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Evolution of Karyotype in Haploid Cell Lines of *Drosophila melanogaster*

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Seven continuous cell lines have been established in vitro from lethal embryos produced by the female sterile mutant mh 1182 of *Drosophila melanogaster*. Six lines show haploid metaphases. Karyotype analysis revealed a high level of aneuploid cells with frequent chromosome fragments. In three lines, haploid cells were quickly overgrown by diploid cells. Two lines were more stable but the proportion of haploid cells decreased with time. One line was stable, showing 80–90% of haploid cells for over 1000 cell generations. Stable haploid clones have been isolated from two lines. Crossing of mh 1182/mh 1182 females with males bearing a ring X chromosome shows that the haploid genome retained in the cells is of maternal origin and that the diploid cells derive from pre-existing haploid cells. The appearance of the diploid cells and the conditions of karyotypic stability are analysed.

One of the main difficulties in the genetic analysis of animal cell lines cultured in vitro lies in the diploid constitution of these cells. Obtaining haploid lines should theoretically make the recovery of recessive mutations and the analysis of somatic hybrids easier.

By using spontaneous or artificial parthenogenesis, some authors have succeeded in culturing cells originating from haploid embryos, in particular from cockroach [23], frog [14] and mouse [12, 20]. Even if sometimes only primary cultures are considered, it seems clear that haploid animal cells are viable, at least in in vitro conditions. Unfortunately, the haploid cells are almost always quickly overgrown by diploid cells. Freed & Mezger-Freed [15] were the first to obtain two continuous animal (frog) haploid cell lines. Such stability in the karyotype therefore represents the main aim in order to use this material for genetic studies.

Haploid cell lines of *Drosophila melanogaster* have been already established in vitro [6] from the progeny of females homozygous for the mutation mh 1182 [16]. We have been following the karyotypic evolution of these lines and of their clones, for 1000 cell generations. In this paper we summarize the numerous karyotypic observations made on these cells, studying more precisely the balance existing between the haploid and diploid cell populations.

MATERIAL AND METHODS

Mutant mh 1182

1182^{ts} mutation, renamed mh 1182 [26], is an X-linked mutation, isolated by Gans et al. [16]. The progeny of homozygous mh 1182/mh 1182 females die during organogenesis. Zalokar et al. [17] have

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shown the existence of haploid embryos in the progeny. Originally, and during the first attempts to establish primary cultures, the mutant stock was temperature-sensitive, embryo development being normal if their mothers were raised at 18°C, and lethal at 29°C; even at 29°C a small number of flies emerged. However, the mutant stock kept in Paris has drifted in such a way that it has become totally lethal at any temperature. There is therefore some ambiguity in the embryo pools used, the first cultures having been made with probably a small proportion of diploid embryos, while the tests made with the actual mutant stock show that all embryos are haploids.

Chromosomal Marker In(1)w^{vC}

D. melanogaster stock in Iw(1)w^{vC} was provided by Dr Gans. The ring X chromosome Iw(1)w^{vC} [18] has been stabilized over many generations in such a way that gynandromorphs can no longer be found.

Primary Cultures

The primary cultures have been made according to a modified version of the technique of Echalier & Ohanessian [10].

Establishing haploid lines. Pools of 200–300 embryos from mh 1182/mh 1182 females are collected. These embryos, 1–18 h of age, are sterilized and dechorionated by a hypochlorite solution, then rinsed in sterile distilled water. They are coarsely ground in culture medium with a glass rod having a flattened end, in order to obtain fragments of a few hundred cells. These clusters are poured off and the supernatant containing yolk is discarded. The cell clusters are then put directly into plastic culture flasks (25 cm^2) with 3 ml of culture medium, at 20°C. The medium is made up of a mixture (half/half) of D_{22} [8] and M3 [29] media, plus 20% of fetal calf serum (FCS). The choice of the serum sample is critical.

Determination of the genome retained in the haploid cells. The embryos are treated following the technique described above but the cell clusters are deposited on a glass coverslip in a Leighton tube. After 3 days of culture the karyotypes are determined.

Continuous Cultures

The established lines are cultivated in cell layers in plastic flasks with the same medium plus 10% FCS. The cells are transferred every week at 23°C, by simple scraping, with a dilution of 1:40 or 1:80. 0.5–1×10^8 cells per flask are obtained.

Karyotypes

Cells are transferred on a glass coverslip into a Leighton tube. Twenty hours later, colchicine (6 μg/ml) is added. Four hours later the glass coverslip is removed and placed in sodium citrate (0.5%). After fixation (acetic acid–methanol 3/1) the chromosomes are stained in acetic orcein. For detailed studies, colchicine is not added. For each chromosome analysis 200 metaphases are observed.

Cell Freezing

5×10^7 cells are placed in 1 ml of culture medium with 10% glycerol. The sample is cooled for 25 min at +4°C, for 35 min at −20°C and for 50 min at −60°C, then immersed in liquid nitrogen.

Cell Cloning

Isolated cells are distributed on a layer of X-ray-irradiated Kc cells into plates with 96 wells, following the technique of Richard-Mollard & Ohanessian [24]. The mean number of cells inoculated per well is around 0.5.

Reference Diploid Cell Line

The Kc cell line, established by Echalier & Ohanessian [9] is used as a reference. This line is mostly diploid (90%) with 10% of tetraploid cells. Numerous sublines and clones isolated from this line are regularly maintained in the laboratory.

RESULTS

Primary Cultures

A few hours after adding the ground embryos to the culture flasks, cells are attached to the bottom. In the best cases, clusters settle and cells multiply. A variety of cell types gradually appear, as described for wild-type cultures [4, 10, 27, 28]. For several weeks these primary cultures remain alive and vigorous muscular contractions can be observed. It is not possible, however, to transfer these cells. In most cases the whole culture eventually degenerates and then dies, after 3–8 weeks.

Colonies of undifferentiated cells which multiply quickly sometimes appear. In this case a first transfer can be attempted. These cultures are still very delicate but some of them can be transferred more and more easily. Nearly one in every ten cultures has produced a continuous line. One can roughly estimate the proportion of embryos involved in the start of a continuous line as being in the order of 10^{-2} or 10^{-3}. There is no sudden change between a primary and a continuous culture. Over a period (from 6 to 12 months) an increasing ease in the transfers has been noticed. Finally, seven continuous lines were obtained: 1182-1, 1182-3a, 1182-3b, 1182-4, 1182-5, 1182-6 and 1182-7b.

Continuous Cultures: Morphology

The seven continuous cultures each have their own particular morphology (fig. 1 *a–e*). This morphology still exhibits a certain heterogeneity which probably implies a non-clonal origin. The line 1182-1 contains two cell types, elongated and round. The line 1182-3a consists of large cells with numerous inclusions. The lines 1182-3b, 1182-4, 1182-6 and 1182-7b have round cells, 1182-6 and 1182-7b forming clusters. The line 1182-5 consists of cells which elongate to an enormous extent after transfer, even so far as to form long bridges linking large clusters. With the exception of 1182-3a, all these lines include cells of clearly smaller diameter (10 μm) than those of normal diploid cells (14 μm for Kc line and clones). There are also many (1–10%) dikaryons or polykaryons, and less frequently (0.01–0.1%) some giant cells (up to 100 μm in diameter) (fig. 1 *f–h*). These giant cells fall into two categories: the first corresponding to very large polykaryons which are incapable of undergoing cytokinesis; the second to cells with one or two very large nuclei.

Chromosome Analysis of the Lines

Gross composition. Six lines reveal haploid metaphases, of the constitution X/2/3/4 (fig. 1 *i–l*). There is a strong correlation between the proportion of haploid metaphases and the proportion of small cells in these six lines. The line 1182-3a is different. Its karyotype is mainly male diploid (XY), with a large proportion (20%) of tetraploids. Haploid cells have never been seen in this line over several thousand metaphases. In the rest of this study we will not consider line 1182-3a.

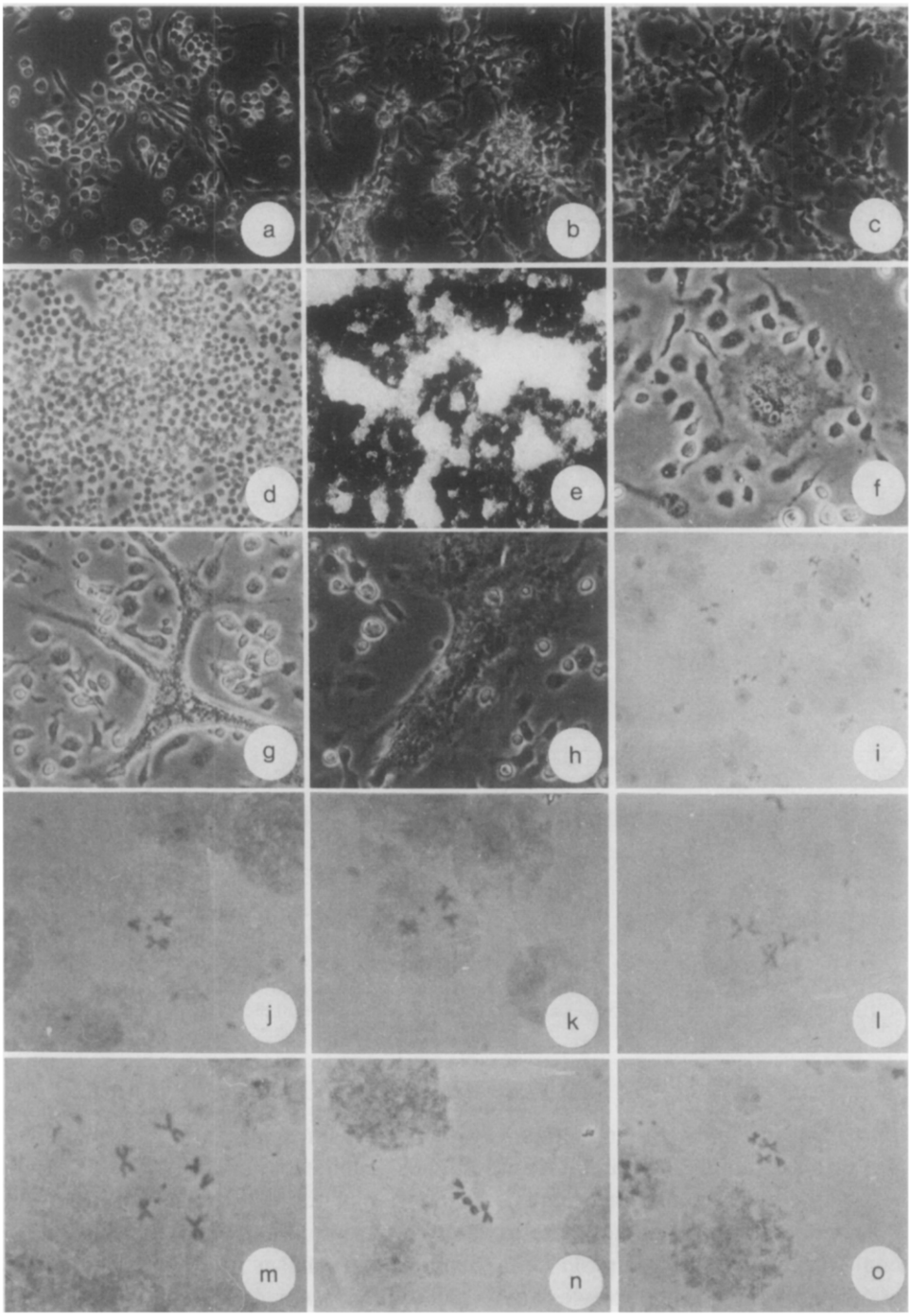
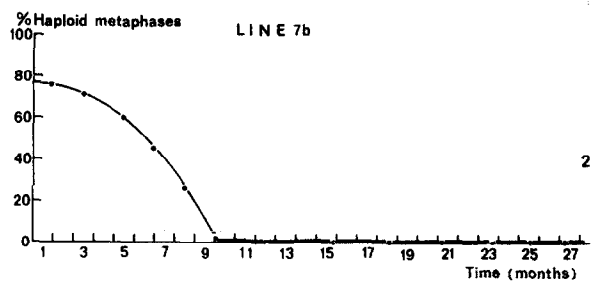
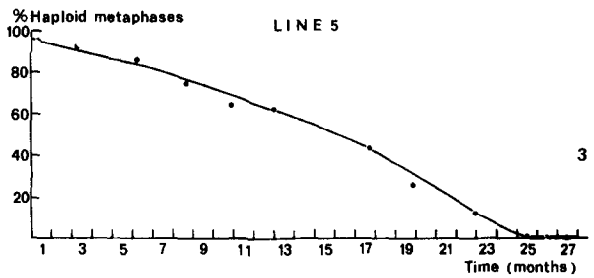
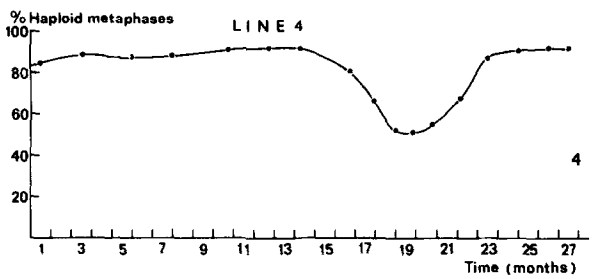


Fig. 1. Line (*a*) 1182-1; (*b*) 1182-7b; (*c*) 1182-6; (*d*) 1182-4; (*e*) 1182-5; (*f, g, h*) giant cells; (*i*) numerous haploid metaphases in line 1182-4; (*j, k, l*) haploid metaphase; (*m*) diploid metaphase; (*n*) aneuploid metaphase XX23; (*o*) aneuploid metaphase X233. (*a–d, i*)×100; (*e*)×30; (*f–h*)×200; (*j–o*)×400.







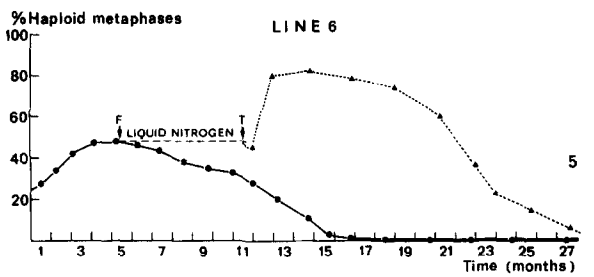


Fig. 2. Evolution of line 1182-7b.
Fig. 3. Evolution of line 1182-5.
Fig. 4. Evolution of line 1182-4.
Fig. 5. Evolutions of the original line 1182-6 and of a sample frozen.

Apart from haploids, other metaphases in the six lines are mainly diploid (fig. 1 *m*), or subdiploid. The haploids and diploids together still represent more than 80% of the cell population. There is a minority of tetraploid cells (from 0.5 to 15%, depending on the line). The number of tetraploid cells in roughly proportional to the number of diploid cells. Triploid metaphases are always very rare (less than 0.1%). No metaphases are found with 5*n*, 6*n* or 7*n* chromosomes. Cells with 8*n* chromosomes can be observed (max. 1%) above all in the lines containing many tetraploid cells. Some cells with 16*n* or 32*n* can occasionally be found.

Detailed analysis. Among those metaphases that we have called diploid, nu-

merous aneuploid cells are found, almost all hypodiploid. These aneuploid cells can be classed in two categories: the most common are monosomics for X and/or chromosome 4. In certain lines the majority of diploid cells are then replaced by X0 and/or 40 cells. Such constitutions are known to be viable, not only with regard to cultivated cells [8, 13], but also in complete organisms. The Kc line is indeed monosomic for the fourth chromosome and certain sublines or clones of Kc spontaneously drifted towards an X0 formula.

The second aneuploid category, which is much rarer (from 0.1 to 0.5%), is made of monosomics of the second or the third chromosome. A large range of different chromosome constitutions can thus be observed, varying from five to seven chromosomes (fig. 1*n*,*o*). Such monosomies are lethal for the fly [21, 31] and have never been observed in normal cell lines. On the contrary, Faccio-Dolfini [13] claims that even in abnormal karyotypes resulting from the complex evolution of in vitro cell lines of *D. melanogaster*, there is always at least the equivalent of a pair of second and third chromosomes. The presence of these aneuploid metaphases is therefore very surprising. It is not known if such cells are undergoing their last mitosis or if they are viable in vitro.

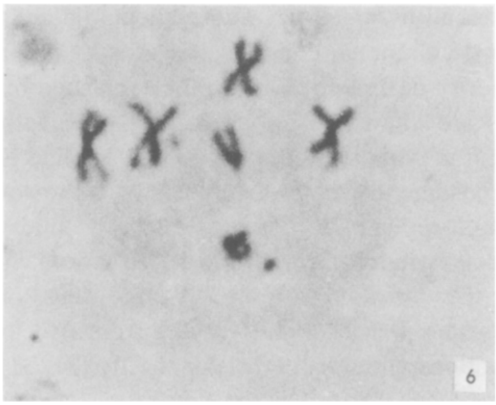
Furthermore, a significant occurrence of chromosome fragments varying from 2 to 20% of the metaphases according to the lines is observed. These fragments can be found in haploids, diploids, aneuploids and polyploids. The other chromosomal accidents are rarer. One of the most representative is the splitting of a metacentric chromosome giving rise to two smaller telocentric chromosomes. Such chromosome abnormalities are much rarer in the Kc line and its derivative clones. To sum up, these six haploid lines show a marked chromosome instability.

Evolution of the Lines

The proportion of haploid cells in a particular line varies with time. This study started from the moment when the lines became easily transferable. The karyotype composition during the first months of culture is uncertain as a result of only limited information. There are three types of evolution.

(1) The lines 1182-1, 1182-3b, 1182-7b show a rapid decline in the percentage of haploid cells during the transfers (fig. 2). The initially high proportion of haploid cells drops dramatically and after 9 months haploid cells disappear. It seems that these three lines are not easily usable.

(2) The lines 1182-5 and 1182-6 are more stable than the lines previously described but again the proportion of haploid cells becomes smaller and smaller (fig. 3). Nevertheless, this decrease is only a few percent per month and takes place over a comparatively long period of time (2 years of 1182-5, 16 months for 1182-6). Thus these lines can already be exploited for genetics. Besides, this interesting period can be extended by freezing. During the first months of culture the percentage of haploid cells increased from 20 to 85% in 1182-5 and from 10 to 55% in 1182-6. This period coincided with progressive ease of transfers.



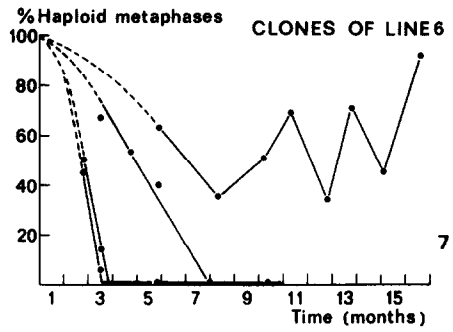


Fig. 6. Embryonic female cell with one rod X and one ring X In(1)w^{vC}.
Fig. 7. Evolutions of clones of line 1182-6.

(3) The line 1182-4 is stable. Over 3 years of culture (involving 150 transfers) this line continually showed a very high percentage (80–90%) of haploid cells (fig. 4). A momentary drop to 50% was, however, observed, followed by a rise to the initial level. In this case, therefore, the haploid cells are not overgrown by diploid cells, and this line is clearly the most useful. Two sublines of 1182-4 growing in a medium without serum were selected. These sublines have the same karyotypic characteristics as 1182-4, in constrast to the reference diploid line (Kc 0%) which under the same conditions shows a high level of heteroploidy.

Numerous samples of these six lines were frozen in liquid nitrogen while they still possessed a high proportion of haploid cells. This method allows maximum exploitation of the interesting period of an unstable line, thawing a new sample each year. We have observed three cell populations in turn, originating from the frozen samples of line 1182-6 (fig. 5). The three types of evolution were virtually the same. Thus a line, once established, maintains precise characteristics of stability. Moreover, the defrozen population had each time a higher proportion of haploid cells than the original line before freezing.

Parental Origin of the Haploid Genome

Chromosomal markers were used in order to recognize the parental origin of the genome found in the cells. Females mh 1182/mh 1182, which possess normal X chromosomes (rod-shape) were crossed with males carrying a ring X chromosome In(1)w^{vC} easily identifiable in a karyotype (fig. 6). Primary cultures were made from embryos resulting from such breeding. After a few days of culture a chromosome analysis was done, giving the following results: (*a*) 98% of the metaphases observed are haploid. All these metaphases possess a rod-shaped X chromosome and thus are of maternal origin. (*b*) 2% diploid metaphases are found, all of them with two rod-shaped X chromosomes. These cells can result

only from diploidization of pre-existing haploid cells. No metaphases with XY or rod X/ring X constitution have been found. Therefore all the embryos were initially entirely haploid (at least with this mutant stock, see Material and Methods). These results could explain the strength of haploid cells in the cultures, diploid cells being completely homozygous and having very similar growth properties. However, the existence of heterozygous diploid cells in these lines cannot be disproven, because the first cultures were made with a mutant fly stock mh 1182 in which the mutation was not fully penetrant. The line 1182-3a illustrates this fact.

Stability of the Clones

The line 1182-6 was cloned when 80% of its cells were haploid. Each clone showed a particular morphology which was much more uniform than the original line. Of ten clones three were purely diploid. The other seven were a mixture of haploid and diploid cells. It is remarkable that most of the clones lost their haploid cells much more rapidly than the original line. Four clones were studied in detail (fig. 7). Complete diploidization was achieved within less than 3 months in two of the clones, and within 7 months for one other clone. Only one clone (clone D) kept a significant level of haploid cells, despite abrupt oscillations. Three years after isolation, this clone still possesses 80% haploid cells, while there are no longer any haploid cells in the original line. Moreover, this clone shows an excellent chromosome stability, with very little loss or breakage.

The clones of 1182-4 line behave differently; out of ten clones, all were of haploid origin, and six showed a karyotypic stability comparable to the original line. The four other clones were less stable and became totally diploid 6–8 months after their isolation. Curiously most of these clones had the same morphological diversity as the 1182-4 line. The plating efficiency of lines 1182-6 and 1182-4 was at 70% and 45% respectively (with the 'feeder-layer'' technique).

DISCUSSION

Drosophila melanogaster Haploid Cells are Viable in in vitro Culture Conditions

This might seem surprising, since the cultures originate from lethal embryos. However, it is well known that the demands of cell multiplication in vitro are much easier to satisfy than those involved in normal development of an organism. Cross & Sang [4] made numerous primary cultures from lethal embryos of *Drosophila*.

Furthermore, a haploid constitution reveals recessive lethal genes normally present in all *Drosophila* stocks. Ripoll [25] has shown that in epidermal cell clones only 9% of lethal zygotic mutations were cell lethal. Lindsley et al. [21] have studied the whole genetic structure of the *Drosophila* genome. They found

one "haplo-lethal" locus (in the aneuploid context) and 52 'haplo-abnormal' loci of which 41 were Minute loci. However, a completely haploid genome is much better balanced, resulting in fewer adverse effects than an aneuploid genome. In particular, haploid cells do not show the Minute phenotype [26]. Some observations already indicate that haploid cells are not cell lethal in *Drosophila*. Very rare haploid/diploid mosaics have been found in *D. simulans* [31] and *D. melanogaster* [2, 3, 19].

Observations of primary cultures show that haploid cells are able to differentiate in vitro. No systematic comparison between primary cultures set up with wild-type embryos or haploid embryos has yet been accomplished. It is possible that the capacity of haploid and diploid cells to differentiate is not exactly the same, analogous to what is known as the 'haploid syndrome' in amphibians [11].

The Haploid Genome is of Maternal Origin

This result found with the ring X chromosome marker agrees with the data of Santamaria & Gans [26]. It is highly probable that the haploid cells of the mutant mh 1182 come from the multiplication of the female pronucleus. The oocyte must, however, be fertilized in order that development can begin. The sperm nucleus is found in the cytoplasm of the egg in a condensed form (Santamaria pers. com.). The abnormalities (polykaryons, giant cells, chromosome loss ...) seen in haploid lines lead one to suspect a defect in the mitosis of the cells. It is very unlikely that the mutation mh 1182 could be *directly* responsible for this, since flies mh 1182/mh 1182 derived from heterozygous mothers mh 1182/mh 1182^{+} are phenotypically normal. Neither is this relevant to the haploid condition, because these abnormalities can also be seen in diploid cells. On the other hand, all these lines possessed at the origin intracellular Rickettsia, the fly stock mh 1182 being itself a carrier of these symbiotic prokaryotes [32]. We now have germ-free lines through antibiotic treatment (tetracycline 100 μg/ml for a month). These abnormalities still remain and therefore are not due to the presence of endosymbionts. Conceivably these abnormalities could be linked with the particular origin of the cells (female pronucleus). Although this is still very controversial, some authors claim that all centrioles of an organism derive from the centriole brought by the fertilizing sperm (the Boveri hypothesis), notably in *Drosophila* [Sonnenblick, 30]. A systematic search for centrioles in 1182 lines showed that one of them (1182-4) is lacking this organelle [7]. This is the first Metazoan cell line without centrioles. The absence of this organelle is not peculiar to haploid cells. It is still too early to attribute the abnormalities observed to the lack of centriole.

The Six Drosophila Haploid Lines show Different Patterns of Evolution

In the frog haploid lines, androgenetic in origin, only two lines out of 150 cultures were stable [15]. The male genotype employed was then considered to be exceptionally vigorous. The *Drosophila melanogaster* strain used here is genetically speaking much more homogeneous. It is noticeable that even our most

unstable lines show a high level of haploid cells over several months of culture. No great difference seems to exist between the division rates of the haploid and diploid cells in *D. melanogaster*. In the haploid/diploid chimaeras, Santamaria & Gans have not observed a cell competition phenomenon analogous to that of Minute/Minute$^+$ mosaics [26]. The single parental origin of the haploid cells and the perfect homozygous condition of the isodiploids must reduce even more this difference. Besides, it is possible that the instability of some lines is due to the presence of 'true' diploid cells derived from embryos which have undergone normal fertilization. Moreover, the tissue origin of all these lines is certainly not the same. For example, only the lines 1182-1 and 1182-6 show very high acetylcholinesterase activity [1].

Crossing females mh 1182/mh 1182 with males bearing a ring X chromosome has shown that diploid cells arise from haploid cells. The occurrence of diploid cells in the clones issued from a haploid cell verifies this hypothesis. Cloning still does not allow one to obtain a pure haploid cell population. Even in stable haploid clones 10–20% of the cells are diploid, similarly to the Kc clones in which 5–10% of the cells are tetraploid. Two creative processes for these isodiploids can be considered, endomitosis or cell fusion. The scarcity of triploids and the absence of $5n$, $6n$ and $7n$ metaphases are not in favor of the cell fusion hypothesis. It is likely that the principal means for genomic doubling in these lines is due to endomitosis. The presence of numerous dikaryons suggests an even more precise mechanism: viz. a lack of cytokinesis followed later by fusion of the nuclei. The stability of these lines and clones depends on a balance between the formation of diploid cells and the corresponding durations of the haploid and diploid cell cycles.

To conclude, some *Drosophila melanogaster* cell lines derived from mh 1182 haploid embryos show a karyotypic stability sufficient for genetic analysis. Even in the case of unstable lines, cloning—and possibly freezing—allows one to recover a high level of haploid cells.

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