Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 6509–6513, September 1986
Developmental Biology

Successful rescue of microsurgically produced homozygous uniparental mouse embryos via production of aggregation chimeras

(imprinting/gametogenesis/inbred strains/biotechnology)

CHRISTOPHER ANDEREGG* AND CLEMENT L. MARKERT†‡

*Department of Biology, Yale University, New Haven, CT 06511; and †Department of Animal Science, North Carolina State University, Raleigh, NC 27695

Contributed by Clement L. Markert, May 19, 1986

ABSTRACT **Homozygous uniparental mouse embryos, produced by microsurgical removal of the male pronucleus from fertilized eggs and diploidization of the female pronucleus with cytochalasin, were surrounded with blastomeres from normal embryos to produce chimeric embryos. A few of these chimeras developed into viable adults, and one female has reproduced using her homozygous uniparental cells as a source of gametes. The production and use of such chimeras in breeding programs could greatly shorten the period required for producing inbred strains of mammals. The data presented demonstrate that a homozygous uniparental mammalian genome, although not lethal to all cells, is extremely deleterious to normal embryonic development. Moreover, the results support the conclusion that the genome is imprinted differently in males and females during gametogenesis so that at fertilization the genomes are not functionally equivalent, and both are required for normal development.**

In the production of inbred strains of mammals, the generation time is of major importance. To produce an inbred strain of mice, for example, requires 20 generations (about 5 years) of brother–sister matings, a breeding program that results in about 98% homozygosity. Achievement of 100% homozygosity, however, is not possible because of the balance that occurs between new mutations and their loss by chance to yield a low steady-state level of heterozygosity. Moreover, most efforts at breeding to homozygosity fail because of inbreeding depression that results in loss of vitality and fertility. Consequently, virtually homozygous strains are available in only a few mammalian species—e.g., mice, rats, hamsters, and rabbits. Furthermore, in mammals such as cattle, with generation times of several years, one could not hope to make an inbred strain in a human lifetime; the scale of effort, time, and cost would make such an undertaking impractical.

In light of these enormous difficulties, the report by Markert and Petters (1) of the successful production of homozygous uniparental (HUP) mouse embryos by microsurgery seemed to open entirely new possibilities for producing inbred strains of mammals. They microsurgically removed either the male or female pronucleus from fertilized eggs and cultured the haploid eggs overnight in the presence of cytochalasin B. This treatment suppresses the first cleavage division and permits the remaining pronucleus to replicate. The cytochalasin was then removed, and the now 100% HUP one-cell eggs were cultured *in vitro* to the blastocyst stage. Such embryos, of course, are all XX and female (the male YY genotype is lethal), and all chromosomes in their genomes are from one parent, either the male or the female. These embryos are not genetically identical to their single parent because even highly inbred strains are not perfectly homozygous. However, all the ova from an HUP female, if such an animal could be produced, would be identical. Therefore, removing the sperm pronucleus from the fertilized egg of such a female and diploidizing the female pronucleus would lead to offspring genetically identical to the mother and to one another and would thus constitute a mammalian clone. Furthermore, HUP female mice could be used to initiate new strains, and such females could be produced in a 3-week gestation period instead of the 5 years required by brother–sister mating.

To exploit this microsurgical strategy, Hoppe and Illmensee (2) repeated the experiment of Markert and Petters (1) and surgically transferred the HUP blastocysts to pseudopregnant recipient females; they reported obtaining seven live-born HUP females, five derived from the maternal and two derived from the paternal genome, as evidenced by coat colors. Many investigators have tried (3), but no one has been able to reproduce the results claimed by Hoppe and Illmensee. Repeating the identical experiment, Surani and Barton (4) not only failed to obtain live-born mice but also found the implanted embryos grossly retarded in development. Mann and Lovell-Badge (5), McGrath and Solter (6), Surani *et al.* (7), and Barton *et al.* (8) have all demonstrated that completion of mouse embryogenesis requires both the maternal and the paternal genomes; all maternal/maternal or paternal/paternal combinations, whether homozygous or heterozygous, die by days 10 or 11 of gestation.

In a different but related series of experiments by Stevens and co-workers (9, 10), diploid parthenotes, similar in genomic composition to the HUP condition produced by microsurgery, were successfully rescued by chimera production. Stevens combined one or two spontaneous parthenotes from the LT/Sv strain of mice with one normally fertilized eight-cell embryo to produce chimeric aggregates. Surani *et al.* (11), using parthenotes that had been activated in medium lacking Ca^{2+} and Mg^{2+} salts, injected parthenogenetic inner cell mass cells into the cavity of normally fertilized blastocysts to produce chimeric embryos. Several of these chimeras developed to term. One of them—a female—reproduced through the use of gametes derived from her parthenogenetic component, thus demonstrating that some parthenogenetic cells can survive through adulthood and can even differentiate into functional gametes (10).

Diploid parthenogenesis in the mouse egg is believed to involve either suppression of the second polar body or doubling of the haploid genome (cf. ref. 12), mechanisms ensuring diploidy but producing different degrees of heterozygosity. However, most mammals of interest to breeders, such as pigs, cows, and sheep, do not produce spontaneous parthenote embryos, nor are their eggs as amenable to experimental activation. How, then, could one

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

Abbreviation: HUP, homozygous uniparental.

‡To whom reprint requests should be addressed at: Department of Animal Science, Box 7621, North Carolina State University, Raleigh, NC 27695-7621.

produce and rescue a HUP embryo as a potential offspring of a prize cow, for example? Perhaps as follows. We report here the successful rescue by chimera production of microsurgically produced 100% HUP mouse embryos with genomes derived entirely from a single female parent.

MATERIALS AND METHODS

In the production of HUP mouse embryos, eggs and cleavage-stage embryos were obtained from females superovulated by intraperitoneal injection of 5 international units of pregnant mare serum gonadotropin followed by 5 international units of human chorionic gonadotropin 48 hr later.

Fertilized eggs for enucleation and subsequent diploidization were obtained by mating albino CD-1 (Charles River Breeding Laboratories) females with black B6D2F_1/J males. CD-1 mice are an outbred strain segregating at the agouti locus. B6D2F_1/J mice are F_1 hybrids derived by crossing the two nonagouti inbred strains C57BL/6J and DBA/2J. From the fertilized eggs, the male pronucleus was removed microsurgically as described by Markert and Petters (1) after 1–6 hr of incubation in the medium of Whitten (13) (modified by omitting the sodium lactate, decreasing the sodium pyruvate to 2.5 mg/100 ml, and increasing the sodium chloride to 5.97 g/liter and the bovine serum albumin to 4.0 mg/ml); to reduce the damage of microsurgery, Colcemid at 0.1 μg/ml (14) and cytochalasin D at 1.0 μg/ml (15) also were added to the medium. Identification of the male pronucleus was based on its larger size and more distant location from the second polar body as compared to the female pronucleus.

Enucleated haploid eggs were diploidized for 14–20 hr overnight in modified Whitten's medium containing cytochalasin D at 0.33–0.50 μg/ml (15). The following morning, the now 100% HUP one-cell eggs were transferred via the ostium to the oviducts of day 1 pseudopregnant C57BL/6J females that had been mated to vasectomized CD-1 males; 48–54 hr later, the oviducts were flushed to obtain HUP eight-cell embryos.

In preparing the embryos for aggregation into chimeras, the zona pellucida was removed by treatment with acidified Tyrode's solution (pH 2.0) for a few seconds (16, 17). Normally fertilized 8- or 16-cell embryos obtained by mating C57BL/6J females to either C57BL/6J or B6D2F_1/J males were dissociated into blastomere pairs by gentle movement into and out of a flame-polished micropipet (diameter ≈ 40 μm) (18). Each whole 8-cell HUP embryo was then placed in a drop of culture medium containing 1% rehydrated Difco phytohemagglutinin P (19) and surrounded with four pairs of blastomeres from an 8-cell embryo or eight pairs of blastomeres from a 16-cell embryo. Each blastomere pair was gently pushed against the HUP embryo by using two small blunt glass probes. After 15–45 min in the aggregation medium, each aggregate was washed in modified Whitten's medium and placed in a microdrop of medium under mineral oil and incubated at 37°C under 5% CO_2/95% air; 24–36 hr later, only those aggregates that appeared to incorporate the HUP embryo and had developed into blastocysts were transferred to the uterine horns of day 3 pseudopregnant CD-1 females. These females were initially allowed to give birth naturally. However, because of the large number of embryo resorptions that occurred, almost half of these females failed to give birth at all. Consequently, in the latter part of this project, all pups were delivered by cesarian section after 20 days *in utero* and transferred to lactating foster mothers.

RESULTS

All the fertilized eggs used for enucleation, if left intact, would have developed into black or agouti animals; no albino animal could arise from such eggs. From a total of 4339 fertilized eggs, 3717 (86%) survived the microsurgical removal of the "black" male pronucleus (Fig. 1 *A–C*). To diploidize the remaining albino female pronucleus (Fig. 1*D*), the eggs were cultured overnight in cytochalasin D [a compound with a greater specificity of action on microfilaments and effective at a much lower concentration than cytochalasin B (15)]. The now 100% HUP one-cell eggs were transferred via the ostium to the oviducts of day 1 pseudopregnant C57BL/6J females; a much greater percentage of HUP eggs proceeded through normal cleavage when put back into the oviduct than if left in culture. C57BL/6J females were used as temporary recipient females instead of CD-1 females to ensure against the possibility of obtaining *in situ* activated albino parthenotes. Two days later these oviducts were flushed to recover the HUP embryos, now at the eight-cell stage. From a total of 3371 HUP eggs transferred to the oviduct, 1108 (33%) were recovered as eight-cell embryos. These embryos were then surrounded with blastomere pairs (Fig. 1*E*) obtained by dissociating blastomeres from normal biparental embryos at the 8- or 16-cell stage; the biparental embryos were flushed from the oviducts of C57BL/6J females that had been mated with C57BL/6J or B6D2F_1/J males. Blastomere pairs from such embryos could contribute only black (not albino) pigmentation to any chimera of which they were a part. Surrounding the HUP embryos with these blastomere pairs also maximized the likelihood that the HUP embryos would contribute to the inner cell mass (as opposed to the trophoblast) of the chimeric blastocyst and, thus, to the embryo proper as well (18, 20, 21).

After a day in culture only those aggregates that appeared to have incorporated all or nearly all of the blastomeres of the HUP embryo and had developed into blastocysts (Fig. 1*F*) were transferred to the uterine horns of day 3 pseudopregnant CD-1 females. From a total of 1059 chimeric aggregates, 770 (73%) developed into such blastocysts, and these were transferred to a total of 31 recipient females. Of the 63 pups born, 24 died and were eaten by the mother before any chimerism could be detected; 39 lived long enough for pigment chimerism to be assessed. Of these 39, 27 appeared nonchimeric in pigmentation, and 12 were identified as overtly chimeric. At birth all 12 chimeras were substantially smaller than their normal-appearing littermates. Of these 12, 2 were stillborns and 6 died 1 or 2 days after birth; these were identified as chimeric by the sectoring of pigmented areas with nonpigmented areas in the retinas (Fig. 1*G*). The remaining 4 of the 12, including 1 female and 3 males, are now healthy adults. The female (Fig. 1*H*, the mouse on the right) is the most extensively chimeric, with about 50% homozygous uniparental-derived white and agouti hairs among the normal biparental black hairs. It should be noted that the appearance of agouti fur results from the migration of melanoblasts from the biparental black component into homozygous uniparental hair follicles, the cells of which, although albino, carry the dominant agouti gene. This female was mated with an albino CD-1 male and has produced 7 litters, for a total of 31 albino pups derived from her HUP cells and 12 agouti pups from her normal biparental cells. One male, littermate of the chimeric female, shows about 10% white fur only, and no agouti hairs. The second male (Fig. 1*H*, the mouse on the left) shows about 40% white and agouti fur, and the third male shows about 5% agouti fur only. Because this last male has no white fur, it is possible that he resulted from a failure of enucleation followed by tetraploidization of the diploid egg and production of a tetradiploid/diploid chimera; such chimeras have been shown to be viable (22). Table 1 summarizes the data.

An important and recurrent finding in these experiments was the great number of resorbing embryos (Fig. 1*I*) in the uterine horns upon cesarian sectioning of the recipient

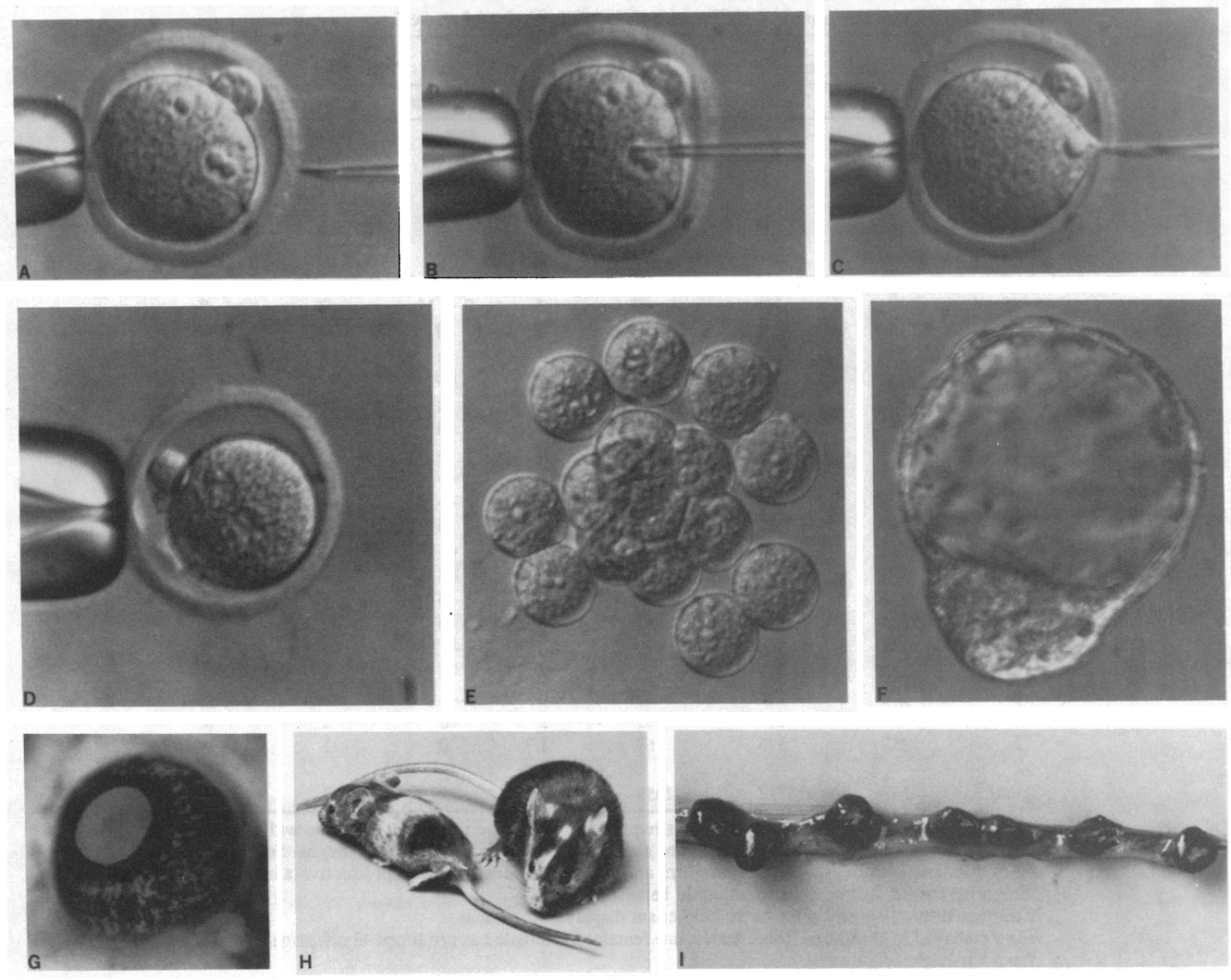

FIG. 1. (*A*) Light micrograph of a fertilized egg. Note the large male pronucleus, showing two nucleoli within, directly opposite the enucleation pipet; the smaller female pronucleus is nearer to the polar body. (*B*) The same egg after penetration by the enucleation pipet. (*C*) The same egg during removal of the male pronucleus. (*D*) A HUP egg after diploidization in the presence of cytochalasin D. Note that the two female pronuclei are about equal in size. (Treatment overnight in cytochalasin D caused a slight shrinkage of the egg.) (*E*) A HUP eight-cell embryo surrounded by four blastomere pairs from a normal biparental eight-cell embryo. (*F*) A chimeric blastocyst after a day in culture of the aggregate in *E*. Note the large inner cell mass in the lower portion of the embryo. (*G*) A chimeric eye from a pup that died shortly after birth. Note the distinctive sectoring of pigmented and nonpigmented areas in the retina. (*H*) Two chimeric adults showing the presence of HUP cells; the female referred to in the text is on the right, a male on the left. (*I*) An opened uterine horn taken from a recipient female after 20 days of gestation, showing seven resorbing embryos implanted along the uterine wall.

females. The abdomens of the females often appeared most swollen at about days 13–15 of gestation and then became substantially thinner as the pregnancy neared term. This suggests that the embryonic death that occurred by day 11 with purely HUP embryos transferred to recipient females (3, 4) also occurred in the vast majority of chimeric aggregates, despite the presence of the normal biparental blastomeres.

Furthermore, out of an average of 25 chimeric blastocysts transferred to each recipient female, an average of only 2 pups (8%) per litter was obtained. Similarly small litters were also obtained by Stevens (10) in rescuing parthenote embryos as components of chimeras. These low rates of success contrast with the much higher rates (about 30–50%) obtained in the uterine transfer of normal chimeras (23–25).

DISCUSSION

What is it, then, about the HUP condition that makes it so deleterious? The explanation cannot lie in the homozygosity *per se*, since biparental heterozygous eggs equipped by microsurgery with two female or two male pronuclei also fail to develop to term (6–8). The evidence is now decisive: both a maternal and a paternal genome must be present in a mammalian egg for development to proceed to term. We must conclude that differential "imprinting" of gamete genomes occurs during gametogenesis in males and females. Even among highly inbred strains of mice, the genomes of eggs and sperm are functionally different by the time of fertilization. These differences, whatever their molecular basis, apparently persist throughout development (7, 26, 27). An illustration of just how critically important these differences are can be found in Searle and Beechey's (28) demonstration that the lack of both a male and female contribution to even one small portion of the genome (i.e., within a single chromosomal pair) is sufficient to prevent embryo development or to abort late pregnancies.

The most probable mechanism for chemically differentiating the chromosomal DNA is based upon differential methylation of cytosine residues (cf. 29). The patterns of

Table 1. Data on the rescue of HUP embryos by chimera manufacture

Exp.	Eggs, no.		Chimera aggregates, no.	Pups, no.			
	Enucleated	Survived		Born	Chimeras	All black	In limbo*
1	85	79	25	0	—	—	—
2	118	107	41	5	0	5	0
3	158	130	43	3	0	3	0
4	154	134	61	2	0	2	0
5	183	144	60	4	0	2	2
6	147	116	45	7	1†	0	6
7	173	113	36	0	—	—	—
8	181	146	82	6	2 + 1†	3	0
9	158	148	23	2	0	0	2
10	146	130	41	4	1	3	0
11	119	113	47	0	—	—	—
12	137	120	18	2	1†	0	1
13	187	122	30	1	0	0	1
14	88	85	11	0	—	—	—
15	208	181	0	0	—	—	—
16	88	86	0	0	—	—	—
17	190	182	33	1	0	1	0
18	217	191	51	0	—	—	—
19	133	117	0	0	—	—	—
20	230	199	36	8	3†	0	5
21	214	199	51	4	0	2	2
22	231	198	87	10	1 + 2†	2	5
23	260	222	91	1	0	1	0
24	178	166	43	0	—	—	—
25	139	114	35	2	0	2	0
26	217	175	69	1	0	1	0
Totals	4339	3717	1059	63	12‡	27	24

Great attrition occurs at two of the steps required to rescue homozygous embryos with all-female genomes. First, only one-third of enucleated eggs are recovered as eight-cell embryos. Second, only 6% of chimeric blastocysts develop to term, and still fewer develop into reproductive adults.

*Eaten by mother before chimerism could be assessed.

†Stillborn or died shortly after birth; chimerism displayed in retinas.

‡Four survived to adulthood (three males, one female; the female has produced 43 offspring, 31 derived from her homozygous cells).

methylation along the DNA can be persistent but changeable, replicable, and erasable when a new generation of gametes is to be produced—characteristics required of any mechanism for differentiating the genome. By differential methylation, initially identical DNA in oocytes and spermatocytes can become functionally different by the time of fertilization. The imprinting does not prevent all genes in an imprinted region from functioning, since the genes do function, whether introduced from the male or female parent, in normal biparental mice. The imprinting clearly is different for eggs and sperm and is in a sense complementary. Parts of homologous chromosomes may be derived from a single sex, and such arrangements commonly prove lethal during embryonic development, or at least alter the final phenotype of the newborn mouse. These observations lead us to conclude that the imprinting is in the noncoding DNA and that the imprinting may affect the interaction among homologous chromosomes to alter the regulation of gene function.

We now know that a completely HUP genome is not lethal in every type of differentiated cell. HUP cells appear in our adult chimeras among pigment and hair follicle cells, in the retinal epithelium, and among gametes. The fact that our female chimera reproduces using her HUP cells as a source of gametes demonstrates that such chimeras can be used to establish new breeds of mammals much more quickly than was previously possible. It is still not possible to produce HUP male embryos because males must have both an X and a Y chromosome—a genetic makeup that cannot be produced from a haploid genome. Even so, the fact that chimeric females can produce eggs derived from HUP cells means that the problem of producing completely homozygous strains has been, in effect, half solved. Since all of the eggs from HUP cells in a chimera are identical, mating of mother to son and then to grandson, etc., would lead to inbred strains containing both sexes much more rapidly than with present breeding regimens in which both the male and female parents are initially heterozygous. Our three adult chimeric males, although containing HUP cells with female genomes, cannot be expected to produce sperm derived from these female cells. Sex chimeras containing both XX and XY cells are known to produce gametes only from the cells with a chromosomal makeup corresponding to the sex phenotype of the adult chimera (30–32).

Since HUP embryos cannot by themselves develop into viable individuals, the data reported here may be interpreted in either of two ways. The few chimeras that developed to term may represent those in which the HUP cells by chance did not populate tissues and organs where their deficiencies could prove lethal (pigment cells and even gametes are not critical to survival). Alternatively, the successes may represent rare alterations in the process of imprinting during gametogenesis so that in these instances a single-sex, uniparental origin of the genome is not lethal, thus permitting survival of the chimera. In either case, biochemical and cytological analysis of these HUP cells will be necessary to begin to resolve the question of what makes homozygous, uniparental cells so deleterious and to reveal the chemical nature of the imprinting of gamete genomes.

The research reported here is part of the Ph. D. dissertation of C.A. at Yale University. The research was supported by National Science Foundation Grant DCB-8417478 to C.L.M. and by National Institutes of Health Medical Scientist Training Program Grant GM-07205 to C.A.

1. Markert, C. L. & Petters, R. M. (1977) *J. Exp. Zool.* **201,** 295–302.
2. Hoppe, P. C. & Illmensee, K. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5657–5661.
3. Markert, C. L. (1982) *J. Hered.* **73,** 390–397.
4. Surani, M. A. H. & Barton, S. C. (1983) *Science* **222,** 1034–1036.
5. Mann, J. R. & Lovell-Badge, R. H. (1984) *Nature (London)* **310,** 66–67.
6. McGrath, J. & Solter, D. (1984) *Cell* **37,** 179–183.
7. Surani, M. A. H., Barton, S. C. & Norris, M. L. (1984) *Nature (London)* **308,** 548–550.
8. Barton, S. C., Surani, M. A. H. & Norris, M. L. (1984) *Nature (London)* **311,** 374–376.
9. Stevens, L. C., Varnum, D. S. & Eicher, E. M. (1977) *Nature (London)* **269,** 515–517.
10. Stevens, L. C. (1978) *Nature (London)* **276,** 266–267.
11. Surani, M. A. H., Barton, S. C. & Kaufman, M. H. (1977) *Nature (London)* **270,** 601–603.
12. Kaufman, M. H. (1983) *Early Mammalian Development: Parthenogenetic Studies* (Cambridge Univ. Press, London).
13. Whitten, W. K. (1971) *Adv. Biosci.* **6,** 129–139.
14. McGrath, J. & Solter, D. (1983) *Science* **220,** 1300–1302.
15. Siracusa, G., Whittingham, D. G. & De Felici, M. (1980) *J. Embryol. Exp. Morphol.* **60,** 71–82.
16. Nicholas, J. S. & Hall, B. V. (1942) *J. Exp. Zool.* **90,** 441–459.
17. Nicolson, G. L., Yanagimachi, R. & Yanagimachi, H. (1975) *J. Cell Biol.* **66,** 263–274.
18. Kelly, S. J. (1977) *J. Exp. Zool.* **200,** 365–376.
19. Mintz, B., Gearhart, J. D. & Guymont, A. O. (1973) *Dev. Biol.* **31,** 195–199.
20. Wilson, I. B., Bolton, E. & Cuttler, R. H. (1972) *J. Embryol. Exp. Morphol.* **27,** 467–479.
21. Hillman, N., Sherman, M. I. & Graham, C. (1972) *J. Embryol. Exp. Morphol.* **28,** 263–278.
22. Lu, T. & Markert, C. L. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 6012–6016.
23. Mintz, B. (1967) *Proc. Natl. Acad. Sci. USA* **58,** 344–351.
24. Bowman, P. & McLaren, A. (1970) *J. Embryol. Exp. Morphol.* **23,** 693–704.
25. Mullen, R. J. & Carter, S. C. (1973) *Biol. Reprod.* **9,** 111–115.
26. Surani, M. A. H. (1985) *BioEssays* **1,** 224–227.
27. Surani, M. A. H., Barton, S. C. & Norris, M. L. (1986) *Cell* **45,** 127–136.
28. Searle, A. G. & Beechey, C. V. (1978) *Cytogenet. Cell Genet.* **20,** 282–303.
29. Razin, A., Cedar, H. & Riggs, A. D., eds. (1984) *DNA Methylation: Biochemistry and Biological Significance* (Springer, New York).
30. Mintz, B. (1968) *J. Anim. Sci.* **27,** Suppl. 1, 51–60.
31. Mystkowska, E. T. & Tarkowski, A. K. (1968) *J. Embryol. Exp. Morphol.* **20,** 33–52.
32. McLaren, A. (1975) *J. Embryol. Exp. Morphol.* **33,** 205–216.