Histochemistry (1984) 81:105–110

Histochemistry

Cytophotometric studies on cells from the ovaries of *otu* mutants of *Drosophila melanogaster*

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Received January 31, 1984 / Accepted May 5, 1984

Summary. Amounts of chromosomal DNA were estimated for Feulgen-stained, ovarian cells from flies carrying certain mutant alleles of the *otu* (ovarian tumor) gene. Epithelial sheath cells and lumen cells were found to contain the diploid (2C) amount of DNA and therefore served as internal, cytophotometric standards. Mitotically active follicle cells over young tumors from homozygous otu^1 females contained either the 2C or 4C amounts of DNA; whereas, the tumor cell population contained 2C, 4C and 8C nuclei and many intermediate values. Egg chambers also occur in homozygous otu^7 females. Follicle cells above these oocytes undergo a maximum of four cycles of endomitotic DNA replication. The accompanying nurse cells (PNC) contain polytene chromosomes. These undergo a maximum of 12 endonuclear replication cycles. The PNCs show the expected levels of DNA for the first 6 cycles and the fraction failing to replicate during subsequent cycles may be as small as 10%. Lower than expected levels of DNA were detected in PNCs from an otu^1/otu^3 ovary, reflecting roughly 20% underreplication. The latter PNCs may have been interrupted before DNA synthesis was concluded. No simple model of genomic underreplication accounts for the several different patterns of DNA behavior observed for various *otu* mutants.

Introduction

Normally each of the egg chambers in a *Drosophila* ovariole contains an oocyte and 15 endopolyploid nurse cells surrounded by a follicular epithelium (King 1970). The oocyte and its accompanying nurse cells arise from a single cell, the germarial cystoblast. This cell and the cystocytes derived from it undergo incomplete cytokinesis, and the cytoplasmic stalks connecting the sibling cells of the clone are converted into canals that subsequently permit the transfer of cytoplasmic components between nurse cells and oocyte (Brown and King 1964; Koch et al. 1967, Figs. 2, 7).

Drosophila melanogaster females homozygous for the *fs(1)231* mutation are sterile because they fail to produce normal egg chambers. When reared at 23° C homozygous females generate ovarian chambers filled with mitotically active cells that resemble cystocytes (King et al. 1978). The mutation was renamed otu^1 because of these ovarian tumors.

King (1979) analyzed reconstructions made from electron micrographs of serially sectioned regions of three adjacent tumors in an otu^1 ovary. About 1.6% of the cells were in mitotic stages. About 35% of the interphase cells were not connected to any other cells, while the remainder were present in clusters generally containing 2–4 cells. These cells were connected by canals, and from the ratio between the number of canals observed and the number of cells sampled it was possible to estimate the average frequency of incomplete cytokinesis. Since the value was 0.47, it follows that cystocytes undergo complete cytokinesis 53% of the time in otu^1 tumors, and this constitutes one of the primary defects of the mutant.

When reared at 18° C otu^1 homozygotes produced chambers containing cells which because of their large nuclei resemble nurse cells. They are called pseudonurse cells (PNC), since their chromosomes are morphologically different from those of wild type nurse cells. In normal nurse cells the endomitotic cycles produce dispersed chromosomes that are of little cytological interest (King 1970, Fig. VI-I). In PNCs, however, the replicating chromatids remain together to form giant polytene chromosomes (Dabbs and King 1980; King et al. 1981). The frequency of PNCs is much higher in the ovaries of females homozygous for certain other independently-induced allelic mutations. This is especially true for otu^3 and otu^7 (King and Riley 1982; Bishop and King 1984), and when otu^1 is combined with either of these alleles, PNCs occur in the ovaries at 23° C, as well as at 18° C.

This paper will describe comparative studies of the replicative behaviors of mutant tumor cells, pseudonurse cells and other ovarian cells utilizing cytophotometric measurements of individual nuclei stained by the Feulgen procedure. Both tumor cells and the follicle cells that surround them are mitotically active. We wished to determine whether these two different populations differed in their replicative behaviors. The follicle cells surrounding vitellogenic oocytes cease dividing and become endopolyploid. We wanted to estimate the maximum number of cycles of DNA replication these mutant cells could undergo. In the case of pseudonurse cells we wished to find the maximum number of DNA replications their giant polytene chromosomes could accomplish and to see whether there was evidence for differential replication of eu- and heterochromatin.

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Materials and methods

In laboratory crosses *otu* mutations are transmitted from generation to generation by matings between *otu* males and *otu*/+ females. The *otu*/+ allele is carried by FM3, a balabcing chromosome marked by the dominant gene *Bar*. FM3 also contains a series of paracentric inversions and two recessive lethal mutations. The X chromosome containing the otu^1 allele also carries the marker genes ct^n and v^{24}, while the otu^3 X only carries v^{24}, and the otu^7 X carries no marker genes. Lindsley and Grell's monograph (1968) contains a description of the FM3 chromosome and the marker genes referred to above. The flies were reared on David's medium (David 1962) at 23° ± 1° C in a normal dark-light cycle.

Feulgen-stained whole mounts were made of ovaries from flies of specified ages and genotypes following the procedure described in King et al. (1957), and slides of squashed, Feulgen-stained ovaries were prepared according to the method of Mulligan and Rasch (1980). Preparations for cytophotometry were mounted in matching refractive index liquids (n_D 1.524–1.532) to minimize nonspecific light loss due to scatter. The amounts of DNA-Feulgen staining in individual groups of chromosomes from each PNC nucleus were determined at 560 nm in relative absorbance units with a Vickers M86 scanning and integrating microdensitometer. Compensation for a glare error of 3% was made by an electronic offset, as recommended by Bedi and Goldstein (1976), after adjustment of the condensor iris diaphragm to match the numerical aperture of the 20X, 40X, or 100X objectives used to measure nuclei of various sizes with the high resolution scanning pattern of this instrument. The Vickers microdensitometer is interfaced to a SOL IIIA microcomputer for rapid data storage, retrieval and statistical processing with custom designed software (Rasch and Rasch 1979).

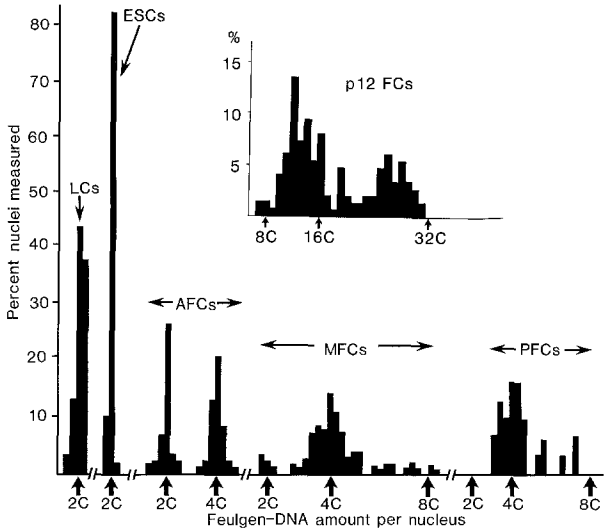

Fig. 1. Frequency distributions of DNA contents (expressed as multiples of the C value) for nuclei from ovarian cells from otu^1 homozygotes. Lumen cells (LCs) and epithelial sheath cells (ESCs) are diploid. Follicle cells show higher C values with age. AFCs, MFCs, and PFCs each represent a sample of the Follicle Cells covering a single tumor located in the Anterior, Middle, or Posterior region of an ovariole, respectively. The figure in the upper right shows the DNA distributions from follicle cells surrounding p12 egg chambers from an otu^7/otu^7 ovary. See text for further discussion

Results and conclusions

Epithelial sheath cells and lumen cells

In earlier studies Mulligan and Rasch (1980) estimated the amounts of DNA in haploid and diploid cells of *Drosophila melanogaster* by DNA-Feulgen cytophotometry, using *Xenopus laevis* erythrocytes as a reference standard. The haploid (or C) value for females was estimated to be 0.20 pg DNA. Mulligan and Rasch also found that terminal filament cells contained the 2C amount of DNA and oocyte nuclei the 4C amount.

The germarium and vitellarium reside within a tubular multinucleate epithelial sheath. This is a contractile structure containing numerous smooth muscle fibrils and abundant tracheoles (King 1970, Fig. I-7, 8). The fluid-filled lumen which extends between the epithelial sheath and the ovariole contains small populations of lumen cells. These plasmatocytes scavenge the fluid and neighboring tissues and also repair the tunica propria (Koch and King 1966). We made measurements on 83 epithelial sheath cells (ESCs) and 40 lumen cells (LCs). These showed little variation, and the peaks of their frequency distributions corresponded to the 2C amounts of DNA (Fig. 1). This being the case, when measurements were made on follicle cell nuclei, tumor cell nuclei, or PNC nuclei from a given ovary, ESCs or LCs in the same preparation were also measured and served as internal standards. By setting the absorbance values for ESCs or LCs from a given specimen equal to 2C, we were able to transform all measurements made on other cells to multiples of the female C value.

Follicle cells

Females homozygous for otu^1 and reared at 23° C contain ovarioles in which almost all chambers are tumorous (Dabbs and King 1980; King and Riley 1982). This statement applies to flies 1–7 days old. PNCs do appear in substantial numbers during the second week of adult life (King et al. 1978). The relative age of a given tumor can be estimated by its position in the ovariole: those closest to the germarium are youngest and those closest to the oviduct are oldest. In an excellent squash preparation of an ovary from a 4 day old otu^1 homozygote it was possible to distinguish follicle cells (FCs) from tumors at anterior, middle and posterior regions of ovarioles. Absorbance estimates were made for 100 FCs from an anterior tumor, 123 FCs from the tumor in the mid region, and 96 FCs from a posterior tumor. The distributions of values obtained from FCs of the 3 classes are shown in Fig. 1.

The follicle cells from the anterior tumor belonged to two equal sized populations of 2C and 4C cells. In the middle tumor most of the 2C cells had shifted into the 4C population, and in the posterior tumor no 2C cells remained. In both of these tumors most FCs belonged to the 4C group, but 8C cells were present and so were intermediate groups.

Normally follicle cells undergo endomitotic DNA replications as they begin the synthetic activities that attend the secretion of the vitelline membrane and chorion. The largest polytene chromosomes occur in mutant chambers belonging to a pseudo 12 (p12) stage (King and Riley 1982, Figs. 2A, 5A). In a normal stage 12 the oocyte has almost reached its maximum volume, and its nurse cells are shrunken and compressed into an anterior cap (King 1970, Fig. II-15). The oocyte is covered by a compartmented endochorion, the dorsal appendages are about a fourth their final length, and the micropylar cone is beginning to form. In a p12 chamber the mutant oocyte is about half the size of a normal stage 12 oocyte, and it is covered by an abnor-

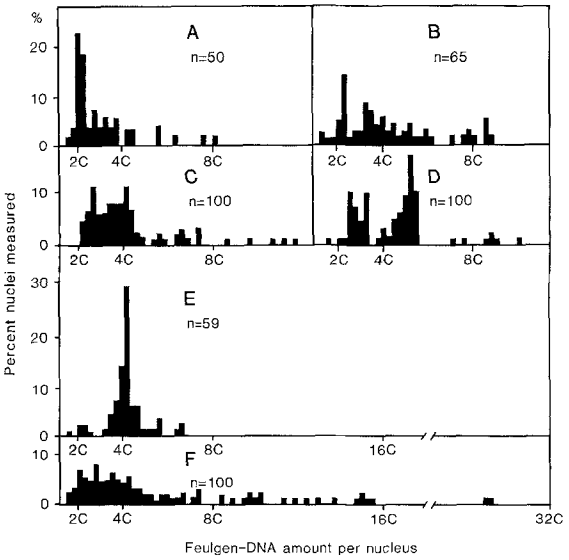

Fig. 2. Frequency distributions of DNA contents (expressed as multiples of the C value) for nuclei from 6 populations of ovarian tumor cells from *otu*^{1} homozygotes. A, small anterior tumor; B, medium sized tumor in mid region of ovariole; C, D, large tumors in mid regions of ovarioles; E, posterior tumor. Tumors A–E were from a 4 day female. F, posterior tumor from a 6 day female. See text for further discussion

mally thick vitelline membrane and a folded endochorion. The dorsal appendages are normal and a micropylar cone is forming (Bishop and King 1984, Fig. 7). The nuclei from 150 follicle cells surrounding two p12 oocytes from a single ovary were also measured. The majority of the cells from both populations were hypo 16C or hypo 32C. Therefore most of the follicle cells surrounding these p12 oocytes were synthesizing DNA after completing replication cycles 2 or 3. The pooled data for these follicle cells are presented in Fig. 1 (p12 FCs).

Tumor cells

Absorbance measurements were made on nuclei from tumors of various ages. In Fig. 2, the frequency distributions are presented in an order (A–F) that is thought to correspond to samples of increasing age. That is, in a given ovariole anterior tumors are younger than posterior tumors, and for a specific location in two or more ovarioles the larger tumors are considered to be older than the smaller ones. Finally, given two posterior chambers, the older one comes from the older fly. Note that all the estimates given in a single frequency distribution are for the cystocytes from a single tumor.

The tumor cells behave very differently from the follicle cells that surround them. In anterior chambers the nuclear values for FCs cluster about the 2C and 4C amounts of DNA (see Fig. 1, AFCs). The enclosed tumor cells (Fig. 2A) show a peak population that is diploid, but there are many cells with intermediate values between 2C and 4C and between 4C and 8C. As the tumors age, the 2C population declines, the 4C population increases, and so does the population of cells containing intermediate values between 4C and 8C (see Fig. 2B–E). Eventually cells appear with DNA values between 8C and 16C (Fig. 2D, F), and there are even a few which almost reach the 32C amount.

The percentage of cells in mitosis varies between 2 and 5% in the tumors. The cells with DNA values between 2C and 4C presumably are in the S phase in preparation for mitosis. Some of the cells with DNA values between 4C and 8C may also be preparing for mitosis, since polyploid mitotic figures have been observed in *otu*^{1} tumors (King 1979; Dabbs and King 1980). However, others are probably entering the endomitotic cycle characteristic of pseudonurse cells. Tumors containing one or several PNCs sometimes occur in *otu*^{1} ovaries (Dabbs and King 1980, Fig. 5). Cells with DNA values between 8C and 32C have probably differentiated into PNCs.

Pseudonurse cells

Measurements were made on squash preparations of ovaries from females of 4 different genotypes: *otu*^{1}/*otu*^{1}, *otu*^{3}/*otu*^{3}, *otu*^{1}/*otu*^{3}, and *otu*^{7}/*otu*^{7}. All females were 7 days old, except the *otu*^{1}/*otu*^{1} female which was 14 days old. Only those chromosomes that could be identified as belonging

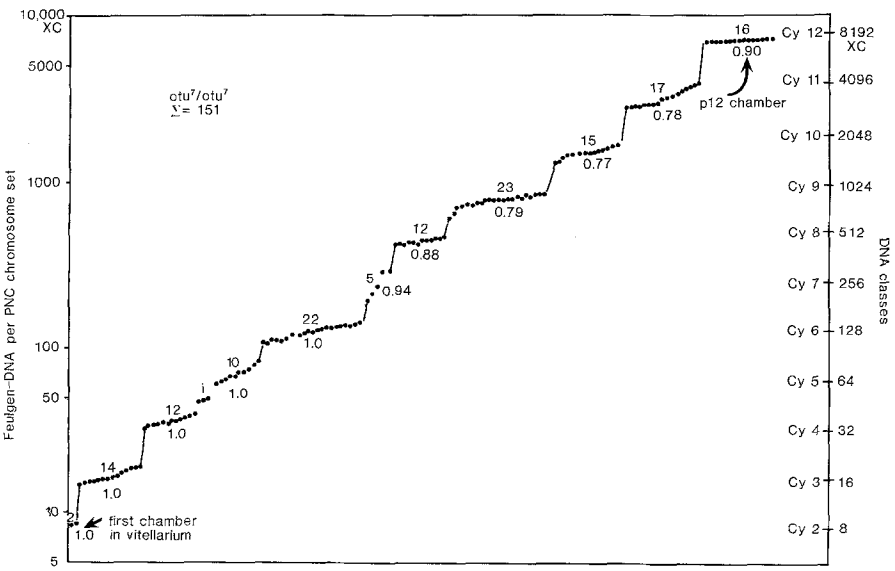

Fig. 3. Measurements of the DNA contents (expressed as multiples of the C value) of 151 sets of pseudonurse cell chromosomes from an *otu*^{7}/*otu*^{7} ovary. The right vertical axis shows the C values expected after 2–12 endomitotic cycles of DNA replication. The numbers above each "step" show the number of estimates in each series. The decimals show the ratios between the medians for the series and the expected DNA values for the appropriate cycles. A cluster of 3 nuclei with DNA values intermediate between the cycle 4 and 5 series is labeled with an *i*. See text for further discussion

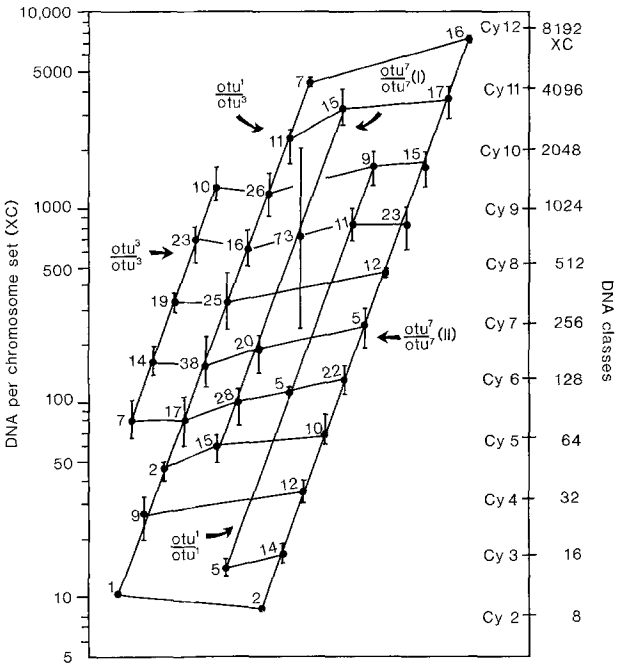

Fig. 4. DNA contents of pseudonurse cells belonging to endomitotic DNA replication cycles 2-12. The curves show the data from 5 different ovaries: otu^3/otu^3 (73), otu^1/otu^3 (152); otu^7/otu^7 (I) (151), otu^1/otu^1 (30), and otu^7/otu^7 (II) (94). The numbers in parentheses give the total measurements for the PNC population from a given ovary. The number opposite each point gives the number in the series. See text for further discussion

to a single nucleus and which were grouped in an area free of follicle cell nuclei were included in the samples.

When the measurements from a single ovary are placed in ascending order, they fall into a series of size classes, where the larger groups are multiples of the smaller ones. In Fig. 3 data consisting of 151 measurements from an otu^7/otu^7 ovary are plotted semilogarithmically. The estimates are given as multiples of the C value, and they range between 8 and 7653C. There are 11 classes representing the endomitotic DNA replication cycles 2 through 12.

In Fig. 4 the combined data (599 measurements in all) from 5 different ovaries are plotted in a way that allows comparisons to be made between estimates from PNCs of different genotypes for a given replication cycle. For each cycle the median value is plotted together with highest and lowest values obtained for that class. The median values for a given cycle for the PNCs from the different populations are joined by horizontal lines.

There are two populations from ovaries of the same genotype (otu^7/otu^7 I and II). Note that in I, the cells in cycles 8, 9, and 10 showed a continuous distribution. The otu^1 ovary contained only 30 PNCs, and only cycles 3, 6, 9 and 10 were represented. In this case and in the otu^3/otu^3 ovary, the PNCs developed only as far as cycle 10.

When otu^1/otu^3 and otu^7/otu^7 PNCs are compared, class by class, one almost always finds larger median DNA values for otu^7. If one divides the median DNA values for the series representing cycles 4, 8, and 12 by the expected DNA values for the cycles (32C, 512C, and 8192C), one gets ratios of 1, 0.88, and 0.90 for otu^7 (see Fig. 3). The values obtained for otu^1/otu^3 for cycles 4, 8 and 12 are 0.84, 0.64, and 0.53, respectively. So in the case of the otu^1/otu^3 ovary relatively less DNA was synthesized in later than earlier cycles.

Discussion

Our cytophotometric measurements indicate that follicle cells and tumor cells behave differently in young tumorous chambers from *otu* mutant flies. Follicle cell nuclei are 2C or 4C, whereas tumor cell populations contain 2C, 4C and 8C nuclei, as well as many nuclei with intermediate DNA values. Follicle cells seem to be more synchronized with respect to cycles of DNA replication than the tumor cells that they enclose (Figs. 1 and 2). This synchrony may be related to the presence of gap junctions between follicle cells, which are known to be electrically coupled in wild-type egg chambers (Caveney and Berdan 1982). There is no evidence, however, that the rapidly dividing cystocytes making up the tumors in *otu* mutants are interconnected in the same way. Follicle cells surrounding young tumors are themselves mitotically active, apparently dividing to keep abreast of the increase in surface area of the growing tumor. Follicle cells surrounding vitellogenic chambers cease to divide, but continue to synthesize DNA (Fig. 1). In otu^7/otu^7, the follicle cells surrounding vitellogenic chambers undergo 4 cycles of endomitotic DNA replication. These cycles seem to be asynchronous, since the cells surrounding the largest oocytes showed a range of DNA values between 8C and 32C (Fig. 1, inset).

In Anopheline mosquitoes, large banded polytene chromosomes are present in both larval salivary gland cells and in ovarian nurse cells (Redfern 1981). In *Anopheles stephensi* the mitotic X chromosome is submetacentric. Its short arm is heterochromatic and its long arm contains a heterochromatic block adjacent to the centromere and a euchromatic segment at its telomeric end. However, the X is represented by a single arm in the polytene chromosomes of the salivary gland cells and the ovarian nurse cells. Redfern (1981) has suggested that the small size of the polytene X chromosome, relative to the autosomes, in comparison to the situation in mitotic chromosomes is due to the underreplication of heterochromatic segments during endomitotic cycles of DNA synthesis.

In the case of the pseudonurse cells of *Drosophila melanogaster* we find no evidence that a *constant* fraction of the DNA fails to replicate during all endomitotic cycles. The otu^7 data (Fig. 3) show expected levels of DNA for the first six endomitotic cycles of DNA synthesis, assuming 100% replication. Although the median values for subsequent cycles are roughly 80%–90% of expected DNA amounts, the highest values for each group cannot be distinguished statistically ($p<0.05$) from levels that reflect virtually complete replication. Using the mean Feulgen-DNA contents determined for 2C nuclei from terminal filament or lumen cells and the coefficients of variation obtained from such sets of measurements (7.5%–9.5%), it is possible to compute projected Feulgen-DNA values over various cycles of replication with different assumed percentages of replicating euchromatin vs non-replicating heterochromatin. For example, if one assumes that there is roughly 25% heterochromatin in the diploid genome and that this DNA fraction does not replicate during subsequent polytenization after achieving the 4C level, the mean relative amounts of DNA in succeeding classes would be 4C, 7C, 13C, 25C, 49C, 97C, 193C, 385C, 769C, 1481C, 3073C and 6145C.

Similarly, if there is only 20% non-replicating heterochromatin, the expected DNA class means would be 4C, 7.2C, 13.6C, 26.4C, 52C, 103.2C, 205.6C, 410.4C, 820C, 1639.2C, 3277.6C and 6554.4C. We found no such consistent patterns in the present study (Fig. 4). Other, more complex models can be derived by assuming that the percentage of replicating DNA varies from cell to cell or that underreplication starts during different cycles in certain cells. Realistic models must also take into account possible differences in rates of DNA synthesis during the later stages of polytenization of PNC chromosomes, a parameter for which no reliable data are presently available.

As discussed by Lakhotia (1984) for polytene nuclei of *Drosophila nasuta,* by Dennhöfer (1982a) for salivary gland nuclei of *Drosophila melanogaster* and by Lamb (1982) for midgut and Malpighian tubule nuclei of the latter species, there is no simple model of underreplication that accounts for all of the patterns of DNA levels that we have found for PNC nuclei from several different *otu* mutants of *Drosophila.* When 95% confidence limits for 2C nuclei are used to estimate expected mean Feulgen-DNA values for highly polytene PNC nuclei in *otu* mutants, many of the observed values fall within the range of the projected 2C doublings. In such cases (Fig. 3), the fraction that fails to replicate may comprise as little as 10%–15% of the total DNA. For populations of PNC nuclei from other *otu* mutants (Fig. 4) the fraction that fails to replicate appears to be as much as 20%–30%, an estimate that agrees with previous findings for normal and mutant nurse cell nuclei by Mulligan and Rasch (1981) and Mulligan et al. (1981).

Since the cytophotometric data presented here were corrected for glare (Bedi and Goldstein 1976) but not for residual distributional error, it is possible that some lowering of apparent integrated absorbance values, particularly in large, highly dispersed nuclei, may be attributed more correctly to this type of systematic error in microdensitometry, rather than construed as unequivocal evidence for substantial underreplication of DNA in all polytene PNC nuclei. Dennhöfer (1982a, b) and Lamb (1982) have discussed in detail other experimental variables such as staging of animals, methods for tissue preparation, conditions for the acid hydrolysis step in the Feulgen reaction and appropriate methods of evaluating cytophotometric data from dipteran cell systems that are actively undergoing polytenization.

Since we generally observed five rod-shaped chromosomes in our squash preparations, it is clear that the arms of chromosomes 2 and 3 were usually torn apart during the squashing procedure. In some preparations of PNC nuclei from younger chambers, however, the pericentric heterochromatin appeared as thin, Feulgen-positive filaments connecting the arms of the two large autosomes. Chromosomes of the latter type often occurred in PNC nuclei with DNA levels that would be expected for 100% replication of 2C amounts. It follows, therefore, that the pericentric heterochromatin and adjacent regions of euchromatin may both be polytene. Observed differences in chromosome morphology and thickness may reflect differences in the coiling behaviors of the component strands between eu- and heterochromatin. As shown by Lakhotia (1984), differences in the nucleotide base sequences of eu- and heterochromatin must also be considered when trying to account for differences in chromosome morphology and total DNA amounts.

Highly repetitive satellite DNA has been localized in the pericentromeric heterochromatin and, in the case of *Drosophila virilis*, such DNA is underreplicated in ovarian cells (Endow and Gall 1975; Renkawitz-Pohl and Kunz 1975). We have observed lower than expected levels of DNA in PNCs from the ovaries of otu^1 and otu^3 homozygotes (Fig. 4). The apparent discrepancy of these data from our findings on otu^7 ovaries may be due simply to intrinsic differences in these specific genotypes, but may also be explained, in part, by assuming that a substantial fraction of the PNCs in ovaries from other *otu* mutants may have been interrupted during the last few replication cycles when these flies were sacrificed to make ovary preparations. The same argument can be applied to observations of DNA underreplication in ovarian cells of *Drosophila virilis.* Since heterochromatin is normally late replicating and continues in the S phase after euchromatin has completed its replication, the freshly eclosed females analyzed in biochemical experiments may have had the replication of some satellite DNA sequences prematurely terminated under the experimental conditions used (Endow and Gall 1975). Finally, as reported by Mahowald et al. (1979), we have also observed lower than expected levels of DNA in follicle cell nuclei from chambers in vitellogenic stages (Fig. 1, inset). These differences between expected and observed DNA levels may also be due either to premature termination of the synthesis of heterochromatin or to its underreplication. Selective amplification of chorion genes has been shown to occur late in oogenesis in normal follicle cells (Spradling and Mahowald 1980).

Acknowledgements. We are grateful to Drs. Madeleine Gans and Anthony Mahowald for supplying initial cultures of otu^3 and otu^7, respectively. Squash preparations of otu^1 tumors were made by Dr. Joseph Cassidy. The technical assistance of Mr. Shawn Riley is gratefully acknowledged. This research was supported by the National Science Foundation (grant PCM 7907597).

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