

results were confirmed by testing the effects of the monoclonal antibody or antigen-antibody complexes on the proliferative response of a Moloney leukaemia virus-specific Lyt-1<sup>+</sup>2<sup>+</sup> cytolytic T-cell clone<sup>7</sup> and a *Leishmania tropica* specific Lyt-1<sup>+</sup>2<sup>-</sup> T-cell clone<sup>8</sup>, neither of which was significantly inhibited (Table 2).

When irradiated spleen cells were 'pulsed' with beef apo-cytochrome *c* before subsequent culture with the T-cell clone 2-16 and the SJL 2-4 monoclonal antibody (Table 3), a similar dose-dependent inhibition of antigen-induced proliferation was observed. These results suggest that the inhibition was not due to an impairment of the uptake and/or appropriate processing of the apo-cytochrome *c* molecules. The SJL 2-4 antibody also inhibited the antigen-induced proliferation of two additional long-term T-cell lines and three clones (of both BALB/c and SJL origin) which are specific for the apo-1-38 fragment of the cytochrome *c* molecule (data not shown). Since the monoclonal antibody was generated by immunization of SJL mice, these results suggest that a similar cytochrome *c* peptide is generated during antigen processing in both SJL and BALB/c strains of mice.

**Table 3** T-cell clone proliferation induced by 'antigen pulsed' spleen cells is inhibited by monoclonal antibodies directed against apo-cytochrome *c*

	Apo-cytochrome <i>c</i> used to pulse spleen cells ( $\mu\text{g ml}^{-1}$ )	Monoclonal antibodies ( $\mu\text{g ml}^{-1}$ )	Proliferation of clone 2-16 (c.p.m. $\times 10^{-3}$ )
Expt 1	500	—	2.2 $\pm$ 0.2
		50	0.9 $\pm$ 0.1 (59)
		100	0.4 $\pm$ 0.1 (100)
		300	0.3 $\pm$ 0.1 (100)
		—	6.0 $\pm$ 1.0
2,000	—	50	5.5 $\pm$ 0.2 (9)
		100	6.5 $\pm$ 0.7 (0)
		300	0.8 $\pm$ 0.1 (87)
		—	5.9 $\pm$ 0.3
Expt 2	500	—	5.9 $\pm$ 0.3
		300	0.8 $\pm$ 0.1 (93)

Irradiated (2,000 rad) BALB/c spleen cells ( $10^6$ ) were incubated for 2 h with different concentrations of apo-cytochrome *c* and washed four times with medium before the addition of  $10^4$  T-cell clone 2-16. Purified SJL 2-4 monoclonal antibodies were used as described in Table 1 legend. The proliferation of control cultures in the absence of added apo-cytochrome *c* was 400 c.p.m. Values in parentheses represent the per cent inhibition of T-cell clone proliferation.

Thus, it should now be feasible to purify and elucidate the biochemical structure of the defined antigenic determinant presented on the macrophage cell surface which subsequently stimulates antigen-specific T-cell clones to proliferate and secrete lymphokines.

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## Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis

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It has been suggested that the failure of parthenogenetic mouse embryos to develop to term is primarily due to their aberrant cytoplasm and homozygosity leading to the expression of recessive lethal genes<sup>1</sup>. The reported birth of homozygous gynogenetic (male pronucleus removed from egg after fertilization) mice and of animals following transplantation of nuclei from parthenogenetic embryos to enucleated fertilized eggs<sup>2,3</sup>, is indicative of abnormal cytoplasm and not an abnormal genotype of the activated eggs. However, we<sup>4</sup> and others<sup>5,6</sup> have been unable to obtain such homozygous mice. We investigated this problem further by using reconstituted heterozygous eggs, with haploid parthenogenetic eggs as recipients for a male or female pronucleus. We report here that the eggs which receive a male pronucleus develop to term but those with two female pronuclei develop only poorly after implantation. Therefore, the cytoplasm of activated eggs is fully competent to support development to term but not if the genome is entirely of maternal origin. We propose that specific imprinting of the genome occurs during gametogenesis so that the presence of both a male and a female pronucleus is essential in an egg for full-term development. The paternal imprinting of the genome appears necessary for the normal development of the extraembryonic membranes and the trophoblast.

Eggs from (C57BL/6J  $\times$  CBA/Ca)F<sub>1</sub> females (henceforth called F<sub>1</sub>) were activated and the haploid parthenogenetic eggs selected at about 5 h post-activation to serve as recipient eggs for either a male or a female pronucleus taken from fertilized eggs (see Table 1). The donor male pronuclei were obtained from F<sub>1</sub>  $\varnothing$   $\times$  MF1  $\delta$  eggs and the female pronuclei from MF1  $\varnothing$   $\times$  F<sub>1</sub>  $\delta$  eggs or F<sub>1</sub>  $\varnothing$   $\times$  MF1  $\delta$  eggs. Reconstituted eggs with two distinct pronuclei were cultured overnight, when all of them cleaved to the two-cell stage. These embryos were transferred to the right oviducts and control MF1  $\times$  MF1 embryos to the left oviducts of day 1 MF1 pseudopregnant mice (day 1 = day of vaginal plug).

The first series of experiments produced, at day 15, two normal embryos from five haploid eggs reconstituted with a male MF1 pronucleus. By contrast, in 29 reconstituted haploid eggs into which a second female MF1 pronucleus was introduced, 15 embryos implanted after transfer of the eggs but all the implantation sites were resorbing without any detectable embryonic derivatives. Therefore, all of the former type of reconstituted eggs were allowed to proceed to term (day 19-20 of pregnancy) while the rest were examined on day 10 of pregnancy to assess the cause of their failure to develop. Day 10 of pregnancy was chosen from our previous experience of development of gynogenetic<sup>4</sup> and parthenogenetic<sup>7</sup> embryos which deteriorate after this time.

The results show that introduction of a male MF1 pronucleus can rescue parthenogenetic haploid eggs, with nearly 38% of them reaching term but a female F<sub>1</sub> or MF1 pronucleus is unable to do so (see Table 1). However, between 50 and 79% of the latter implanted, which shows that most of them had at least developed to the blastocyst stage, but all the decidual swellings at day 10 were about half the volume of their normal controls whether or not they contained embryos. The few embryos present were small or retarded with poor extraembryonic membranes and trophoblast. The most advanced embryo was perfectly formed but it was very small; for a brief description of other embryos see Fig. 1 legend.

**Table 1** Development of haploid activated eggs reconstituted with a male or a female pronucleus

Type of reconstituted egg*	No. of eggs		Embryos†				
	With fused karyoplasts/total operated	Transferred to recipients that became pregnant/total transferred	Implanted	Normal	Small	Retarded and abnormal	None
(1) Pn+Ak MF1	30/65	24/30	—	9 Live young‡			
(2) Pn+Gk MF1	57/96	48/57	38	0	8	12	18
(3) Pn+Gk F <sub>1</sub>	38/73	24/38	12	0	0	4	8
(4) Pn+Pnk	15/22	7/15	4	0	0	0	4
(5) Unoperated fertilized MF1×MF1	—	106/162	88	65	4	2	17

Cumulus masses containing unfertilized eggs were recovered 16.5–17.0 h after an injection of 5 IU human chorionic gonadotropin (HCG) in animals which had been injected previously with 5 IU pregnant mare's serum (48 h before injection of HCG) to induce superovulation. The cumulus cells were dispersed by incubation in 300 IU ml<sup>-1</sup> hyaluronidase (ovine testis) in phosphate-buffered saline (PBI)<sup>19</sup> plus 0.4% bovine serum albumin (BSA) for 3–5 min at 37 °C. Eggs were activated by culture at room temperature for 7 min in M16 plus 0.4% BSA plus 7% absolute ethanol<sup>20</sup>, washed 6–9 times in M16 plus 0.4% BSA<sup>21</sup> and cultured in this medium under paraffin oil at 37 °C in humidified 5% CO<sub>2</sub> in air. The second polar body was extruded and a single pronucleus was detected after ~5 h in 70–90% of the parthenogenetic activated eggs. Fertilized eggs were obtained at the same time from F<sub>1</sub>♀×MF1♂ and MF1♀×F<sub>1</sub>♂ matings. The transfer of donor pronuclei from fertilized eggs to haploid parthenogenetic eggs was carried out on a Leitz micromanipulator<sup>22</sup> essentially as described previously<sup>23</sup>. The donor and recipient eggs were placed in PBI+0.1% BSA containing the cytoskeletal inhibitors cytochalasin D and Nocodazole (Sigma) at a final concentration of 1 µg ml<sup>-1</sup> and 0.05 µg ml<sup>-1</sup> respectively, dissolved in 1.5 µl dimethyl sulphoxide. After 30–45 min at 37 °C, the eggs were suspended in hanging drops for micromanipulation. Using a 25-µm bevelled needle, donor pronuclei were drawn into the needle and the membrane pinched off to make a small karyoplast fragment, followed by approximately 6 pl of β-propiolactone-inactivated Sendai virus<sup>24,25</sup> (3,000 haemagglutination units ml<sup>-1</sup>) to induce fusion<sup>26</sup>. Virus and karyoplast were then gently released into the perivitelline space underneath the zona pellucida of the recipient haploid parthenogenetic eggs, usually at the opposite end to the polar body. The recipient eggs with adhering karyoplasts were washed twice in PBI plus 0.1% BSA and cultured in M16 plus 0.4% BSA at 37 °C as described above. The eggs were checked for fusion of the karyoplast ~4 h after the completion of manipulation on the last group of eggs. Fusion was confirmed by the disappearance of the karyoplast and the appearance of two pronuclei inside the recipient egg. The fusion rate was between 30 and 65%. The diploid reconstituted eggs were separated from the haploid ones with unfused karyoplasts, washed nine times in equilibrated M16 plus 0.4% BSA and cultured overnight in this medium at 37 °C in 5% CO<sub>2</sub> in air under paraffin oil. The following morning all the eggs had cleaved, and they were transferred to the oviducts of day 1 MF1 outbred albino pseudopregnant mice.

\* (C57BL×CBA)F<sub>1</sub> haploid activated eggs: Pn (parthenogenone) were used as recipients for a male pronucleus, Ak MF1, from F<sub>1</sub>♀×MF1♂ eggs (1). The haploid eggs were also used as recipients for a female pronucleus, Gk MF1, from MF1♀×F<sub>1</sub>♂ eggs (2), or Gk F<sub>1</sub>, from F<sub>1</sub>♀×MF1♂ eggs (3). In a few haploid eggs, a second pronucleus from activated eggs, Pnk, was introduced (4).

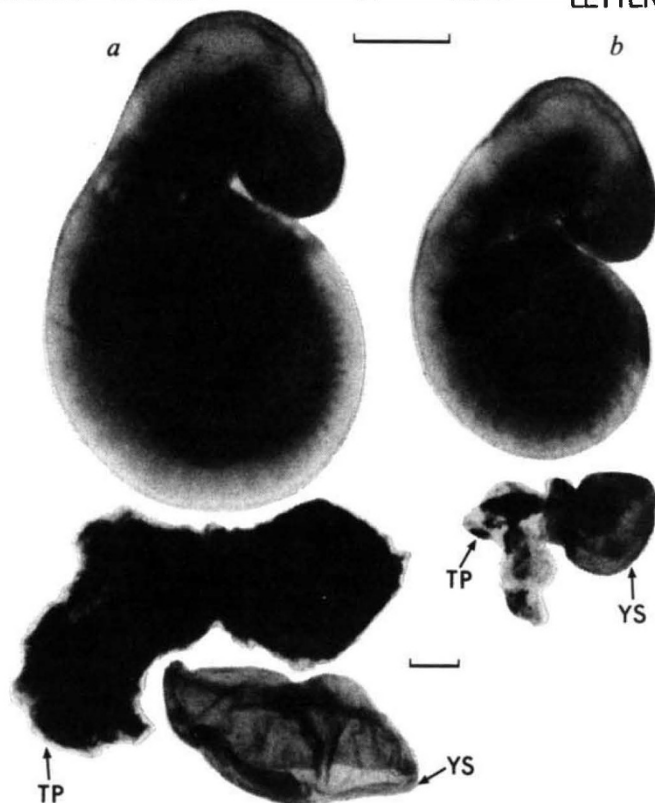
† The F<sub>1</sub> animals are non-albino, homozygous for glucose phosphate isomerase (GPI bb) while the MF1 are outbred albino (GPI aa). The live young and the other experimental and control embryos were typed for GPI<sup>27</sup>. The live young (1) and the experimental embryos in (2) were GPI ab, the experimental embryos in (3) were GPI bb and the controls (5) were GPI aa. All the embryos were examined on day 10 of pregnancy apart from those in group (1) which were allowed to go to term.

‡ Five females and four males were obtained. All the animals have now reached adulthood and they have bred normally.

Carry-over of a small amount of fertilized egg cytoplasm (2–5% of the total egg volume) and plasma membrane cannot account for these results as it occurred to an equal degree in both male and female pronuclei. It is unlikely that a specific cytoplasmic region from fertilized eggs was included with the male pronucleus because the site of sperm entry into the egg is random and the pronuclei migrate continuously from the periphery to the centre of the egg. We cannot exclude entirely the possible transfer of specific extragenetic components closely associated with the male pronucleus. However, when the eggs are introduced into the cytoskeletal inhibitors before micromanipulation (see Table 1), they lose their rigidity, becoming more 'fluid' so that considerable mixing of the cytoplasmic contents occurs during the extraction of the donor pronuclei. Furthermore, we have not obtained development of activated eggs to term by introducing cytoplasm from fertilized eggs (unpublished observations), nor of diploid gynogenones<sup>4</sup> and, from preliminary results, of heterozygous gynogenetic embryos. Taken together, the results on the rescue of parthenogenones by a male pronucleus do not support the notion that the cytoplasm of activated eggs is aberrant<sup>2,3</sup>. The failure of development to term of heterozygous reconstituted eggs having two female pronuclei also does not support the contention that homozygosity is a primary cause of the death of gynogenones<sup>4</sup> and parthenogenones<sup>1,6,7,8</sup>. Although a male and a female pronucleus are presumably equivalent in their genetic contribution to the embryo, the crucial difference lies in their origin as the former is derived after spermatogenesis and the latter after oogenesis. We propose that specific imprinting of the paternal and maternal genomes occurs during gametogenesis, which is perhaps why both of them are required in an egg for full-term development.

There is good evidence for paternal imprinting of the mouse genome and some paternal genes are expressed as early as the two-cell stage<sup>9</sup>. In the extraembryonic membranes and trophoblast of female embryos, the paternal X chromosome is preferentially inactivated<sup>10–12</sup>. There are conflicting results on X-inactivation in these tissues in parthenogenetic embryos<sup>13,14</sup>, but variable X-inactivation has been suggested as a cause of their poor development<sup>13</sup>. In addition, studies on DDK mutant mice suggest that the cytoplasm of eggs from this strain of mice cannot activate some specific paternal genes from alien males, essential for the development of embryos after implantation and especially for the normal development of the trophoblast<sup>15</sup>. On the basis of these results, reconstituted eggs having two female pronuclei and lacking a paternal genome are likely to be affected in a similar way, which they apparently are.

If the primary defect in embryos having the maternal genotype is in the extraembryonic membranes, this may explain the apparent discrepancy between the ability of these embryonic cells to proliferate and differentiate in ectopic sites<sup>16,17</sup> and chimaeras<sup>8,18</sup>, and their poor development when relying on their own extraembryonic tissues to sustain them. Development of embryos with two female pronuclei in this study is no different from that of gynogenetic<sup>4</sup> or parthenogenetic embryos<sup>7</sup>. Nearly 25% of the parthenogenetic embryos can reach about this level of development but deteriorate rapidly thereafter (ref. 7 and S.C.B. and M.A.H.S., unpublished). This is perhaps not surprising as the embryo increasingly relies on the extraembryonic tissue for nutrition, which develops particularly poorly in the absence of the paternal genome. However, there could also be other less obvious effects of the genetic constitution of eggs on embryonic development.



**Fig. 1** Two embryos obtained on day 10 of pregnancy (scale bar, 0.5 mm): *a*, control MF1 x MF1 embryo (GPI *aa*); *b*, the most advanced embryo obtained from a reconstituted parthenogenetic haploid egg with an MF1 female pronucleus (GPI *ab*). Their corresponding trophoblasts (TP, mural trophoblast and ectoplacental cone) and yolk sacs (YS) are shown directly underneath the embryos at a reduced magnification (scale bar, 0.5 mm). The experimental embryo, apart from its small size, was apparently normal, with about 25 somites and a beating heart. Note the greatly reduced trophoblast tissue obtained with the experimental embryo. Nine live-born non-albino mice (GPI *ab*) were obtained from reconstituted haploid parthenogenetic F<sub>1</sub> eggs (GPI *b*) with an MF1 male pronucleus (GPI *a*) (see Table 1). All the embryos resulting from the reconstituted eggs with a female F<sub>1</sub> or MF1 pronucleus were cramped inside tiny yolk sacs and surrounded by very sparse trophoblast. There were two 20–25-somite embryos, three 15-somite embryos and three 6–8-somite embryos. All the embryos were less than half the volume of their normal controls of comparable somite stage, and all in very deficient membranes. There were two very abnormal embryos (one tail part only, one contorted deteriorating neural tissue), and a further 14 tiny yolk sac vesicles, often with a prominent Reichert's membrane. The remaining 26 implantation sites contained no embryonic derivatives.

Our results are in contrast to the reported birth of gynogenetic and parthenogenetic mice<sup>2,3</sup>. One explanation could be that in rare cases enough trophoblast develops to carry the embryos to term. Incomplete enucleation of some eggs also apparently occurred in previous studies<sup>2,3</sup> but whether this permits sufficient participation by the paternal genome to ensure development to term is unknown. Nevertheless, our studies suggest that specific imprinting of the genome during gametogenesis is essential for full-term development in the mouse. At some point during embryonic development and formation of primordial germ cells, previous influences on the genome are presumably lost and new ones initiated during the production of gametes.<sup>28</sup>

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## Maternal $T^{hp}$ lethality in the mouse is a nuclear, not cytoplasmic, defect

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The  $T^{hp}$  mutation, an allele of the mouse  $T/t$  complex, differs from other known mutations in that its effects are determined by the sex of the parent from which it is inherited; when inherited from the female parent, it is invariably lethal at the embryonic stage, but, most embryos which inherit the mutation from the male parent survive<sup>1,2</sup>. Thus most heterozygous embryos carrying the maternally derived mutation die in the second half of pregnancy, while the exceptional embryos surviving to parturition give oedematous, cyanotic individuals that die within 24 h<sup>3,4</sup>. The lethal maternal effect of  $T^{hp}$  may be transmitted either through the cytoplasm of the ovum (oogenic defect) or through the female pronucleus (embryogenic defect). Here we have sought to decide between these possibilities by performing reciprocal nuclear transplantations<sup>5</sup> between one-cell embryos from  $T^{hp}/+$  and  $+/+$  females. Our observation that this maternally inherited lethal effect of  $T^{hp}$  persists when  $T^{hp}/+$  pronuclei are transplanted into  $+/+$  cytoplasm suggests that the defect responsible for the pattern of inheritance lies in the pronuclei and not the cytoplasm.

One cell-stage embryos obtained from  $+/+$  albino females mated to  $+/+$  albino males were used in reciprocal nuclear transplantations with one-cell stage embryos obtained from pigmented  $T^{hp}/+$  females previously mated to pigmented  $+/+$  males. Nuclear transplant embryos were transferred to the oviducts of day 1 pseudo-pregnant females and allowed to develop to term. The number, coat colour phenotype and tail length of the progeny were observed, as  $T^{hp}$  causes a shortening of the tail when heterozygous.

Of 197 successful nuclear transplant embryos (Table 1) in which wild-type pronuclei from albino females mated to albino males were introduced into the cytoplasm of enucleated embryos from pigmented  $T^{hp}/+$  females, 45 (20 females and 25 males) normal-tailed albino progeny resulted (23%). Among 206 successful nuclear transplant embryos in which pronuclei from pigmented  $T^{hp}/+$  females mated to pigmented males were introduced into the cytoplasm of enucleated embryos from albino females, 18 (10 females and 8 males) pigmented progeny were born (9%). Thus, a significantly greater proportion of nuclear transplant embryos, in which the egg cytoplasm was derived primarily from  $T^{hp}/+$  females, developed to term (23%) than those embryos in which egg cytoplasm was derived primarily from  $+/+$  females (9%) ( $\chi^2=8.99$ ;  $P<0.01$ ). We