GENETICS

Visualization and Cytogenetic Analysis of Second Polar Body Chromosomes Following Its Fusion with a One-Cell Mouse Embryo

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Submitted: April 28, 1994
Accepted: June 30, 1994

Purpose: *This study was designed to visualize the second polar body (2PB) chromosomes using its electrofusion with a one-cell-stage mouse embryo to approach preconception diagnosis of chromosomal disorders.*
Results: *Eighty to 90% hybridization efficiency has been achieved by electrofusion of 2PB with mouse zygotes. 2PB chromosomes were visualized in 40–50% of hybrids. Sixty-five percent of 2PB chromosomes were visualized when fused with the cytoplast obtained microsurgically by removing pronuclei from a one-cell embryo. As much as 33–43% of these resulting metaphases appeared to contain chromosomal aberrations. The follow-up of the development of the reconstructed one cell-stage hybrids in vitro revealed a significant decrease in their viability. The hybrid embryos resulting from 2PB electrofusion with enucleated zygotes did not develop beyond the two-cell stage.*
Conclusion: *Electrofusion is an efficient approach for hybridization of 2PB with a one-cell mouse embryo and may be useful for visualization and cytogenetic analysis of 2PB chromosomes. The visualization rate of 2PB chromosomes is higher if 2PB is fused with enucleated zygotes. However, the method induces over 30% of chromosomal aberrations and may lead to a significant decrease in the viability of the resulting one-cell embryos.*

KEY WORDS: second polar body (2PB); 2PB chromosomes; preimplantation diagnosis; electrofusion.

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INTRODUCTION

First polar body sampling has recently been introduced as an approach for preimplantation diagnosis (PD) of single-gene disorders (1,2). There has also been progress in approaching PD of chromosomal disorders by attempting visualization of the chromosomes of the second polar body (2PD) (3,4). Modlinsky and McLaren (3) microsurgically transplanted mouse 2PB into fertilized egg and demonstrated the possibility of transformation of 2PBs in a presumably haploid group of mitotic chromosomes. However, the success rate was low, and even when 2PB chromosomes were visualized, they were unsuitable for karyotyping. Dyban and collaborators (4) visualized 2PB chromosomes by treating one cell-stage mouse embryos with okadaic acid (a specific inhibitor of phosphates 1 and 2A), which induced the breakdown of the 2PB nucleus, making it possible to visualize analyzable chromosomes in 80% of cases. The visualized chromosomes of 2PBs were unichromatid G1 premature condensed chromosomes of good quality, suitable for differential staining.

The purpose of the present study was to investigate the possibility of visualization of 2PB chromosomes following its hybridization with a one cell-stage mouse embryo, using an electrofusion system which was demonstrated to be highly efficient for fusion of blastomeres (5,6).

MATERIALS AND METHODS

Eight- to 10-week-old female and 10- to 12-week-old male mice were obtained from The Jackson

1058-0468/94/0300-0123$07.00/0

Laboratory (Bar Harbor, ME). Two mouse strains were used: hybrid B6CBAF1/J (C57BL/6J × CBA/J) mice with a normal karyotype and inbred mouse strain CBA/CaH-T6/J, homozygous for reciprocal chromosomal translocation T(14;15)Ca with T6 marker chromosomes (7).

Superovulation was induced by sequential (44–48 hr apart) injection of 10 IU of pregnant mare serum gonadotropin (PMSG; Sigma) and human chorionic gonadotropin (hCG; Serono). After hCG injection females were caged with males, examined 17 hr later for the presence of vaginal plugs, and killed by cervical vertebral dislocation.

The ampular part of the oviduct was torn by needles and cumulus masses with oocytes were released in M2 medium (8) containing hyaluronidase (100 IU/ml; Sigma). Oocytes freed of attached cumulus cells were transferred to M2 medium without hyaluronidase and examined by interference phase-contrast optics (Nikon Diaphot). Only one cell-stage embryos with extruded 2PBs and recognizable pronuclei were selected for micromanipulation experiments. The experiments were performed on embryos at the early pronuclear (obtained 19–22 hr after hCG) and the middle pronuclear (recovered 23–26 hr post hCG) stage.

It was impossible to find out the exact time of the 2PB extrusion in a very heterogeneous population of one cell-stage embryos obtained after in vivo fertilization. Preliminary observations, however, had shown that the 2PB is present in many embryos 16 hr after hCG injection or 1 hr later in all one cell-stage embryos. Therefore, the age of the 2PB was estimated as hours post hCG, bearing in mind that 2PB were fully formed no later than 16–17 hr after hCG injection.

The 2PBs were isolated by micromanipulation (9) in drops of M2 medium, transferred to drops of M16 medium under mineral oil, and kept under standard culture conditions (at +37°C in a mixture of 90% N_2, 5% O_2, and 5% CO_2) until fusion with one cell-stage embryos.

2PB and/or pronuclei transplantation was performed using a modified technique proposed previously for mouse blastomere fusion (5,6). The sensor-type cell electrofusion system was custom-made by Bams Manufacturing Co., Inc. (Chicago, IL). Platinum electrodes were prepared from wire (diameter, 120 μm; Fisher). The following two electrofusion procedures were used.

(1) Eggs were placed for 7–10 min in a 0.3 *M* manitol solution in pure (18-MΩ) water supplemented with 0.5% polyvinylpyrrolidone (MW 360,000; Sigma), 0.05 m*M* $CaCl_2$, and 0.1 m*M* $MgSO_4$. A chamber with parallel-wire electrodes was used and the eggs were individually treated at room temperature with an electric current (single square pulse, 1.0 kV/cm; duration, 500 μsec). This procedure gave a high rate (80–90%) of fusion between a karyoplast and a one cell-stage embryo or between such an embryo and its own 2PB.

(2) Via a slit in the zona pellucida the 2PB was removed from the egg by micropipette and the isolated foreign 2PB was transferred into the perivitelline space by another micropipette. The produced pairs (foreign 2PB + egg) were placed in the chamber with needled electrodes filled with medium (9 parts M2 and 1 part pure water). The electrode tips (diameter 10–15 μm) were oriented in such a way that one of the tips was touching the zona pellucida above the place of the 2PB location, and the other the opposite side of the egg. Then the eggs were individually treated with an electrical current (one direct pulse, 68 V; duration, 40 μsec).

After treatment with electrical current the eggs were washed in M2, placed in drops of M16 under oil, and incubated for 30–50 min under standard culture conditions. Then the cells in which the 2PB had already been fused with an egg were selected and cultured for a further 8–12 hr in drops of M16 with colcemide (0.035 μg/ml). The chromosomal preparations from fusion products were made according to the technique described by Dyban (10), stained with a 2% Giemsa solution, and examined and photographed under a Nikon Microphot (oil immersion objectives, 60 and 100×).

After a short incubation in M2 medium supplemented with cytochalasin B (5 μg/ml; Sigma), pronuclei were removed from one cell-stage embryos using the technique described by McGrath and Solter (11) and cytoplasts or karyoplasts were placed in drops of M16 under oil and kept until further experiments under standard culture conditions. For pronucleus transplantation the first variant of the electrofusion technique (see above) was used. Then the manipulated and control eggs were incubated for 24–72 hr in drops of M16 under standard culture conditions, studied live under an inverted microscope (Diaphot, Nikon), and fixed. The air-dried preparations were made according to Dyban's (10) method and the number of nuclei was counted on slides stained with a 2% Giemsa solution.

RESULTS

Nuclear Changes in 2PBs Induced by the Cytoplasm of One Cell-Stage Embryos

Cytogenetic studies were carried out 8–12 hr following fusion of 2PBs with fertilized eggs, i.e., when one-cell embryos proceeded into mitosis, with their pronuclei being transformed into metaphase plates. In all experiments, an identical reaction of the 2PB nucleus to the recipient zygote cytoplasm was observed. A total of 248 products of fusion containing the pronucleus metaphase plates has been analyzed. Additional metaphase plates originating from the 2PB were present in 119 of these hybrids (47.9%), the rest containing a prematurely condensed chromatin resulting from 2PB nucleus envelope breakdown (NEBD). These 2PB nuclei were transformed into compact or granular chromatin masses, containing prematurely condensed chromosomes (PCC) of the G1, S, and G2 types (the most frequent type being S-PCC), described previously in interphase somatic nuclei transplanted in the mitotic cell cytoplasm (for review see Ref. 12).

Fusion of One-Cell Embryos with Their Own 2PB

As shown in Table I, of 124 hybrids of this type, 2PB metaphases were present in 42 of them (34%), the rest containing different types of PCC (Fig. 1). The efficiency of visualization of 2PB chromosomes depended on the age of the recipient one-cell embryo: Half of the hybrids with visualized 2PB chromosomes resulted from their fusion with early pronuclear-stage embryos (19–21 hr after hCG), 8% of 2PB chromosomes were visualized following fusion with older embryos (23 hr after hCG), and no 2PB chromosomes were present when fused with embryos obtained 24 hr after hCG, NEBD and PCC, mainly of the S type, being observed in such hybrids. Seventeen (40%) of 42 visualized 2PB metaphases had chromosome and chromatid aberrations, observed more frequently with an increase in the age of the recipient one-cell embryos (Fig. 2).

Fusion of 2PBs with Foreign One Cell-Stage Embryos

For this series of fusion experiments, 2PBs were obtained from T(14;15)Ca homozygous mouse embryos, so that the marker T6 chromosome could be identified in the resulting hybrids without chromosome banding (Fig. 3). As shown in Table II, of 78 hybrids with metaphases, 2PB chromosomes were visualized in 24 cases (31%). As in previous experiments the rest of the embryos (69%) had 2PB NEBD and PCC, mainly of the S type. Up to half of the 2PB metaphases were visualized following 2PB fusion with recipient embryos of corresponding ages, irrespective of the age of the PB. For example, the 2PBs of earlier ages (18–19 hr after hCG) fused with embryos of older ages resulted in only 19% of 2PD metaphase plates, 2PB NEBD and PCC being found in the other 81% of the hybrids. Accordingly, no metaphases were observed when the

Table I. Fusion of the One-Cell-Stage Mouse Embryo with Its Own 2PB

Analyzed embryos			Transformation of the 2PB nucleus into						
			Metaphase plate		NEBD and different types of PCC				
						Chromatin mass		PCC	
Group[a]	Age[b]	No.	Total No. (%)	No. with aberrations (%)[c]	Total No. (%)	Compact	Granular & fibrous	S	$S–G_2$
1	19	11	5 (45)	—	6 (55)	1	3	2	
2	20–21	16	9 (56)	—	7 (44)	2		5	
3	20–21	28	14 (50)	8 (55)	14 (50)	7	3	4	
4	22	27	12 (44)	7 (58)	15 (56)	10	2	3	
5	23	23	2 (8)	2 (100)	21 (82)	6	3	10	2
6	24	19	—		19 (100)	6	3	10	
1 & 2		27	14 (52)	—	13 (48)				
3–6		97	28 (29)	17 (61)	69 (71)				
Total		124	42 (34)	17 (40)	82 (66)	32	14	34	2

[a] Groups 1 and 2, eggs from a C_{57}BL/CBA mouse; Groups 3–6, eggs from a CBA/T_6T_6 mouse.
[b] Age of one cell-stage embryos (at the moment of fusion with 2PB) was estimated (hr) from hCG injection.
[c] Percentage from number of metaphase plates.

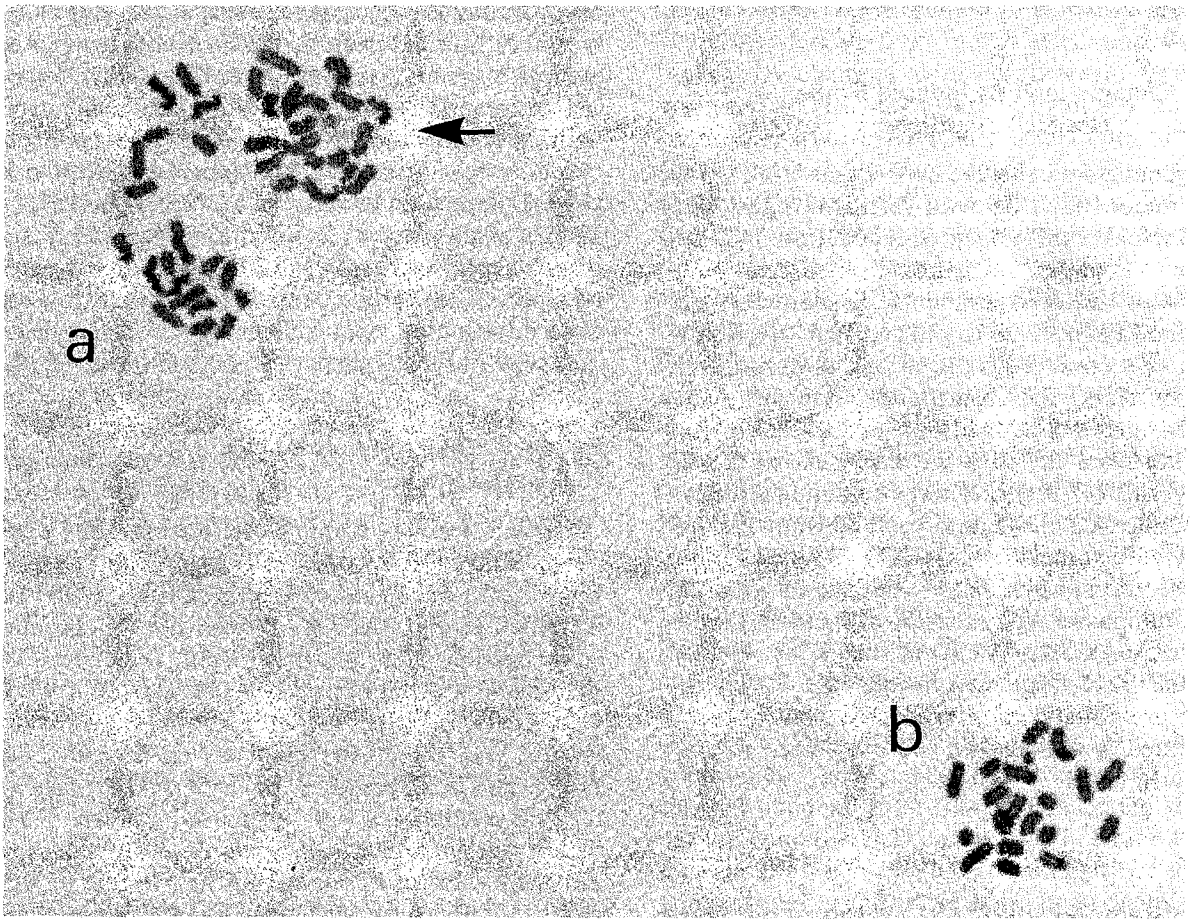

Fig. 1. Triploid one cell-stage embryo produced by fusion of the T6/+ zygote with its own 2PB: three haploid metaphase plates (n = 20) derived from (a) the paternal pronucleus and (b) the maternal pronucleus and 2PB nucleus (arrow). Metaphases of maternal origin have a T6 marker chromosome. Air-dried preparation stained with Giemsa Original magnification, ×930.

older 2PBs (23 hr after hCG) fused with younger embryos (21 hr after hCG), all hybrids showing 2PB NEBD and PCC.

As in previous experiments, 30% of all the 2PB metaphases identified in the resultant hybrids by the presence of a T6 marker chromosome had chromosome and chromatid breaks, more frequently observed with an increase in the age of the recipient embryos.

Fusion of 2PBs with One Cell-Stage Cytoplasts

For these experiments, both pronuclei from the recipient fertilized oocytes were removed before their fusion with 2PBs. Of 81 cybrids obtained in this way (Table III), 65% had 2PB metaphases (from 35 to 93–100% in different groups) (Fig. 4). Metaphases were visualized more frequently from "younger" 2PBs fused with cytoplasts of a corresponding age (groups 1 and 2 in Table III). With the age of 2PB and the age of the cytoplasm, the number of metaphases decreased considerably, with a corresponding increase in NEBD and PCC (groups 4 and 5 in Table III).

Preimplantation Development of the Reconstructed Mouse Eggs Produced by 2PB Transplantation

Results of these experiments are presented in Table IV. The first column shows the development of intact (control) diploid embryos, 95% of which developed to the late morula and blastocyst stage following 80 hr in culture, 65% of them being expanded and hatched by 85 hr. On the contrary, only 46% of the reconstructed diploid embryos in which the maternal pronucleus had been substituted with one of the 2PB (column 2) reached the four-cell stage, and those 32% that developed to the morula

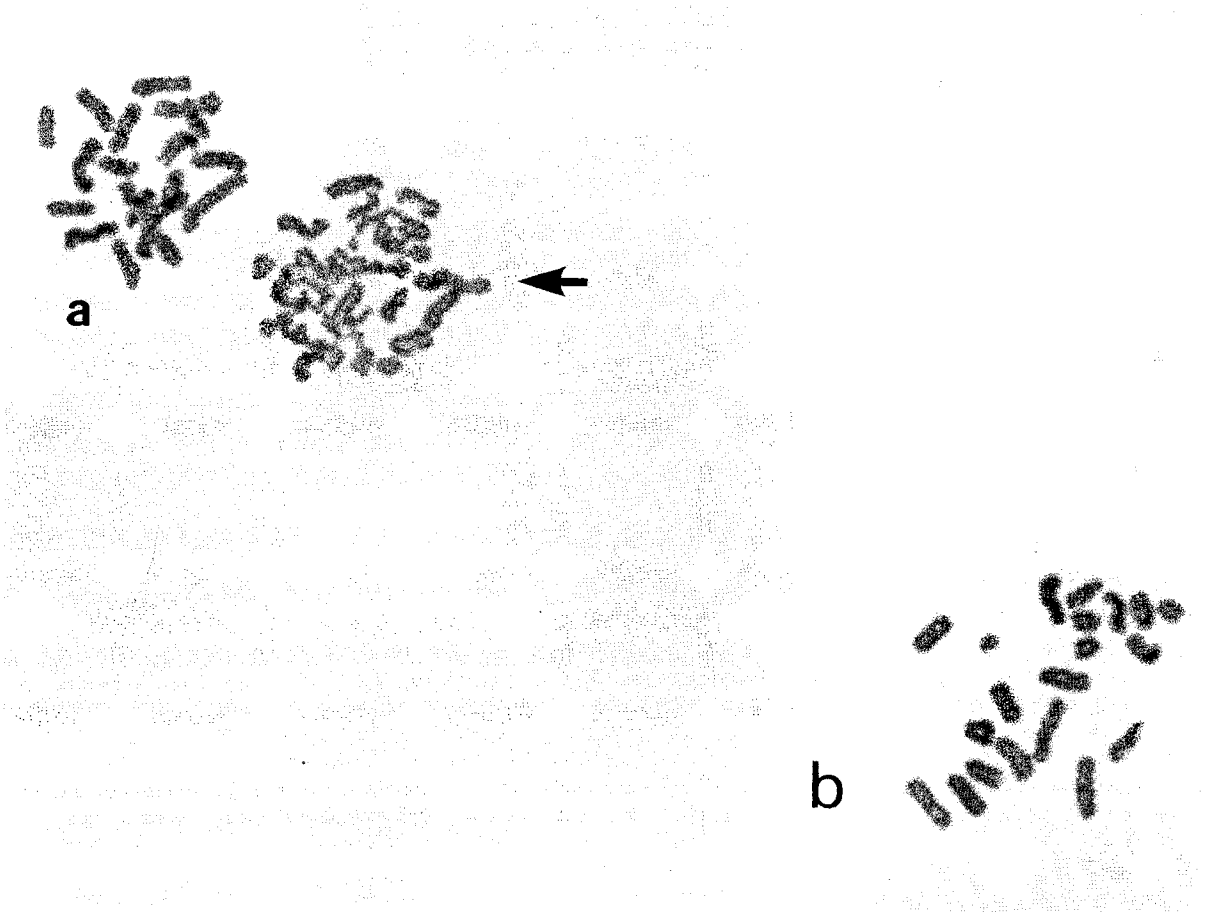

Fig. 2. Hybrid one cell-stage embryo obtained by fusion of a T6/T6 2PB with a B6CBAF$_1$ zygote: (a) paternal pronucleus and (b) maternal pronucleus and 2PB nucleus (arrow) transformed into haploid metaphases. Multiple chromatid and chromosome breaks are present in the 2PB nucleus. Air-dried preparation stained with Giemsa. Original magnification, ×1160.

and blastocyst stage contained fewer cells than the control embryos.

A similar developmental effect was observed in the reconstructed triploid embryos containing 2PB (column 4), in comparison with the control triploid embryos originating from additional maternal pronuclei (column 3). Only 54% of the triploids with a 2PB developed to the late morula and blastocyst stage, suggesting that the 2PB contribution to the triploids may not be an adequate substitute for the maternal pronucleus.

Finally, those hybrid embryos which resulted from 2PB fusion with cytoplasts did not develop beyond the two-cell stage (column 5). As can be seen from column 6 the viability of these gynogenetic haploids is even lower than that of androgenetic haploids, 40% of which developed at least to the four-cell stage, 29% of them even forming abnormal morulae and pseudoblastocysts.

DISCUSSION

Our results on electrofusion of 2PB with one-cell mouse embryos are in agreement with the data obtained by microsurgical transplantation (3) and confirm that 2PB chromosomes can be visualized by the effect of zygote cytoplasm. The fact that our experiments resulted in up to 90% hybridization suggests that electrofusion is an efficient approach for visualization of 2PB chromosomes. Over one-third of such hybrids contained 2PB metaphases, identified by the presence of a T6 chromosome. Therefore, the method may be suitable for cytogenetic analysis of 2PBs, although it needs to be improved considerably, before its application to possible prediction of chromosomal aneuploidy in fertilized oocytes. First, less than half of the 2PBs were transformed into metaphase chromosomes, the rest being represented by premature interphase

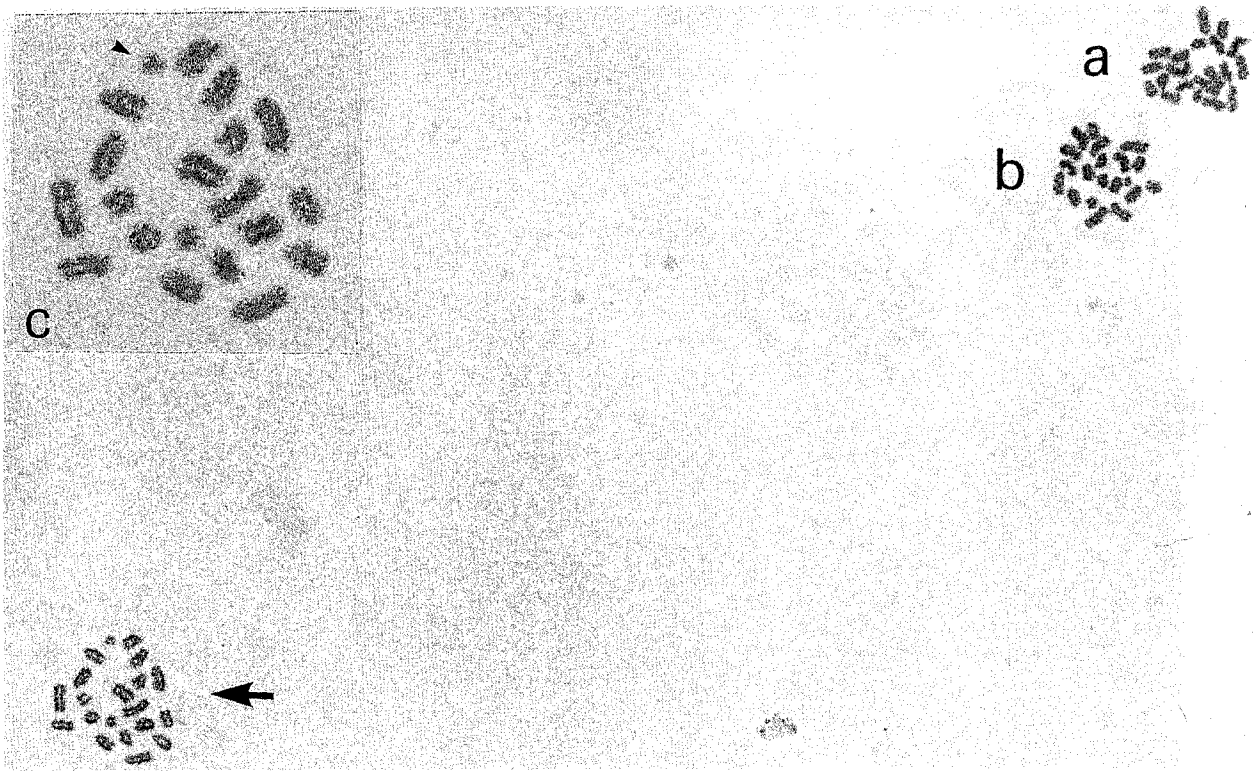

Fig. 3. Hybrid one cell-stage embryo obtained by fusion of a T6/T6 2PB with a B6CBAF$_1$ zygote: (a) paternal pronucleus and (b) maternal pronucleus and 2PB nucleus (arrow) are transformed into haploid (n = 20) metaphase plates. Air-dried preparation stained with Giemsa. Original magnification, ×820. (c) Enlarged metaphase derived from a 2PB nucleus containing a T6 marker chromosome (arrowhead). Original magnification ×1550.

chromosome condensation. It is of interest that almost all 2PB nuclei entered into mitosis following their fusion with cytoplasts, suggesting possibilities for the improvement of the technique.

Our data show that the older the 2PB used for fusion, the higher the frequency of PCC or structural abnormalities in 2PB metaphases, irrespective of the age of the recipient zygote. This is actually in agreement with the well-known fact that the success of transplantation of embryonic nuclei into mammal oocytes depends on the precise synchrony of cell cycles of the donor and the recipient (13–17). For example, PCC has been described in embryonic nuclei transplanted into oocytes, zygotes, or two-cell

Table II. Fusion of the 2PBs Isolated from CBA/T$_6$T$_6$ Mouse Eggs with C$_{57}$BL/CBA One-Cell-Stage Embryos (EMB)

			2PB nucleus transformed into								
						NEBD and different types of PCC					
Analyzed embryos				Metaphase plate			Chromatin mass		PCC		
	Age[a]			Total	No. with						
Group[a]	2PB	EMB	No.	No. (%)	aberrations (%)[b]	Total	Compact	Granular, fibrous	G_1–S	S	S–G_2
1	19	19	22	9 (41)	1 (11)	13	2	1		7	3
2	20–21	19–20	20	10 (50)	3 (30)	10		2		6	2
3	18–19	22.5	26	5 (19)	4 (80)	21	5	7	1	6	2
4	23	21	10	0		10	1	1		6	2
Total			78	24 (31)	8 (33)	54	8	11	1	25	9

[a] Age of 2PB (at the moment of isolation) and age of EMB (at the moment of fusion with 2PB) were estimated in hours from hCG injection.
[b] Percentage calculated from the number of metaphase plates.

Table III. Fusion of Isolated 2PBs with Cytoplasts (CPL) Obtained from One-Cell-Stage Mouse Embryos

Analyzed cybrids			Transformation of the 2PB nucleus into							
						NEBD and different types of PCC				
	Age[b]			Metaphase plate			Chromatin mass		PCC	
Group[a]	2PB	CPL	No.	Total No. (%)	No. with aberrations (%)	Total No. (%)	Compact	Granular, fibrous	S	$S-G_2$
1	18	19	15	14 (93)	—	1 (7)			1	
2	19–20	19	11	11 (100)	5 (45)	—				
3	19–20	21	21	16 (72)	9 (56)	5 (27)	2	3		
4	23–24	24	23	8 (35)	5 (62)	15 (65)	9	2	3	1
5	25–26	24	11	4 (36)	4 (100)	7 (64)	2	2	3	
Total			81	53 (65)	23 (43)	28 (35)	13	7	7	1

[a] Group 1, 2PB and CPL from C_{57}BL/CBA mouse eggs; groups 2–5, 2PB from CBA/T_6T_6 eggs fused with CPL from C_{57}BL/CBA eggs.
[b] Age of 2PB (at the moment of isolation) and age of CPL (at the moment of zygote enucleation) were estimated (hr) after hCG injection.

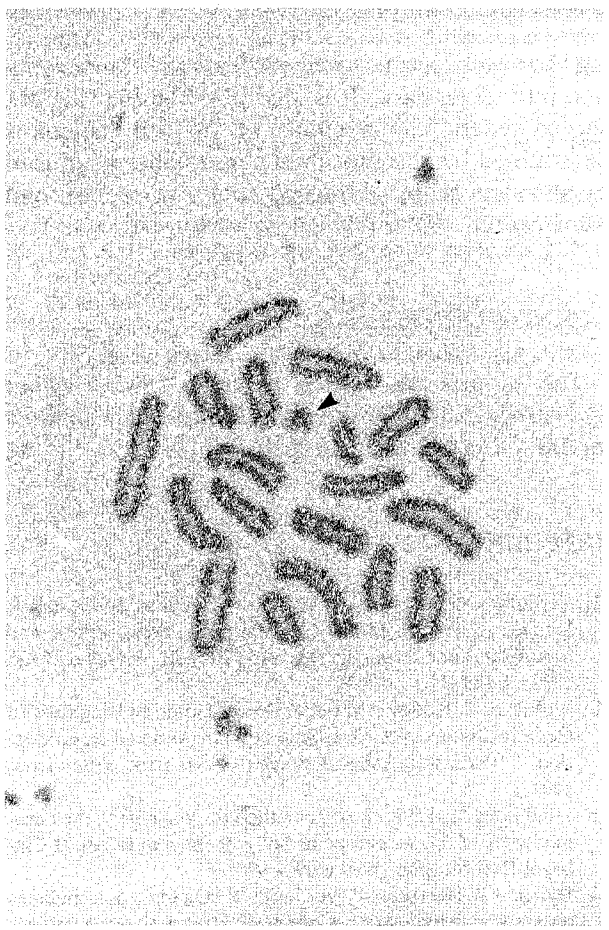

Fig. 4. Cybrid produced by fusion of a T6/T6 2PB with a cytoplast from an enucleated B6CBAF_1 zygote: the 2PB nucleus transformed into a haploid metaphase plate (n = 20) contains a T6 marker chromosome (arrowhead). Air-dried preparation stained with Giemsa. Original magnification, ×1550.

embryos in mouse (13–15), cow (16), and rabbits (17). It is known that PCC may be induced by transplanting interphase nuclei into recipient cytoplasm with an increased activity of maturation promoting factor (MPF) (for review, see Refs. 18–20). Incomplete DNA replication leading to chromosomal aberrations and death of cloned embryos in the transplanted embryonic cells was also described (for review, see Refs. 21–23).

Therefore, a high frequency of PCC and structural chromosomal abnormalities in our data may be determined by the lagging behind of the transplanted 2PB cell cycle from that of the recipient pronucleus, i.e., the pronucleus already enters mitosis with a high activity of MPF (24,25) in the cytoplasm, while the 2PB is still in S or G2. In those cases when the 2PB nucleus enters mitosis without completing DNA synthesis, chromatid and chromosome breaks are induced in the late-replicating chromosomal segments.

It is known that, contrary to the cell cycle of zygotes, the mouse 2PB cell cycle never completes DNA synthesis (for review, see Ref. 20), the fact that constitutes the basis for the failure of synchronization between 2PB and zygote cell cycles. In addition, the heterogeneity of the population of the recipient one-cell embryos used in our study also contributed to the difficulties in synchronization of cell cycles. Therefore, to improve the effectiveness of the visualization of 2PB chromosomes and to avoid structural chromosomal abnormalities, the cycles of the isolated 2PBs and enucleated zygotes need to be synchronized as much as possible.

Our data on the viability of the reconstructed hybrids are not in agreement with previous reports

Table IV. Development of Reconstructed One-Cell-Stage Mouse Embryos in Vitro

Embryos				Stage after culturing				
				24 hr	36 hr	80 hr–85 hr		
		Constitution		2-cell	4-cell	Morulae and blastocysts		
Group	No.	Classes	Origin of chromosomes			Total No.	Mean No. of cells	No. of blastocysts
1	921	Diploid zygote	PPN + MPN	894 (97%)	ND	877 (95%)	33.9 ± 0.5	601 (65%)
2	81	Reconstructed diploid	PPN + 2PB	78 (96%)	37 (46%)	26 (32%)	21 ± 1	9 (12%)
3	38	Digynic triploid	PPN + MPN + MPN	38 (100%)	38 (100%)	38 (100%)	22 ± 2	12 (32%)
4	69	Digynic triploid	PPN + MPN + 2PB	57 (83%)	48 (70%)	37 (54%)	18 ± 3	10 (17%)
5	28	Gynogenetic haploid	2PB	27 (96%)	0	0		0
6	110	Androgenetic haploid	PPN	108 (98%)	44 (40%)	32 (29%)	12.7 ± 7	0

[a] PPN: paternal pronucleus; MPN: maternal pronucleus; 2PB: second polar body nucleus.

(26–29). First, only a few of our hybrids in which the maternal pronucleus was substituted with a 2PB were able to develop to the blastocyst stage. Genetic incompetence of the 2PB nucleus was particularly obvious when fused with enucleated zygotes, the resulting hybrids being unable to develop beyond the two-cell stage. This is different from the data available, as the haploid embryos obtained by the removal of the paternal pronucleus were shown to be able to develop in vitro similarly to parthenogenetic haploids (for review, see Refs. 30 and 31). It is not clear why the chromosomal sets of 2PB and maternal pronucleus in our experiments were found to be genetically unequal. A possible explanation of that genetic inequity may be related to the processes of the isolation of the 2PB from the oocyte. It was demonstrated that the digynic triploid embryos obtained through the inhibition of 2PB extrusion, and even digynic diploids, i.e., the 2PB inhibited triploids from which the maternal pronucleus was removed, not only were able to cleave, but even resulted in the birth of normal mice (28,29). Therefore, it may be speculated that the retention of both sister chromatids in oocytes following second meiotic division does not interfere with each group of chromatids forming the maternal pronuclei, which together with the paternal pronuclei, may ensure appropriate pre- and postimplantation development. However, the extrusion of 2PB resulting in the formation of a 2PB nucleus probably leads to irreversible changes hampering adequate development of the hybrid embryos, although not preventing the sister chromosome set from the transformation into metaphase. It is also possible that the observed genetic incompetence of the 2PB nucleus is determined by chromosomal abnormalities induced by an asyinchrony of the cell cycles of zygotes and transplanted 2PBs.

ACKNOWLEDGMENTS

The authors are grateful to Drs. Andrey Dyban and Anver Kuliev for their advice and criticism during the preparation of the paper.

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