2 μM leupeptin; pepstatin A and phenylmethylsulfonylfluoride were dissolved in ethanol and added to give final concentrations of 1.4 μM and 1 mM, respectively). The suspension was mixed with urea, ampholytes (pH 2-4, Serva) and dithiothreitol to give final concentrations of 9 M, 2% and 70 mM, respectively. The mixture was subjected to sonication in a waterbath (Sonorex RK 510, Brandelin electronic KG, Berlin FRG) for 6×10 seconds using glass beads. The homogenate was centrifuged at 145 000 ***g*** for 30 minutes. The supernatant was used for 2-DE. Samples containing about 0.150 mg protein were applied to the gel.

For 2-DE of proteins, the modified version (Klose, 1983) of the method described by Klose (1975) was employed. The size of the gels used for separating the proteins in the second dimension was 30 cm (running direction)×23 cm×0.75 mm. The protein spots were revealed by silver staining (Heukeshoven and Dernick, 1985).

Proteins separated by 2-DE were blotted onto glassybond membranes (Biometra, Göttingen, FRG) as described (Jungblut et al., 1990). Protein spots selected for microsequencing were cut out from the membrane and processed in a 470 A gas phase sequencer (Applied Biosystems, Foster City, CA, USA) (Eckerskorn et al., 1988).

DNA and RNA methods

DNA and RNA was isolated following standard procedures (Sambrook et al., 1989). DNA samples (10 μg) were digested with restriction enzymes as recommended by the supplier (NBL). When using methylation-sensitive enzymes, an aliquot of the digest was incubated with phage λ DNA and completeness of the digest was monitored on small gels. Digested samples were electrophoresed through 0.8-1% agarose gels and blots were hybridised and washed at high stringency (Sambrook et al., 1989). The inserts of probes BS62 and BS655 (Al-Shawi et al., 1989, and references therein) were labelled by random priming.

Total cytoplasmic RNA was electrophoresed through 5.4% formaldehyde gels. Blots were hybridised with the cDNA clone *Mup 11* (Al-Shawi et al., 1989) and washed at high stringency (Sambrook et al., 1989). For *BL 1*-specific hybridisation, an oligonucleotide (sequence CACATAGTTTTGCAAACC) complementary to the *BL 1* sequence from position 536 to 553 was made. The *BL 1* gene has an A in position 544 whereas all other group 1 genes have a C in this position (McIntosh and Bishop, 1989). The oligonucleotide was labelled with T4 polynucleotide kinase and γ[^{32}P] ATP (Sambrook et al., 1989). Hybridisation and washing was essentially as described by McIntosh and Bishop (1989). Briefly, hybridisation was in 0.5 M $NaHPO_4$ (pH 7.2), 7% SDS, 2 mM EDTA at 43°C, and washing was at the same temperature in 0.5 M $NaHPO_4$ (pH 7.2), 5% SDS, 1 mM EDTA for 2× 10 minutes and in 0.5 M $NaHPO_4$ (pH 7.2), 1% SDS, 1 mM EDTA for 2× 20 minutes.

Statistics

Body weights of males of the nucleocytoplasmic hybrid group (6

BDB, 5 DBD) and 3 control groups (11 B6×D2 cultured), (5 D2×B6 cultured), (4 BBD) were determined in animals that were more than 7 months old. The experimental group (mean=26.18 g, s.d.=2.26; s.e.m.=0.68; n=11) was significantly different from all 3 control groups (B6×D2: mean=32.46 g, s.d.=4.57, s.e.m.=1.38, n=11; D2×B6: mean=38.80 g, s.d.=7.43, s.e.m.=3.32, n=5; BBD: mean=34.99 g, s.d.=1.22, s.e.m.=0.61, n=4, P<0.01, Mann Whitney U-test) whereas the control groups did not differ significantly from each other. The 3 control groups were therefore combined.

RESULTS

Aberrant gene expression in nucleocytoplasmic hybrids

Zygotes from the inbred strains C57BL/6 (B6) and DBA/2 (D2) were used to study the effects of interactions between the egg cytoplasm and the maternal genome. From fertilised eggs of the B6 type (B6×B6 mating), the female pronucleus was removed at 22-24 hours postinjection of human chorionic gonadotrophin and replaced by a female pronucleus from a D2×D2 egg (Fig. 1). The zygote thus produced is a nucleocytoplasmic hybrid carrying a B6-type cytoplasm, D2 maternal chromosomes and B6 paternal chromosomes (termed BDB). In a mirror-image experiment, zygotes were produced that have a D2 cytoplasm, B6 maternal chromosomes and D2 paternal chromosomes (DBD nucleocytoplasmic hybrid). From 55 BDB and 95 DBD experimental embryos transferred, respectively, 11 female and 7 male BDB offspring and 2 female and 7 male DBD offspring were obtained. One DBD male was very small and died at the age of 7 weeks. All other animals grew up initially without any obvious phenotypic abnormalities. To examine gene expression in the nucleocytoplasmic hybrids, a collective analysis of polypeptide expression by 2-dimensional electrophoresis (2-DE) of liver proteins was performed. Initially 8 females of the BDB and 2 females of the DBD type at an age of 12 to 13 weeks were analysed. As an initial control, we analysed 2-DE patterns in age-matched D2×B6 (D2 mother and B6 father) and B6×D2 female hybrids, as these are genotypically identical to BDB or DBD animals, respectively (Fig. 1).

On first inspection of the gels, a striking difference in polypeptide expression was seen between the nucleocytoplasmic hybrid group and the control animals. A group of at least three acidic polypeptides of approximate relative molecular mass 20×10^3 was prominently expressed in all controls analysed (Figs 2,3A; Table 1) but was virtually absent in all experimental females, except in one DBD animal (Fig. 3B; Table 1).

We next analysed liver polypeptides in adult males, comparing BDBs and DBDs with controls. In control males, the same group of polypeptides was found to be expressed considerably more abundantly than in control females (Fig. 4), thus revealing a sexual dimorphism in the expression pattern. However, the male BDB and DBD pattern was substantially reduced in comparison with male controls (Fig. 4). While the level of residual expression was higher in BDB/DBD males than in BDB/DBD females, the actual extent of repression in BDB/DBD males and females was quite comparable (Fig. 4).

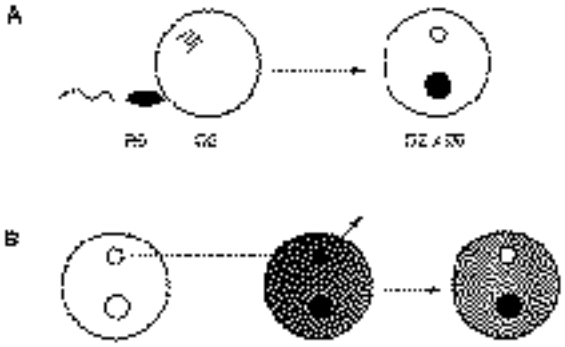

Fig. 1. Construction of nucleocytoplasmic hybrids. (A) In a natural hybrid between a DBA/2 (D2) female and C57BL/6 (B6) male, the cytoplasm and the maternal chromosomes are of the same genotype (D2). (B) From a B6×B6 egg, the maternal pronucleus was removed and replaced by a maternal pronucleus from a D2 egg. The experimental zygote thus produced (termed BDB) is identical in genotype to the D2×B6 egg, but has a B6 cytoplasm. DBD hybrids were produced by the reciprocal transfer of the maternal pronucleus.

Hence, the great majority of the experimental animals (25 out of 26) showed repression of this particular group of liver polypeptides when compared to natural hybrid controls (0 out of 27, Table 1). While a small number of other much less obvious differences in polypeptide expression were also detected between experimental animals and controls, overall the great majority of polypeptides were found to be expressed normally in nucleocytoplasmic hybrids. Indeed, we monitored a number of liver polypeptide spots that showed a characteristic developmental profile during the first 13 weeks of age. The nucleocytoplasmic hybrids showed appropriate activation of all these stage-specific polypeptides (data not shown), indicating that the general programme of tissue-specific gene expression is on schedule in those animals.

Expression of polypeptides in controls

The repression of liver polypeptides in BDB and DBD animals as compared to D2×B6 and B6×D2 natural hybrids can be attributed to several factors that differ between the two groups of animals. First, the experimental embryos are exposed to a different intrauterine environment as they are carried in (B6×CBA) F_1 foster mothers, whereas the natural hybrids develop in a B6 or D2 uterus. Second, the experimental embryos were isolated at the one-cell stage, cultured in vitro for several hours and subjected to micromanipulation during transplantation of their female pronucleus. And finally, as a result of this pronuclear transplantation, the cytoplasmic and female pronuclear genotypes are different in BDB and DBD embryos.

We sought to discern the influence of these factors by performing a number of embryo manipulation experiments (Fig. 4; Table 1). Thus D2×B6 and B6×D2 one-cell embryos were isolated and cultured in vitro for a few hours or overnight and were then transferred to (B6×CBA) F_1 recipients on their first day of pseudopregnancy. Furthermore, zygotes were made in which the female pronucleus was removed from a B6×D2 egg and replaced with a B6 female pronucleus coming from another B6×D2 egg. This restores

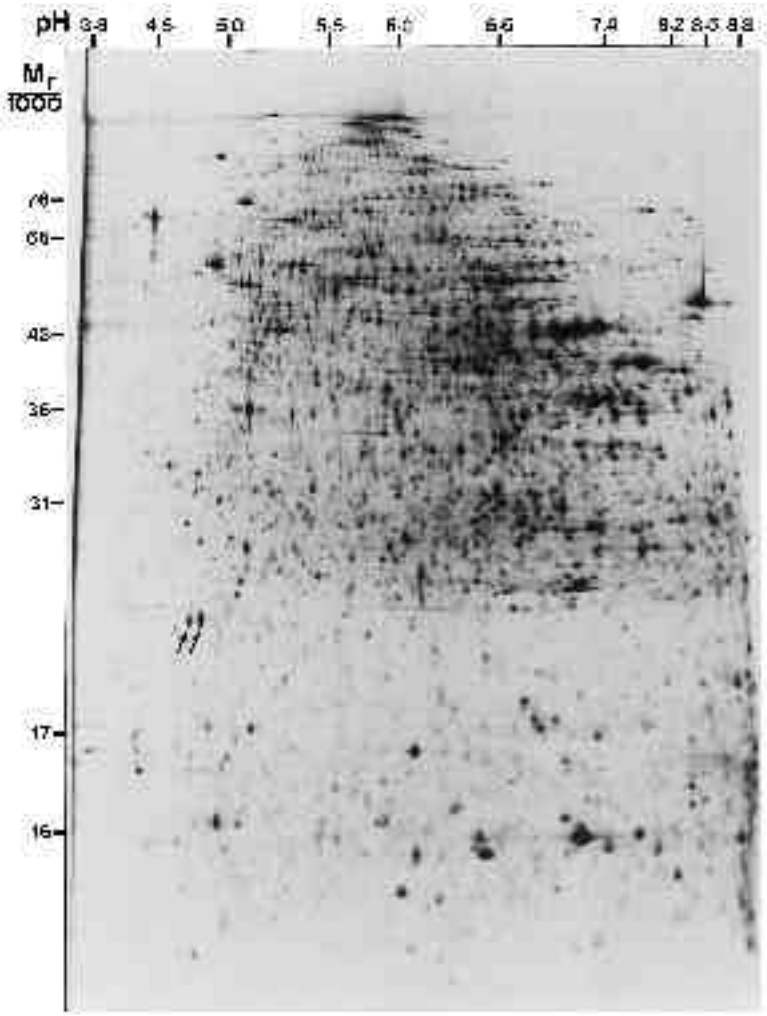

Fig. 2. Polypeptide expression in liver of C57BL/6 (B6) control mice. Urea extracts of livers were prepared from females (older than 12 weeks) and separated by 2-dimensional electrophoresis as described in Materials and methods. Protein samples were applied to the acidic side of the isoelectric focusing gel. Protein spots were revealed by silver staining. Arrows indicate the major urinary protein spots.

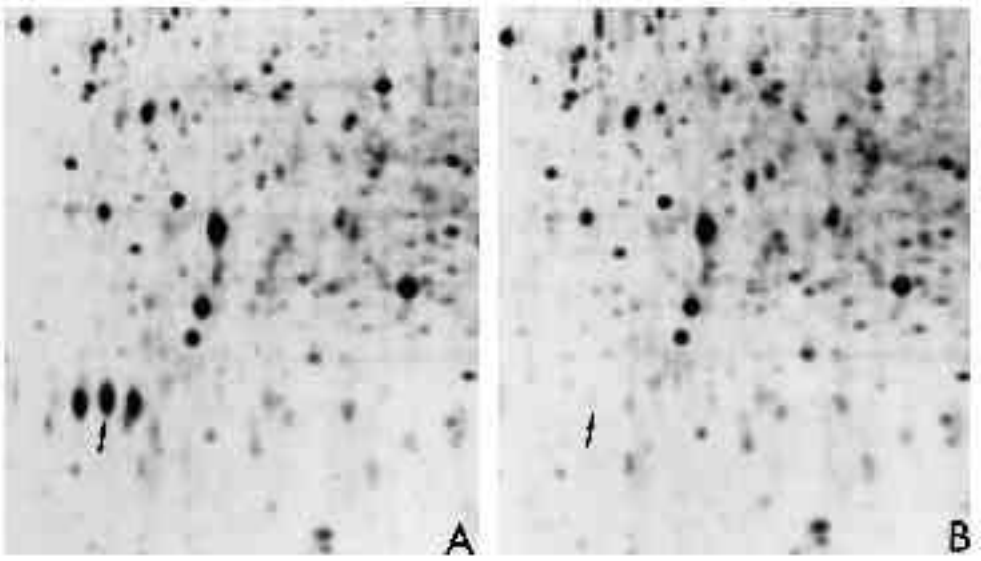

Fig. 3. Polypeptide expression in livers of nucleocytoplasmic hybrids and control mice. (A) A section of the lower molecular weight acidic region from a D2×B6 natural hybrid. (B) The same section from a nucleocytoplasmic hybrid (BDB). Arrows indicate the major urinary protein spots.

the B6×D2 genotype; these zygotes were thus termed BBD (B6 cytoplasm, B6 female pronucleus, D2 male pronucleus). In 10 D2×B6, 11 B6×D2, and 6 BBD females resulting from these experiments, the polypeptide expression pattern (or the DNA methylation pattern, see below) was determined by 2-DE of liver proteins at 12-13 weeks of age as described before. 25 animals, including the 6 BBDs, showed a pattern indistinguishable from unmanipulated controls (Fig. 4; Table 1). Two animals from the B6×D2 group however, had an aberrant pattern similar to the BDBs (Table 1). In the

Table 1. Liver polypeptide (major urinary protein, MUP) expression and DNA methylation in nucleocytoplasmic hybrids and control mice

		Number of animals analysed			Total number of animals analysed	Number of animals with aberrant MUP phenotype
		MUP phenotype + DNA methylation	DNA methylation	MUP expression		
BDB	♀	8	3		11	11
	♂	5	2		7	7
DBD	♀	2			2	1
	♂	4	2		6	6
B6	♀		2	9	11	0
	♂		2	5	7	0
D2	♀		2	7	9	0
	♂		2	5	7	0
B6×D2	♀		2	5	7	0
	♂		2	4	6	0
D2×B6	♀		2	5	7	0
	♂		2	5	7	0
B6×D2 transferred	♀	9	2		11	2
	♂		5		5	0
D2×B6 transferred	♀	5	2	3	10	0
	♂	2		2	4	0
BBD	♀	6			6	0
	♂	2	2		4	0

MUP phenotype was determined by 2-dimensional electrophoresis (MUP expression) and methylation-sensitive restriction enzyme digestion (DNA methylation) in animals in the first column, and by either methylation digest or 2-DE in the second and third columns, respectively. Phenotypes were classified as normal or aberrant as shown in Figs 3, 4 and 6. Since expression and methylation were always concordant in animals in which both were analysed, the total phenotype scores were combined.

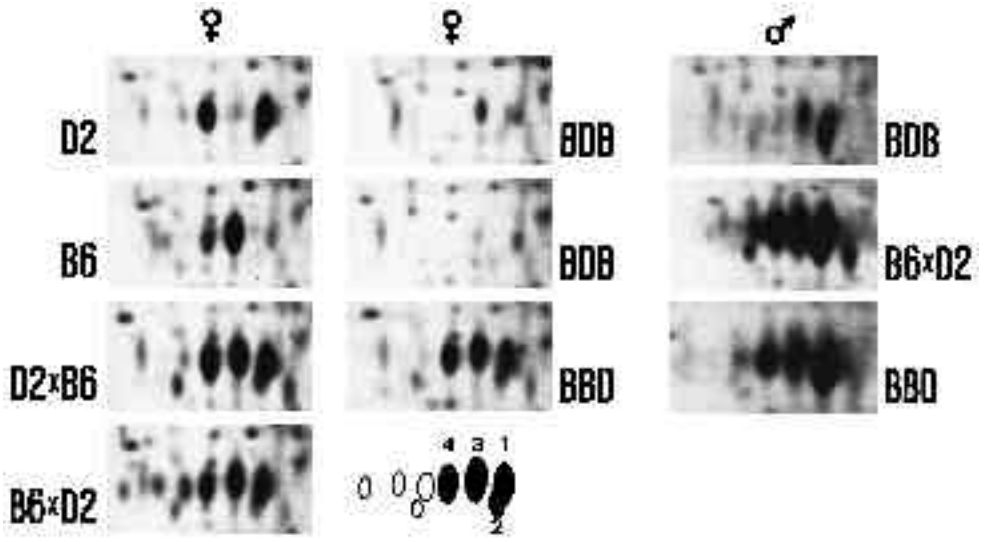

Fig. 4. Major urinary protein (MUP) expression in livers of nucleocytoplasmic hybrids and control mice. Shown are magnified 2-DE sections of the affected group of polypeptides, which were identified, by microsequencing, as MUPs (see Fig. 5 and text). The nomenclature for the MUP spots follows that of Knopf et al. (1983) and Held et al. (1987). Shown are patterns of MUP expression in females and males from the parental strains used in this study, C57BL/6 (B6) and DBA/2 (D2), from their natural hybrids (D2×B6 and B6×D2), from the nucleocytoplasmic hybrids (BDB) and from the nuclear transplant controls (BBD). All animals were more than 12 weeks old. The 2 female BDB patterns shown represent the ones with the highest and the lowest level of MUP expression found in this study. In the male BDB group, there was 1 animal with slightly higher levels of spot 4 so that the whole group looked similar to that of a natural hybrid female. The male DBD and female DBD (1 animal) patterns were identical to the respective BDB patterns.

male group, 4 D2×B6 and 5 B6×D2 (cultured and transferred) and 4 BBD controls were examined and were found to have the normal male-type pattern (Fig. 4; Table 1).

This analysis demonstrates that the aberrant polypeptide expression in BDB and DBD livers is due to an inappropriate combination of egg cytoplasm and maternal pronucleus. However, such aberrant phenotypes also occur, albeit at a much reduced frequency (2 out of 40), in animals developing from embryos that were cultured in vitro and transferred to recipient foster mothers (Table 1). Hence, in vitro culture of embryos can apparently have a long-lasting effect on phenotype. The precise effects of specific culture conditions on later phenotype remain to be determined, and some of these experiments are now underway.

The major urinary protein genes are affected: analysis of epigenetic modification and transcription

Amino acid sequence

To identify the genes whose expression is affected in the

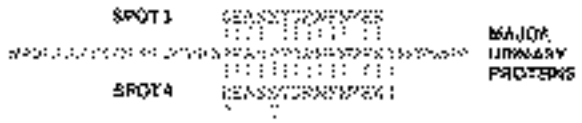

Fig. 5. N-terminal amino acid sequence of the affected liver polypeptides. The amino acid sequences of spots 3 and 4 (see Fig. 3) identify the major urinary proteins. The first 18 amino acids are a signal peptide cleaved off in the liver (Kuhn et al., 1984).

nucleocytoplasmic hybrids, N-terminal amino acid sequences were obtained from polypeptides 3 and 4 (Fig. 4), and unequivocally identify them as belonging to the major urinary proteins (MUPs, Fig. 5). The MUPs are a group of closely related small acidic proteins that are expressed in a number of secretory tissues in the mouse. These proteins are encoded by a family of approximately 40 genes, 15 of which constitute a subfamily of group 1 *Mup* genes that are expressed in mature liver (Shaw et al., 1983; Held et al., 1987; Shahan et al., 1987; Al-Shawi et al., 1989). All of these genes are located on the middle part of chromosome 4, and two co-dominant alleles are known in inbred strains of mice. The two strains used in this study, D2 and B6, carry the *Mup-1*^{a} and *Mup-1*^{b} alleles respectively, which determine a distinct electrophoretic pattern of the liver MUPs (Fig. 4, Finlayson et al., 1963). Synthesis of some of the individual liver proteins that can be resolved by 2-DE has been attributed to specific group 1 genes (Held et al., 1987). Synthesis of MUPs is regulated during postnatal development and begins a few weeks after birth (Barth et al., 1982; Shaw et al., 1983). Our analysis of liver MUPs by 2-DE showed them to become fully expressed at 6 to 8 weeks of age (data not shown).

Analysis of DNA methylation

There are a number of examples of transgenes in the mouse where epigenetic modification post-fertilisation leads to characteristic patterns of DNA methylation and transgene expression in later foetal or indeed in adult life. We therefore sought to establish the patterns of DNA methylation of group 1 *Mup* genes in livers of BDB and DBD animals and controls. A representative group 1 probe was chosen (BS 655) and DNA methylation of group 1 genes was analysed by digestion with *Bam*HI and *Hpa*II, and hybridisation with BS 655 (Fig. 6). A number of group 1 genes were found to be significantly more methylated in all BDB and DBD females analysed as compared with both embryo-transferred and BBD controls (Fig. 6B; Table 1). While the pattern of methylation was different in males, it was again evident that in BDB and DBD males a number of the group 1 genes were more methylated than in embryo-transferred and in BBD controls (Fig. 6B; Table 1).

The group 1 genes fall into distinct subgroups that are characterised by the presence of specific restriction enzyme sites (Al-Shawi et al., 1989). Hence, from the sizes of the

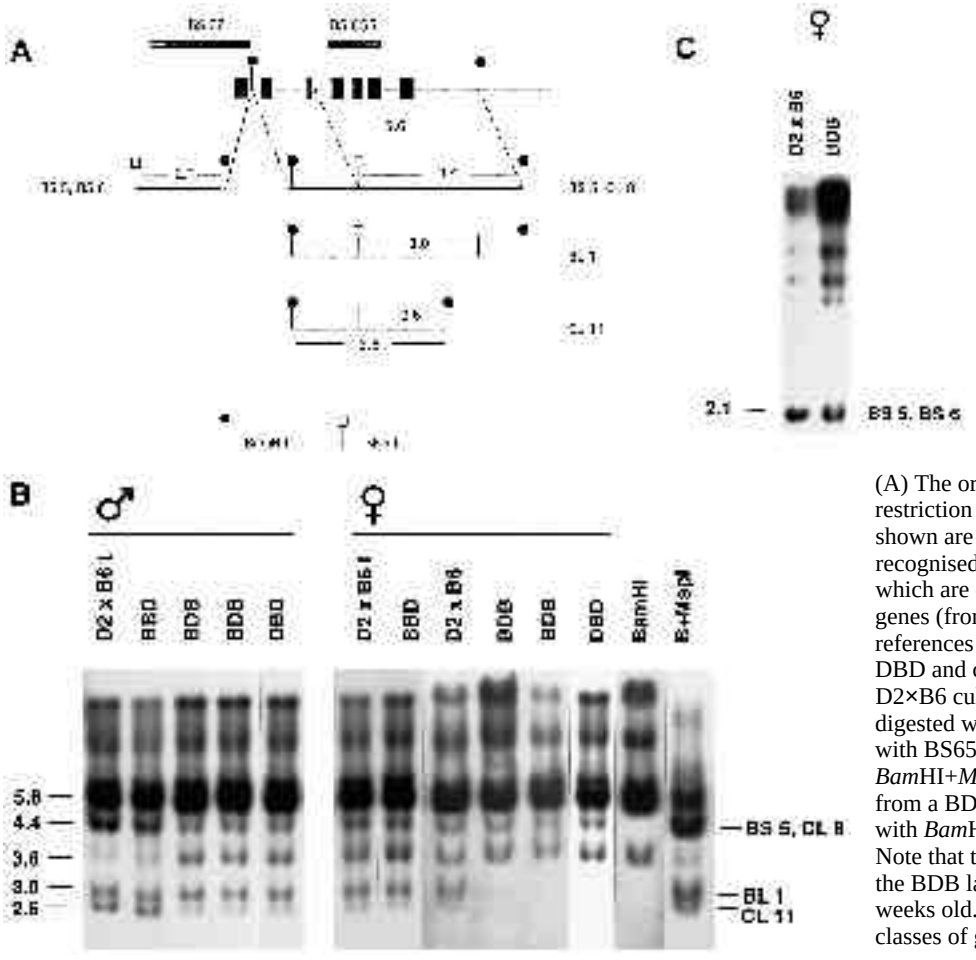

Fig. 6. Methylation of *Mup* genes in BDB, DBD and control livers. (A) The organisation of group 1 genes, restriction enzyme sites and probes used. Also shown are the *Bam*HI-*Msp*I fragments that are recognised by probes BS62 and BS655 and which are diagnostic for particular group 1 genes (from Al-Shawi et al., 1989, and references therein). (B) Liver DNAs from BDB, DBD and control animals, including BBD and D2×B6 cultured and transferred (t), were digested with *Bam*HI and *Hpa*II and probed with BS655. Control digests with *Bam*HI and *Bam*HI+*Msp*I are also shown. (C) Liver DNAs from a BDB and a D2×B6 female were digested with *Bam*HI and *Hpa*II, and probed with BS62. Note that there is considerably more DNA in the BDB lane. All animals were more than 12 weeks old. Size markers are in kb. The different classes of group 1 genes are indicated.

bands produced by digestion at *Bam*HI and *Hpa*II sites, it is possible to deduce the identity of some of the genes that are more heavily methylated in BDB and DBD animals (Fig. 6A,B). It is clear from Fig. 6 that genes belonging to the *BL 1* class and the *CL 11* class are more methylated in livers of BDB/DBD females (*BL 1* and *CL 11* are 5-fold more methylated as determined by scanning of autoradiographs), whereas the *CL 11* and the *BS 5* and *CL 8* classes are more methylated in BDB/DBD males (*CL 11* is 10-fold and *BS 5*/*CL 8* are 5-fold more methylated). Thus, at least for the *BL 1* gene, whose protein product migrates in the position of spot 1 on 2-DE gels (Held et al., 1987; D. Johnson, personal communication), we found a correlation between DNA methylation of a specific *Mup* gene and repression of the corresponding polypeptide in our nucleocytoplasmic hybrids (compare with Fig. 4). We extended the methylation analysis to *Hpa*II restriction sites situated 5′ of the *Mup* genes by using probe BS 62 (Fig. 6A,C). As before, it was found that some of the group 1 genes were more methylated in BDB livers than in controls.

As a control, we examined the DNA methylation pattern of the albumin gene and found it to be demethylated to the same extent in nucleocytoplasmic and control liver DNA (data not shown). The albumin gene has previously been shown to undergo liver-specific demethylation during postnatal development (Tratner et al., 1988).

Transcription of *Mup* genes

We next sought to determine whether the methylation of particular *Mup* genes observed in BDB animals affected their transcription. This analysis is difficult because the genes are so similar (the pairwise identity between group 1 mRNA sequences is about 99.6%). Fortunately, the *BL 1* gene, which was found to be affected by DNA methylation, differs by one nucleotide (in position 544 of the transcribed sequence) from all other group 1 genes (see Materials and methods). Using an oligonucleotide probe (an 18-mer) complementary to this part of the *BL 1* sequence, it is possible to examine transcription of the *BL 1* gene alone (McIntosh and Bishop, 1989). Transcription of *BL 1* was detected in control liver RNAs whenever the D2 genome was present (Fig. 7A; the B6 genotype serves as a negative control because it does not contain a *BL 1* gene, Kuhn et al., 1984; Shahan et al., 1987) but was considerably reduced in BDB liver (approximately 10-fold; Fig. 7A). Hence, transcription of the methylated *BL 1* gene in BDB liver is substantially diminished. As mentioned above, the *BL 1* gene encodes a MUP product that migrates in the position of spot 1 on 2-DE gels (Held et al., 1987; D. Johnson, personal communication), consistent with the repression of this spot in all the BDBs (Fig. 4). We have so far not been able to extend this analysis to other genes methylated in BDBs (such as *CL 11*), because there is insufficient sequence information or sequences do not show unique differences such as used to examine *BL 1*. However, we determined the total amount of group 1 mRNA by hybridisation with a group 1 cDNA clone (*Mup 11*) and found it to be reduced by at least 7-fold in BDB versus control liver (Fig. 7B). It appears therefore that the repression of MUPs seen in BDB and DBD animals is largely, if not exclusively, at the level of transcription.

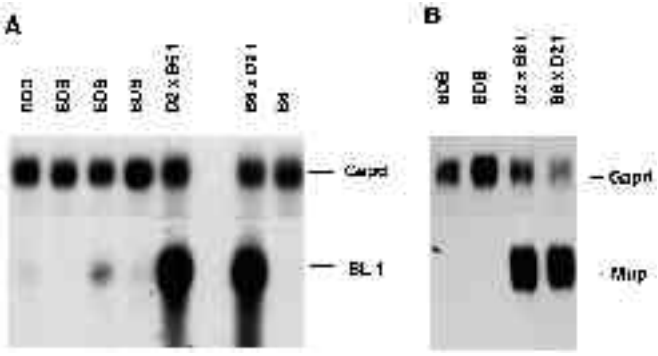

Fig. 7. Transcription of the *BL 1* gene in nucleocytoplasmic hybrids. The transcribed sequence of the *BL 1* gene has a single nucleotide difference in position 544 in comparison to all other group 1 genes (Clark et al., 1985). (A) An oligonucleotide (18-mer) complementary to this part of the *BL 1* sequence (position 536-553) was hybridised to a Northern blot of total liver RNA (~10 μg per lane) of female BDB and control (cultured and transferred) mice, under conditions of specific hybridisation to *BL 1*. No hybridisation was observed to RNA from B6 as the B6 genotype does not have a *BL 1* allele, but has all other group 1 genes. Note the substantial decrease of *BL 1* RNA in BDB liver. (B) A representative group 1 cDNA probe (*Mup 11*, Al-Shawi et al., 1989)) was hybridised to a Northern blot of total liver RNA of female BDB and control mice. Transcription of group 1 *Mup* genes was found to be substantially repressed in BDBs. Similar results were obtained with three additional BDB females: although some variability was found, the extent of *Mup* repression was at least 7-fold as compared to controls. RNAs were quantitated by hybridisation with *Gapd* probes.

Additional phenotypes in nucleocytoplasmic hybrids: growth retardation

Our study does not directly address the question of whether *Mup* genes are epigenetically modified in BDB and DBD embryos as an early, and perhaps primary, consequence of altered nucleocytoplasmic interaction. It is conceivable that an altered MUP expression pattern arises secondarily to changes in other gene products, for example in hormones that are known to influence *Mup* gene transcription. We observed earlier that overall BDB and DBD animals appeared to be quite normal; however during the course of these experiments we noticed that their growth lagged behind that of normal controls. Because most BDB females were killed as young adults (12-13 weeks), the following analysis is exclusively based on males and includes 6 BDB males and 5 DBD males. These animals were weighed at ages ranging from 7 months to more than 20 months and their weights were compared to those of an age-matched control group consisting of B6×D2 (11 animals) and D2×B6 hybrids (5 animals) that had been cultured in vitro and transferred to recipient foster mothers. Also included were 4 BBD controls. A striking difference in weights was found, with a mean of 26.18 g (s.d.=2.26 g, s.e.m.=0.68, n=11) in the experimental group and a mean of 34.55 g (s.d.=5.49, s.e.m.=1.23, n=20) in the control group (Fig. 8). This difference is significant (P<0.001, Welch test; P<0.005, Mann Whitney U-test). We note that weights are more variable in the control group; we attribute this to a few considerably lighter animals in this group. At what stage in development differences in growth first become apparent remains to be determined.

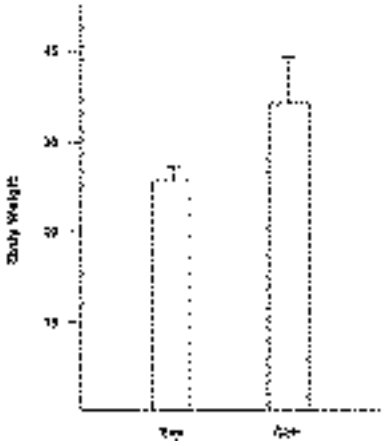

Fig. 8. Growth deficiency in nucleocytoplasmic hybrids. Body weights of adult males (more than 7 months old) were compared between the experimental group (11 animals) and initially 3 control groups (B6×D2 cultured and transferred, 11 animals; D2×B6 cultured and transferred, 5 animals; BBD, 4 animals). The experimental group was found to be significantly different from each of the control groups, whereas the control groups did not differ significantly from each other. Controls were therefore combined. The means (26.18 g, 34.55 g) and standard deviations (2.26, 5.49) of the experimental and the control group, respectively, are shown. Weights are in grams. See text and Materials and methods for details.

DISCUSSION

The most general conclusion from this study is that aspects of adult phenotype in the mouse can depend on some form of extragenetic programming in the embryo. This programming is affected markedly by nucleocytoplasmic factors and to a lesser extent by environmental factors in vitro. We call this programming epigenetic simply to indicate that the phenotype observed is not predicted by genotype; this does not imply any particular molecular mechanism for the phenomenon that we have observed.

All nucleocytoplasmic hybrids made between the strains C57BL/6 and DBA/2 in which the genotype of the cytoplasm differed from that of the female pronucleus (except one DBD female) showed transcriptional repression and methylation of major urinary protein (*Mup*) genes in adult liver. We were able to show for one particular *Mup* gene, *BL 1*, that there was complete concordance of DNA methylation, transcriptional repression, and absence of the corresponding polypeptide in nucleocytoplasmic hybrids. In control experiments in which the female pronucleus was transplanted but the normal genotypic constitution was restored (BBD), phenotype was found to be normal. Clearly, the only operation that invariably led to an altered phenotype was that in which the cytoplasmic genotype differed from that of the maternal genome.

The polypeptides found to be repressed in adult liver of nucleocytoplasmic hybrids, the MUPs, belong to a superfamily of proteins, including for example retinol-binding protein, that bind hydrophobic substances (Sawyer, 1987). While their biological function is unknown, a role for the renally excreted MUPs in mating competence has been suggested (Shaw et al., 1983; Böcskei et al., 1992). Difficulties with mating have been reported for animals that do not express MUPs (Eicher and Beamer, 1976) and it may be pertinent that our nucleocytoplasmic hybrids are very reluctant to mate, and offspring are only very occasionally produced (unpublished results).

Phenotypes other than MUP expression are also affected in BDB and DBD animals. A small number of other liver polypeptides were found to be expressed differently in nucleocytoplasmic hybrid versus control animals (unpublished results). In addition, all BDB and DBD males (females were killed too early to be tested) showed a visible phenotype in that they were deficient in growth and hence in adult weight (on average their adult body weight was 75% of that of controls). This phenotype is unlikely to result from MUP repression alone. Indeed, deficiencies in growth factors such as growth hormone and thyroxine lead to repression of MUPs (Knopf et al., 1983; Shaw et al., 1983). MUP repression could therefore be a secondary phenomenon. However, the great majority of polypeptides detected in liver appeared to be expressed normally in nucleocytoplasmic hybrids. In particular, a number of stage-specific polypeptides made their appropriate appearance on schedule in the nucleocytoplasmic hybrids, suggesting that there was no unspecific developmental retardation in these animals.

The molecular mechanism that underlies these alterations in phenotype is at present unknown. There is however evidence to show that the egg cytoplasm can induce alterations in gene expression in transplanted nuclei (see Gurdon, 1986; Howlett et al., 1987 for details). Exposure of a nucleus to a genetically different egg cytoplasm could lead to an altered phenotype later in development because maternally provided components can persist and influence expression of the embryonic genome at later stages of development. Such components include maternal proteins and mitochondria that are present in the egg cytoplasm. Alternatively, exposure of pronuclei to a different egg cytoplasm might induce heritable alterations in nuclear function as a result of epigenetic modification. For example, exposure of a paternal genome to DBA/2 egg cytoplasm in the mouse results in poor preimplantation development of androgenetic embryos even when the paternal genome is then transplanted back into a B6 cytoplasm (which normally leads to relatively good preimplantation development of androgenones (Latham and Solter, 1991)). This suggests that the function of the nucleus has been heritably altered by a relatively brief exposure to a cytoplasm from a particular genotype. It is likely that this alteration is brought about by products of genotype-specific modifier genes present in the egg cytoplasm.

In the mouse there are several examples of transgenes that become methylated in a predictable fashion in response to genotype-specific modifier genes, and this methylation occurs after fertilisation (Sapienza et al., 1989; McGowan et al., 1989; Allen et al., 1990; Reik et al., 1990; Chaillet et al., 1991; Engler et al., 1991). In one example, methylation of the transgene occurs early in development at around the 8- to 16-cell stage when the methylating allele of the modifier gene has been introduced through the female line (Allen and Mooslehner, 1992). Methylation therefore occurs quite rapidly in response to the product of the modifier gene and is, we assume, somatically heritable from that stage on. Similarly, in *Drosophila* position-effect variegation is

brought about by heritable chromatin modification, which involves the products of genotype-specific modifier genes encoding, for example, non-histone heterochromatin proteins (Eissenberg et al., 1990). Homologues of these genes have recently been characterised in mammals (Singh et al., 1991; Reik et al., 1992) and it will be interesting to see whether their products are present in the egg cytoplasm. Because these proteins are thought to aggregate in macromolecular complexes to form heterochromatic domains, chromatin modifications may be quite sensitive to external disturbances. Indeed, variegating position-effect arrangements are notoriously susceptible to environmental influences, such as for example temperature, and the effect on expression of the variegating arrangement is often observed much later than the actual temperature shift (Spofford, 1976). Interestingly, expression of a transgene in the mouse controlled by genotype-specific modifiers has also been found to be influenced by culture conditions during preimplantation development (Kothary et al., 1992). It may be pertinent that in one of our control groups, in which embryos were cultured and transferred, two animals were obtained that also showed repression of MUPs (see Table 1). We are now assessing whether any particular treatment during the culture period, such as shifts in temperature, can exaggerate the phenotypic variability.

Whether methylation and repression of *Mup* genes or of other genes is a direct consequence of an altered epigenetic programming remains to be elucidated. However, it has been noted previously that some otherwise functional *Mup* genes are not expressed in the animal and are located in DNase I-resistant chromatin domains (Shi et al., 1989; Rodriguez and Derman, 1992), suggesting that *Mup* genes might become a target for epigenetic modification and inactivation. It is desirable to extend our experiments with nucleocytoplasmic hybrids to other combinations of genotypes, in particular to combinations that are evolutionarily less related, such as subspecies of *Mus musculus*. These experiments may reveal additional examples of aberrant gene expression and development. They may also begin to provide a better understanding of nucleocytoplasmic interactions, epigenetic modification of the genome and the genomic targets of such modification.

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