structural gene for testis differentiation located on the X is induced by a controlling element on the Y; or, (ii) the structural gene is on the Y, its controlling element on the X (*8*). In our family the X-borne locus (*8*) is mutant and fails in the interaction with the locus on the Y. The analysis of this rare genetic disorder indicates also that a single human X, although sufficient for differentiation of ovary, is insufficient for the sustenance into adult life of normal oogenesis.

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References and Notes

1. J. L. Simpson, *Disorders of Sexual Differentiation* (Academic Press, New York, 1976).
2. _____, A. C. Christakos, M. Horwith, F. S. Silverman, *Birth Defects, Orig. Artic. Ser.* **7** (No. 6), 215 (1971).
3. J. L. Simpson and J. German, *Am. J. Hum. Genet.* **22**, 24a (1970).
4. J. L. Simpson, R. L. Summitt, I. R. Merkatz, J. German, *Gynecol. Invest.* **7**, 37 (1976).
5. W. H. Sternberg, D. L. Barclay, H. W. Kloepfer, *N. Engl. J. Med.* **278**, 695 (1968); E. A. Espiner, A. M. O. Veale, V. E. Sands, P. H. Fitzgerald, *ibid.* **283**, 6 (1970); J. Chemke, R. Carmichael, J. M. Stewart, R. H. Geer, A. Robinson, *J. Med. Genet.* **7**, 105 (1970); B. Ionescu and C. Maximilian, *ibid.* **14**, 194 (1977).
6. Three families have been reported [J. Baron, T. Rucki, S. Simm, *Gynecologia* **153**, 298 (1962); M. L. Barr, D. H. Carr, E. R. Plunkett, H. C. Soltan, R. E. Wiens, *Am. J. Obstet. Gynecol.* **99**, 1047 (1967)] which do not fit into the scheme we advance here to explain XY gonadal dysgenesis. The individuals with abnormal sexual development did have the chromosome complement 46,XY, and at least one affected person in each family had streak gonads with an otherwise normal female development, that is, had XY gonadal dysgenesis. However, another abnormal sib in each family had poorly formed testes along with genital ambiguity and Müllerian derivatives. At present, these families defy explanation; conceivably the mutant gene is leaky. In a fourth family [S. Allard, M. Cadotte, Y. Boivin, *Un. Méd. Canad.* **101**, 448 (1972)], transmission of the trait through a normal male appears to contradict X linkage, the pattern of inheritance suggested in all other known families with multiple affected persons.
7. Defined here as development of the sex opposite to that expected from the chromosome constitution.
8. Conceivably the mutant gene is located on an autosome because autosomal dominant inheritance with effect in males cannot be excluded in such families.
9. R. P. Singh and D. H. Carr, *Anat. Rec.* **155**, 369 (1966); J. E. Jirasek, in *Disorders of Sexual Differentiation*, J. L. Simpson, Ed. (Academic Press, New York, 1976), pp. 51–110.
10. D. H. Carr, R. A. Haggar, A. G. Hart, *Am. J. Clin. Pathol.* **49**, 521 (1968).
11. J. L. Hamerton, *Nature (London)* **219**, 910 (1968).
12. J. L. Simpson, in *Genetic Mechanisms of Sexual Development*, I. H. Porter, Ed. (Academic Press, New York, in press).
13. R. A. McFeely, W. C. D. Hare, D. J. Biggers, *Cytogenetics* **6**, 242 (1967).
14. E. H. Goldberg, E. A. Boyse, D. B. Bennett, M. Scheid, E. A. Carswell, *Nature (London)* **232**, 478 (1971); E. H. Goldberg, E. A. Boyse, M. Scheid, D. B. Bennett, *Nature (London) New Biol.* **238**, 55 (1972); S. S. Wachtel, *Immunol. Rev.* **33**, 33 (1977).
15. J. German, J. L. Simpson, G. A. McLemore, Jr., *Ann. Genet.* **16**, 225 (1973).
16. The only published report of H-Y antigen testing in a purported case of XY gonadal dysgenesis is the following, in which the affected person was found H-Y antigen positive: E. Dorus, A. P. Amarose, G. C. Koo, S. S. Wachtel, *Am. J. Obstet. Gynecol.* **127**, 829 (1977).
17. K. Fredga, A. Gropp, H. Winking, F. Frank, *Nature (London)* **261**, 225 (1976).
18. M. F. Lyon, *Proc. R. Soc. London Ser. B* **187**, 243 (1974).
19. S. Ohno, *Sex Chromosomes and Sex-linked Genes* (Springer-Verlag, Berlin, 1967).
20. W. J. Welshons and L. B. Russell, *Proc. Natl. Acad. Sci. U.S.A.* **45**, 560 (1959).
21. B. M. Cattanach, *Genet. Res.* **3**, 487 (1962).
22. M. F. Lyon and S. G. Hawker, *ibid.* **21**, 185 (1973).
23. S. Ohno, C. Stenius, L. Christian, in *Chromosomes Today*, C. D. Darlington and K. R. Lewis, Eds. (Oliver & Boyd, London, 1964), pp. 182–187.
24. *Mus musculus* conceivably is different, in that 40,XY germ cells which find themselves in the ovary of experimentally produced chimeric females (40,XY/40,XX) may undergo meiosis [C. E. Ford and E. P. Evans, *J. Reprod. Fertil.* **49**, 25 (1977)].
25. S. M. Gartler, R. M. Liskay, N. Gant, *Exp. Cell Res.* **82**, 464 (1973); B. R. Midgeon and K. Jelalian, *Nature (London)* **269**, 242 (1977).
26. I. J. Kerber, *Southern Med. J.* **63**, 623 (1970).
27. Supported by NIH grants HD 04134 and HL 09011. We thank Dr. William Sternberg of New Orleans who reviewed the gonadal histological preparation from individual III.11.
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Manufactured Hexaparental Mice Show That Adults Are Derived from Three Embryonic Cells

Abstract. *Two female chimeric mice have been produced by aggregates of three genetically marked eight-cell embryos. All three embryonic genotypes are clearly expressed in the pigment pattern of the adults. These hexaparental mice together with their littermates demonstrate that, in the 64-celled blastocyst, at least three cells, and probably only three, are the source of all adult tissues.*

Chimeric mice produced by aggregating cleavage-stage embryos in vitro are being extensively used in studies of mammalian development (*1*). Genetic markers such as coat color mutants are usually incorporated in the embryos in order to make chimerism in the adult obvious. Thousands of such chimeric mice have now been produced in many laboratories. However, all adult chimeras so far manufactured have manifested only two genotypes—that is, have been derived from just two embryos. We report here the first adult chimeric mice derived from three aggregated embryos, and therefore having six genetic parents—hexaparental mice.

The coat color phenotypes used in this investigation were yellow, black, white, and dilute brown. In genotype, all were non-agouti (*aa*). Recessive yellow (*e/e*, *B/B*) mice are yellow because yellow is epistatic to black. These mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. An albino strain was segregated in our laboratory from CD-1 (ICR) mice (Charles River Breeding Laboratories, Wilmington, Mass.) to provide the albino embryos (*c/c,a/a, B/B, E/E*). Homozygous albino animals produce no pigment. Albino females mated to black males produced the black embryos used in these experiments. These embryos were heterozygous for albinism (*c/+*) but homozygous black (B/B). Dilute brown embryos (*a/a, b/b, d/d*) were obtained from a stock maintained in our facility.

Two series of experiments were conducted: one aggregating three embryos (black, white, and yellow) and another aggregating four embryos (black, white, yellow, and dilute brown). The results from the experiments aggregating three embryos are discussed first.

In manufacturing the chimeric mice, embryos composed of four or eight cells were first flushed from the oviducts of hormonally superovulated females. After the zonae pellucidae were removed with pronase, black, albino, and yellow embryos were placed in a triangular configuration in microdrops of phytohemagglutinin (PHA)-containing medium (*2*) under oil for 15 minutes and cultured in vitro without PHA for 24 hours. These trios were periodically observed with an inverted microscope in a 37°C constant temperature room. A gas phase of 5 percent CO_2 in air was maintained over the culture medium, except during the observation periods. The embryos were transferred to pseudopregnant females for gestation. Mosaicism was scored after formation of hair pigmentation—about 7 to 10 days after birth.

Preliminary experiments were conducted to ensure that the embryos of all three genotypes (black, white, and yellow) were equal in developmental ability. Pairwise combinations of the three genotypes were constructed. Three yellow ↔ white, six black ↔ white, and two black ↔ yellow chimeras were produced, indicating that the three geno-

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types were developmentally equal and compatible and therefore should make equivalent contributions to any chimera derived from aggregating the three embryos.

Most of the three-embryo aggregates formed single embryos, triple the usual size (Fig. 1). By the end of the in vitro culture period, the embryos had developed small blastocoels (Fig. 1d). Forty of these triple embryos survived the in vitro culture period and were transferred to two pseudopregnant females, 20 to each female.

Both foster mothers gave birth to offspring (Table 1). All three pairwise combinations were obtained: black ↔ yellow, yellow ↔ white, and black ↔ white. In addition, one individual was a mosaic of all three colors. This triple (black ↔ yellow ↔ white) chimera is shown on the cover.

The pigment patches on this hexaparental mouse are obvious and show some evidence of clonal origin from single cells arranged linearly from head to tail (*3*). The albino fur represents the smallest proportion of the coat (about 10 percent) while yellow and black hairs populate the remaining areas of the fur more or less equally. The patches of black, yellow, and white hairs demonstrate unequivocally that this individual arose from three different embryos.

The experiments aggregating four genetically marked embryos (black, white, yellow, and dilute brown) yielded a second triple chimera (black ↔ yellow ↔ white), four double chimeras (one yellow ↔ white, one black ↔ yellow, two dilute brown ↔ yellow), and three nonchimeric offspring (all dilute brown).

During mouse embryonic development the egg first undergoes a series of cleavages until about 64 cells have formed. Morphogenetic movements then shape these 64 cells into a hollow ball—the blastocyst—with a concentration of about 15 cells, the inner cell mass (ICM), on one side (*4*). The wall of the blastocyst is the trophoblast and gives rise exclusively to tissues of the placenta. The ICM is the forerunner of the embryo proper and also of the cells of several extraembryonic structures such as the yolk sac, allantois, and amnion. How many of these ICM cells actually contribute to the tissues of the embryo rather than to extraembryonic tissues is at issue. The determinative event that selects a few cells of the ICM to be the exclusive progenitor cells of the embryo must occur prior to the differentiation of primitive ectoderm and endoderm, probably when the ICM is composed of about 15 cells, or shortly thereafter.

Table 1. Offspring from experiment aggregating three genetically marked embryos.

Phenotype	No.
Black ↔ yellow ↔ white	1 ♀
Black ↔ yellow	2 ♂; 1 ♀
Black ↔ white	1 ♀
Yellow ↔ white	2 ♀
Black	2 ♀
Yellow	0
White	1 ♂

However, the fact that chimeric mice can be produced at all demonstrates that at least two cells in the ICM participate in forming the adult. From her extensive data with allophenic mice, Mintz (*5*) has suggested that just three cells in the ICM are determined to form the entire embryo. All other cells are channeled into extraembryonic structures. Her argument stems from the finding that approximately 75 percent of aggregated double chimeric embryos yield chimeric adults. The remaining 25 percent develop into parental types showing no evidence of chimerism. If the cells of each of the two combined embryos have an equal chance to contribute to the chimeric adult, then the predicted frequency of chimeric mice —based on a three-cell model—would be 75 percent. This prediction is arrived at by expanding the binomial: $(W + B)^3 = W^3 + 3W^2B + 3WB^2 + B^3$, where, for example, W is an albino strain and B is a black strain; together these two strains would produce a conspicuous mosaic pigment pattern. Moreover, by this three-cell model, 25 percent of the adults would be derived from the cells of

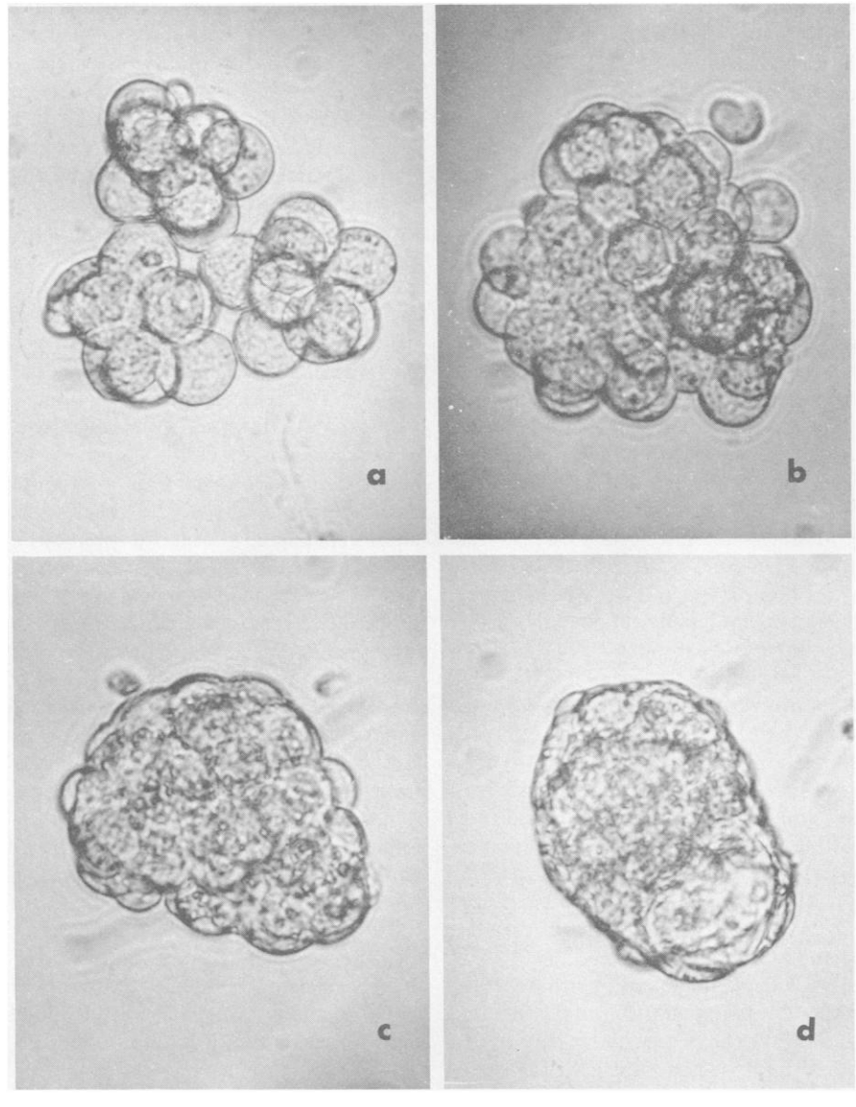

Fig. 1. Aggregation of three eight-cell mouse embryos. Hours are time elapsed after aggregation. (a) At 1 hour, a few blastomeres from each embryo are in contact. (b) At 8 hours, individual embryos are less distinct and the blastomeres are beginning to form a single compact embryo. (c) At 19 hours, morula are three times normal size. (d) At 25 hours, a small blastocoel has formed. Chimeric embryos were transferred to foster mothers at this stage.

only one of the two embryos initially aggregated.

Another method for producing chimeric mice is by injection of cells from one embryo into the blastocyst cavity of another. The injected cells can become incorporated in the ICM and go on to participate in the formation of the embryo. The degree of chimerism produced by such injections of single cells supports the conclusion that the number of ICM cells destined to give rise to the adult is probably small. Injections of two cells (*6*) and single cells (*7*, *8*) have resulted in mice with extensive chimerism; approximately one-third of the chimeric mouse was frequently derived from a single injected cell. In fact Illmensee and Mintz (*7*) report that about 30 percent of the blastocysts that received a single additional cell by injection eventually developed into chimeras. These percentages are consistent with the hypothesis that three cells of the ICM are set aside for the formation of the embryo at the time the ICM is composed of 10 to 15 cells.

The assumption that the cells from two aggregated embryo will contribute equally to the formation of the blastocyst and to the ICM is commonly correct, but there are many exceptions. The intrinsic developmental capacity of cells, as stipulated by genotype, together with the relative positions of the cells when aggregated will influence the proportion of each cell type in the ICM and in the later adult chimera. The data do suggest a three-cell model, but purely statistical arguments do not prove the hypothesis. Our manufacture of chimeric mice from three genetically marked embryos demonstrates that at least three cells in the ICM are allocated to form the adult organism. However, while this result sets a lower limit of three, it does not stipulate the maximum number of cells that might be involved.

By expanding the trinomial, $(W + Y + B)^3$, and collecting the appropriate terms, we can calculate the expected proportions of each type of progeny for a three-cell model when aggregating three embryos and compare these proportions with the actual data. This calculation assumes that the cells of each embryo (W, Y, and B) have an equal chance of contributing to the adult. Each pairwise combination ($W \leftrightarrow Y$, $W \leftrightarrow B$, $Y \leftrightarrow B$) and the triple combination are expected with a frequency of 0.22 (total chimeras, 88 percent), while each parental type would be expected with a frequency of 0.036 (total nonchimeric mice, 11 percent). Since there are three possible pairwise combinations, three times more pairwise chimeras than triple chimeras should be present in the progeny. The data from our experiment are six double chimeras, one triple chimera, and three nonchimeric parental types. Our data vary from expectations based on a three-cell model by showing too few triples and too many singles (parental types) which may reflect the small sample size. However, it is possible that some of the double chimeras are actually triples since small patches of yellow or albino hair would be difficult to distinguish on a mosaic background. Some of the parental types might be cryptic chimeras failing to show pigment cell mosaicism, but chimeric in other tissues.

The expected proportions of the various progeny derived from chimeric embryos would change greatly as the number of ICM cells participating in embryo formation increased. For example, one can extend the trinomial calculation to consider a four-cell model, $(W + Y + B)^4$. When this is done, the expected frequency of double chimeras is 0.52, and for triples the expected frequency is 0.44. Parental types would be expected only 4 percent of the time. Therefore, if just four cells in the ICM are set aside to form the embryo proper, the chimeric progeny from aggregating three embryos should be distributed in approximately equal numbers among double (52 percent) and triple (44 percent) chimeras. Our limited data, showing too few triple chimeras and too many parental types, are not consistent with the predictions of the four-cell model. Moreover, the proportion of nonchimeric parental types from aggregates of two embryos should be 13 percent for a four-cell model—far from the actual data, which show parental types to be approximately 25 percent of the total.

We realize that arguments based on binomial or trinomial calculations are probably oversimplifications (*1*, *9*). The arrangement of cells in the inner cell mass is not random (*10*) and so each of the three genotypes will not have an equal chance of being represented. This fact may explain the small number of triples and the overrepresentation of parental types in our results. Further, differential cell death or abnormal cell movements may distort the contributions of each aggregated embryo to the ICM. For these reasons and others, one must be cautious in interpreting the data on the expected frequency of double or triple chimeras. We wish only to point out that our data lend support to the conclusions based on a three-cell model.

Our argument that the embryo is derived from three cells of the ICM also leads to the conclusion that extensive, imprecise cell movements take place during development. Chimerism is commonly rather fine-grained, and large discrete patches of one genotype are rare. The fixed positional mosaicism characteristic of insect systems is noticeably lacking in chimeric mice.

The patterns of mosaicism within tissues of chimeric mice have been used to estimate the number of primordial cells giving rise to each tissue. For instance, the number of melanocyte progenitors has been estimated from the striped pigment patterns in chimeras to be 34 (*3*); however, others have suggested much larger numbers (*11*). Triple chimeric mice might provide a finer resolution than double chimeras in revealing the patterns of clonal growth of melanocytes. The distinction between clone size and patch size may be clarified in hexaparental mice since more genotypes are involved.

The germ cells appear to arise in the primitive ectoderm and then migrate into the yolk sac, which is derived from primitive endoderm (*12*). The discussion of Falconer and Avery (*9*) predicts a positive correlation between gametic output and coat mosaicism since both cell types are thought to be derived from the same embryonic tissue—the primitive ectoderm. Accordingly we may expect our triple chimera, a fertile female, to produce eggs from all three component genotypes. Results of breeding tests of this mouse are not yet available.

Our data and that of others suggest that three cells and only three cells in the ICM of the blastocyst are the sole source of the cells making up the adult. The vast majority of cells in the blastocyst never contribute to the tissues of the adult.

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References and Notes

1. A. McLaren, *Mammalian Chimaeras* (Cambridge Univ. Press, London, 1976),
2. B. Mintz, J. D. Gearhart, A. O. Guymont. *Dev. Biol.* **31**, 195 (1973).
3. B. Mintz, *Proc. Natl. Acad. Sci. U.S.A.* **58**, 344 (1967).
4. R. L. Gardner, in *The Developmental Biology of Reproduction*, C. L. Markert and J. Papaconstantinou, Eds. (Academic Press, New York, 1975), pp. 207–236.
5. B. Mintz, *Symp. Int. Soc. Cell Biol.* **9**, 15 (1970).
6. R. L. Gardner, *Nature (London)* **220**, 596 (1968).
7. K. Illmensee and B. Mintz, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 549 (1976).
8. C. E. Ford, E. P. Evans, M. D. Burtenshaw, H. M. Clegg, M. Tuffery, R. D. Barnes, *Proc. R. Soc. London Ser. B* **190**, 187 (1975).
9. D. S. Falconer and P. J. Avery, *J. Embryol. Exp. Morphol.* **43**, 195 (1978).
10. W. Garner and A. McLaren, *ibid.* **32**, 495 (1974); S. J. Kelly, *J. Exp. Zool.*, in press.
11. L. Wolpert and D. Gingell, *J. Theor. Biol.* **29**, 147 (1970); J. D. West, *J. Embryol. Exp. Morphol.* **35**, 445 (1976).
12. R. L. Gardner and J. Rossant, *Ciba Found. Symp.* **40**, 5 (1976).
13. Supported in part by NSF grant PCM76-23036, NIH grant 5 R01 HD07741, and (to R.M.P.) PHS postdoctoral training grant 5 T01 HD00032-14.

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