

Establishment of pluripotential cell lines from haploid mouse embryos

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SUMMARY

Eggs from 129 SvE and (C57BL × CBA)F₁ hybrid female mice were activated parthenogenetically following their exposure to a 7 % solution of ethanol in PBS. Only the haploid class which developed a single pronucleus following second polar body extrusion was examined further. These eggs were transferred to suitable recipients and 'delayed' blastocysts subsequently recovered. The 'delayed' blastocysts were explanted into tissue culture and a total of four haploid-derived pluripotent cell lines established from individual embryos. Chromosome analysis of morulae revealed that over 80 % contained only haploid mitoses. However, chromosome analysis of early passage cell lines revealed that all were diploid with a modal number of 40 chromosomes. When transplanted into syngeneic hosts, all lines formed well-differentiated teratocarcinomas. This technique provides a source of homozygous diploid cell lines of parthenogenetic origin.

INTRODUCTION

It has been suggested that the direct isolation of pluripotential cells from early embryos might provide sources of pluripotential cells with a karyotype unchanged from that of the embryo from which it was derived (Evans, 1981). In addition to their relative ease of production from blastocysts, the principal advantage that such EK cell lines have over most currently available embryonal carcinoma (EC) cell lines derived from tumours is that, at least initially, they do have a normal karyotype (Evans & Kaufman, 1981).

Recent studies in which pluripotential cell lines have been established from a considerable number of individual fertilized mouse embryos with both a normal and abnormal chromosome complement (Martin, 1981; Evans, Robertson, Bradley, Handyside & Kaufman, unpublished) have stimulated us to attempt to

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establish similar pluripotential cell lines from haploid parthenogenetically-derived embryos.

MATERIALS AND METHODS

i. *Ethanol activation*

Eight- to 12-week-old 129 SvE and (C57BL×CBA) F_1 hybrid female mice were superovulated with 5 i.u. PMSG followed 48 h later by 5 i.u. HCG. The females were autopsied 17 h later and the cumulus masses recovered from the oviducts released into a freshly prepared 7% (v/v) solution of Analar quality ethanol in Phosphate Buffered Saline (PBS) containing both Ca^{2+} and Mg^{2+} , and retained in this solution for about $4\frac{1}{2}$ min. Cumulus masses from four to six females were pooled together, treated as a single group, and this and the following washing procedures were carried out at room temperature. The cumulus masses were washed through three changes of ethanol-free PBS and through two changes of embryo culture medium (Whittingham, 1971). Individual cumulus masses were then transferred to separate drops of medium under paraffin oil and incubated for 4–5 h at 37°C in an atmosphere of 5% CO_2 in air. The adherent cumulus cells were then removed with hyaluronidase, and the overall activation frequency determined and the various classes of parthenogenone induced separated into different groups (Kaufman, 1978a). Only those activated oocytes that developed a single haploid pronucleus following second polar body extrusion were used in this study. A more detailed description of this ethanol activation technique has been published elsewhere (Cuthbertson, Whittingham & Cobbold, 1981; Kaufman, 1982).

A proportion of the pronucleate-stage 1-pronuclear haploid eggs were transferred to the oviducts of recipients (Tarkowski, 1959) anaesthetized with Avertin on the afternoon of the first day of pseudopregnancy (i.e. on the day in which the vaginal plug had earlier been observed, following mating of the female with a vasectomized male), while others were retained in culture.

The recipients were divided into two groups. The first group was ovariectomized on the afternoon of the 4th day of pseudopregnancy and, while they were still under the influence of the anaesthetic, given a subcutaneous injection of 1 mg Depo-Provera (Upjohn). This group of females was subsequently autopsied 4–5 days later, the uterine horns removed and flushed with PBS in order to recover delayed blastocysts. The second group of recipients was autopsied at about midday on the 4th day of pseudopregnancy and the reproductive tract flushed with PBS. About half of the recovered embryos, which were mostly at the morula stage, were then incubated for about 3 h in medium containing 1 $\mu g/ml$ Colcemid. These embryos were then examined by the air-drying technique (Tarkowski, 1966), and the preparations stained with Giemsa. It was possible to classify almost all of the embryos with cells in division into three distinct groups, namely i. haploid, ii. haploid-diploid mosaics or iii.

diploid, according to the number of chromosomes present in the individual metaphase plates. The embryos from this group of recipients that were not examined by air drying were allowed to develop to the blastocyst stage in culture, then transferred to the uterine horns of other recipients in order to recover delayed blastocysts.

The embryos that were retained in culture from the 1-cell stage were subsequently transferred, at about 74–75 h after activation, into medium containing 1 μ g/ml Colcemid for 3–4 h, and air-dried preparations made as described above. The ploidy of this group of embryos was also determined.

ii. *Establishment of pluripotential cell lines from delayed blastocysts*

Individual delayed blastocysts, many of which contained large clearly delineated inner cell masses (ICMs) (Fig. 1A) were transferred to tissue culture

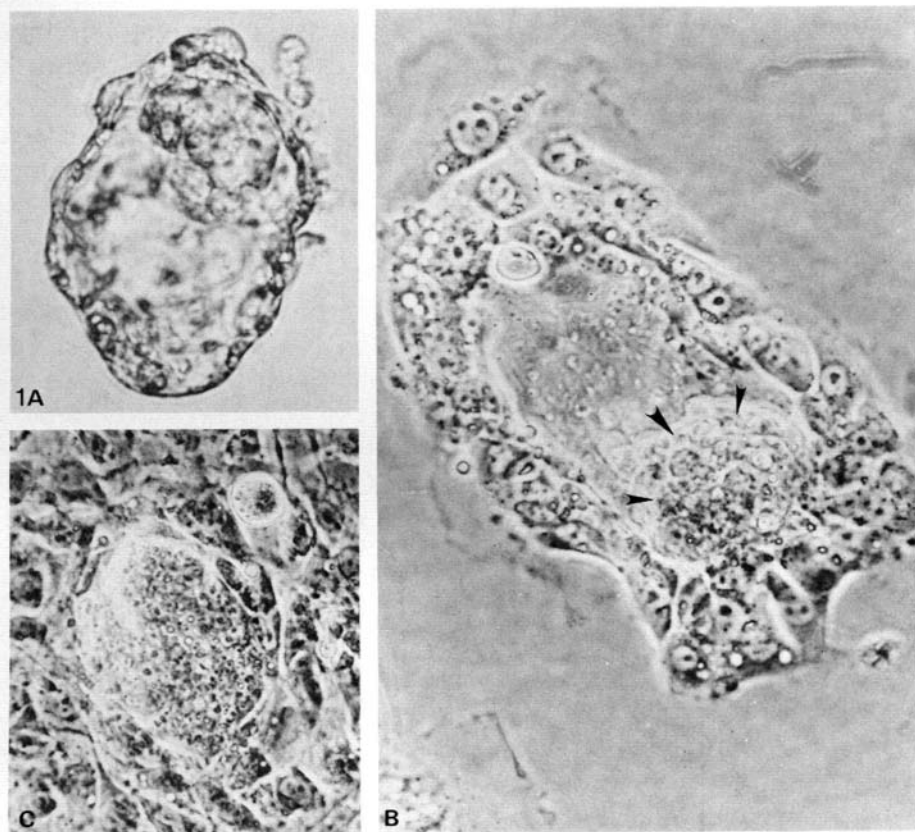


Fig. 1. A. 'Delayed' 129 SvE blastocyst shortly after its explantation into tissue culture medium. Note the large inner cell mass. B. Appearance of 'implanted' blastocyst at approximately 60 h after explantation. Note centrally-located clump of inner-cell-mass-derived cells (arrow). C. A group of haploid-derived cells, growing on a feeder layer, shortly after their establishment in culture.

dishes containing Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% foetal calf serum and 10% newborn calf serum (Evans & Kaufman, 1981). After blastocyst attachment, which usually occurred within 48 h of explantation (Fig. 1B), the ICM-derived cell clumps were selectively removed following an additional 4 days of culture. The ICM clumps were disaggregated in 0.25% (w/v) trypsin, 0.04% (w/v) EDTA and replated onto feeder layers of mitomycin-treated fibroblasts. In successful cultures nests of stem cells appeared following two rounds of cell growth and trypsinization. These cells have a distinctive morphology in culture (Fig. 1C) closely resembling other established tumour-derived and embryo-derived pluripotential cell lines. The haploid-derived (HD) lines were subsequently maintained on feeder layers and subcultured at 4–6 day intervals.

iii. *Testing of differentiation ability of the cell lines*

The differentiation ability of the 129 SvE lines was tested by inducing tumour formation in syngeneic host animals. For each line 10–12 male 129 SvE mice were inoculated subcutaneously with approximately 10^6 cells. Tumour masses were retrieved after 4 to 6 weeks and fixed in Bouin's solution, dehydrated and subsequently serially sectioned at a nominal thickness of 7 μm . Alternate slides were then either stained with haematoxylin and eosin or with Masson's trichrome.

iv. *Chromosome analysis of cell lines*

Chromosomal analysis was performed on early passage cell lines. This was usually carried out within five to ten passages following the original disaggregation of the ICM-derived cell clumps. The chromosomes were analysed by G-banding (modification of the A.S.G. procedure of Gallimore & Richardson, 1973), and karyograms arranged according to the nomenclature of Nesbitt & Francke (1973).

RESULTS

A. *Observations on the activation rate and incidence of the various classes of parthenogenone induced*

Observations on the incidence of the various classes of parthenogenone induced when 129 SvE and (C57BL \times CBA) F_1 hybrid oocytes isolated at 17 h after the HCG injection for superovulation were stimulated by exposure to a 7% solution of ethanol in PBS for about 4½ min are presented in diagrammatic form in Fig. 2. The data included in this figure are the combined results of all the activation studies carried out over a period of several months involving these two strains of mice, and in all represent the results of isolated experiments carried out on more than 10 separate occasions.

In both strains, the highest proportion of the activated population consisted of haploid parthenogenones which had developed a single (haploid) pronucleus

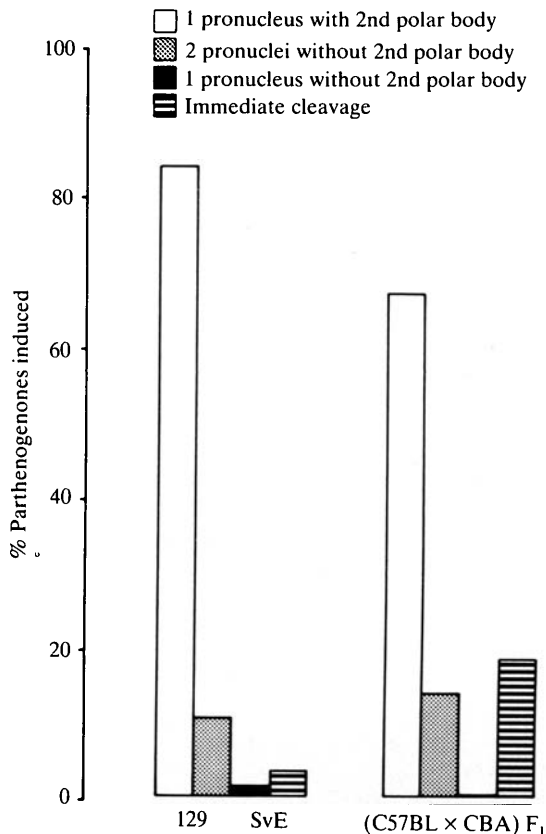


Fig. 2. Incidence of different types of parthenogenones induced when eggs from 129 SvE and (C57BL \times CBA) F_1 hybrid mice were briefly incubated in 7% ethanol in PBS. Cumulus masses were released at 17 h after HCG and observations made 4–5 h later. The total number of activated eggs examined in the 129 SvE series was 1351, and the activation frequency was 80.0%. In the F_1 series, 1211 activated eggs were examined, and the activation frequency was 95.7%.

following extrusion of the second polar body. The overall activation rate in both strains was high, with about 80–95% of the oocytes exposed to the ethanol treatment being stimulated to develop parthenogenetically. As indicated in the Methods section, only the 1-pronuclear haploid embryos were used subsequently in the present study.

B. Chromosome analysis at about 75–77 h after activation

i. In vivo series

Activated haploid eggs from 129 SvE and (C57BL \times CBA) F_1 hybrid females which had previously been transferred to the oviducts of suitable recipients at the 1-cell stage, were isolated at about midday on the 4th day of pseudopregnancy. The embryos, which were largely at the morula stage, were then incubated for about 3 h in medium containing 1 $\mu\text{g}/\text{ml}$ Colcemid, and subsequently examined

by the air-drying technique described by Tarkowski (1966). As only about half of the recovered embryos were used to assess the ploidy, the others being transferred to additional recipients in order to obtain delayed blastocysts, only details of the fixed embryos with cells in division will be presented here (see Table 1). In the haploid-diploid mosaic embryos only one or two diploid metaphases were usually present, and almost all of the mitoses observed in this group were haploid. In the 129 SvE series 82 %, and in the (C57BL × CBA)_F₁ series 85 % of the embryos examined had only haploid mitoses present (see Fig. 3).

ii. *In vitro series*

In a parallel series of experiments, (C57BL × CBA)_F₁ hybrid oocytes were activated *in vitro* with ethanol and the 1-pronuclear haploids retained in culture until about midday on the 4th day (about 73–74 h after activation), then those that had progressed beyond the 4-cell stage were transferred to medium containing 1 µg/ml Colcemid for 3–4 h. Out of an initial total of 174 1-cell activated eggs, 157 embryos had more than four cells present by the early afternoon on the 4th day, but by this time most of the embryos were at the morula stage of development. Air-dried preparations were made as described above. In 12 of these embryos no cells were in division, in 141 embryos one or more mitoses were present, and in 4 embryos virtually all of the cells were in division and it was considered impossible to make an assessment of the ploidy because of extensive overlapping of mitotic figures. Of the 141 embryos with cells in division, 102 (72 %) had only haploid mitoses, 35 (25 %) had both haploid and diploid mitoses present, while 4 (3 %) had only diploid mitoses present (see Table 1). The mean number of cells (\pm s.e.) in the haploid, haploid-diploid mosaic and diploid embryos in this series was 18.2 ± 0.6 , 16.4 ± 0.9 and 14.0 ± 4.3 , respectively, while the mean number of cells in mitosis in each of these groups of embryos was 5.2 ± 0.3 , 6.6 ± 0.6 and 4.0 ± 1.7 , respectively. Following the 3–4 h period of incubation in medium containing Colcemid, approximately 30–40 % of the blastomeres in these embryos were therefore blocked in mitosis at the time of analysis. In the haploid-diploid

Table 1. *Chromosome analysis of 1-pronuclear haploid embryos at the morula stage of development*

Group	Strain	Total embryos with mitoses	Ploidy		
			Haploid	Haploid-Diploid	Diploid
Oviduct transfer embryos	129 SvE	17	14 (82%)	2	1
	(C57BL × CBA) _F ₁	78	66 (85%)	5	7
<i>in vitro</i> culture	(C57BL × CBA) ₁	141	102 (72%)	35	4

1-cell embryos were transferred to the oviducts of recipients on the afternoon of the first day and isolated at midday on the 4th day of pseudopregnancy.

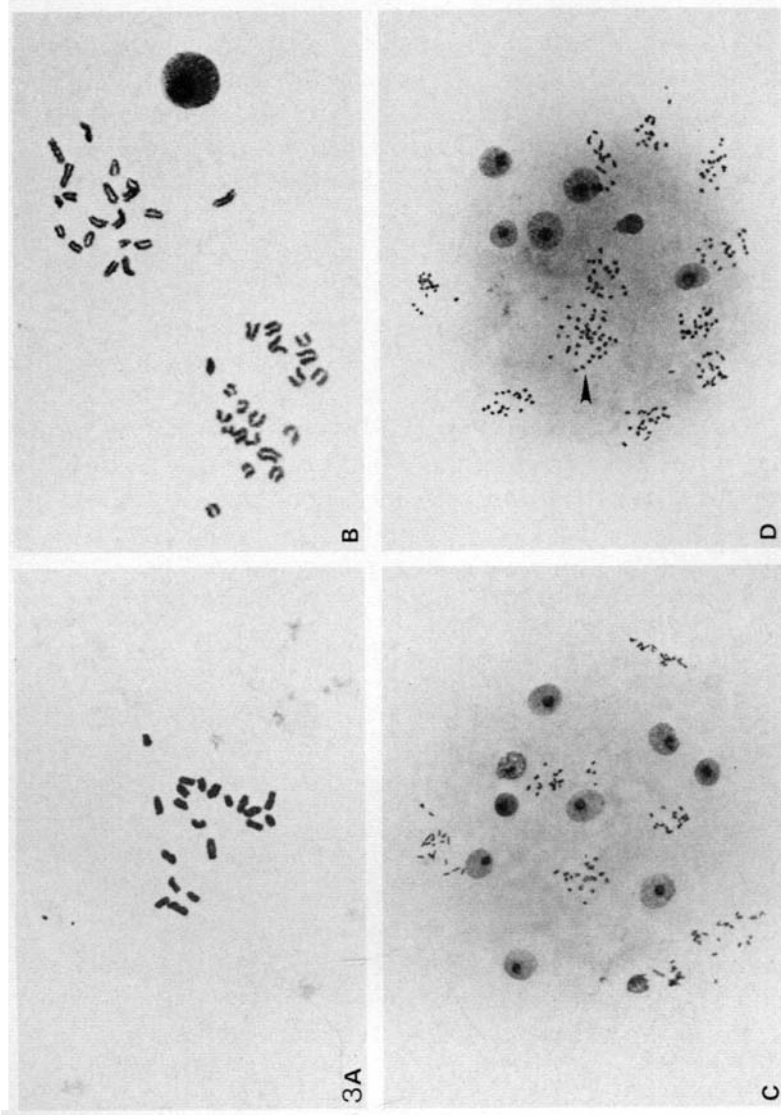


Fig. 3. Air-dried preparations stained with Giemsa. A. Haploid first cleavage metaphase spread with 20 chromosomes present. B. Two haploid metaphases from a 17-cell haploid morula. C. Morula with 7 haploid mitoses. D. Haploid-diploid morula with 10 haploid and a single diploid mitosis (arrow).

mosaics, only one or occasionally two diploid mitoses were observed, and almost all of the mitoses present were haploid.

C. Recovery of delayed blastocysts

Out of an initial total of 327 129 SvE and 627 (C57BL \times CBA) F_1 hybrid pronucleate-stage 1-pronuclear haploid eggs transferred to the oviducts of

recipients on the afternoon of the first day of pseudopregnancy, 64 129 SvE (20%) and 104 F₁ hybrid (17%) delayed blastocysts were subsequently recovered. The delayed blastocysts were then transferred to tissue-culture medium supplemented with serum. After 72–96 h, when the majority of embryos had ‘implanted’, the inner-cell-mass-derived lumps were either disaggregated in an attempt to determine their ploidy (see Section F), or retained in culture to establish pluripotent cell lines (see Section D).

D. Establishment of cell lines in culture

Four haploid-derived cell lines have so far been established. These lines were derived on three separate occasions over a period of several months from both 129 SvE and (C57BL × CBA)F₁ hybrid delayed blastocysts (Table 2). The origin of the various lines was confirmed by GPI isozyme analysis, as the 129 SvE-derived lines were homozygous for the Gpi-1^a isozyme, and the F₁ derived lines homozygous for the Gpi-1^b isozyme of glucose phosphate isomerase.

Table 2. *Haploid-derived pluripotent cell lines*

	Strain of origin	Lines established	Modal chromosome number
1.	129 SvE	HD1	40
2.	..	HD2	40
3.	(C57BL × CBA)F ₁	HD3	40
4.	..	HD4	40

E. Differentiation ability of pluripotent cell lines

Both the HD1 and HD2 cells formed typical well-differentiated teratocarcinomas when injected subcutaneously into syngeneic hosts. A wide range of easily recognizable cell types were present (Fig. 4A–F), in addition to nests of undifferentiated embryonal carcinoma cells. *In vitro*, all four lines formed typical simple and cystic embryoid bodies following suspension culture of cell aggregates. Cells from lines HD3 and HD4 have recently been injected into syngeneic hosts, but the results have yet to be analysed.

F. Chromosome analysis of pluripotent cell lines

Repeated attempts to determine the chromosome constitution and ploidy of the ICM-derived clumps between 72 and 96 h after blastocyst explantation have so far been unsuccessful. Despite prolonged culture in Colcemid (6–12 h), no cells have been observed in division. Parallel observations on fertilized material at similar stages of development have also failed to demonstrate cells in division (authors, unpublished observations). This appears to be a technical problem, either because the cells are not in division at the time of analysis, or because of problems associated with the disaggregation of the small clumps of cells which

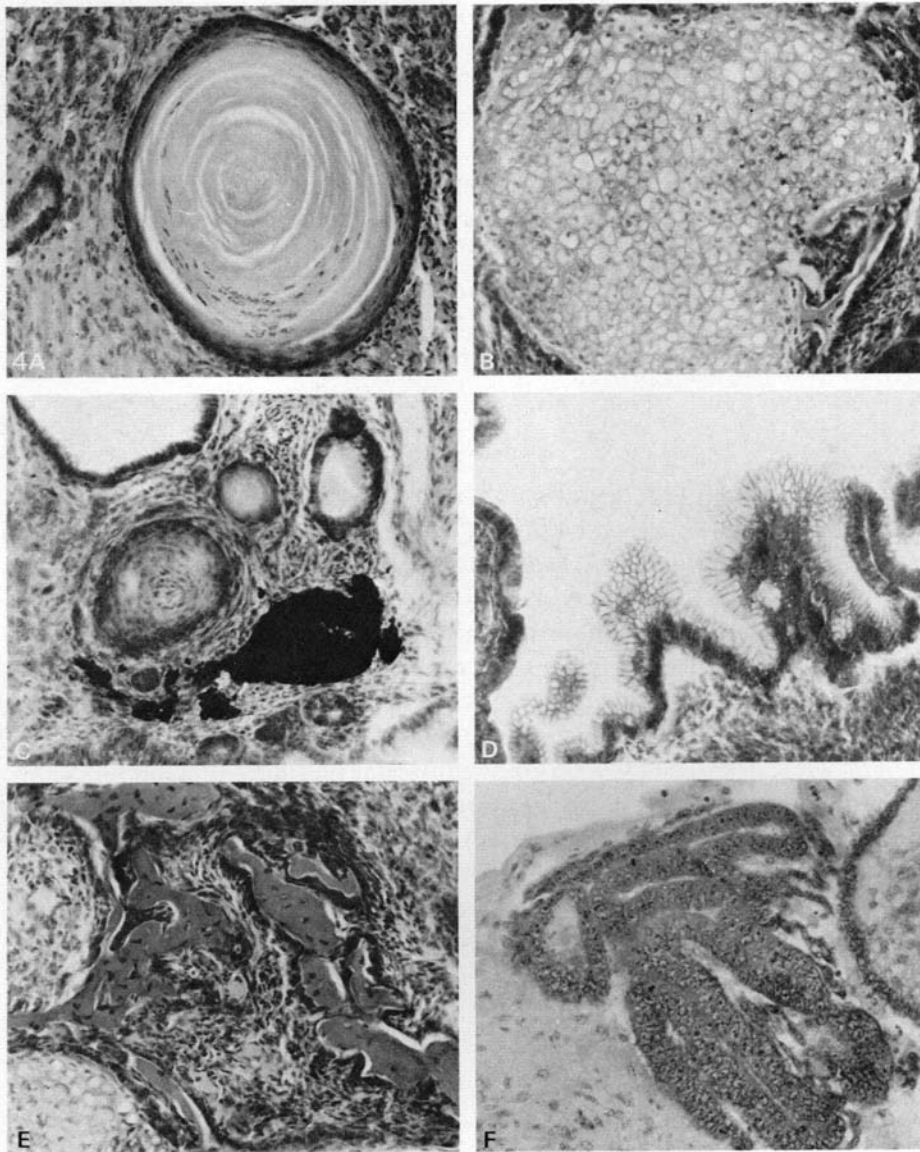


Fig. 4. Representative regions through a well-differentiated 129SvE haploid-derived teratocarcinoma. Sections stained with Masson's trichrome. A. Keratin whorl. B. Cartilage nodule. C. Region showing areas of melanin pigmentation, precartilaginous nodule and epithelial-lined tubules. D. Detail from wall of large cyst lined by secretory epithelial cells. E. Area showing tissue interspersed with yolk-sac-like material. F. Organized structure formed from folded layers of columnar epithelial cells.

were tightly adherent and failed to separate following standard disaggregation techniques.

Chromosome analysis of early passage cultures revealed that all cells observed

in division at this stage were diploid – no haploid cells were detected. All four cell lines proved to have a modal number of 40, as expected. G-banding studies of 30–35 metaphase spreads from each of the lines examined confirmed that all the cell lines had a normal diploid autosomal complement. Interestingly, all the lines at the time of karyotyping, were characterized by the possession of a deletion of the distal end of the X chromosome. However, in the HD4 line, of the 31 banded spreads which were karyotyped shortly after its establishment in culture, this abnormality was only present in 16 of the metaphase spreads.

Karyograms from the HD4 line are presented in Fig. 5. In Fig. 5A, a normal karyogram is observed, whereas in Fig. 5B the karyogram showing a deletion of approximately 25 % of the distal part of a single X chromosome is presented.

DISCUSSION

We have demonstrated that it is technically feasible to establish pluripotential cell lines from haploid embryos. These cells which were derived from haploid parthenogenones from various strains have all the properties expected in that, once established in culture, they can be induced to differentiate both *in vivo* into typical teratocarcinomas with a wide variety of cell types present, and *in vitro*.

Previous attempts to establish haploid teratocarcinomas and to derive cell lines from these sources were only partially successful in that while tumours were derived from the ectopic transfer of haploid parthenogenones (Iles *et al.*, 1975; Graham, McBurney & Iles, 1975), no permanent pluripotential lines have been reported. Lines have, however, been established from spontaneous teratocarcinomas occurring in the ovaries and testes of LT/Sv strain mice, but these are undoubtedly diploid (Martin *et al.*, 1978) and some lines appear to be restricted in their differentiation (Gachelin, cited in Nicholas *et al.*, 1976). However, in the only published report in which LT-derived teratocarcinoma cells were injected into blastocysts, Illmensee (1978) reported that in one instance out of eight chimaeric individuals obtained, the tumour-derived cells not only took part in normal tissue differentiation but even contributed to the germ line.

Chromosomal analysis carried out at different stages in the establishment of the lines reported in this paper indicated that 15–18 % of the embryonic population at the morula stage contained at least a proportion of diploid cells. In the *in vivo* series no significant difference was observed between the 129 SvE- and F₁-derived embryos in the numbers of haploid vs. haploid–diploid and diploid mitoses. A difference is apparent, however, between the *in vivo* and *in vitro* series in this regard, since more diploid mitoses were seen in the latter group (see Table 1). This may be a reflection of the fact that conditions *in vitro* may be suboptimal compared to those *in vivo* for the maintenance of haploidy during the early preimplantation period.

Several attempts to determine the chromosome constitution of the delayed blastocysts within 3–4 days after their isolation and explantation into culture

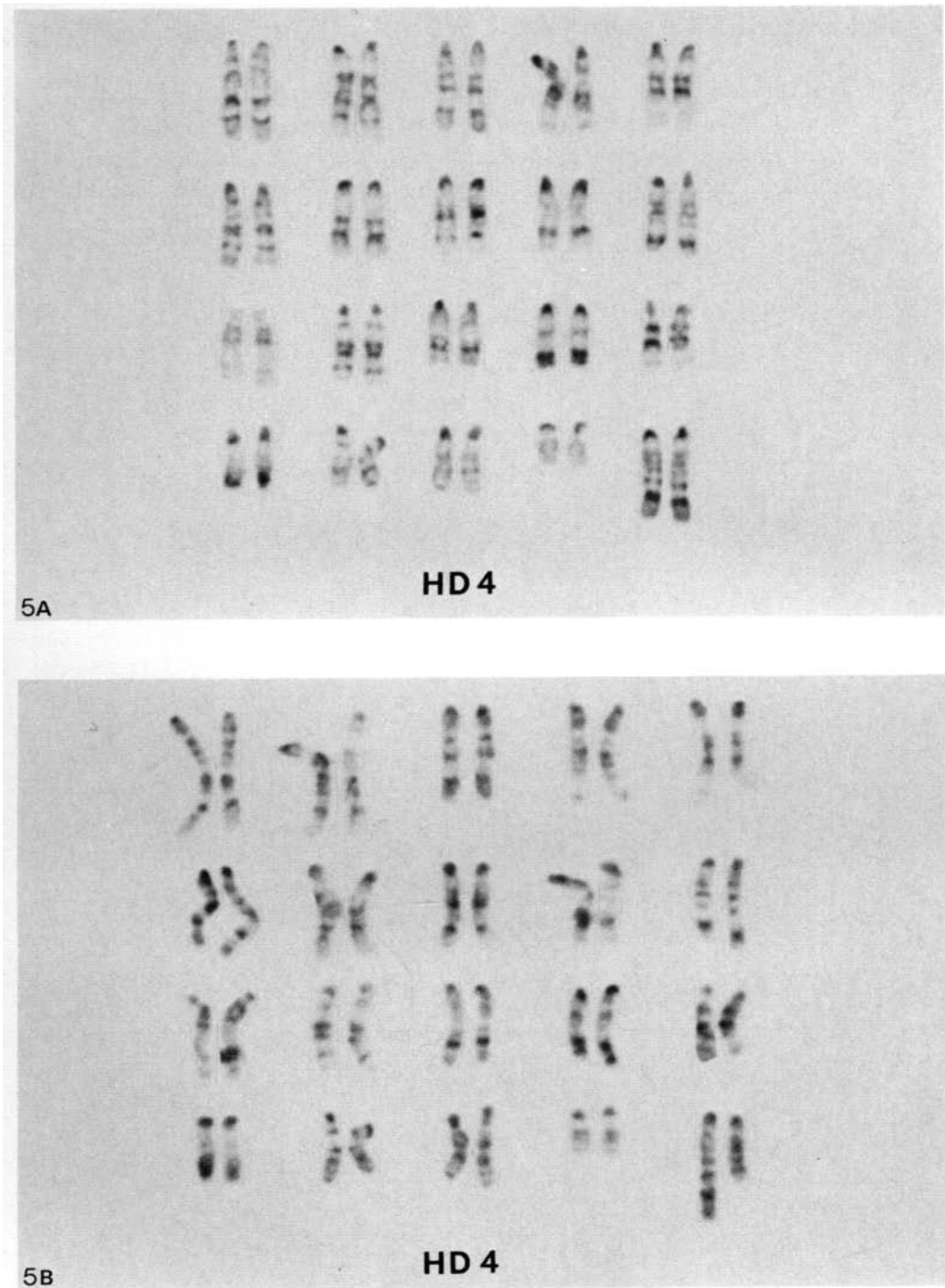


Fig. 5. Karyograms from HD4 line. A. Showing normal XX euploid chromosome complement. B. Showing deletion of approximately 25% of the distal region of one of the X chromosomes.

were unsuccessful, as no mitoses were observed in the inner-cell-mass-derived cells. In the earliest stages at which the cells were successfully karyotyped (after the establishment of mass cultures), all the cells were found to be diploid.

The chromosome constitution of early passage cultures was normal. However, with subsequent culture, partial deletions of one of the X chromosomes was evident, though this had no apparent effect on their differentiation. The extent of this deletion varies between HD lines, but the observation that the position of the break point is constant within a given line strongly suggests that, firstly, this phenomenon occurs early in their isolation and, secondly, that it does not arise by progressive deletion. It is interesting to note that the ESC stem cell line isolated by Martin (1981) is also reported as having a deletion of a single X chromosome. A more detailed analysis of the cytogenetic characteristics of these and other parthenogenetically-derived EK lines is currently being prepared (Robertson, Evans & Kaufman, 1983).

To date, no haploid mitoses have been observed in the established lines, and we can only speculate at which stage diploidization is occurring. We believe, from the morula studies indicated above, and from previous analyses of intact egg cylinders derived from haploid embryos (Kaufman, 1978*b*) that at least a proportion of the cells at explantation and shortly thereafter are still haploid.

While the success rate of establishing haploid-derived lines by the technique reported here is rather low, because of inevitable losses at each stage of the isolation procedure, attempts are being made to modify the explantation and cell isolation techniques in order to increase the chance of establishing both homozygous diploid as well as haploid pluripotential cell lines from this source. The HD lines reported here, which have been established from 1-pronuclear 'uniform' haploid embryos (Kaufman, 1981), clearly demonstrate that it is now possible to establish homozygous diploid pluripotent cell lineages of parthenogenetic origin which, at least initially, appear to be karyotypically normal, and capable of a full range of cellular differentiation.

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