PANEL QUESTIONS EMBRYO RESEARCH

By HAROLD M. SCHMECK Jr.
Published: June 4, 1983

A special committee of scientists convened by Jackson Laboratory in Bar Harbor, Me., has recommended that Dr. Karl Illmensee and Dr. Peter C. Hoppe repeat some of their widely publicized experiments with mouse embryos to confirm the legitimacy of the original findings.

Dr. Illmensee, an internationally known Swiss embryologist, is best known for reports of successful cloning of mice. His recent experiments are under investigation by the University of Geneva, and the National Cancer Institute in Washington has held up a grant of about $63,000 to him pending results of the Swiss investigation.

The Jackson Laboratory's inquiry was prompted by allegations, made by members of Dr. Illmensee's staff, that there were irregularities in his recent research reports. The laboratory began a review by a committee of its own and other scientists because of the importance of the research to the understanding of embryonic development and related human health issues, and because the Swiss scientist has worked at the laboratory, collaborating with Dr. Hoppe there. No Fraud Is Found

Two types of experiments, one of them involving cloning with mice, were reviewed by the committee. In the other type, mice were reported born with genes from only one parent. No other researchers have reported success with such experiments.

In its report, the committee said it "searched for evidence of possible fraud, and none was found." It said the experiments should be repeated "using an experimental design which will give unequivocal results." Dr. Hoppe has unsuccessfully tried to do so in some of the experiments, said the report completed Thursday.

In reports on his cloning experiments, Dr. Illmensee said that mice had been grown successfully from fertilized mouse egg cells in which the nuclei had been replaced, early in embryonic development, by nuclei from cells of other mouse embryos. After the transplantations, the embryos were implanted in female mice. Embryos Placed in Mice

Some such embryos were successfully grown in their foster mothers and were born normally, the Swiss scientist has reported. Comparable feats have been achieved in frogs, but no team other than Dr. Illmensee's has reported success in producing living mice by such experiments.

One of the prime criteria for validity in scientific research is confirmation of the results by other scientists who repeat the experiments, using the same methods. Thus, prolonged failure to confirm a scientist's results tends to cast a shadow on the research.

The main focus of the inquiry in Switzerland is reportedly recent research in which Dr. Illmensee has tried to grow mice from embryos in which the original nuclei of egg cells were replaced with nuclei from cells of an unusual type of cancer called teratocarcinoma. Success in such experiments have been reported only by Dr. Illmensee, according to American scientists.

In its May 19 issue, the scientific journal Nature reported that an internal committee at the University of Geneva found no evidence of "systematic fraud." The university has recently set up an external review committee for further investigation.

Proc. Natl. Acad. Sci. USA
Vol. 74, No. 12, pp. 5657–5661, December 1977
Genetics

Microsurgically produced homozygous-diploid uniparental mice

(androgenesis/gynogenesis/removal of pronucleus/cytochalasin B/isogeneic mice)

PETER C. HOPPE* AND KARL ILLMENSEE†

* The Jackson Laboratory, Bar Harbor, Maine 04609; and † Department of Biology, University of Geneva, Geneva, Switzerland

Communicated by Robert W. Briggs, October 10, 1977

ABSTRACT **Shortly after fertilization, either the male or the female pronucleus was microsurgically removed from 202 F_1 hybrid eggs derived from crosses of two inbred strains. Subsequent incubation of these haploid eggs in medium containing cytochalasin B, which inhibits cytokinesis but not nuclear division, enabled the remaining pronucleus to become diploid. After nuclear diploidization and transfer to regular culture medium, cleavage commenced normally, and a total of 135 successfully manipulated eggs continued in development and yielded 93 morulae and blastocysts. These embryos were surgically transferred to the uteri of pseudopregnant foster mothers who gave birth to seven live female offspring. Five of the females were derived from the maternal genome (gynogenesis) and the remaining two mice inherited only the paternal genes (androgenesis), depending on whether the female or male pronucleus had been retained in the egg, respectively. Homozygosity for a number of genetic loci positioned on different chromosomes and effecting the coat color phenotype and strain-specific allelic variants of several enzymes, urinary and plasma proteins, and hemoglobins could be demonstrated unequivocally in all instances. Chromosomal analysis revealed a normal diploid karyotype including two X chromosomes. Thus far, six of the seven homozygous-diploid (isogeneic) females have proved to be fertile and have given birth to progeny corresponding only to the pronuclear genotype of the mother.**

Development of the *fertilized* egg in which only the maternal (gynogenesis) or paternal (androgenesis) genome is retained seems to occur infrequently in the animal kingdom. The same holds true for another type of reproduction that involves differentiation controlled exclusively by the maternal genes of the *unfertilized* but activated egg (parthenogenesis). Nevertheless, each event can happen spontaneously or be initiated experimentally in a number of vertebrate species (1) and insects (2). Although different in origin, these uniparental animals with either a hemizygous-haploid or homozygous-diploid genome are of considerable interest for analysis of gene function during early development.

Various attempts to generate uniparental mice experimentally from fertilized and unfertilized eggs have so far been limited to embryonic and early postimplantation stages (3). Similarly, most recent efforts using microsurgical techniques either to remove one pronucleus shortly after fertilization (4) or to bisect the fertilized egg at the pronuclear stage (5) rarely have resulted in the production of haploid blastocysts. Because mouse embryos with only one set of chromosomes are apparently nonviable, full restoration of the genome by diploidizing haploid embryos in the presence of cytochalasin B has recently been tried. Thus far, only blastocysts have been obtained, and no genetic markers were introduced to allow testing for homozygosity at the preimplantation stage (6). By using similar techniques, young uniparental axolotls have already been generated, thereby demonstrating the value of such an experimental scheme (A. J. Brothers, personal communication).

In the present report, we describe the successful development of adult homozygous-diploid mice, demonstrate their isogeneity, and discuss their possible applicability in studying gene action during mammalian embryogenesis.

MATERIALS AND METHODS

Mouse Strains. Mice from the inbred strains C57BL/6J (black), C57BL/10Wt (black), SJL/J (albino), and BALB/cWt (albino) and F_1 hybrid mice of the crosses C57BL/10Wt × SJL/Wt (agouti), C57BL/6J × DBA/2J (black), and C57BL/6J × BALB/cWt (agouti) were bred at the Jackson Laboratory. BL6 and B10 females were selected in proestrus (7) and mated with SJL and BALB males. Occasionally, reciprocal crosses were also used. The next morning, those females with vaginal plugs were killed by cervical dislocation and their fertilized eggs were harvested.

Collection and Culture of Eggs. Both oviducts were removed from the mated females and transferred to a depression slide containing 1 ml of modified Whitten's medium (8) supplemented with bovine testis hyaluronidase (Sigma) at 460 NF units/ml. After the ampullae were punctured with a fine needle to release the clutch of eggs, they were incubated at 37° for about 10 min to facilitate removal of the cumulus cells. Fertilized eggs showing two pronuclei and polar bodies were pooled from several females of the same genotype and carefully washed in fresh Whitten's medium. Denuded eggs with their intact zona pellucida were then placed in petri dishes in Whitten's medium containing cytochalasin B (Aldrich) at 5 μg/ml dissolved in 95% ethanol (10 mg/ml). The eggs were stored in a drop of medium under paraffin oil in an atmosphere of 5% CO_2/5% O_2/90% N_2 (Ohio Medical Products) at 37° for approximately 1 hr. After this incubation, about five eggs were taken from the petri dish, transferred in a small drop of the same medium onto a special microscopic slide, and covered with halofluorocarbon oil (Voltalef 10 S) for subsequent operation.

Micromanipulation. Extreme care was taken in preparing the enucleation pipets. Pyrex tubing (1.2 mm outside diameter; Corning Glass) was first pulled to an 8-μm (OD) tip which was then sharpened on a special grinding wheel and a De Fonbrune microforge. After thorough cleaning in sulfuric acid, the pipets were siliconized (Siliclad) and stored under sterile conditions. For enucleation, a pipet was connected to a syringe (Hamilton) containing paraffin oil, and the distal part was filled with halofluorocarbon oil (Voltalef 3S). The holding pipet with a blunt tip (60 μm OD) was connected to another syring system and its distal half was filled with Whitten's medium. Both glass

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: BL6, C57BL/6J; BL10, C57BL/10Wt; SJL, SJL/J; BALB, BALB/cWt; B6D2F_1, C57BL/6J × DBA/2J; B10SJLF_1, C57BL/10Wt × SJL/Wt; B6BALBF_1, C57BL/6J × BALB/cWt.

Table 1. Homozygous-diploid mice produced via microsurgical removal of one pronucleus from fertilized F_1 hybrid eggs

Egg genotype	Eggs		Fragmentation	Cleavage arrested	Morula	Blastocyst	Embryos transferred	Mice born*
	Manipulated	Survived						
SJL × BL10	75	54	18	12	11	13	25†	1[a]
BL10 × SJL	52	24	5	2	4	13	17	2[b]
SJL × BL6	5	4	1	—	—	3	3	—
BL6 × SJL	56	46	2	2	25	17	44†	3[c]
BALB × BL6	14	7	1	2	3	1	4	1[d]
Total	202	135	27	18	43	47	93	7

* a, BL10 androgenetic ♀; b, BL10 gynogenetic ♀ and SJL androgenetic ♀ in same litter; c, BL6 gynogenetic ♀♀ in two different litters; d, BALB gynogenetic ♀.

† One and two advanced-cleavage embryos, respectively, had been included in the total number of embryos transferred.

pipets could be controlled by Leitz micromanipulators equipped with a Zeiss inverted UPL microscope.

The enucleation pipet was inserted into the egg, previously attached to the holding pipet, until it touched the pronucleus which was slowly sucked into the capillary (Fig. 1). After enucleation, the pipet had to be withdrawn very gently from the egg in order to minimize internal damage. These eggs were subsequently transferred to fresh Whitten's medium. Only those eggs that were still intact about 1 hr after microsurgery were further cultured in glass tubes (Kimble) containing 1 ml of Whitten's medium with cytochalasin B (5 μg/ml) in a 5% CO_2/5% O_2/90% N_2 atmosphere. Under these culture conditions, the enucleated (and therefore haploid) eggs became diploid in the presence of cytochalasin B which allowed nuclear division but inhibited cytokinesis (9, 10). After overnight culture, the diploidized eggs, all remaining at the one-cell stage, were carefully washed in Whitten's medium *without* cytochalasin B and then cultured to the morula or blastocyst stage. As controls, nonmanipulated B10SJLF_1 embryos were grown under the same *in vitro* conditions. The manipulated embryos were cultured 24 hr longer than the control embryos. This additional culture period was required because of the developmental delay resulting from inhibited cytokinesis during cytochalasin B incubation.

About five experimental and seven control embryos at days 4 and 5 were surgically transferred to the uterus of a pseudopregnant B10SJLF_1 or B6D2F_1 foster mother [day 3 or 4 postcoitus (11)]. These females had previously been selected in proestrus and mated to sterile (vasectomized) B10SJLF_1 males.

Analyses of Tissue Genotypes. Strain-specific allelic variants of glucosephosphate isomerase (*Gpi-1* locus on chromosome 7) in blood cell lysates were determined by cellulose acetate electrophoresis (12). Diffuse and single adult hemoglobins (*Hbb* locus on chromosome 7) in erythrocyte hemolysates and plasma proteins (*Plp* locus on chromosome 17) were separated electrophoretically on cellulose acetate plates (B. Whitney, personal communication). Variants of the major urinary protein complex (*Mup-1* locus on chromosome 4) were analyzed by acrylamide gel electrophoresis (13). The separation of allelic forms of carbonic anhydrase (*Car-2* locus on chromosome 3) was performed on Cellogel plates (14). Strain-specific variants of plasma esterases (*Es-1* locus on chromosome 8) were separated by starch gel electrophoresis (15).

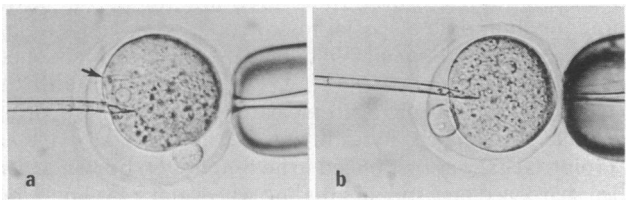

FIG. 1. Microsurgical removal of either the male (*a*) or female (*b*) pronucleus from a fertilized SJLBL6F_1 hybrid egg. About 9 hr after fertilization, both pronuclei are normally located at the egg periphery, the smaller female pronucleus near the polar body and the larger male pronucleus in the opposite position. (*a*) The micropipet has been inserted into the egg opposite to the polar body and is placed near the male pronucleus. Note the sperm tail located next to the pronucleus (arrow). (b) After the egg is properly attached to the holding pipet, a sharply tipped micropipet is pushed through the zona pellucida and plasma membrane above the polar body into the egg adjacent to the female pronucleus. Subsequently, this pronucleus with its distinct nucleolus (part of which has already been sucked into the pipet) is gently withdrawn from the egg. (×500.)

Karyotypic Analysis. Chromosome preparations from blastocysts were obtained by the air-drying method (16). Blood of all the postnatal mice at the age of about 6 weeks was collected from either the tail vein or the retro-orbital sinus and leukocytes were cultured *in vitro* (17). Chromosomes were counted on Giemsa-stained preparations. Individual chromosomes could be identified by means of a modified G-banding technique (18).

RESULTS

In control series, the effect of cytochalasin B during cleavage had been verified at the chromosomal level by analyzing air-dried preparations of early embryos (16). After overnight incubation in cytochalasin B, normal diploid eggs developed into tetraploid blastocysts and some of the haploid eggs derived from microsurgical removal of one pronucleus advanced normally to diploid blastocysts. On the other hand, several of the experimentally produced haploid eggs progressed to haploid blastocysts in regular culture medium *without* cytochalasin B, thereby corroborating the successful removal of one pronucleus.

The survival rate of the manipulated eggs was increased significantly by preincubating the fertilized eggs in the presence of cytochalasin B for 1 hr before micromanipulation. Under these conditions, about 65% of the enucleated eggs remained intact.

Approximately 70% of the eggs surviving microsurgery and diploidization with cytochalasin B became morulae or blastocysts on culture *in vitro*. The survival rate appeared to be greater with BL6 eggs than with eggs from other strains as far as enucleation and culture were concerned (Table 1). Developmental failure usually occurred early in embryogenesis and was generally characterized by either fragmentation immediately after removal from cytochalasin B or arrest predominantly at the two- or four-cell stage. This latter death may have been a consequence of withdrawing the female pronucleus from the egg and leaving the male pronucleus derived from a Y chromosome-bearing sperm. Embryos with no X chromosome do not develop beyond early cleavage (5).

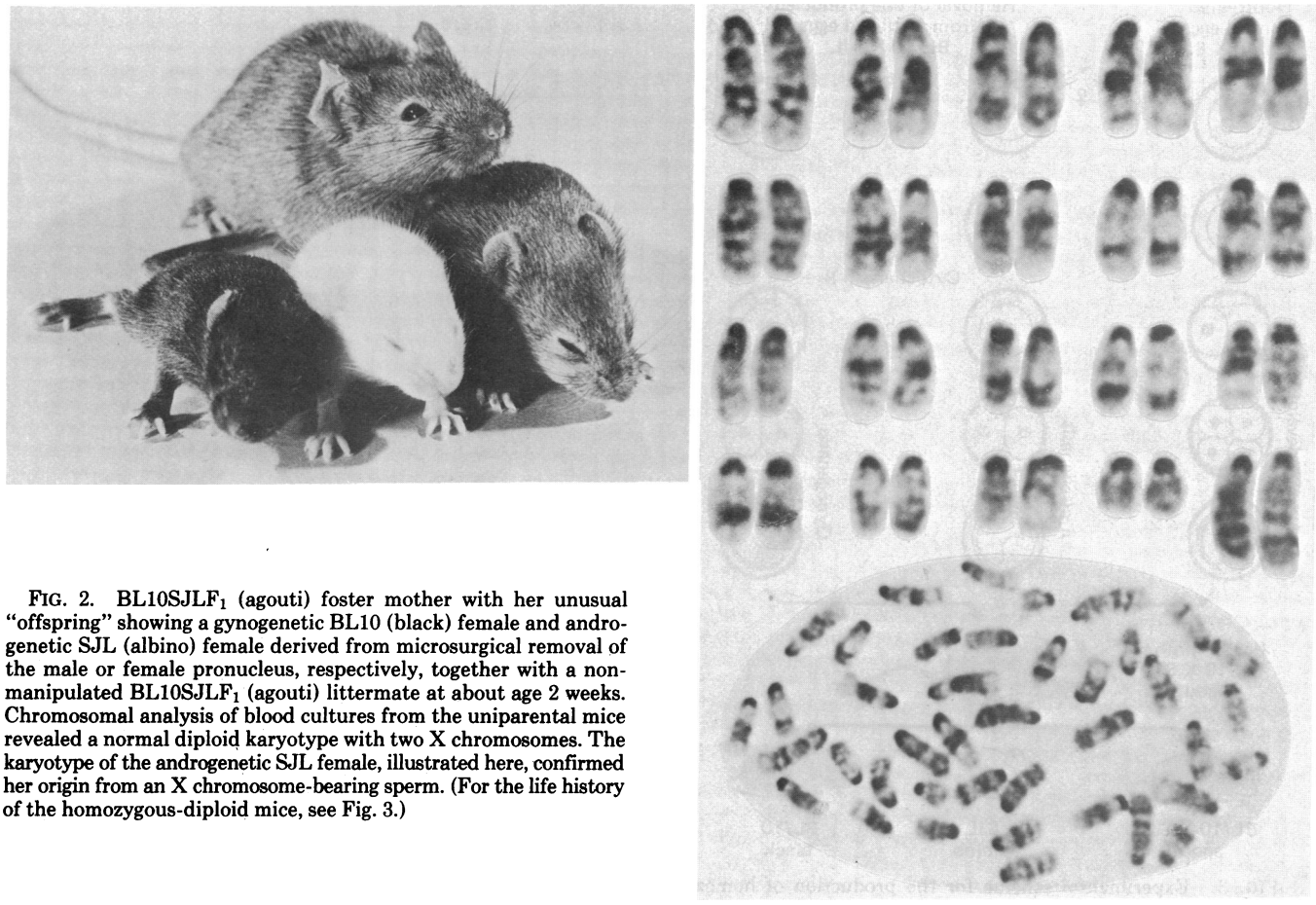

FIG. 2. BL10SJLF$_1$ (agouti) foster mother with her unusual "offspring" showing a gynogenetic BL10 (black) female and androgenetic SJL (albino) female derived from microsurgical removal of the male or female pronucleus, respectively, together with a nonmanipulated BL10SJLF$_1$ (agouti) littermate at about age 2 weeks. Chromosomal analysis of blood cultures from the uniparental mice revealed a normal diploid karyotype with two X chromosomes. The karyotype of the androgenetic SJL female, illustrated here, confirmed her origin from an X chromosome-bearing sperm. (For the life history of the homozygous-diploid mice, see Fig. 3.)

From a total of 93 diploidized embryos transplanted to foster mothers, seven female offspring, either black or albino, were born together with the expected agouti control littermates (Fig. 2). By comparing the coat color phenotype of these females with the original genetic composition of the eggs before microsurgery, it was possible to confirm retrospectively whether the female or male pronucleus had been removed (Fig. 3). Two of the seven experimental mice were derived from the paternal genome and the remaining ones carried only maternal genes (Table 1). The low postnatal recovery (about 8%) of the homozygous-diploid mice probably resulted from unfavorable implantation conditions in the foster mothers; only 14 offspring were born from 107 transferred control embryos (13%). Although significantly smaller than normal babies during the first 2 postnatal weeks, the uniparental females gradually reached normal size at weaning age.

In addition to using coat color as a genetic marker for identifying the experimental progeny, the blood of these mice was analyzed for glucose phosphate isomerase, hemoglobins, plasma proteins and esterases, and carbonic anhydrase; the urine was screened for the major urinary protein complex. All of the presumed isogeneic mice exhibited only the enzymatic form or protein profile of one of the inbred parents (Fig. 4; without the *Car-2* and hemoglobin pattern). The occurrence of pure strain-specific variants of these different gene products clearly demonstrated that, after microsurgical removal of one pronucleus, the initial F_1 hybrid egg was still able to develop normally with the genome of the residual pronucleus. Furthermore, all seven uniparental mice showed normal diploid karyotypes with two X chromosomes, thus substantiating their diploid genetic constitution at the chromosomal level (Fig. 2).

The experimental females at age 2 months were paired with sexually mature males of the corresponding inbred strain. So far, four of the gynogenetic BL10 and BALB females and the two androgenetic BL10 and SJL females (Table 1) have given birth to a total of 82 healthy offspring, males and females, all showing the coat color phenotype of their parents.

DISCUSSION

Thus far, experimentally produced gynogenetic and androgenetic mouse embryos have not progressed beyond the preimplantation stage (4–6). As we report here, viable homozygous-diploid mice can now be generated by microsurgically removing one pronucleus from the fertilized egg and diploidizing the residual one in the presence of cytochalasin B. These mice are expected to be females, because diploidized eggs left with only a Y chromosome bearing pronucleus should eventually develop into Y/Y embryos which usually die during early cleavage (5). The uniparental mice carry either the maternal or paternal genome, depending on whether the female or male pronucleus has been retained in the egg. Confirmation of such a process seems to be absolutely essential at the genetic level and, in fact, ought to be considered as *onus probandi* in order to reveal the gynogenetic or androgenetic origin of the experimental mice. First, homozygosity for a number of gene loci positioned on different chromosomes has adequately been demonstrated in all instances by using coat color differences and allelic variants of several enzymes and specific proteins as phenotypic criteria. Second, chromosomal analysis showed a normal diploid karyotype with two X chromosomes. Third, the coat color phenotype of the offspring derived from uniparental females always corresponded with the respective genotype of

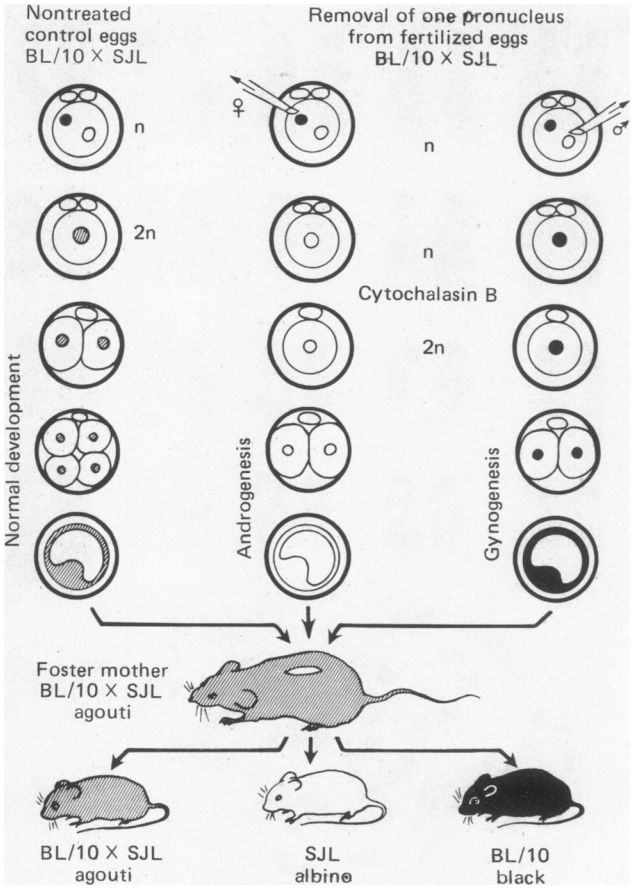

FIG. 3. Experimental scheme for the production of homozygous-diploid (isogeneic) mice derived from either the paternal or maternal genome. Fertilized BL10SJLF$_1$ hybrid eggs at the pronuclear stage were placed in modified Whitten's medium containing cytochalasin B. Subsequently, either the female or male pronucleus was removed microsurgically. The resulting haploid eggs were diploidized with cytochalasin B and cultured in regular medium to the morula or blastocyst stage. These embryos, as well as nonmanipulated controls, were then transferred to the uteri of pseudopregnant foster mothers and allowed to develop to term. Live born albino (SJL) or black (BL10) offspring indicated either androgenetic or gynogenetic development, depending on whether only the male or only the female pronucleus had been left in the egg, respectively. Agouti (BL10SJLF$_1$) progeny originated from nonmanipulated eggs.

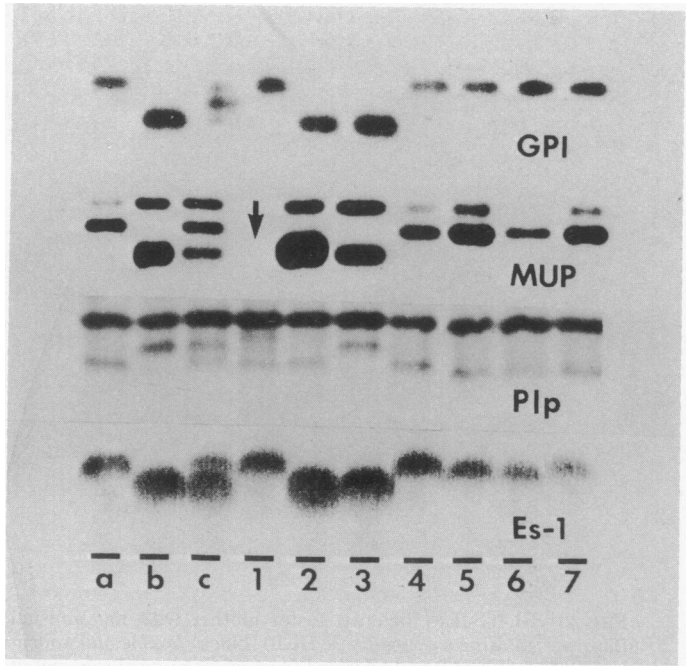

FIG. 4. Electrophoretic analysis of strain-specific allelic variants of glucose phosphate isomerase (GPI), major urinary protein complex (MUP), plasma protein (Plp), and plasma esterase (Es-1) from the seven homozygous-diploid mice. The first three lanes represent control samples from BL10 (a), SJL (b), and B10SJLF$_1$ (c). The remaining lanes document the experimental animals as follows: (1) BL10 androgenetic ♀; (2) BALB gynogenetic ♀; (3) SJL androgenetic ♀; (4) BL10 gynogenetic ♀; (5, 6, and 7) BL6 gynogenetic ♀♀. All homozygous-diploid mice reveal only one of the two parental type variants and demonstrate, at the gene product level, the successful removal of the particular pronucleus carrying the genetic information for the other parental form of the proteins analyzed. Any failure to remove one pronucleus would be signaled by the occurrence of F$_1$-type hybrid patterns shown in control lane c. Only in the MUP test, the genotype of the BL10 androgenetic ♀ cannot unequivocally be determined because of consistently weak banding (arrow). Because BALB has the same allelic variant for Plp as B10, the adult hemoglobin test (not illustrated) was used to verify the isogeneity of the gynogenetic female in lane 5.

only one of the genetically different pronuclei expressing either the black or albino phenotype.

Optimal culture conditions during collection and incubation of fertilized mouse eggs (8) undoubtedly are necessary prerequisites for successful microsurgery and subsequent development of the manipulated eggs and will definitely increase the number of embryos that advance to the blastocyst stage. Taking into consideration the expected loss of androgenetic embryos with two Y chromosomes and inevitable damage to some of the eggs caused by micromanipulation, the survival rate of the diploidized embryos during preimplantation culture resembled propitiously that of nonmanipulated embryos. On the other hand, the postnatal recovery of the experimental mice (8%) seemed to be somewhat reduced when compared with the birth rate of control mice (13%). The unusually low recovery of transplanted embryos in this series, in comparison with previous recovery rates (8), was probably due to unfavorable uterine conditions for experimental as well as control embryos. We believe, therefore, that the observed decrease in postimplantation development of homozygous-diploid embryos does not result predominantly from the effects of deleterious genes or gene combinations being uncovered in these isogeneic mice. The latter possibility has been suggested to explain the early death of parthenogenetically activated mouse embryos (3). The present results indicate that alternative explanations need to be considered in order to understand fully the developmental failure of parthenotes. It should be pointed out that our isogeneic mice differ at least in one respect from true parthenotes in that they are derived from *fertilized* eggs after sperm penetration and already may contain paternal contributions.

Microsurgical production of homozygous-diploid mice potentially provides a new and perhaps useful means for analyzing the developmental consequences of parthenogenesis. It may also be beneficial in studying recessive mutations, lethal or X-linked genes, and maternal *vs.* paternal gene expression during mammalian differentiation. Additionally, accelerated inbreeding should be facilitated by backcrossing F$_1$ males to their isogeneic mother.

We thank Drs. L. C. Stevens and W. K. Whitten for generous support, Drs. E. M. Eicher, L. C. Skow, B. A. Taylor, J. B. Whitney, and

L. P. Kozak for helpful advice, and D. Dorr, G. C. McKay, and K. L. Triman for excellent technical assistance. P.C.H. thanks Dr. C. L. Markert for encouragement and discussion. This investigation was supported by National Institutes of Health Grants CA 02662 to L.C.S., HD 04083 to W.K.W., and by RR 05545 to the Jackson Laboratory. The Jackson Laboratory is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

1. Beatty, R. A. (1967) in *Fertilization*, eds. Metz, C. B. & Monroy, A. (Academic Press, New York), Vol. 1, pp. 413–440.
2. Astaurov, B. L. (1967) *Adv. Morphog.* **6**, 199–257.
3. Graham, C. F. (1974) *Biol. Rev.* **49**, 399–422.
4. Modlinski, J. A. (1975) *J. Embryol. Exp. Morphol.* **33**, 897–905.
5. Tarkowski, A. K. (1977) *J. Embryol. Exp. Morphol.* **38**, 187–202.
6. Markert, C. L. & Petters, R. M. (1977) *J. Exp. Zool.* **201**, 295–302.
7. Champlin, A. K., Dorr, D. L. & Gates, A. H. (1973) *Biol. Reprod.* **8**, 491–494.
8. Hoppe, P. C. & Pitts, S. (1973) *Biol. Reprod.* **8**, 420–426.
9. Carter, S. B. (1967) *Nature* **213**, 261–264.
10. Snow, M. H. L. (1973) *Nature* **244**, 513–515.
11. Mullen, R. J. & Carter, S. C. (1973) *Biol. Reprod.* **9**, 111–115.
12. Eppig, J. J., Kozak, L. P., Eicher, E. M. & Stevens, L. C. (1977) *Nature* **269**, 517–518.
13. Wilcox, F. H. (1975) *Biochem. Genet.* **13**, 243–245.
14. Eicher, E. M., Stern, R. H. Womack, J. E., Davisson, M. T., Roderick, T. H. & Reynolds, S. C. (1976) *Biochem. Genet.* **14**, 651–660.
15. Ruddle, F. H., Shows, T. B. & Roderick, T. H. (1969) *Genetics* **62**, 393–399.
16. Tarkowski, A. K. (1966) *Cytogenetics* **5**, 394–400.
17. Triman, K. L., Davisson, M. T. & Roderick, T. H. (1975) *Cytogenet. Cell Genet.* **15**, 166–176.
18. Gallimore, P. H. & Richardson, C. R. (1973) *Chromosoma* **41**, 259–263.

Cell, Vol. 37, 179–183, May 1984, Copyright © 1984 by MIT

0092-8674/84/050179-05 $02.00/0

Completion of Mouse Embryogenesis Requires Both the Maternal and Paternal Genomes

James McGrath and Davor Solter
The Wistar Institute of Anatomy and Biology
36th Street at Spruce
Philadelphia, Pennsylvania 19104

Summary

Transplantation of pronuclei between one-cell-stage embryos was used to construct diploid mouse embryos with two female pronuclei (biparental gynogenones) or two male pronuclei (biparental androgenones). The ability of these embryos to develop to term was compared with control nuclear-transplant embryos in which the male or the female pronucleus was replaced with an isoparental pronucleus from another embryo. The results show that diploid biparental gynogenetic and androgenetic embryos do not complete normal embryogenesis, whereas control nuclear transplant embryos do. We conclude that the maternal and paternal contributions to the embryonic genome in mammals are not equivalent and that a diploid genome derived from only one of the two parental sexes is incapable of supporting complete embryogenesis.

Introduction

It is generally believed that both parental sexes contribute equivalent nuclear information to the zygote nucleus since in many animal species viable parthenotes exist. Equivalency of the maternal and paternal contributions to the zygote genome in mammalian species, however, is uncertain. Mammalian parthenotes are inviable, and although experimentally activated mouse eggs have developed as far as the 25-somite stage (Kaufman et al., 1977), most die shortly after implantation.

Possible causes for the death of mammalian parthenotes (Graham, 1974) may be either nuclear or cytoplasmic deficiencies. Nuclear deficiencies would include nonequivalent parental contributions to the zygote nucleus and/or homozygosity for lethal alleles. Alternatively, cytoplasmic deficiencies, such as the lack of an extragenetic contribution by the fertilizing spermatozoon or the inability of the parthenogenetic stimulus to mimic appropriately the stimulus provided by the spermatozoon, could result in the death of mammalian parthenotes. Cells derived from parthenogenetic embryos are viable (Iles et al., 1975), and parthenogenetic and normal embryos can combine to form viable chimeras in which many tissues, including functional germ cells, originated from the parthenogenetic embryo (Stevens, 1978). The full-term development of mouse diploid uniparental embryos (Hoppe and Illmensee, 1977) and of nuclear-transplant embryos that received a nucleus from an inner cell mass cell of a parthogenetic embryo (Hoppe and Illmensee, 1982) argued against a nuclear defect's causing death of the parthenotes. Although these last reports suggest that the lack of development of parthenogenetic embryos is due to the absence of an extragenomic contribution from the spermatozoon, similar attempts by other investigators to produce uniparental mice have not resulted in the birth of live progeny (Modlinski, 1980; Markert, 1982).

More recently, uniparental diploid gynogenetic mouse embryos were derived by the microsurgical removal of the paternal pronucleus following suppression of second polar-body extrusion (Borsuk, 1982; Surani and Barton, 1983). These embryos, however, like parthenotes, died shortly after implantation (Surani and Barton, 1983). Thus uniparental diploid gynogenetic embryos derived by inhibition of the first mitotic division are reportedly viable (Hoppe and Illmenssee, 1977), whereas those derived by inhibition of the second meiotic division are not (Surani and Barton, 1983). The reason(s) for this apparent discrepancy is not known.

To determine whether the maternal and paternal contributions to the zygote are equivalent, we transplanted single pronuclei between one-cell-stage embryos using a recently described nuclear-transplantation technique (McGrath and Solter, 1983). Removal of a single pronucleus, followed by the introduction of a second pronucleus from another embryo, was used to construct biparental diploid gynogenetic and androgenetic and control (one male and one female pronucleus) nuclear-transplant embryos. These embryos were transferred into the oviducts of day 1 pseudopregnant females and permitted to develop to term. On the basis of our results, we conclude that maternal and paternal genomic contributions are not functionally identical and that both are essential to complete embryogenesis.

Results

From a total of 1528 embryos, 1475 (97%) survived the microsurgical removal of one or two pronuclei, 1452 (98%) of the resulting pronuclear karyoplasts were successfully injected, along with inactivated Sendai virus, into the perivitelline space of the recipient embryos, and 1355 (93%) of the karyoplast:recipient embryo pairs underwent fusion. The overall efficiency of the nuclear-transplantation procedure was 89%.

In an initial experimental series, biparental gynogenones, androgenones, and control nuclear-transplant embryos were made from embryos obtained from C57BL/6J females mated to BALB/c males. The maternal/paternal origin of the pronuclei was assigned, and one of the pronuclei was removed and replaced with a second pronucleus from another embryo. In a separate experimental series, nuclear transplantation was performed with embryos obtained from BALB/c females mated to C57BL/6J males. In both cases, embryos that survived microsurgery and incorporated the donor pronucleus were introduced into the oviducts of day 1 pseudopregnant females and allowed to develop to term. Since C57BL/6J and BALB/c mice differ in their coat color and glucose phosphate

isomerase-1 (GPI-1) alleles, the maternal/paternal origin of the pronuclei could be verified in progeny by determination of these phenotypic characteristics.

The results (Table 1) show that of 239 putative control embryos, seven agouti male progeny (3%) were born. The agouti phenotype in mice is consistent with their being derived from zygotes possessing one male and one female pronucleus. Of 207 putative gynogenetic and 189 putative androgenetic embryos, one offspring was obtained (Table 1), an agouti male. The agouti phenotype, however, shows that this male possesses a normal maternal/paternal genotype and must have resulted from an incorrect assignment of the parental origin of the pronucleus during microsurgery. Using the proportion of control progeny born, and correcting among the androgenetic embryo population for the expected proportion of lethal YY androgenetic embryos (25%), we should expect the birth of six gynogenetic and four androgenetic offspring. None, however, was observed ($X^2 = 7.93$; $p < 0.01$). Thus nuclear-transplant embryos that possess one male and one female pronucleus developed to term, whereas embryos receiving two male pronuclei or two female pronuclei did not.

Because the gynogenetic and androgenetic embryos produced in this initial experimental series are either C57BL/6J or BALB/c homozygotes while their control counterparts are F1 hybrids, we tested the possible effect of homozygosity on gynogenetic and androgenetic embryo development by removing a pronucleus from an embryo obtained from the C57BL/6J♀ × BALB/c♂ mating and replacing it with a pronucleus obtained from a BALB/c ♀ × C57BL/6J♂ mating and vice versa. Maternal/paternal control embryos in this experimental series are therefore C57BL/6J or BALB/c homozygotes, while the gynogenetic and androgenetic embryos are F1 hybrids. The results (Table 2) show that from 109 putative control embryos, 11 progeny (10%) were born (two albino males, two albino females, and seven black males). The coat-color phenotype of all progeny verifies their maternal/paternal origin. No progeny possessing the androgenetic or gynogenetic agouti phenotype were observed. Putative gynogenetic (132) and putative androgenetic (139) embryos were similarly produced, from which four progeny were born. Of the three mice that survived to adulthood, all were albino (two males and one female) and are therefore descended from an embryo possessing one male and one female pronucleus. The fourth mouse, which died within 24 hr of birth, was pigmented, and electrophoretic analysis showed it to be homozygous for the GPI-1^b allele (i.e., C57BL/6J homozygote; data not shown). Thus all four progeny exhibited the control parental phenotype and are instances in which the assigned maternal/paternal origin of the transplanted pronucleus was incorrect. Using the proportion of control progeny born (10%) and correcting for the expected proportion of lethal YY androgenetic embryos, we should expect the birth of 13 gynogenetic and 10 androgenetic offspring. None, however, was observed ($X^2 = 19.4$; $p < 0.001$). Furthermore, the combined results of Tables 1 and 2 show that from a total of 348 control embryos, 18 progeny (5%) were born. From the latter figure, we would anticipate the birth of 19 gynogenones (from 339 embryos) and 14 androgenones (from 328 embryos), for a total of 33 progeny. None, however, was observed ($X^2 = 27.15$; $p < 0.001$). We conclude that gynogenetic and androgenetic embryos cannot complete normal embryogenesis and that their inability to develop is not the result of homozygosity.

Table 1. Progeny Resulting from Single-Pronucleus Transplantation Using *Either* Embryos from C57BL/6J♀ × BALB/c♂ Matings *or* BALB/c♀ × C57BL/6J♂ Matings

	No. of Embryos	Expected Phenotype of Progeny	No. of Progeny	Observed Phenotype of Progeny
C57BL/6J♀ × BALB/c♂				
gynogenones	140	black GPI-1B	0	—
androgenones	129	albino GPI-1A	0	—
maternal/paternal	154	agouti GPI-1AB	2	agouti males
BALB/c♀ × C57BL/6J♂				
gynogenones	67	albino GPI-1A	1	agouti male
androgenones	60	black GPI-1B	0	—
maternal/paternal	85	agouti GPI-1AB	5	agouti males

Table 2. Progeny Resulting from Single-Pronucleus Transplantation *Between* Embryos from C57BL/6J♀ × BALB/c♂ *and* BALB/c♀ × C57BL/6J♂ Matings

	No. of Embryos	Expected Phenotype of Progeny	No. of Progeny	Observed Phenotype of Progeny
gynogenones	132	agouti GPI-1AB	2	albino (1 male, 1 female)
androgenones	139	agouti GPI-1AB	2	1 albino male 1 GPI-1B
maternal/paternal	109	black GPI-1B *or* albino GPI-1A	11	7 black males 4 albino (2 males, 2 females)

To determine the fate of gynogenetic and androgenetic embryos during the preimplantation and postimplantation stages of development, nuclear transplantation was performed and the embryos were either cultured in vitro or transferred into the oviducts of day 1 pseudopregnant females. Those pseudopregnant females receiving embryos were sacrificed on gestational days 6–11, their uteri excised, and deciduoma subjected to histological analysis. The results of the in vitro culture of embryos (Table 3) show that 100% of the maternal/paternal control nuclear transplant embryos, 88% of putative gynogenetic, and 64% of putative androgenetic embryos developed to the morula-blastocyst stage. Thus diploid embryos possessing exclusively maternal or exclusively paternal pronuclei can successfully develop to the blastocyst stage in vitro. The significantly greater death of androgenetic embryos occurred primarily at the 8-cell stage, presumably the result of YY embryos in this population. Histological analysis of the postimplantation embryos on gestational days 6–11 (Table 4) shows approximately the same number of gynogenetic, control nuclear-transplant, and unmanipulated control embryos implanted. The number of implanted androgenetic embryos was approximately half that observed in other groups, probably due to the presence of YY embryos. Very few biparental gynogenetic and androgenetic embryos survive implantation for very long. The most advanced embryo observed in those groups had 11 somites and was, at the time of sacrifice, approximately 1 day delayed in development. These results indicate that biparental gynogenetic and androgenetic embryos die during the early postimplantation period.

The proportion of control single-pronucleus transplant embryos that successfully developed to term in this study (5%) is less than that obtained in a previous investigation (23%), in which we transplanted both pronuclei (McGrath and Solter, 1984). To determine the basis of this reduction in developmental potential, two additional experiments were performed. Transplantation of both pronuclei from one embryo to another was performed using C57BL/6J and BALB/c mice and these embryos were transferred to the oviducts of pseudopregnant females. From 45 successful double-pronuclei transplants, 16 progeny (36%) were born. This proportion of progeny born from transfer of two pronuclei agrees with our earlier studies and indicates that our results are not a function of strain differences or a technical difficulty peculiar to this investigation. The second experiment was designed to detect any deleterious effect peculiar to the transfer of a single pronucleus. One pronucleus was removed but then returned to the embryo from which it had been obtained. Of 98 such nuclear-transplant embryos, 20 progeny were born (20%). The proportion of progeny born from embryos in which one pronucleus was removed but then returned to the same embryos is not significantly different from the proportion of progeny born from the double pronuclear transfers ($X^2 = 3.34$; $p > 0.05$), but is significantly greater than the proportion of progeny obtained from the control single-pronuclear transplants listed in Tables 1 and 2 (5%) ($X = 16.23$; $p < 0.001$). Although the control embryo population almost certainly contains some inviable gynogenones and androgenones, the calculated decrease of viable embryos (based on the proportion of control progeny among the putative gynogenones and androgenones) does not exceed 1%, and is not sufficient to explain the decreased developmental potential of single-pronuclear-transfer embryos.

Table 3. In Vitro Development of Gynogenetic, Androgenetic, and Control Embryos

Putative Genotype	No. Degenerate	No. Morula	No. Blastocyst
gynogenones	3	4	19
androgenones	10	3	15
maternal/paternal	0	0	8
unmanipulated control	0	1	13

Table 4. Postimplantation Development of Gynogenetic, Androgenetic, and Control Embryos

Putative Genotype	No. of Embryos Transferred	No. of Implantations (%)[a]	No. of Resorptions[b]	No. of Embryos
gynogenones	54	19 (35)	17	2[c]
androgenones	42	7 (17)	7	0
maternal/paternal	39	13 (33)	3	10[d]
unmanipulated controls	46	16 (34)	2	14[d]

[a] Foster mothers were sacrificed between days 6–11 of gestation.
[b] On histological examination, deciduoma contained massive bleeding and a few giant trophoblastic cells. No remnants of embryos were observed.
[c] One embryo, from a mother sacrificed on day 8 of gestation, was a morphologically normal egg cylinder with three germ layers. The other embryo, from a mother sacrificed on the day 11 of gestation, was morphologically normal, although approximately half the size of corresponding control embryos. It contained 11 somites.
[d] All embryos were morphologically normal and corresponded developmentally to their gestational age.

Discussion

Shortly after fertilization the haploid maternal and paternal genomes unite to form the zygote nucleus. While both parental sexes contribute equivalent nuclear information to the zygote, this genetic information is not necessarily functionally equivalent. For example, the paternal X chromosome is preferentially inactivated in murine extraembryonic tissues (Takagi and Sasaki, 1975; Harper et al., 1982). It is, however, still unknown if homozygosity for either the maternal or paternal X chromosome results in embryonic lethality. Another example of this phenomenon occurs in embryos with the T^{hp} mutation. Heterozygous embryos that inherit the T^{hp}-deleted chromosome 17 from their male parents are viable, whereas embryos that receive the same T^{hp} chromosome from their female parents are inviable (Johnson, 1974). This lethality does not appear to

result from a cytoplasmic defect but from differential functioning of a portion of chromosome 17 during embryogenesis depending on its parental origin (McGrath and Solter, 1984). Normal embryogenesis therefore requires a functional region of maternal (but not paternal) chromosome 17.

The concept of differential functioning of maternal and paternal genomes is further extended and generalized by the present results. After obtaining 18 progeny from 348 control embryos (5%), but no progeny from 339 gynogenetic and 328 androgenetic embryos, we conclude that the maternal and paternal contributions to the embryonic genome are not equivalent and that they differ sufficiently to result in early postimplantation embryonic lethality when either component is absent. Histological analysis reveals that the lethal period of biparental gynogenetic and androgenetic embryos occurs shortly after implantation. This has also been described for uniparental gynogenetic embryos (Markert, 1982; Surani and Barton, 1983) and parthenotes (Graham, 1974). In a recent investigation, Surani and Barton (1983) concluded that the inviability of parthenotes could not be explained by the lack of an extragenomic contribution by the fertilizing spermatozoon since uniparental diploid gynogenones derived from fertilized ova are also inviable. The authors concluded that death may have resulted from lethal homozygosity. The results of the present study also imply a nuclear and not a cytoplasmic defect as a probable cause of the death of mammalian parthenotes. However, we suggest that parthenogenetic embryos die because of a lack of a paternal contribution to the embryonic genome. The comparison of the development of biparental gynogenetic and androgenetic embryos with that of control nuclear-transplant embryos that possess both a male and a female pronucleus permits us to account for any possible deleterious effect from the technical manipulations.

The results presented here are in conflict with those presented by Hoppe and Illmensee (1977). For reasons mentioned above, we do not believe that uniparental gynogenetic embryos can complete development. In addition, the need for a functional maternal portion of the chromosome 17 (McGrath and Solter, 1984) would preclude the development of uniparental androgenetic embryos. One possible explanation for the discrepancy between our results and those reported by Hoppe and Illmensee (1977) might be the incomplete removal of pronuclei because of the small-bore pipette used by those investigators. If a sufficient and appropriate part of the pronucleus remains, it might support the embryos during a critical stage, resulting in a small number of viable progeny. Variations in GPI-1 patterns observed in nuclear-transfer preimplantation embryos (Illmensee and Hoppe, 1981; Hoppe and Illmensee, 1982) are compatible with the possibility of incomplete enucleation.

Two general mechanisms for differential functioning of the maternal and paternal genomes during mammalian embryogenesis may be envisioned. A specific gene(s) may be inherited in a functional form from one parent but in a nonfunctional form from the other. In this model, androgenones and gynogenones should each express a subset of normal gene products, which, when combined, should exactly equal those products found in normal embryos. Alternatively, embryonic gene expression might require interaction between the maternal and paternal genomes (Markert, 1982). In this model both gynogenones and androgenones would again express only a subset of gene products found in normal embryos. However, the combined gene products of gynogenones and androgenones would not equal the spectrum of gene products in normal embryos since the necessary interaction of maternal and paternal genes did not occur. Such a requirement for interaction might also bear on the decreased development of the maternal/paternal control embryos observed in the present study, i.e., the requisite communication and/or synchrony between the resident and newly introduced pronuclei shortly after fertilization might be impaired. However, it is also possible that selective death of female control embryos underlies the decrease in the number of viable controls. Among the 22 control nuclear-transplant progeny that survived to adulthood, 19 were phenotypically male. The significance of this distorted sex ratio and its possible contribution to the decreased development of control embryos is uncertain.

Regardless of the mechanism involved, the differential functioning of parental genomes might be essential for development of embryonic or extraembryonic parts of the conceptus or for both. Recombination of the normal inner cell mass with biparental gynogenetic or androgenetic trophoblast, and vice versa, should address this question. Our model implies that the paternal and/or maternal genome (whole or in part) are somehow conditioned/altered during gametogenesis and that this conditioning is completely reversible (as is the case for the X chromosome). A logical consequence of differential functioning of parental genomes is the unlikelihood of successful cloning of mammals using somatic cell nuclei, since presumably the sex-related conditioning of the paternal and maternal genome has already been reversed in those cells.

Experimental Procedures

Embryo Isolation

All embryos were obtained from reciprocal spontaneous matings of C57BL/6J (Jackson Laboratory, Bar Harbor) males and females with BALB/c (Jackson Laboratory, Bar Harbor) males and females. One-cell-stage embryos were isolated by puncturing the ampullae of oviducts from mated females (day 1) in HEPES-buffered Whitten's medium (Whitten, 1971) containing 500 U/ml bovine hyaluronidase (Sigma). Embryos were washed through 4 drops of modified Whitten's medium containing 100 μM Na_2-EDTA (Abramczuk et al., 1977).

In Vitro Culture

Embryos were cultured under silicone oil (Dow Corning 2000 Fluid; Kurt Lesker, Pittsburgh, PA) in modified Whitten's medium in an atmosphere of 5% O_2, 5% CO_2, and 90% N_2 at 37°C. Before microsurgery, groups of embryos (five to ten) were cultured for 15–30 min at 37°C in Whitten's medium supplemented with 5 μg/ml cytochalasin B (Hoppe and Illmensee, 1977) and 0.1 μg/ml demecolcine (Sigma) (McGrath and Solter, 1983).

Microsurgery

Microsurgery was performed on a Leitz Laborlux II fixed-stage microscope using Leitz micromanipulators. Embryos were placed singly in hanging drops of HEPES-buffered Whitten's medium supplemented with 5 μg/ml cytochalasin B and 0.1 μg/ml demecolcine. Nuclear transplantation was performed as previously described (McGrath and Solter, 1983). Determination of the parental sex of the pronuclei was based on their cytoplasmic location, the pronucleus located beneath the second polar body was judged as maternal in origin, while the more eccentrically located pronucleus was judged as paternal. Embryos in which the maternal/paternal origin of the pronuclei could not be decided were discarded. Subsequent to microsurgery, embryos were washed through 4 drops of modified Whitten's medium and returned to the incubator. Embryos that fused with the pronuclear karyoplast were either cultured in vitro in modified Whitten's medium as described above, or transferred to the oviducts of day 1 albino CD-1 (Charles Rivers) pseudopregnant females that had previously mated to vasectomized CD-1 males. Transfer of one-cell-stage embryos into oviducts was performed using a small-bore glass pipette.

Histological Analysis of Embryos

Pseudopregnant females receiving nuclear-transplant embryos were sacrificed on successive days, their uteri excised, and the deciduoma dissected free. Some deciduoma were examined under the dissecting microscope for the presence of embryos, but the majority were fixed in formalin, embedded, serially sectioned, and hematoxylin/eosin stained. Sections were examined under a light microscope.

Statistical Analysis

The number of progeny resulting from gynogenetic and androgenetic embryos was compared to the number of progeny developed from control embryos using the X^2 contingency test. In all statistical comparisons involving androgenones, 25% of the androgenetic embryos were excluded in order to compensate for the expected production of lethal YY embryos.

Acknowledgments

This investigation was supported by grants CA-10815 and CA-25875 from the National Cancer Institute and by grants HD-12487 and HD-17720 from the National Institute of Child Health and Human Development.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 9, 1984

References

Abramczuk, J., Solter, D., and Koprowski, H. (1977). The beneficial effect of EDTA on development of mouse one-cell embryos in chemically defined medium. Dev. Biol. *61,* 378–383.

Borsuk, E. (1982). Preimplantation development of gynogenetic diploid mouse embryos. J. Embryol. Exp. Morphol. *69,* 215–222.

Graham, C. F. (1974). The production of parthenogenetic mammalian embryos and their use in biological research. Biol. Rev. *49,* 399–422.

Hoppe, P. C., and Illmensee, K. (1977). Microsurgically produced homozygous-diploid uniparental mice. Proc. Nat. Acad. Sci. USA *74,* 5657–5661.

Hoppe, P. C., and Illmensee, K. (1982). Full-term development after transplantation of parthenogenetic embryonic nuclei into fertilized mouse eggs. Proc. Nat. Acad. Sci. USA *79,* 1912–1916.

Harper, M. I., Fosten, M., and Monk, M. (1982). Preferential paternal X inactivation in extraembryonic tissues of early mouse embryos. J. Embryol. Exp. Morphol. *67,* 127–135.

Iles, S. A., McBurney, M. W., Bramwell, S. R., Deusen, Z. A., and Graham, C. F. (1975). Development of parthenogenetic mouse embryos in the uterus and in extrauterine sites. J. Embryol. Exp. Morphol. *34,* 387–405.

Illmensee, K., and Hoppe, P. C. (1981). Nuclear transplantation in Mus musculus: developmental potential of nuclei from preimplantation embryos. Cell *23,* 9–18.

Johnson, D. R. (1974). Hairpin-tail: a case of post-reductional gene action in the mouse egg? Genetics *76,* 795–805.

Kaufman, M. H., Barton, S. C., and Surani, M. A. H. (1977). Normal postimplantation development of mouse parthenogenetic embryos to the forelimb bud stage. Nature *265,* 53–55.

Markert, C. L. (1982). Parthenogenesis, homozygosity, and cloning in mammals. J. Hered. *73,* 390–397.

McGrath, J., and Solter, D. (1983). Nuclear transplantation in the mouse embryo by microsurgery and cell fusion. Science *220,* 1300–1302.

McGrath, J., and Solter, D. (1984). Maternal T^{hp} lethality in the mouse is a nuclear, not cytoplasmic, defect. Nature, in press.

Modlinski, J. A. (1980). Preimplantation development of microsurgically obtained haploid and homozygous diploid mouse embryos and effect of pretreatment with cytochalasin B on enucleated eggs. J. Embryol. Exp. Morphol. *60,* 153–161.

Stevens, L. C. (1978). Totipotent cells of parthenogenetic origin in a chimaeric mouse. Nature *276,* 266–267.

Surani, M. A. H., and Barton, S. C. (1983). Development of gynogenetic eggs in the mouse: implications for parthenogenetic embryos. Science *222,* 1034–1036.

Takagi, N., and Sasaki, M. (1975). Preferential inactivation of the paternally derived X chromosome in the extraembryonic membranes of the mouse. Nature *256,* 640–642.

Whitten, W. K. (1971). Nutrient requirements for the culture of preimplantation embryos *in vitro*. Adv. Biosci. *6,* 129–139.