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Full-term development after transplantation of parthenogenetic embryonic nuclei into fertilized mouse eggs

(nuclear transplantation/parthenogenesis/LT/Sv mice)

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ABSTRACT **Diploid parthenogenetically activated oocytes were obtained after gonadotropin-induced ovulation of virgin females of the LT/Sv (LT) inbred mouse strain. These oocytes cleave spontaneously and develop into blastocysts which implant in the uterus but die within a few days. We examined the developmental potential of nuclei from parthenogenetic embryos after transplantation into fertilized eggs. The inner cell mass (ICM) and trophectoderm (TE) of LT parthenogenetic blastocysts were mechanically isolated and dissociated into single cells. Their nuclei were then injected into fertilized C57BL/6J eggs from which the male and female pronuclei were removed. Of 94 eggs injected with TE cell nuclei, 4 embryos developed to the morula stage; all 4 showed abnormalities and subsequently became arrested in development. Enzyme analysis of these embryos revealed that TE cell nuclei could neither independently initiate nor support preimplantation development. However, of 54 eggs injected with nuclei from ICM cells, 3 morulae and 3 blastocysts developed and enzyme analyses of them confirmed that the preimplantation development of 2 embryos was supported by transplanted parthenogenetic nuclei. In another experimental series, 3 morulae and 4 blastocysts developed from 107 eggs injected with ICM nuclei and were transferred to uteri of foster mothers to ascertain their postimplantation development. Four female offspring were born and all of them showed a diploid karyotype and expressed enzyme activity of only the LT genotype. One female proved to be fertile and transmitted the parthenogenetic genome to the next generation. These results demonstrate that the nucleus from LT parthenogenetic blastocysts contains a complete genome necessary to support development of an adult mouse. Therefore, the early postimplantation death of parthenogenetic embryos does not seem to be related to an aberrant genotype but rather to undefined mechanisms associated with fertilization and normal morphogenetic processes.**

Parthenogenesis—i.e., development without fertilization—occurs most frequently in invertebrate species but is also observed, although to a limited extent, in reptiles (1), fishes (2), and birds (3). Amphibian oocytes can be activated experimentally to commence cleaving, and a small proportion of the developing embryos reach the adult stage (4). Artificial activation of mammalian oocytes results in only limited development with death of the embryo occurring within a few days after implantation (5, 6). In females of the inbred mouse strain LT/Sv (LT), approximately 10% of the oocytes from spontaneous and gonadotropin-induced ovulation cleave in the absence of fertilization and develop into diploid blastocysts (7–9). The mechanism for activation and subsequent diploidy is unknown. These blastocysts can implant when transferred to uteri of pseudopregnant foster mothers but die soon after implantation. Experimentally activated mouse oocytes also die shortly after implantation (5, 6, 10, 11).

On the other hand, when the activated LT oocytes do not ovulate but remain in the ovary, they can develop into ovarian teratomas (7). These tumors are usually benign and differentiate into various tissues derived from the three germ layers. Only occasionally do malignant tumors occur within the ovary. After being injected into genetically different blastocysts, single cells from these teratocarcinomas are able to contribute to the development of several organs of chimeric mice (12).

Parthenogenetic LT embryonic cells have been "rescued" by aggregating them with fertilized embryos (8, 13) or by injection of parthenogenetic embryonic cells into normal blastocysts (9). The resulting chimeric mice exhibited the parthenogenetic genotype (LT) in their coat color and glucosephosphate isomerase activity in several adult tissues. One chimera gave birth to offspring whose phenotypes were derived from the parthenogenetic genome (8). This suggests that the early mortality of LT parthenogenetic embryos may involve organismic rather than genetic factors. In these experiments, however, cells from parthenogenetic embryos have always been associated with normal cells of the chimeric tissues which, in principle, could compensate for cell defects due to parthenogenesis.

In the present study, we examined the developmental potential of nuclei from cells of spontaneously activated LT parthenogenetic blastocysts by transplanting them into fertilized but enucleated eggs. This experimental approach unequivocally demonstrated that the parthenogenetic genome in inner cell mass (ICM) cells can fully support normal development.

MATERIALS AND METHODS

Recipient Zygote Collection. Recipient C57BL/6J (B6) fertilized eggs, approximately 10 hr after ovulation, were collected in culture medium (14) containing bovine testis hyaluronidase (Sigma) at 1 mg/ml for removing cumulus cells. The pronuclear-stage eggs were washed in fresh medium to remove residual hyaluronidase and cumulus cells and were incubated at 37°C in culture medium with 5 μg of cytochalasin B (Aldrich) per ml under paraffin oil in an atmosphere of 5% CO_2/5% O_2/90% N_2 for at least 1 hr before microsurgical manipulation.

Donor Cell Isolation. Donor parthenogenetic embryos were obtained from LT mice after ovulation was induced with pregnant mare's serum and human chorionic gonadotropin. Approximately 10% of ovulated LT oocytes cleave spontaneously (7). Parthenogenetic morulae or early blastocysts were flushed from the uteri on day 4 (approximately 80 hr after administration of the gonadotropin) and cultured overnight. This culture period was necessary in order to allow the parthenogenetic blas-

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Abbreviations: B6, C57BL/6J; LT, LT/Sv; ICR, ICR/Swiss; TE, trophectoderm; ICM, inner cell mass; GPI, glucosephosphate isomerase (EC 5.3.1.9).

tocysts to expand fully and to facilitate the mechanical separation of the ICM from the trophectoderm (TE). The ICM or TE was dissociated into individual cells by a short incubation in Ca^{2+}- and Mg^{2+}-free Hanks' solution (GIBCO) containing 1% pancreatin (GIBCO) and 0.25% trypsin (GIBCO) followed by rapid pipetting of the cells in Ca^{2+}- and Mg^{2+}-free Hanks' solution containing 5 μg of DNase per ml. The isolated cells were incubated at 37°C in Dulbecco's modified Eagle's medium plus 10% fetal calf serum under paraffin oil in an atmosphere of 5% CO_2/5% O_2/90% N_2 until transplantation of their nuclei into recipient eggs.

Micromanipulation. Usually 5 zygotes and about 15–20 donor cells were transferred to a welled microscope slide containing a drop of culture medium with cytochalasin B under halofluorocarbon oil (Voltalef 10S). A fertilized egg was firmly attached to a holding pipette and an ICM or TE cell was sucked into the transfer pipette whose diameter was smaller than that of the cell, causing its rupture but leaving the intact nucleus within the pipette. The nucleus was immediately injected into the egg cytoplasm and both pronuclei were sucked into the same pipette before its withdrawal from the egg (15). Eggs surviving nuclear transplantation were subsequently cultured (14) during their preimplantation development.

Preimplantation and Postimplantation Development. Participation of the transplanted ICM or TE nucleus in preimplantation development was determined by analyzing the strain-specific allelic variants of glucosephosphate isomerase (GPI) by using cellulose acetate electrophoresis (16). Individual embryos at 5 days after nuclear transplantation were treated for enzyme analysis as described (15). Postimplantation development of day 5 nuclear transplant embryos occurred after their transfer into uteri of day 3 ICR/Swiss (ICR) pseudopregnant foster mothers who were allowed to carry the embryos to term. Offspring were analyzed for coat color and GPI expression to determine whether development was exclusively derived from the transplanted LT parthenogenetic nucleus. For chromosome analysis, short-term skin and organ cultures were established from the nuclear transplant mice. The cultured cells were further processed for karyotyping as described (15).

RESULTS

Preimplantation Development. Data in Table 1 (series A) illustrate the proportion of recipient B6 eggs surviving the injection of either ICM or TE cell nuclei from LT parthenogenetic blastocysts and the subsequent embryonic development of these eggs after 5 days in culture. Fifty-two percent of the eggs survived transplantation of an ICM cell nucleus and removal of pronuclei whereas only 30% of the eggs remained intact after TE cell nuclear injection. A larger proportion of the ICM than the TE surviving nuclear transplant eggs cleaved (57% versus 41%), although no difference was observed in the proportion of cleaving eggs developing to morulae or blastocysts between the two groups (38% versus 36%). These results are similar to those of earlier studies using nuclei from ICM or TE cells of fertilized blastocysts (15). In order to investigate whether the transplanted nuclei from TE or ICM cells had promoted preimplantation development, some of the resulting embryos (Fig. 1) were prepared for GPI assay.

Table 1. Preimplantation development supported by nuclei from ICM or TE cells from parthenogenetic mouse LT blastocysts after transplantation into fertilized but enucleated B6 eggs

Donor cell type	Eggs, no.			No. reaching various development stages		
	Operated	Cultured	Cleaved	2–8 cells	Morula	Blastocyst
			Series A			
ICM	54	28	16	10	3	3
TE	94	27	11	7	4	—
			Series B			
ICM	107	38	25	18	3	4

As comparison and control, see our previously published data with ICM and TE nuclei from normal blastocysts (15).

Enzyme analysis was carried out on seven preimplantation stage embryos (three 4- to 8-cell embryos and four morulae, all of which appeared to be abnormal) that developed after transplantation of TE cell nuclei. Three embryos (Fig. 2A, lanes 4, 5, and 7) expressed only the B6 parental GPI variant as a result of incomplete enucleation. Four embryos (Fig. 2A, lanes 1, 2, 3, and 6) exhibited both parental and the heteropolymeric hybrid bands, probably due to a possible coexistence between a residual egg genome and the transplanted nucleus. Alternatively, the egg-specific GPI contribution may have been derived from a maternal cytoplasmic mRNA or enzyme pool. No embryos developed exclusively from TE cell nuclei of parthenogenetic blastocysts, as judged from our GPI analysis. Similar restrictions in developmental potency of transplanted TE cell nuclei have also been observed in normal blastocysts (15).

Eight embryos (two 4- to 8-cell embryos, four morulae, and two blastocysts) developing after ICM nuclear transplantation were analyzed for their GPI activity (Fig. 2B). Two early embryos expressed only the GPI variant of the B6 strain (lanes1 and 2), presumably due to incomplete enucleation. These embryos arrested at the 4- and 5-cell stages, respectively. Two embryos revealed both the LT and B6 variants with the heteropolymeric hybrid band (lanes 4 and 6) as a result of incomplete enucleation or maternally derived mRNA, giving rise to GPI heterodimers during translation. Two embryos exhibited both parental variants without the hybrid band (lanes 3 and 5). Such a GPI pattern may have originated from incomplete enucleation and subsequent segregation of the remaining egg nuclear genome into one cleavage blastomere and functional integration of the transplanted nucleus into the other blastomere, thus giving rise to two different cell lineages. The remaining two embryos showed only the LT specific band (lanes 7 and 8). The presence of a faint recipient egg-specific enzyme band in these two embryos most likely resulted from residual GPI of maternal origin. This enzyme analysis of early embryonic stages demonstrates that ICM cell nuclei of LT parthenogenetic blastocysts support normal preimplantation development after their transplantation into fertilized eggs.

Postimplantation Development. In this experiment (Table 1, series B), the proportion of eggs surviving after transplantation of an ICM cell nucleus from LT parthenogenetic blastocysts was less (36% versus 52%) than observed in the preimplantation studies (Table 1, series A). However, a larger proportion of the surviving eggs cleaved (66% versus 57%) although fewer cleaving eggs developed to morulae or blastocysts (28% versus 38%). Seven experimental embryos and 23 control albino ICR embryos were transferred to the uteri of three pseudopregnant ICR foster mothers (Table 2). Two of the three females became pregnant; one foster mother gave birth to one dark-eyed female and seven albino control mice. The other pregnant female gave birth prematurely to three dark-eyed female babies and five albino control fetuses that died shortly after birth.

The dark-eyed baby from the first foster mother subsequently developed the light coat color characteristic of the LT strain (Fig. 3), and analysis of a blood cell lysate showed only the LT strain-specific variant of GPI (Fig. 4, lane 1). Matings

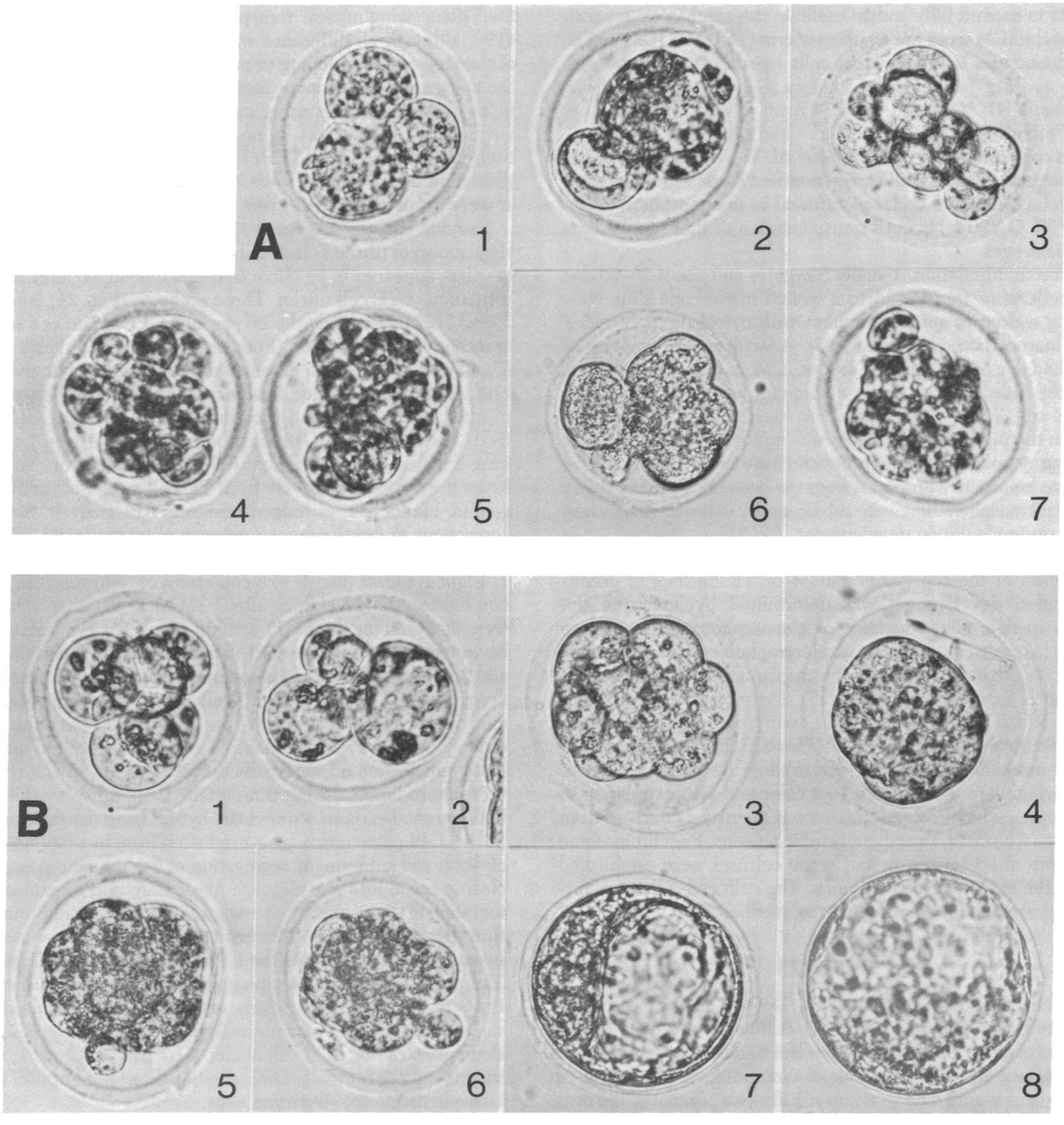

FIG. 1. Nuclear transplant embryos at various preimplantation stages of development. Nuclei from TE and ICM cells of LT parthenogenetic blastocysts were transplanted into fertilized but enucleated B6 eggs. (*A*) TE series: (*1* and *2*) 4-cell stage; (*3*) 8-cell stage; (*4–7*) early morulae. All embryos appear to be morphologically abnormal. (*B*) ICM series: (*1* and *2*) 4- to 5-cell stage; (*3–6*) morulae; (*7* and *8*) blastocysts apparently normal morphologically. For each embryo, the corresponding GPI pattern is shown in Fig. 2.

of this female to males of the LT strain have produced 33 (15 female and 18 male) normal LT offspring. Enzyme analysis of tissue homogenates from the other three dark-eyed females expressed only the LT variant of GPI (Fig. 4, lanes 2, 3, and 4). Chromosomal analysis revealed that all four nuclear-transplant mice had a diploid karyotype indicating two X chromosomes. Our data demonstrate that the nucleus of the ICM cell of the LT parthenogenetic blastocyst will support full-term development when transplanted into the fertilized egg.

DISCUSSION

Mouse oocytes can be parthenogenetically activated either experimentally (5) or as occurs spontaneously in the LT inbred strain (7). Parthenogenetic oocytes cleave and can develop into normal-appearing blastocysts which implant but die within a few days (10, 11). There is no evidence confirming the birth of a mouse parthenote developing from an unfertilized egg. On the other hand, chimeric mice derived from aggregation of diploid LT parthenogenetic and normal fertilized embryos exhibit significant cellular contributions, including the germ line, from the parthenogenetic embryonic cells (7, 8, 13). Apparently these cells do survive to the adult stage when associated with normal cells. Similarly, cells of parthenogenetic embryos proliferate and differentiate into adult-appearing cells when transplanted to extrauterine sites such as the testis (7) or kidney (17).

In LT females, parthenogenetic oocytes remaining within the

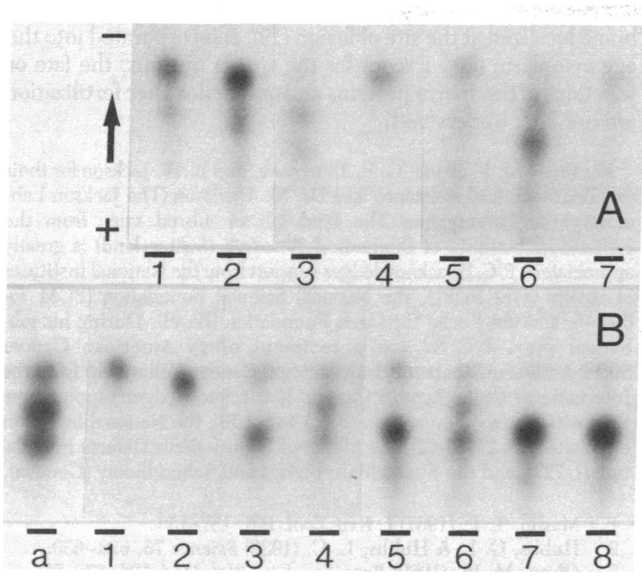

FIG. 2. GPI analysis by microelectrophoresis of total cell lysates of single preimplantation stage embryos developing after transplantation of nuclei from TE (*A*) or ICM (*B*) from LT parthenogenetic blastocysts into fertilized but enucleated B6 eggs. A blood cell lysate control (lane a) of an F_1 hybrid mouse (LT × B6) shows the fast-migrating GPI pattern of the B6, the slower migrating GPI variant of the LT, and the heteropolymeric band. (*A*) From seven embryos developing after transplantation of nuclei from TE cells, three express only the B6 parental GPI (lanes 4, 5, and 7) and the four other embryos show the GPI pattern of the F_1 hybrid (lanes 1, 2, 3, and 6). (*B*) In contrast, two embryos developed from the transplanted ICM cell nuclei as evidenced by their GPI expression of only the LT parental type (lanes 7 and 8). The faint egg-specific band observed in these two blastocysts most likely resulted from a persisting maternal GPI pool. Two embryos exhibited only the B6 pattern (lanes 1 and 2), two embryos showed both parental bands but not the heteropolymeric band (lanes 3 and 5), and two embryos expressed the characteristic F_1 hybrid pattern (lanes 4 and 6). For each GPI test, the corresponding embryo is shown in Fig. 1.

ovary develop into ovarian teratomas which are usually benign and are composed of differentiated cells derived from all three germ layers (7). Infrequently these tumors are malignant, and

Table 2. Postimplantation development supported by ICM cell nuclei from LT parthenogenetic blastocysts transplanted into fertilized but enucleated B6 eggs

Genotype of embryo	Embryos transferred	Mice born	
		No.	%
LT nuclear transplant	7*	4†	57
ICR control	23	12	52

* Embryos at the morula and blastocyst stage from the experimental series B (see Table 1) were transferred with control ICR embryos into uteri of three ICR foster mothers.
† Three female mice died after premature birth.

injection of these LT teratocarcinoma cells into blastocysts results in the birth of chimeric mice in which the injected cell participated in normal development and differentiation (12). The contribution or mechanism by which parthenogenetic cells can be developmentally "rescued" when in contact with normal embryonic cells or adult somatic cells has not yet been determined. The birth of isodiploid mice developed from the genome of only one parent, either maternal or paternal in origin (18), demonstrates that the cause of early parthenote death is not a consequence of containing only the maternal genome. Development of these completely homozygous mice also argues against the possible expression of recessive lethal genes as the primary cause of parthenogenetic embryonic mortality in inbred strains of mice. Although it cannot be ruled out that interaction of normal cells with parthenogenetic cells allows further development beyond the usual lethal period by compensating for possible genetic defects, the evidence suggests that the arrested development of LT parthenogenetic embryos may involve aberrant organismic rather than genetic factors. It is therefore desirable to ascertain the developmental capacity of the genome of parthenogenetic embryos directly by transplantation of their ICM and TE cell nuclei into fertilized but enucleated mouse eggs.

The results from nuclear transplantation experiments demonstrate that nuclei from the ICM of parthenogenetic embryos supported development of full-term offspring. Of 107 eggs injected with nuclei and subsequently enucleated, four female

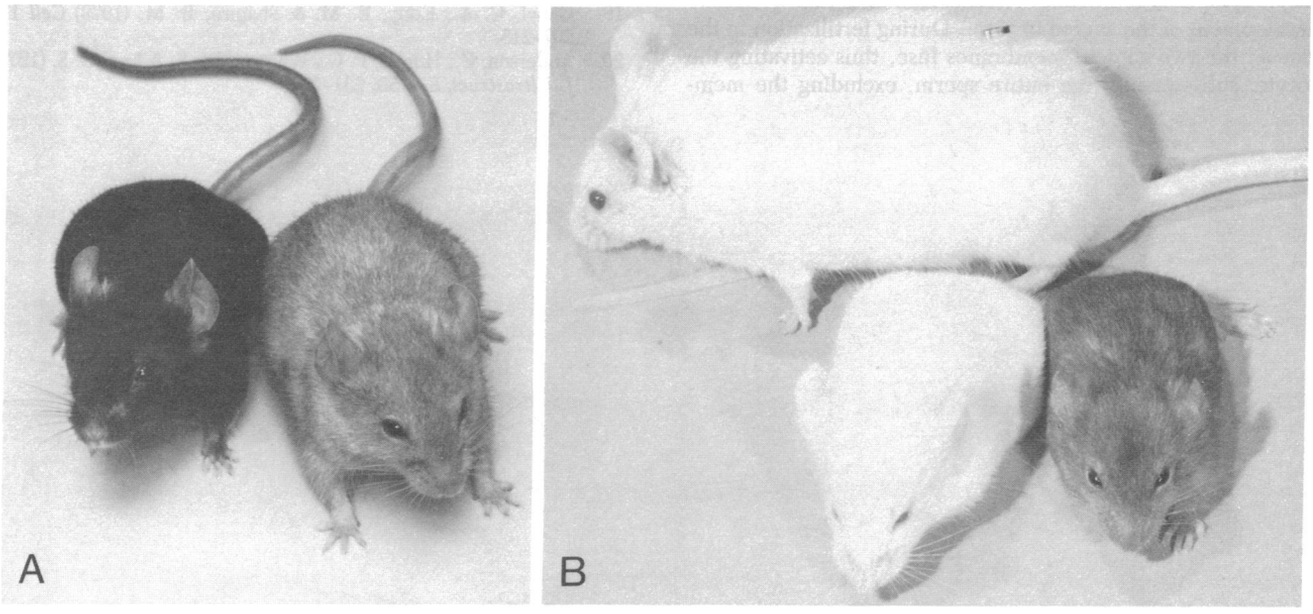

FIG. 3. (*A*) Mice from the recipient B6 and donor LT inbred strains, showing their different phenotypes in coat color. (*B*) ICR foster mother with an albino ICR control offspring and a female whose coat color demonstrates that her development was supported by the transplanted nucleus of an ICM cell from an LT parthenogenetic blastocyst.

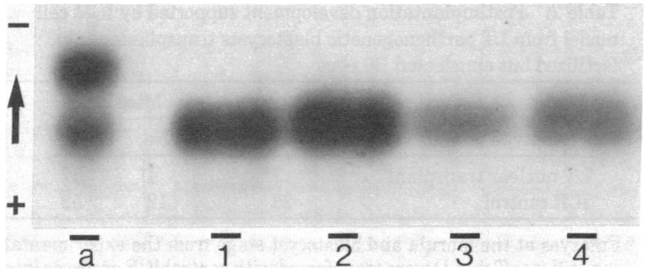

FIG. 4. GPI analysis of postnatal mice derived from transplanted nuclei of the ICM cells of LT parthenogenetic blastocysts. Cellulose acetate electrophoresis of GPI from a control blood cell lysate mixture is illustrated in lane a, showing the faster-migrating GPI band of the egg recipient B6 strain and the slower migrating enzyme variant of the LT nuclear donor type. A blood cell lysate (lane 1) or tissue homogenates (lanes 2, 3, and 4) of four female offspring all show only the slow-migrating band of the donor nucleus genotype.

offspring were born which is not significantly different from earlier nuclear transplantation studies using nuclei from ICM cells of fertilized embryos, 3 offspring born from 148 injected eggs (15). Further evidence of the similarities in developmental potential of nuceli from parthenogenetic and fertilized embryos was obtained by an experimental series involving the transplantation of TE cell nuclei from parthenogenetic blastocysts. Enzyme analysis of preimplantation stage embryos showed that in no case was development supported exclusively by the transplanted TE cell nucleus. This developmental restriction was also observed after transplantation of TE cell nuclei from normal embryos (15) and demonstrates that parthenogenetic blastocysts differentiate similarly in this respect.

We have shown that the diploid nucleus of the LT parthenogenetic embryo supports full-term development when transplanted into the cytoplasm of the fertilized egg and therefore we conclude that the death *in utero* of mouse parthenotes cannot be caused primarily by genomic abnormalities as previously suggested (reviewed in ref. 5).

Although it is based on indirect evidence, we assume that the sperm may provide a stimulus, substance, or organelle to the oocyte cytoplasm at the time of fertilization which allows normal development of the zygote to term. During fertilization in the mouse, the two gamete membranes fuse, thus activating the oocyte; subsequently the entire sperm, excluding the membrane localized at the site of fusion (19), is incorporated into the egg cytoplasm (20). Except for the sperm nucleus, the fate or functions of the sperm proteins and organelles after fertilization are not fully understood.

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