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Electrofusion of mouse embryos results in uniform tetraploidy and not tetraploid/diploid mosaicism

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Summary

Some previous attempts to produce tetraploids experimentally have resulted in a proportion of treated embryos becoming 2n/4n mosaics at a frequency which may be as high as 20%, when using cytochalasin B as a fusigenic stimulus and cytogenetic techniques to identify putative tetraploid embryos. To investigate the possible occurrence of 4n/2n mosaicism, tetraploid embryos were produced by electrofusion, a process which allows adjacent blastomeres at the 2-cell stage to fuse following exposure to electric field pulses. Embryos used for electrofusion were hemizygous for a transgene consisting of approximately 1000 copies of the mouse β-globin gene. After *in situ* hybridization, one hybridization signal is expected per diploid genome. Tetraploid cells in 7·5-, 8·5-, 9·5- and 10·5-day-old conceptuses were distinguished from diploid cells by performing *in situ* hybridization on histological sections. The frequency of nuclei with two hybridization signals in the 'hemizygous' tetraploid embryos was compared to diploid embryos which were either hemizygous or homozygous for the β-globin transgene. Comparison of the frequency of nuclei with two hybridization signals between tissues of 'hemizygous' tetraploid conceptuses and homozygous diploid conceptuses showed no significant difference, which implies that the tissues in the tetraploid conceptuses were uniformly tetraploid. No evidence was found to suggest that electrofusion results in 2n/4n mosaicism.

1. Introduction

The phenomenon of polyploidy is a well documented natural occurrence in plants, invertebrates and some vertebrates (Beatty & Fischberg, 1949), which are able to tolerate and compensate for polyploidy throughout the whole organism, but it appears to be a lethal or sublethal condition in mammals, including man. Tetraploidy frequently occurs in amniotic fluid cells and some instances of partial hydatidiform mole (Surti *et al.* 1986), as well as in other tissues (Wilson *et al.* 1988).

Although somewhere in the region of 1·3% of first trimester abortuses are tetraploid (Scarbrough *et al.* 1984), very few cases of liveborn infants have been reported (Golbus *et al.* 1976; Pitt *et al.* 1981; Scarbrough *et al.* 1984; Shiono *et al.* 1988; Pajares *et al.* 1990). Several theories have been proposed to describe the events which may give rise to a tetraploid conceptus; trispermic (3 haploid sperm) or dispermic (1 haploid and 1 diploid sperm) fertilization of a haploid ovum (Sheppard *et al.* 1982; Surti *et al.* 1986). Alternatively fertilization by either two haploid or one diploid sperm after suppression of first or second maternal meiotic division (Scarbrough *et al.* 1984). The sex chromosome complement of reported tetraploid cases is generally either XXYY or XXXX, and this fact along with information obtained by performing chromosome banding techniques (Kajii & Niikawa, 1977) on abortuses and their parents strongly suggests that tetraploidy principally arises due to suppression of cell division at the first cleavage division of the zygote (Pitt *et al.* 1981). However, the majority of human liveborn tetraploid cases reported have been mosaics with a tetraploid and another cell line, usually 4n/2n (Scarbrough *et al.* 1984; Warburton *et al.* 1991). Mosaicism may arise due to failure of cytoplasmic cleavage at a later mitotic division. In some cases 4n/2n mosaic individuals are only diagnosed after presenting with a well defined clinical

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syndrome such as Ullrich–Turner or Noohan (Wilson *et al.* 1988).

Several methods have been devised to induce tetraploidy experimentally in mammalian embryos in order to develop a suitable animal model to study the behaviour of polyploid cells within the embryo as a whole. Three techniques have been described to date which involve: (1) the suppression of cell division using colchicine (Edwards, 1958) or cytochalasin B (Snow, 1973, 1975, 1976; Tarkowski *et al.* 1977), (2) microsurgical transfer of nuclei from morula cells to fertilized one cell eggs (Modlinski, 1978) or (3) blastomere fusion at the 2-cell stage. Previously the production of tetraploids by cell fusion involved either virally-assisted blastomere fusion employing inactivated Sendai virus (Graham, 1971; O'Neill *et al.* 1990), or aggregation of 4-cell blastomeres into pairs using phytohaemagglutinin (PHA), then treating with 45% w/v polyethylene glycol (PEG) (Eglitis, 1980). More recently, the technique of electrofusion has been developed, which causes degradation of the cell membrane following exposure to electric field pulses, allowing adjacent cells to fuse (Kubiak & Tarkowski, 1985; Ozil & Modlinski, 1986; Kurischko & Berg, 1986; Kaufman & Webb, 1990; Petzoldt, 1991). The advantages of using electrofusion over other methods to produce tetraploids are that, unlike using cytochalasin B, it is not necessary for the cells to be at any particular stage in the cell cycle, nor are the embryos exposed to toxic chemicals. The time of exposure to the fusigenic stimulus is also very short, in the range of milliseconds.

Up to 20% of tetraploid embryos produced by the suppression of the second cleavage division may undergo reversion to 2n/4n mosaics, when using cytochalasin B (Tarkowski *et al.* 1977). Identification of putative tetraploids in the past has relied on cytogenetic techniques performed on embryonic and extraembryonic tissues. This method can only be applied to dividing cells, which constitute a small proportion of the total number of cells in the conceptus, so that it is difficult to say whether the sample is typical of the particular tissue studied, or of the embryo as a whole. The ideal solution to the problem would be a cellular marker through which it was possible to distinguish between diploid and tetraploid cells in histological sections, thereby determining the exact location and distribution of tetraploid cells over the whole conceptus. One such system employs a transgene, consisting of approximately 1000 copies of the mouse β-globin gene, which has been inserted into the mouse genome and may be detected by *in situ* hybridization (Lo, 1983).

By producing tetraploids from embryos carrying the transgene, tetraploid cells may be distinguished from diploid cells by the number of hybridization signals present in the nucleus after performing *in situ* hybridization. In this paper, the technique of *in situ* hybridization is used to investigate tetraploid conceptuses produced by electrofusion. By determining whether, and what percentage of, fused embryos are prone to mosaicism, it will be possible to decide whether the process of electrofusion is consistent. The technique will also facilitate estimation of the proportion of diploid:tetraploid cells, as well as their distribution within the embryo.

2. MATERIALS AND METHODS

(i) *Embryo collection*

Female mice (see below) were superovulated by injection of pregnant mare's serum gonadotrophin (PMSG), followed by human chorionic gonadotrophin (hCG) 2 days later (Hogan *et al.* 1986). The females were mated to males of the required strain, and checked for vaginal plugs the following morning. The plug date is equivalent to day 0·5 p.c. For the collection of morula or blastocyst stage embryos at 3·5 days, both PMSG and hCG were injected at midday, and for two cell stage embryos at 1·5 days, the hCG was administered at 16·30 h. This delay in hCG injection was to ensure that the embryos were not in the process of dividing during the electrofusion procedure.

(ii) *Tetraploid production*

Embryos for electrofusion were obtained by crossing (C57BL/Ws × CBA/Ca) F_1 females to strain 83 males (Lo, 1983), which are homozygous for a transgene comprising approximately 1000 copies of the mouse β-globin gene. Two cell stage embryos were flushed from the oviducts and electrofusion was employed, as described by Kaufman & Webb (1990) to generate tetraploid embryos. Fused reconstituted '1-cell stage' embryos were transferred to the oviducts of pseudopregnant F_1 females on the first day of pseudopregnancy (on the morning of the presence of a vaginal plug after mating to a vasectomized male).

(iii) *Diploid controls*

Two series of diploid controls, homozygous and hemizygous, were generated to estimate the extent of developmental retardation in the tetraploid embryos and to compare the percentages of labelled nuclei in the tetraploid embryos with those in homozygous diploid and hemizygous diploid embryos. Homozygous diploid controls were generated by mating strain 83 females to strain 83 males, resulting in diploid embryos with two copies of the β-globin transgene (*Tg/Tg*). F_1 females mated to strain 83 males produced hemizygous (single copy) diploid embryos (*Tg/−*). Morula or blastocyst cell stage embryos were flushed from the oviduct or uterus

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(Pratt, 1987) and the embryos were transferred immediately to the uterus of pseudopregnant F_1 females.

(iv) *Histology*

Putative tetraploid conceptuses were isolated at 7·5, 8·5, 9·5 and 10·5 days of gestation, diploids at 6·5, 7·5 and 8·5 days, and fixed in 3:1 (ethanol:acetic acid) at 4 °C for a minimum of 6 h. After transfer to 70% ethanol it was possible to store the embryos for several weeks at 4 °C, prior to processing, with several changes of 70% ethanol. The processing procedure involved dehydration through ethanol, followed by Histo-Clear (National Diagnostic), then immersion in a 50:50 Histo-Clear:paraffin wax mixture, before paraffin wax, under vacuum. After processing, the samples were embedded in wax and stored at 4 °C. Serial sections of the tetraploid and diploid conceptuses were cut at 7 and 5 μm respectively (see Results), and floated onto TESPA (3-amino propyl triethoxysilane) coated slides.

(v) In situ *hybridization*

The DNA probe, pM$\beta\delta$2, used to detect the β-globin sequences in the strain 83-derived cells of the tetraploids was provided by Dr John Ansell, Institute of Cell, Animal and Population Biology, University of Edinburgh. The probe plasmid, pM$\beta\delta$2, is derived from the plasmid pMJ, which is inserted into the transgenic strain 83 mice (Lo, 1983). The plasmid was linearised using the restriction enzyme *Eco*R I and labelled by random primed incorporation of digoxigenin-labelled deoxyuridine-triphosphate, using a Nonradioactive DNA Labelling and Detection Kit (Boehringer, Mannheim). Routinely 1 μg was labelled using the kit reagents to produce a stock concentration of 20 ng/μl (Keighren & West, 1992).

Hybridization procedures were modified from those of Morris *et al.* (1990) and Dr Reinald Fundele (personal communication). Before performing *in situ* hybridization, the slides were dewaxed by immersion in Histo-Clear, rehydrated through a series of graded alcohols, then washed in PBS. Endogenous peroxidase activity was inhibited by immersion for 30 min in 3% hydrogen peroxide in methanol, and the DNA present in the sections was denatured using 1 mM-NaOH at 70 °C, followed by PBS at 4 °C. The probe was denatured by boiling for 10 min, then removing to ice for a further 5 min. After prehybridization for 15 min at 60 °C in a humid box, hybridization took place under sealed hydrophobic coverslips (Gel Bond), overnight at 60 °C, using the β-globin probe at a concentration of 20 ng/slide.

After hybridization the slides were washed in a series of solutions of SSC of increasing stringency with 0·1% Triton-X detergent added, and then with 5% BSA added to block non-specific conjugation of the antibody used to detect the digoxygenin-labelled probe. The antibody used (Boehringer, Mannheim) was an anti-digoxygenin antibody from sheep; Fab fragments conjugated with horseradish peroxidase (HRP). After binding of the anti-digoxygenin antibody the slides were washed and flooded with the development reagent which contained the peroxidase substrate diaminobenzidine. The product of the reaction is a brown, water and ethanol insoluble precipitate, which can be viewed under bright field, phase contrast or dark-field light microscopy. The sections were counterstained with haematoxylin and eosin, dehydrated through graded alcohols, followed by immersion in Histo-Clear, before being mounted with Histo-Mount (National Diagnostic).

3. Results

(i) *Section thickness*

Histological sections were analysed in preference to cell spreads so that it was possible to test whether there were tissue specific differences in the proportions of the diploid and tetraploid cells within the conceptus. The section thickness has a crucial bearing on the percentage of nuclei scored as positive for the hybridization signal, as well as the proportions scored as having one or two signals. If sections are too thin compared to the nuclear diameter, some cells will lack a section of nucleus, creating a higher incidence of false negative results. If the sections are too thick, overlapping of nuclei occurs, making scoring difficult (Thomson & Solter, 1988). Tetraploid sections were cut at 7 μm, but since the diploid nuclei are expected to be smaller than tetraploid nuclei, thinner sections were used to try and ensure that a similar proportion of the nucleus was present in the sections. The ratio of nuclear diameters (4n:2n) was estimated in two ways. Firstly, assuming that the tetraploid:diploid nuclear volume ratio is 2:1 (Henery & Kaufman, 1992), the corresponding ratio for nuclear diameters can be calculated as 1·26:1 (Epstein, 1986), given that the volume of a sphere is $4/3\ \pi r^3$. Thus, to ensure that a comparable proportion of the nucleus was included in histological sections, the section thickness for tetraploid samples should be 1·26× greater than for diploid tissue. For tetraploid samples cut at 7 μm, the appropriate thickness for diploid sections would be 5·56 μm. A second calculation was made, based on observed nuclear diameters, in matched material. The nuclear length and breadth was measured in 8 areas of the embryo, 4 areas of parietal endoderm, 4 areas of ectoplacental cone and 4 areas of trophoblast of a 7·5-day tetraploid conceptus and a 6·5-day hemizygous diploid conceptus. The average of the two values was calculated for each cell to estimate the nuclear diameter and the mean for each tissue calculated. The mean

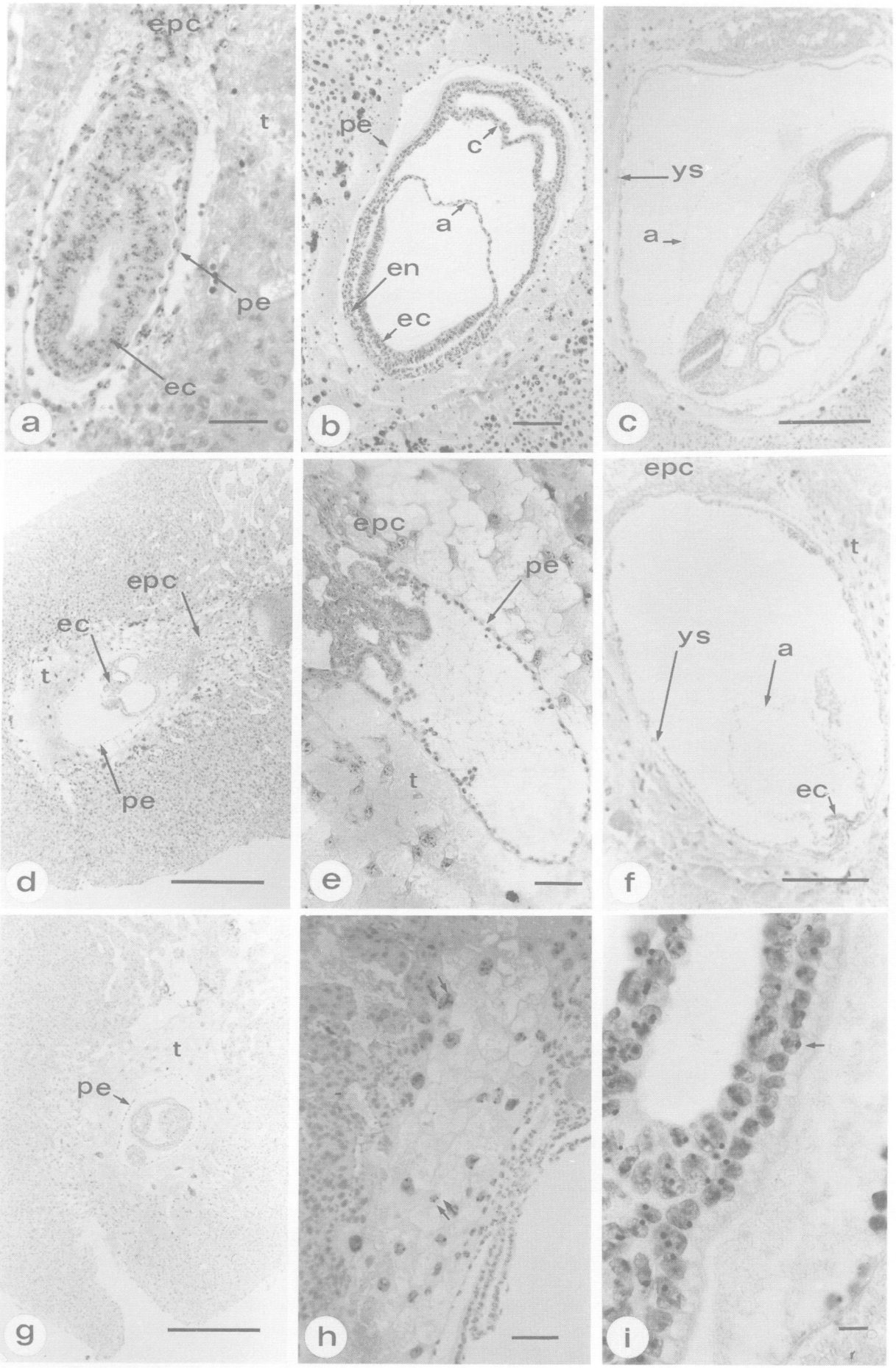

Fig. 1. For legend see opposite.

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Table 1. *Comparison of mean nuclear diameter of tetraploid and diploid cells*

Tissue	No. of nuclei	Mean (±S.E.) nuclear diameter (μm)		Mean ratio 4n/2n
		Tetraploid	Diploid	
Embryo	8	11·64±0·58	7·10±0·46	1·64
Parietal endoderm	4	10·02±1·08	6·35±0·36	1·58
Ectoplacental cone	4	10·88±2·12	7·70±0·68	1·41
Trophoblast	4	11·96±0·71	7·70±0·26	1·55
Total mean ratio (all tissues)				1·56

Table 2. *The proportion and percentage of hybridization signals in various tissues of tetraploid embryos of different developmental ages detected by* in situ *hybridization*

Tissue	Age (days)	No. of embryos scored	Total nuclei scored	Nuclei with 0 signal	Nuclei with 1 signal	Nuclei with 2 signals	% positive nuclei with 2 signals
Tetraploid embryos, hemizygous (7 μm sections)							
Embryo	7·5	3	86	23	23	40	63·5
	8·5	4	101	19	34	48	58·5
	9·5	4	133	29	56	48	46·1
	10·5	3	90	26	36	28	43·7
Parietal endoderm	7·5	3	97	18	32	47	59·5
	8·5	4	249	53	74	122	62·2
	9·5	4	482	107	145	230	61·3
	10·5	3	163	41	70	52	42·6
Ectoplacental cone	7·5	3	46	11	11	24	68·6
	8·5	2	25	3	11	11	50·0
	9·5	2	24	5	8	11	57·9
	10·5	2	24	4	9	11	55·0
Yolk sac (whole)	9·5	2	502	111	180	211	54·0
Yolk sac endoderm*	9·5	1	24	2	14	8	36·4
Yolk sac mesoderm*	9·5	1	18	2	5	11	68·7
Trophoblast	7·5	1	64	†	33	31	48·4
	8·5	1	9	†	5	4	44·4

* From same embryo.
† All trophoblast cells scored were positive (hybridization signal distinguished them from maternal decidua cells).

ratio between tetraploid and diploid nuclear diameters was calculated for each tissue along with the ratio for all tissues (Table 1). The mean ratio of 1·56, was used to calculate a section thickness for the diploid embryos of 4·48 μm (equivalent to 7 μm for the tetraploid). The actual section thickness used for the diploid embryos was 5 μm, which is close to both the theoretical (5·56 μm) and calculated (4·48 μm) values.

(ii) *Morphology*

The development of the tetraploid embryos was retarded with respect to the diploid control conceptuses which agrees with previous studies of tetraploid development (Snow, 1975; Tarkowski *et al.* 1977). Fig. 1 *a–c* (6·5, 7·5 and 8·5 days old respectively) shows histological sections of diploid embryos

Fig. 1. Histological sections of diploid (*a–c*, *i*) and tetraploid (*d–h*) mouse conceptuses, after *in situ* hybridization to detect the transgenic β-globin sequence (*Tg*), showing poor embryonic development of tetraploids. Bar is 10 μm in (*i*) 50 μm in (*a*), 100 μm in (*b*, *e*, *h*) and 500 μm in (*c*, *d*, *f*, *g*). (*a*) Normal 6·5-day homozygous, *Tg*/*Tg* diploid; (*b*) normal 7·5-day heterozygous, *Tg*/− diploid; (*c*) normal 8·5-day homozygous, *Tg*/*Tg* diploid. (*d–g*) 'Hemizygous', *Tg*/*Tg*/−/− tetraploid conceptuses at (*d*) 7·5 days, (*e*) 8·5 days, (*f*) 9·5 days and (*g*) 10·5 days. (*h*) Part of a 10·5-day 'hemizygous', *Tg*/*Tg*/−/− tetraploid showing trophoblast giant cells with two hybridization signals (arrows). (*i*) High power of part of normal 7·5-day heterozygous, *Tg*/− diploid conceptus (*b*), showing one hybridization signal in most nuclei. Abbreviations: a, amnion; c, chorion; ec, embryonic ectoderm; en, embryonic endoderm with subjacent mesoderm; epc, ectoplacental cone; pe, parietal endoderm; t, trophoblast; ys, visceral yolk sac.

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Table 3. *The proportion and percentage of hybridization signals in various tissues of diploid hemizygous and homozygous embryos of different ages detected by* in situ *hybridization*

Tissue	Age (days)	No. of embryos scored	Total nuclei scored	Nuclei with 0 signal	Nuclei with 1 signal	Nuclei with 2 signals	% positive nuclei with 2 signals
			Diploid embryos, homozygous (5 μm sections)				
Embryo	6·5	3	123	21	46	56	54·9
	7·5	2	98	21	31	46	59·7
	8·5	3	142	25	54	63	53·8
Parietal endoderm	6·5	2	65	14	28	23	45·1
	7·5	3	193	55	67	71	51·4
	8·5	3	327	70	138	119	46·3
Ectoplacental cone	6·5	3	63	14	21	28	57·1
	7·5	2	43	12	17	14	45·2
	8·5	3	64	16	20	28	58·3
Amnion	8·5	1	78	22	31	25	44·6
			Diploid embryos, hemizygous (5 μm sections)				
Embryo	6·5	3	121	46	72	3	4·0
	7·5	3	124	49	72	3	4·0
	8·5	1	50	10	38	2	5·0
Parietal endoderm	6·5	3	91	39	51	1	1·9
	7·5	3	167	53	111	3	2·6
	8·5	1	154	57	92	5	5·1
Ectoplacental cone	6·5	3	57	27	29	1	3·3
	7·5	2	39	17	22	0	0
	8·5	1	26	11	14	1	6·7
Yolk sac (whole)	8·5	1	45	11	34	0	0
Trophoblast	7·5	1	16	6	9	1	10

displaying increasing complexity with age. By comparison, Fig. 1*d–g* (7·5, 8·5, 9·5 and 10·5 days old, respectively) illustrates typical morphology of tetraploid conceptuses. Extraembryonic membranes survived better than embryonic tissues which were much reduced and disorganized. The parietal endoderm, yolk sac and trophoblast were usually visible (Fig. 1*g*), and were the least affected structures in the tetraploid conceptuses. This agrees with the observations of Tarkowski *et al.* (1977), who also reported that the major problem during tetraploid development was the lack of mesoderm, and that mesoderm present was limited to the fetal membranes. In some cases the tetraploid conceptuses were surrounded by blood islands outside the parietal endoderm.

(iii) *Scoring hybridization signals*

In situ hybridization was carried out on all slides which had sections containing the embryo. Sections showing labelled nuclei after *in situ* hybridization (indicating the presence of embryonic tissue) were noted and a section near the middle of the range was chosen for scoring (Fig. 2). By counting the number of hybridization signals visible in the nuclei of different tissues, it was possible to determine whether the tissue was hemizygous for the transgene, one spot per nucleus or homozygous (two spots). Tetraploid embryos produced from diploid hemizygous (*Tg*/−) embryos should have two copies of the transgene per nucleus (*Tg*/*Tg*/−/−) (Fig. 2*a*), as the homozygous diploid (*Tg*/*Tg*) embryos do (Fig. 2*d*). The number of

Fig. 2. (*a*, *b*) 'Hemizygous', *Tg*/*Tg*/−/− tetraploid mouse conceptuses, after *in situ* hybridization to detect the transgenic β-globin sequence (*Tg*) in histological sections, at (*a*) 7·5 days and (*b*) 10·5 days (dark field photograph of conceptus shown in Fig. 1*g*). Each conceptus has nuclei with two hybridization signals, including some trophoblast giant cells (small arrows). A mitotic figure indicated in (*a*) (heavy arrow) is shown in more detail in (*g*). (*c*, *d*) High-power photographs of nuclei from normal diploid embryos after *in situ* hybridization. (*c*) Nuclei with one hybridization signal in the parietal endoderm of a 7·5-day heterozygous, *Tg*/− diploid conceptus; (*d*) neurectoderm of an 8·5-day homozygous, *Tg*/*Tg* diploid conceptus with some nuclei with two hybridization signals. (*e–g*) High-power photographs of nuclei from 'hemizygous', *Tg*/*Tg*/−/− tetraploid embryos after *in situ* hybridization. (*e*) Parietal endoderm from 9·5-day tetraploid and (*f*) trophoblast giant cell from 10·5-day tetraploid, each with two signals per nucleus. (*g*) 'Hemizygous' tetraploid mitotic figure from 7·5-day conceptus, showing segregation of two pairs of labelled chromosomes (arrows indicate the two hybridization signals moving to each pole). Bar is 10 μm in (*c–g*), 50 μm in (*a*) and 100 μm in (*b*). Abbreviations as in Fig. 1.

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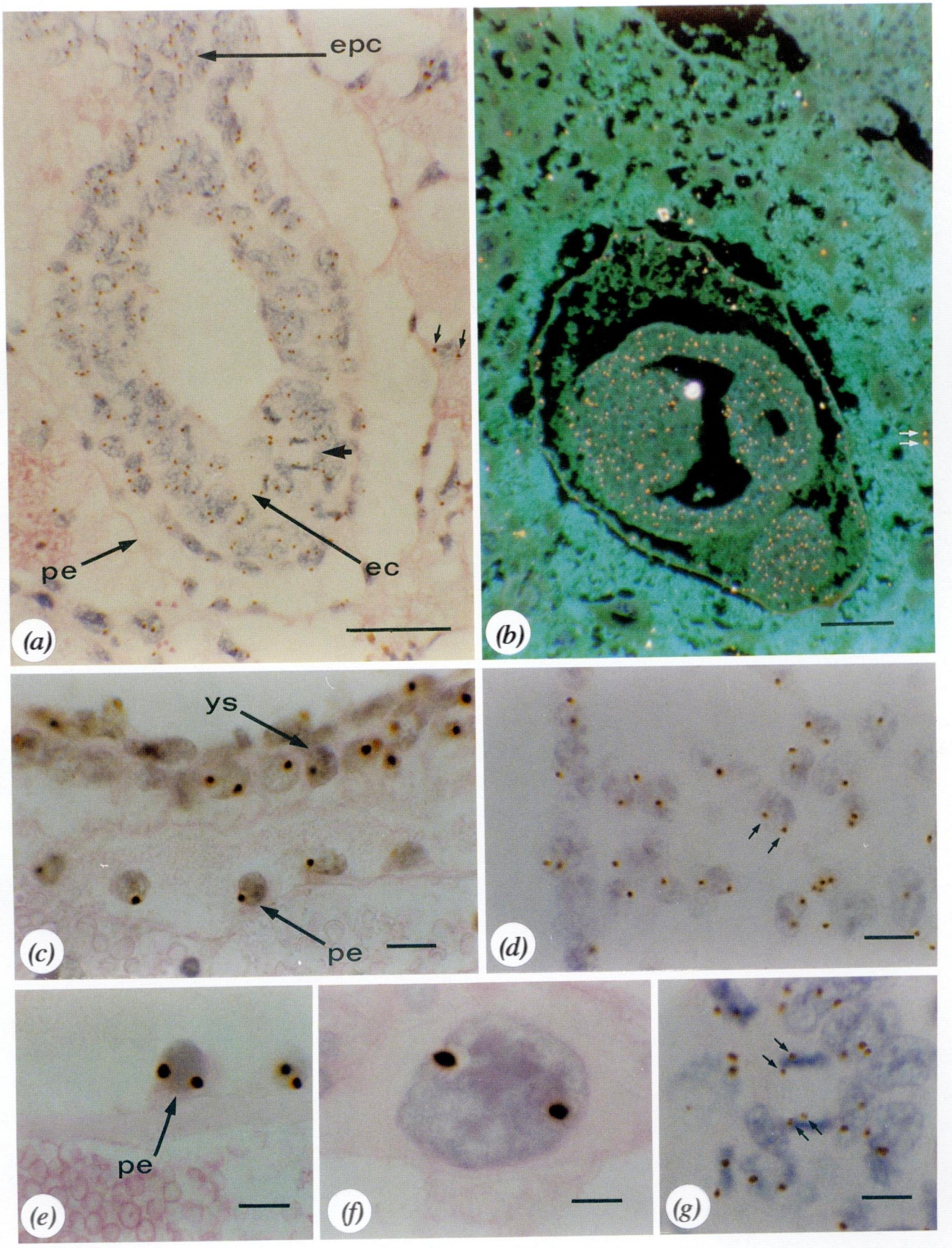

FIGURE 2.

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Table 4. *Mean frequencies of nuclei with 0 or 2 hybridization signals in the embryo, parietal endoderm and ectoplacental cone*

	Expected genotype					
	2n hemizygote		2n homozygote		4n 'hemizygote'	
Tissue	proportion	%	proportion	%	proportion	%
Overall proportion of nuclei with no signal						
Embryo	105/295	35·6	67/363	18·5	97/410	23·7
Parietal endoderm	149/412	36·2	139/585	23·8	219/991	22·1
Ectoplacental cone	55/122	45·1	42/170	24·7	23/119	19·3
Overall proportion of positive nuclei with 2 signals						
Embryo	8/190	4·2	165/296	55·7	164/313	52·4
Parietal endoderm	9/263	3·4	213/446	47·8	451/772	58·4
Ectoplacental cone	2/67	3·0	70/128	54·7	57/96	59·4

Table 5. *Statistical significance (χ^2 test*) of the differences, in frequency of hybridization signals per nucleus, between tetraploid and both groups of diploid conceptuses*

	No signal		2 signals	
	χ^2	P	χ^2	P
2n hemizygous versus 4n 'hemizygous'				
Embryo	11·95	*$P = 0{\cdot}0005$*	119·86	*$P < 0{\cdot}0001$*
Parietal endoderm	29·76	*$P < 0{\cdot}0001$*	238·09	*$P < 0{\cdot}0001$*
Ectoplacental cone	18·25	*$P < 0{\cdot}0001$*	51·92	*$P < 0{\cdot}0001$*
2n homozygous versus 4n 'hemizygous'				
Embryo	3·12	$P = 0{\cdot}0775$	0·69	$P = 0{\cdot}4075$
Parietal endoderm	0·58	$P = 0{\cdot}4468$	12·96	*$P = 0{\cdot}0003$*
Ectoplacental cone	1·16	$P = 0{\cdot}2812$	0·49	$P = 0{\cdot}4835$

* Yates correction was applied when expected frequencies fell below 10.
Differences were considered significant if $P < 0{\cdot}05$ (shown in italics).

spots per nucleus counted in the tetraploid embryos were compared with those counted in control hemizygous and homozygous diploid embryos. A uniformly 'hemizygous' tetraploid tissue (*Tg/Tg/−/−*) would be expected to have a similar proportion of double-spot nuclei as the homozygous (*Tg/Tg*) controls. A mosaic tissue which was a mixture of *Tg/Tg/−/−* and *Tg/−* cells would have a lower percentage of nuclei with 2 spots.

The tissues scored were: embryo, parietal endoderm, ectoplacental cone and trophoblast (Fig. 1 *a–g*). All four of these structures were easy to distinguish in early stage conceptuses. Embryonic and extra-embryonic tissues were included in the scoring system to ensure that if 4n/2n mosaicism was confined to one developmental lineage, it would still be detected. Apart from the parietal endoderm where the whole structure was scored, areas of tissue to be counted were delineated under a Leitz Diaplan microscope using an eye-piece with 1 mm grid. Using a ×25 objective and ×12·5 (20 mm) eye-piece, a single square, (corresponding to 50 × 50 μm on the slide) was selected and each of the nuclei contained in that area was scored as: 0 signal; 1 signal; 2 signals or unscoreable. One area each of trophoblast tissue and ectoplacental cone tissue was scored for each embryo, and two areas of embryonic tissue.

The mean and the standard error for the percentage of each of the categories in the scoring system: 0 signal; 1 signal; 2 signals or unscoreable, were calculated for each age of embryo, for both the tetraploid and diploid series. The number of embryos and the total number of nuclei scored for each age and tissue can be seen in Tables 2 and 3 (tetraploid and diploid respectively). The proportion of nuclei with no signal gives an indication of the percentage of false negatives which occurred due to failure of the *in situ* technique. Also calculated was the proportion of positive nuclei with two signals. The total proportion of nuclei with no signal and positive nuclei with two signals for each tissue type, disregarding age, is shown in Table 4. The results were tested statistically using a χ^2 test (Table 5). The data for the 'hemizygous' tetraploid embryos was tested statistically against both the hemizygous and homozygous diploid values. Tables 4 and 5 show that the 'hemizygous' tetraploid

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Table 6. *Statistical significance (χ^2 test*) of the differences, in frequency of hybridization signals per nucleus, between tissues in tetraploid conceptuses*

	Proportion of nuclei with no signal		Proportion of nuclei with 2 signals	
Comparison	χ^2	P	χ^2	P
Embryo versus parietal endoderm	0·40	0·5251	3·29	0·0697
Embryo versus ectoplacental cone	0·99	0·3206	1·44	0·2300
Parietal endoderm versus ectoplacental cone	0·48	0·4891	0·03	0·8578

* Yates correction was applied when expected frequencies fell below 10.
No differences were considered statistically significant (none with $P < 0·05$).

group had significantly fewer nuclei with no signal and significantly more nuclei with two signals than the hemizygous diploid group. Comparison of 'hemizygous' tetraploid and homozygous diploid groups revealed no significant difference for the embryo or ectoplacental cone. Although a significant difference in the frequency of nuclei with two signals was detected in the parietal endoderm this does not provide evidence for mosaicism in the 'hemizygous' tetraploid group because the frequency of nuclei with two signals was higher in the 4n than the homozygous diploid parietal endoderms. χ^2 tests were also carried out between the different tissues scored in tetraploid embryos (Table 6). There was no significant difference between the different tissue types tested. This supports the conclusion that the discrepancy in the parietal endoderm χ^2 values is not biologically significant.

4. Discussion

The development of the tetraploid embryos generated by this particular combination of strains was poorer than that previously reported by Kaufman & Webb (1990). Viable embryos have been recorded as late as 17·5 (Snow, 1975) and 16 days of gestation (equivalent to normal embryos of about 14–14·5 days p.c., Kaufman, 1991 *a*). There are two possible explanations for this observation; either the development of tetraploid embryos varies according to the strain combination (genetic background) or that the presence of the transgene is somehow interacting to hamper development in the tetraploid embryo in a way not observed in diploids generated from the same strain. The former explanation would seem more plausible, since strain dependent effects on development have been reported in several different instances for both tetraploidy (Tarkowski *et al.* 1977; Dyban & Baranov; 1987; Webb *et al.* 1992) and triploidy (Wroblewska, 1971; Niemierko, 1981). In the series of tetraploid mouse embryos described by Snow (1975), the embryonic ectoderm was commonly reduced and disorganized, the cells being loosely arranged and fewer in number than diploids of the same age. Tarkowski *et al.* (1977) noted that growth of tetraploid conceptuses was generally slower than in normal diploid embryos. By 8·5 days of gestation the embryonic portion of the conceptus appeared underdeveloped with either an absence of, or vestigial embryonic structures. The type of morphological changes observed in tetraploid conceptuses have also been seen in embryos with other chromosome abnormalities (Dyban & Baranov, 1987). For instance, the triploidy syndrome described by Wroblewska (1971) involved a general retardation of growth, but was also strain dependent, since in appropriate strain combinations, limb bud stage diandric and digynic triploid embryos are observed (Kaufman, 1991 *b*), though more advanced stages of development have so far not been reported. By around 8–9 days of gestation the embryonic region of the triploidy syndrome conceptus was small in comparison to the extra-embryonic region. The primary germ layers were less differentiated, with fewer layers and rounded cells. These observations closely resemble morphology seen in tetraploid embryos, although the tetraploid phenotype manifests earlier and often results in no more than a trophoblastic vesicle (Graham, 1971; Snow, 1976). Tetraploid embryos have been recovered at between 10 days of gestation (Tarkowski, 1977), which resembles more closely the conceptuses collected in this study, and 17·5 days of gestation reported by Snow (1975). However, recent attempts to repeat the success achieved by Snow, have resulted in embryos no older than 16 days of gestation (Kaufman, 1991 *a*). There may be two explanations for these differences. Firstly, that the differences in development are due to the strain combinations used, or that Snow's tetraploids were perhaps 4n/2n mosaics and that the cytogenetic analysis failed to identify them as such. Even a small proportion of diploid cells in a mosaic embryo may have been sufficient to partially rescue the embryo and allow development to proceed further than in a solely tetraploid embryo.

A low incidence of nuclei with two positive signals was observed in all tissues of the hemizygous diploid ($Tg/-$) conceptuses (Table 3). There are two possible

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explanations for this observation. Firstly, that the cells are genuinely tetraploid, as observed, for example in the trophoblast. Alternatively, the apparent polyploid cells observed may be technical artefacts, due to overlapping cells, despite the fact that the sections were cut at 5 μm to compensate for the smaller nuclear diameter of the diploid cells. The latter seems less likely since closely packed cells or cells which did not have obvious nuclear borders were counted as unscoreable.

The incidence of 2 signals per nucleus in homozygous diploid (and 'hemizygous' tetraploid) conceptuses is only ~ 50%, when, theoretically, the expected value is 100%. This is most likely to be an artefact attributable to the use of histological sections, because some target sequences will be excluded from part of the sectioned nuclei particularly in thin sections. The ability to detect mosaicism is reduced by the failure to detect both hybridization signals in about 50% of the nuclei expected to have two copies of the transgene. Nevertheless it should have been possible to recognize the presence of diploid cells in the tetraploid embryos, if they comprised at least 15% of the total nuclei scored, as explained below.

If the proportion of all nuclei scored that had one hybridization signal in the embryonic region of the 4n group was 177/410 (43·2%), instead of 149/410 (36·3%; Table 2), then this would have been significantly higher than the corresponding proportion for the homozygous diploid embryos; 131/363; 36·1% ($\chi^2 = 4{\cdot}03$; $P = 0{\cdot}045$). This higher proportion of nuclei with one signal could have been accounted for by 11·1% of diploid nuclei in the 'tetraploid' embryos; as calculated by:

$$\left[1-\left(\frac{0+2 \text{ spots in 4n}}{\text{All nuclei in 4n}}\Big/\frac{0+2 \text{ spots in 2n homozygotes}}{\text{All nuclei in 2n homozygotes}}\right)\right]\times 100.$$

This formula is based on that used to calculate the number of induced mutations (Lyon, 1970) and, when used for the above example, resolves as

$$\left[1-\left(\frac{233}{410}\Big/\frac{232}{363}\right)\right]\times 100 = 11{\cdot}1\,\%.$$

Therefore, on this basis, it would have been possible to detect the presence of diploid cells in the tetraploid embryos if they composed at least 11·1% of the total nuclei scored. A similar calculation, ignoring the nuclei with no hybridization signals, predicted that 2n/4n mosaicism would be detected if at least 14·6% of the nuclei were diploid. Thus, diploid cells in the tetraploid embryos should have been detected, by a statistically significant increase in the proportion of nuclei with only one signal, if they comprised at least 15% of the total nuclei scored.

The frequency of nuclei with no hybridization signal was higher in the hemizygous diploid series. This was expected since there is a greater chance of failing to include the target sequence in a section when there is only one target per nucleus, compared to two sequences in both the homozygous diploid and 'hemizygous' tetraploid series. The frequency of positive nuclei with two signals in 'hemizygous' tetraploid ($Tg/Tg/-/-$) conceptuses was not significantly lower than that observed in the homozygous diploid (Tg/Tg) conceptuses. This implies that the tissues in the tetraploid conceptuses are uniformly 4n, and consequently that there is no evidence that the electrofusion technique results in 4n/2n mosaicism.

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