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Relationship between chromosome content and nuclear diameter in early spermatids of *Drosophila melanogaster*

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Summary

We have studied, using light microscopy, the relationship between chromosome content and nuclear diameter in early spermatids of males carrying different combinations of wild-type and compound chromosomes in *Drosophila melanogaster*. By using these genotypes we have been able to observe spermatid nuclei bearing various numbers of chromosomes ranging from only one sex chromosome and no major autosomes to almost twice the normal chromosome complement. We have found that variations in the chromosome content are accompanied by increasing the variance in early spermatid nuclear diameter; the more gametic classes produced, the higher the variance of nuclear diameters. These results indicate that measuring nuclear diameters in early spermatids represents a useful way to estimate the levels of meiotic non-disjunction and thereby to improve the characterization of lethal or male sterile mutants in which analysis of meiotic chrosome non-disjunction cannot be achieved by conventional genetic methods.

1. Introduction

The fact that the sperm nuclear size varies as a result of the amount of DNA it carries was first described in *Drosophila* by Herskowitz & Muller (1954). In an attempt to ascertain whether chromosomes were tandemly arranged inside the sperm nucleus, these authors measured the length of sperm heads in males bearing both sex chromosomes attached to the same centromere ($\overline{XY}$) in which half of the gametes do not carry sex chromosomes while the other half carry the compound chromosome. Although they failed to find the bimodal distribution they expected, it was possible to conclude that the variance of sperm head length was greater in $\overline{XY}$ than in wild-type males.

Since then, several attempts have been made to estimate the amount of DNA contained in the mature sperm nucleus as a function of its volume (Sidhu, 1964; Beatty & Sidhu, 1967; 1970; Beatty & Burgoyne, 1971). Such a relationship was clearly established by Hardy (1975). By means of three-dimensional reconstruction of serial sections of mature sperm heads, observed by electron microscopy, Hardy obtained an accurate estimate of their shape and volume, finding that the latter changes linearly with varying chromosome content while the former remains unaffected. This correlation between nuclear size and DNA amount could be used to estimate chromosome content by measuring nuclear volume. Such measurements would provide information about errors in chromosome segregation during meiosis, thus providing a way to improve the characterization of lethal or male sterile mutants in which this cannot be achieved by conventional genetical methods.

Although very accurate, Hardy's approach is not particularly practical as a systematic method since it needs thin sections, electron microscopy, and computer processing. In addition, it requires spermiogenesis to proceed until sperm maturation. We have tried an alternative method studying the nuclear sizes of early spermatids by light microscopy. Instead of the small needle-shaped, highly condensed nuclei of mature sperm, early spermatids have larger, spherical nuclei, which are easy to measure under phase-contrast optics. There is a general agreement, but no proof, that variations in nuclear size in early spermatids reflect variations in their chromosomal content. This variation has been taken to indicate abnormalities in chromosome segregation during meiosis (Lifschytz & Hareven, 1977; Ripoll *et al.* 1985; Regan & Fuller, 1988; Gonzalez *et al.* 1988; Sunkel & Glover, 1988). Nevertheless, since DNA accounts for only a very small fraction of the large nuclear mass of early spermatids, there is no *a priori* reason to believe that Hardy's (1975) observations on mature sperm can be extrapolated to other stages of spermiogenesis.

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The aim of this work is to ascertain whether a correlation between nuclear volume and chromosome content also exists in early spermatids. Like previous authors, we have taken advantage of the large number of chromosome rearrangements available in *Drosophila*. In the present work, we have measured the diameter of spermatids carrying different combinations of wild-type and compound chromosomes, ranging from those having only one sex chromosome and no major autosomes to those having almost twice the normal amount of DNA carried by a wild-type sperm. We have found that the variance of early spermatid nuclear diameters correlates to the variance of DNA content in those cells, thereby offering a method to estimate meiotic non-disjunction in lethal or male-sterile mutants in which cysts of germline cells in the testes develop to the 'onion-stage'.

2. Materials and methods

(i) Drosophila *strains*

Flies were reared in standard *Drosophila* medium at 25 °C. Three different strains were used: *red* (for a description see Lindsley & Grell, 1968) which does not have any cytologically visible rearrangement, was used as wild type; *C(2)EN, c bw* consisting of two second chromosomes sharing a single centrometere (Novitski, 1976; Novitski *et al.* 1981); and *C(2L)RM, dp; C(2R)RM, px; C(3L)RM, h; C(3R)* in which each pair of homologous autosome arms are joined together at a common centromere (see Holm, 1976).

(ii) *Cytology*

Late pupal testes were squashed following the protocol described by Hardy *et al.* (1981). Testes were dissected in 0·7% NaCl, on a siliconized slide, cut with a pair of tunsgsten needles and covered with a non-siliconized cover slip. The excess of liquid was removed with a piece of blotting paper. Observations were made in a Zeiss Universal Microscope under phase-contrast optics (neofluar ph2 40/0·75 objective; S-Kpl 10 × /18 ocular; 2 × optovar. Photographs were taken on 36 mm Kodak Technical Pan 100 ASA film, and the magnification factor of the camera was 0·25 ×. The diameters of the nuclei were measured on the negative with a Nikon profile projector 6CT2.

Measurements of early spermatid nuclei were performed during the 'onion-stage' (Lindsley & Tokuyasu, 1980), shortly after meiosis-II and before the beginning of elongation. This stage may be further sub-divided by using electron microscopy (Tates, 1971; Tokuyasu, 1974*a*, *b*). Under the light microscope however, such distinctions cannot be spotted.

To minimize variations in nuclear sizes due to slightly different developmental stages, nuclear diameters were measured in spermatids from eight

Table 1. *Kinds and frequencies of gametes produced by (a) wild-type; (b)* C(2)EN; *and (c)* C(2L); C(2R); C(3L); C(3R) *males.* maa, *major autosome arm*

Male karyotype	Segregants	Relative frequency	DNA content (*maa*)
(*a*) $\frac{x}{y}\,\frac{2}{2}\,\frac{3}{3}$	One sex chromosome + one chromosome II + one chromosome III	1	5
(*b*) $\frac{x}{y}\,\frac{C(2)EN}{C(2)EN}\,\frac{3}{3}$	One sex chromosome + one C(2)EN chromosome + one chromosome III	1/2	7
	One sex chromosome + one chromosome III	1/2	3
(*c*) $\frac{x}{y}\,\frac{C(2)L\ C(2)R}{C(2)L\ C(2)R}\,\frac{3}{3}$	One sex chromosome	1/16	1
	One sex chromosome + one compound chromosome	4/16	3
	One sex chromosome + two compound chromosomes	6/16	5
	One sex chromosome + three compound chromosomes	4/16	7
	One sex chromosome + four compound chromosomes	1/16	9

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different testes. About 15 nuclei per testis in the cases of wild type and *C(2)EN* and 40 in the case of *C(2L)*; *C(2R)*; *C(3L)*; *C(3R)* were scored.

(iii) *Estimation of chromosome content*

To quantify chromosome content, we have used the arbitrary unit *maa* (major autosome arm) equal to the chromosome content of one arm of one of the major autosomes. The aim of using such a simple estimation is twofold. First, one *maa* provides enough resolution to quantify the number of chromosomes, thereby allowing the study of its relation to nuclear size. Secondly, it is our goal to present this method in a way that can be widely used to characterize the effect of mutants affecting chromosome segregation in meiosis, and one *maa* provides an easy estimation of chromosome content. For practical purposes, we have made the following assumptions (which will be discussed later). (1) The fourth chromosome does not make any noticeable contribution to the total amount of DNA. (2) There are no major differences in size between autosome arms. (3) The contribution of each sex chromosome can be considered about the same, and similar to one chromosome arm.

Spermatids produced by the flies used in this work may then be considered to contain quantities of chromosomal material equivalent to between 1 and 9 major autosome arms (*maa*). The chromosome content of the different types of spermatids produced by males of each strain is summarized in Table 1.

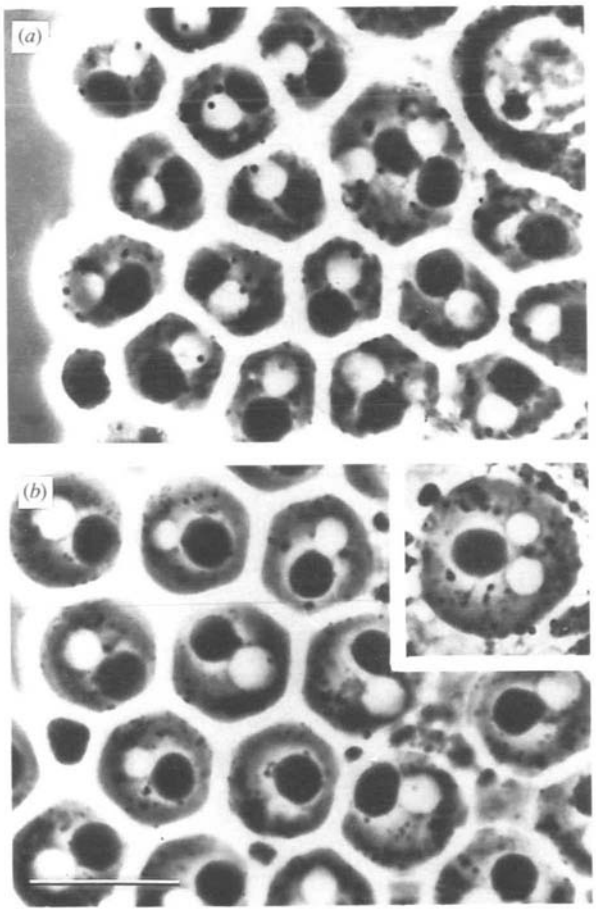

Fig. 1. (*a*) Wild-type and (*b*) *C(2L)*; *C(2R)*; *C(3L)*; *C(3R)* early spermatids in the 'onion-stage'. The black bodies are mitochondrial derivatives (nebenkerns) and the white ones are nuclei. The inset in (*b*) shows a binucleate spermatid. Scale bar = 25 μm.

3. Results

Spermiogenesis in *Drosophila melanogaster* begins after meiosis-II (see Tates, 1971, and Lindsley & Tokuyasu, 198p for a detailed description). During meiosis, the spindle is intermingled with membranes from the endoplasmic reticulum and with mitochondria which are distributed among the resulting spermatids (Church & Lin, 1982). In early spermatids, the mitochondria coalesce into a lamellate structure known as the nebenkern. This stage has been termed 'onion stage' after the appearance of the nebenkern in cross-section. Fig. 1 shows the 'onion stage' in wild type (*a*) and *C(2L)*; *C(2R)*; *C(3L)*; *C(3R)* (*b*) testes. At this stage cysts of 64 cells are present in the wild-type testis. The cells within a cyst each contain a translucent spherical body (the nucleus), associated with a phase-dark body, the nebenkern. The size of both structures is fairly constant among the cells of a cyst, nebenkerns being slightly larger than nuclei (Tates, 1971; Tokyuasu, 1975). This is not the case in the *C(2L)*; *C(2R)*; *C(3L)*; *C(3R)* 'onion-stage' cysts, where the nuclear sizes are clearly variable, and occasionally more than one nucleus is associated with a single nebenkern. The 'onion-stage' in *C(2)EN* males shows a somewhat intermediate situation (data not shown).

To ascertain whether the variations in nuclear size during the 'onion-stage' in *C(2)EN* and *C(2L)*; *C(2R)*; *C(3L)*; *C(3R)* males correlate with their differences in chromosome content, we measured the nuclear diameters of early spermatids of wild-type, *C(2)EN* and *C(2L)*; *C(2R)*; *C(3L)*; *C(3R)* males. The results are shown in Fig. 2. Wild-type spermatids show a normal distribution of nuclear diameters, with a range (r) = 1·5 μm. The distribution of nuclear diameters is more disperse in *C(2)EN* (r = 3·1 μm), where two different gametic classes are expected, and even more so in *C(2L)*; *C(2R)*; *C(3L)*; *C(3R)* males (r = 5·3 μm) where five gametic classes are produced. In the latter case, the distribution becomes clearly asymmetrical, skewed towards smaller sizes. Whereas the values of the means for the three genotypes are almost identical, the variances are clearly different (Fig. 2). A one-tailed F test renders the following values for the differences between these variances: 2·8, $P < 0{\cdot}005$ between *C(2)EN* and wild type, and 3·2, $P < 0{\cdot}001$ between *C(2L)*; *C(2R)*; *C(3L)*; *C(3R)* and *C(2)EN*. Thus, the more gametic classes expected in a given genotype, the bigger the variance of nuclear diameters in early spermatids. Consequently, an increase in the variance in 'onion-stage' spermatid nuclear diameters

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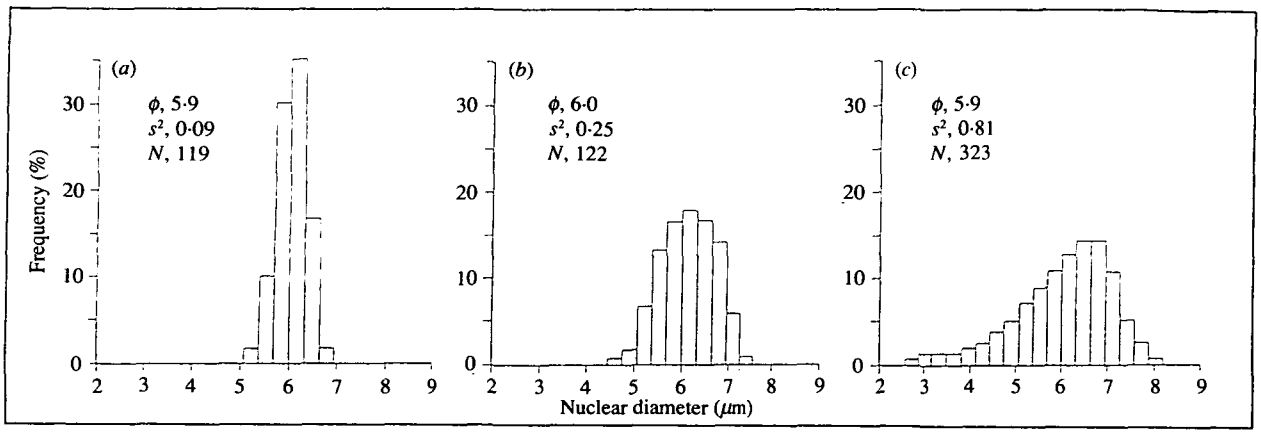

Fig. 2. Frequency distributions of nuclear diameters in 'onion-stage' spermatids from (*a*) wild-type, (*b*) *C(2)EN* and (*c*) *C(2L)*; *C(2R)*; *C(3L)*; *C(3R)* males. The data have been grouped into classes of nuclear diameters corresponding to the standard deviation of the distribution of the wild-type males. ϕ, mean nuclear diameter; s^2, variance; *N*, sample size.

Table 2. *Diameters of nuclei of early spermatids in wild type,* C(2)EN *and* C(2L); C(2R); C(3L); C(3R) *males*

	Chromosome content (*maa*)	Expected frequency		Mean nuclear size	
		Rel.	Abs.	Diameter (μm)	Volume (μm³)
Wild type	5	100·00	119	5·9	107·5
C(2)EN	3	50·00	61	5·5	87·1
	7	50·00	31	6·3	130·9
C(2L); C(2R); C(3L); C(3R)	1	6·25	19	3·7	26·5
	3	25·00	73	5·1	69·5
	5	37·50	109	6·1	118·8
	7	25·00	73	6·7	157·5
	9	6·25	19	7·3	203·7
	2	—	30	4·4	44·6

The values presented in Fig. 2, were grouped into as many classes as expected by genetic criteria, and the mean diameters and volumes of each class were estimated. The class with a chromosome content of 2 in *C(2L)*; *C(2R)*; *C(3L)*; *C(3R)* males corresponds to micronuclei defined as the smaller nuclei in those instances in which a pair of nuclei are attached to one mitochondrial derivative.

relative to the control is indicative of a variable chromosome content in those nuclei.

Considering the dispersion found in wild type as the result of natural variations and measuring errors, the significantly increased dispersion found in *C(2)EN* and *C(2L)*; *C(2R)*; *C(3L)*; *C(3R)* males is likely to be due to the different kinds of spermatids they can produce. Nevertheless, the three distributions are monomodal, and therefore discrete classes of diameters attributable to the different kinds of spermatids present in these samples were not detected. This could be due to overlap between the different populations of nuclei. However it should be possible to subdivide the three samples into as many classes as genetically expected, according to their gametic frequencies. The mean diameter obtained from these subsets of data might then resemble the actual mean diameter of nuclei with the corresponding genetic constitution.

Genetic data allow us to infer the number of expected postmeiotic nuclei belonging to each gametic class in a given genotype (Table 1). Compound chromosomes are known to segregate at random in *Drosophila* males (Holm *et al.* 1967; Holm & Chovnick, 1975) independently of other compounds and non-rearranged chromosomes, because they do not have a homologue to pair with and there is no distributive pairing in *Drosophila* males (Grell 1976). As a result all the possible combinations are found in gametes of compound bearing males. Therefore it is possible to split the populations of nuclei into size classes according to their expected frequencies. Since *C(2)EN* individuals produce two types of spermatids with equal frequency (Novitsky *et al.* 1981), we subdivided our data into two groups. One half of the nuclei, those having a size below the mean, should correspond to nullo-2 spermatids (having a chromosome content equivalent to 3 *maa*) whereas the larger nuclei should correspond to diplo-2 spermatids, thus having a chromosome content equivalent to 7 *maa*. Similar reasoning with *C(2L)*; *C(2R)*; *C(3L)*; *C(3R)* spermatids allows the subdivision of the population of

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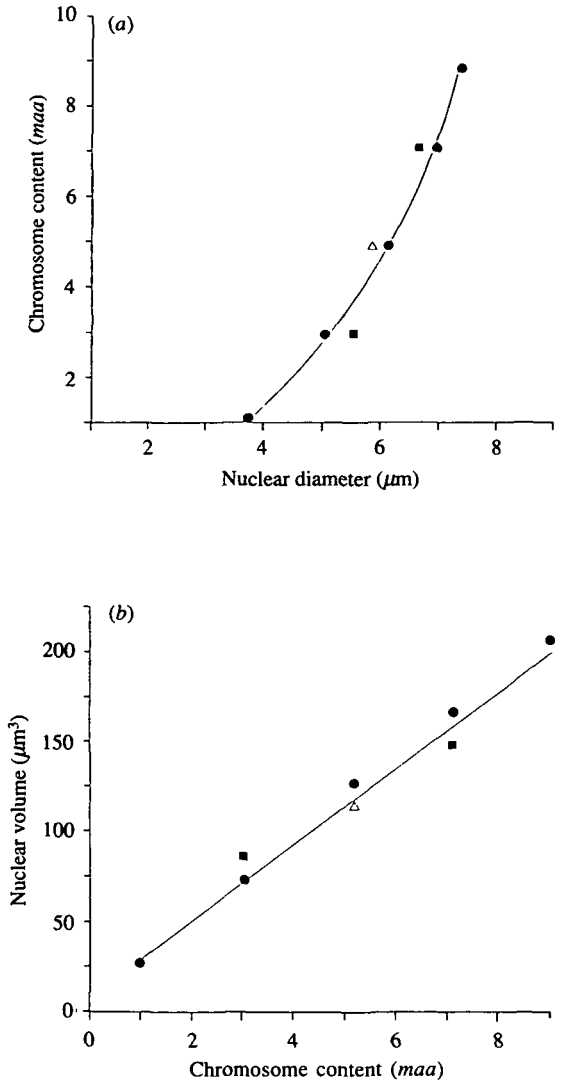

Fig. 3. Relationship between chromosome content and nuclear size in early spermatids. The points used to draw the curve were obtained from wild-type (△), *C(2)EN* (■), and *C(2L); C(2R); C(3L); C(3R)* (●) males. (*a*) Chromosome content versus mean nuclear diameter. These data fit to a power curve of the kind: $maa = 0{\cdot}01\phi^{3{\cdot}42}$ ($R^2 = 0{\cdot}97$). (*b*) Mean nuclear volume versus chromosome content. These data fit to a straight line of the kind $vol = 20{\cdot}6\ maa + 8{\cdot}96$ ($R^2 = 0{\cdot}98$). *maa*, major autosome arms; ϕ, mean nuclear diameter in μm; *vol*, mean nuclear volume in μm^3.

nuclei into five size categories according to the expected frequency of each gametic class, which contain 1, 3, 5, 7 and 9 *maa* respectively.

The results are shown in Table 2 and plotted in Fig. 3. Gametes containing 3, 5 and 7 chromosome arms are present in more than one of the genotypes under study thus offering two independent estimates of the diameters of nuclei having this chromosomal complement. After studying the correlation between nuclear diameters and their corresponding theoretical chromosome content, we found that the equation that best fits these data points is a curve of the kind $maa = 0.01\phi^{3{\cdot}42}$ ($R^2 = 0{\cdot}97$), where 'ϕ' represents nuclear diameter and R^2 is the linear regression coefficient (Fig. 3*a*). That the exponent is close to 3 suggests a linear correlation between DNA content and volume. Such an exponential relation between nuclear diameter and chromosome content explains the asymmetry found in the distribution of *C(2L); C(2R); C(3L); C(3R)* males (Fig. 2*c*). Since, for a given nucleus, chromosome content is linearly proportional to the cubic root of its diameter, the bigger a nucleus is, the smaller the variation in diameter produced by changing its chromosome content by one unit. This results in diameter distributions which are wider towards smaller sizes.

As expected, when the diameters are used to calculate the nuclear volume, assuming the nuclei are spherical, the correlation between nuclear volumes and chromosome content becomes a highly correlated straight line. The equation found is $vol = 20{\cdot}6\ maa + 8{\cdot}96$ ($R^2 = 0{\cdot}98$) where *vol* refers to nuclear volume measured in μm^3 and R^2 is the linear regression coefficient. According to this equation, when *maa* is equal to 0, nuclear volume is 8·96 μm^3, a very small volume indeed. This result provides a reasonable argument that chromosome content largely determines early spermatid nuclear volume.

Confirmation of the validity of these results comes from measurement of supernumerary micronuclei. Binucleated spermatids appear when bivalent formation during the first meiotic prophase is disrupted (Sandler & Braver, 1954; Hardy, 1975). Micronuclei have been shown by Hardy to bear only one chromosome. Since compound chromosomes lack a homologue with which to pair, they are likely to produce micronuclei. These micronuclei will contain only one of the compound chromosomes, the non-rearranged ones not being likely to do so since they have a homologue to pair with. Only in *C(2L); C(2R); C(3L); C(3R)* males is the frequency of supernumeracy nuclei high enough to allow an accurate estimate of their mean diameter. In the sample of spermatids studied in *C(2L); C(2R); C(3L); C(3R)* males we have found 293 mononucleated, 30 binucleated and 4 with three nuclei. Therefore, each univalent independently results in the formation of supernumerary nuclei in about 0.25% of the spermatids. The mean diameter of the 30 micronuclei was 4·42 μm (Table 2). According to the equation previously derived, this corresponds to 1·7 *maa*, in agreement with these nuclei bearing one compound chromosome (2 *maa*; Fig. 3).

For the wild type, the mean diameter of the nuclei in 'onion-stage' spermatids is about 6 μm, which is in agreement with the value found by electron microscopy (Tates, 1971; Tokuyasu, 1975). Assuming sperical shape, this value gives a volume of around 113 μm^3. A comparison between this volume and the volume of the mature sperm head (0·5 μm^3) indicates that the condensation undergone by the spermatid nucleus until maturation is around 226 times. According to Hardy (1975), around 18% of the total volume of the mature sperm head is DNA. Since there

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is no change in DNA amount between the two stages, it can be concluded that DNA represents around 0·08% of the actual volume of the nuclei of early spermatids. It is for this reason that, when compared to mature sperm, small variations in DNA amount in the 'onion-stage' spermatid lead to large variations in nuclear size, thus facilitating the estimation of the relationship between these two parameters.

4. Discussion

The end result of meiosis is the production of haploid spermatids, so that diploidy will be restored after fertilisation. The uniform nuclear size of the postmeiotic cells in wild-type *Drosophila melanogaster* males reflects their uniform chromosomal complement. In males where classical genetics indicates high levels of meiotic non-disjunction (hence the production of aneuploid gametes) the nuclear size of early spermatids is not uniform. This is the case, for instance, in *cry* (Hardy *et al.* 1984), *asp* (Ripoll *et al.* 1985), and *polo* (Sunkel & Glover, 1988) males. By extrapolation, differences in nuclear size have been taken to reveal abnormalities in chromosome segregation when, due to sterility or lethality, genetic analysis is not feasible (Lifschytz & Hareven, 1977; Fuller, 1986; Regan & Fuller, 1988; Gonzalez *et al.* 1988; Sunkel & Glover, 1988).

We have found that an increase in the number of gametic classes produced is accompanied by an increase in the variance of the nuclear size in the 'onion-stage' cysts and that nuclear sizes are highly correlated with the amount of DNA carried by the nucleus measured in *major autosome arms* (*maa*).

The use of one *maa* as a unit is based on the assumptions that both sex chromosomes are similar in size (1) to each other and (2) to any arm of a major autosome. That the *X* chromosome and any major autosomal arm are similar is based on their equivalent length during metaphase (Kaufman, 1934; Cooper, 1965), the number of bands they show in polytene chromosomes (see Lefevre, 1986), and their meiotic length in centiMorgans (see Lindsley & Grell, 1968). The same criteria apply for regarding the fourth chromosome as non significant in terms of its amount of DNA. The cytological criterion can also be used for the *Y* chromosome. Besides, Hardy (1975) could not find any noticeable difference in nuclear volume between sperm heads carrying the *X* and those carrying the *Y* chromosome, in spite of following a very precise, albeit laborious procedure to determine nuclear volume.

By using one *maa* as a unit, the different kinds of gametes produced by the males we have studied can be grouped into 5 classes containing 1, 3, 5, 7 and 9 *maa*, the normal haploid content being 5 *maa*. With this combination of rearrangements we can observe spermatids containing from as little as a single sex chromosome up to as much as almost twice the normal DNA amount of a wild-type gamete. Furthermore we can observe the size of spermatids carrying essentially the same amount of DNA in gametes of different karyotypes, as is the case of 5 *maa* (observed in wild-type and in *C(2L)*; *C(2R)*; *C(3L)*; *C(3R)* males) and 3 and 7 *maa* (observed in *C(2)EN* and *C(2L)*; *C(2R)*; *C(3L)*; *C(3R)* males), thus providing an additional way to confirm the consistency of the data. For these reasons, we consider that the combination of chromosomal rearrangements we have chosen offers a simple way to obtain a fairly representative range of DNA contents.

It is worth recalling that in the case of *C(2)EN* males it has been shown that either few or no offspring are obtained bearing the compound chromosome, but this effect is clearly post-elongation, probably embryonic, and does not affect the relative frequency of the two kinds of early spermatids produced by those males (Peck, 1980; Novitski *et al.* 1081).

The fact that the distribution of sizes found in *C(2L)*; *C(2R)*; *C(3L)*; *C(3R)* males is monomodal and not polymodal (one mode for each gametic class), indicates that the resolving power of this method is about at its limit when considering the differences in size between populations which differ in 1 *maa*. Nevertheless, since this is the minimal difference likely to be found as a result of meiotic non-disjunction, this technique provides a reliable quantitative method of detecting the results of meiotic nondisjunction for practical purposes. When compared to electron microscopy or other light microscopical methods, such as spectrocytophotometry, our method has the advantage of simplicity, since it does not require sophisticated systems.

Spermatids are in a transitional stage leading to condensation, and this, together with natural variations and measuring errors, does represent a source of variation unrelated to chromosome content that could account for the dispersion found in the size of wild-type spermatid nuclei. Nevertheless, since the extent of background variance introduced by those factors is likely to be the same in the different genotypes studied in this work, the fact that the variance in early spermatid nuclear sizes increases in a statistically significant fashion with the number of gametic classes produced indicates that chromosome content largely determines spermatid nuclear diameter.

By working out a calibration curve, like the one presented in Fig. 3, and the distribution of nuclear diameters of a mutant under study, the method described can be easily used to estimate chromosome non-disjunction. This will be particularly useful in cases of lethal alleles of mitotic mutants, most of which reach the third larval instar or even pupation, when onion-stage cysts are already present in the testes.

Quantification of the variations in the nuclear diameter of early spermatids has already been

performed in three mutant strains defective in mitotic and meiotic chromosome segregation: *abnormal spindle* (*asp*, Ripoll *et al.* 1985); *polo* (Sunkel & Glover, 1988) and *merry-go-round* (*mgr*, Gonzalez *et al.* 1988). These mutations result in lethality, but with *asp* and *polo* occasional fertile male escapers are recovered and chromosomal non-disjunction has been studied by genetic methods. Both, *asp* and *polo* males present high levels of meiotic non-disjunction for any chromosome pair, and accordingly nuclear diameters corresponding to chromosomal contents ranging from 2 to 10 *maa* have been found among their spermatids. Nuclei smaller than 1 *maa* have also been observed, most probably corresponding to spermatids bearing only the small fourth chromosome. In the case of *mgr* most of the spermatids have a nuclear diameter equivalent to four times the normal haploid chromosomal content, in agreement with the cytological evidence suggesting that these nuclei are tetraploid (Gonzalez *et al.* 1988). The results obtained with these mutants are a practical demonstration of the utility of this method, which offers a way to improve the characterization of the increasing number of cell division mutants which are being identified in *Drosophila melanogaster*.

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