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Review

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Underreplication During Polytenization?

Recent Cytophotometric DNA Determinations and Related Biochemical Results Concerning Polytene Salivary Gland Nuclei of *Drosophila melanogaster*

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Summary. Recent cytophotometric DNA determinations and results of labeling experiments are compared with results of biochemical experiments concerning larval polytene salivary gland nuclei of *Drosophila melanogaster*. Recent publications (Dennhöfer 1981; 1982 a, b) demonstrate that methodological errors both in hydrolysis of the DNA before Feulgen reaction and in interpretation of the cytophotometric values give raise to the hypothesis of heterochromatic underreplication during polytenization. It is concluded also that methodological difficulties cause the absence of polytene SAT-DNA in biochemical centrifugation experiments since, because of different solubilities of eu- and heterochromatic DNA, the latter is not resolved in DNA isolation procedures from polytene nuclei.

Key words: *Drosophila* – Polytene nuclei – Underreplication – Polytenization – Cytophotometry – Heterochromatin

Introduction

The publications of Rudkin and Schultz (1961) and Rudkin (1964, 1965, 1969) report an underreplication rate of 20–30% in polytene salivary gland chromosomes of *Drosophila melanogaster* during polytenization. This percentage agrees with the heterochromatic portion of the diploid karyotype (Perreault et al. 1968). Other authors do not confirm the result of underreplication, because they find complete doublings of the DNA quantities (Swift and Rasch 1954; Rasch 1970; Rodman 1967). Recent cytophotometric DNA determinations show that the values of ^{3}H-thymidine non-incorporating salivary gland nuclei of *Drosophila melanogaster* alternate with those of labeled nuclei (Dennhöfer 1981). The extinction integrals of non-labeled polytene salivary gland nuclei are exact doublings of the 4 C value of the corresponding karyotype. This is true also for aberrant karyotypes, which differ in the number of the Y-chromosome (Dennhöfer, in press). Further, it is demonstrated that the number of nuclei which fall into complete DNA size classes is dependent on the temporary state of the replication cycle. This process, however, is strongly correlated with the larval development (Dennhöfer, in preparation). These recent cytophotometric results contradict the hypothesis of underreplication during polytenization in *Drosophila melanogaster*.

The methodological details of the cytophotometric DNA determinations and labeling experiments, and results of in-situ-hybridizations are comprehensively discussed (Dennhöfer, in press). The most important argument is that the DNA of each nucleus should be quantitatively determined. This is possible using an optimum method of hydrolysis before Feulgen reaction, which quantitatively depurinates the DNA both in eu- and heterochromatin resulting in maximum DNA values of each nucleus.

Recent cytophotometric DNA determinations and related labeling experiments in polytene salivary gland nuclei do not agree with biochemical results: up to now no SAT-fractions of heterochromatic DNA has been found in polytene nuclei by centrifugation experiments. First it is necessary to review the biochemical facts concerning polytenization in order to discuss the discrepancies between the results of cytophotometric and biochemical experiments.

Diploid Non-polytene DNA

Biochemical characterization of isolated DNA by centrifugation in a CsCl-gradient separates the nucleotide sequences in a buoyant pattern according to their specific gravity. The main fraction of non-polytene

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embryonic DNA of *Drosophila melanogaster* is accompanied by six satellite fractions (SAT-DNA), one of them is heavier (i.e. GC-rich) and five are lighter (i.e. AT-rich) than the main fraction. Each satellite represents 3–4%, all six together represent 20–25% of the total DNA in diploid nuclei, and they are mainly located in the heterochromatin. The brilliant publications of Endow et al. (1975), Brutlag et al. (1977b), Peacock et al. (1978) ad Wollenzien et al. (1977) summarize all that is known about DNA satellites in diploid DNA of *Drosophila melanogaster* to date. Earlier publications (Laird and McCarthy 1968a, b, 1969; Botchan et al. 1971; Lee and Thomas 1973; Peacock et al. 1973) report only about one or three satellites, representing 5–15% of the total embryonic respectively adult DNA quantity. The relationship between that small quantity of satellite DNA and the heterochromatic proportion of 20–30% in metaphase chromosome sets is often discussed.

Isolation of diploid DNA with different numbers of nucleolus organizers show no proportional increase in the quantities of AT-rich fractions with respect to the heterochromatic amounts (Entingh 1970; Blumenfeld and Forrest 1971, 1972; Perreault et al. 1973; Wollenzien et al. 1977), the amount of rRNA (Ritossa and Spiegelmann 1965; Spear and Gall 1973; Spear 1974, 1977), or the number of ribosomal gene repeats (Endow and Glover 1979). Polytene X/X-DNA yields the same result as polytene X/O-DNA (Spear 1974; Endow and Glover 1979). In all these publications it is concluded that the replication of rDNA is controlled in a way different from that of the other DNA fractions.

Polytene DNA

Polytene DNA from larval salivary gland nuclei has been analysed for *Drosophila melanogaster* (Gall et al. 1971; Lee and Thomas 1973; Spear 1977; Steinemann 1978), for *D. virilis* (Gall et al. 1971; Endow and Gall 1975; Steinemann 1978), for *D. hydei* (Dickson et al. 1971; Hennig 1972), and for *D. nasutoides* (Cordeiro et al. 1975). Although centrifugation of embryonic DNA results in six satellites, no distinct satellite peaks have been seen in polytene DNA up to now, except some barely seen bumps (Gall et al. 1971; Hennig 1972; Steinemann 1978).

Papers dealing with the polytene DNA of salivary gland nuclei have been published recently by Steinemann (1978), Schmidt (1980), and Schmidt et al. (1980). Steinemann finds large satellite peaks in the polytene DNA of *Chironomus melanotus*. In polytene DNA of *Drosophila virilis* satellite fractions are barely detectable. In order to test the methods of DNA isolation and quantitative centrifugation in a neutral CsCl-gradient, Steinemann demonstrates the buoyant density pattern of diploid non-polytene ganglia nuclei. Schmidt (1980) finds SAT-fractions in diploid as well as in polytene DNA of nuclei from *Glyptotendipes barbipes* by centrifugation in a neutral CsCl-gradient. In polytene DNA of *Chironomus thummi* and *Ch. piger* Schmidt et al. (1980) cannot detect any SAT-fractions in a gradient. They only find such a fraction by differential melting. This AT-rich fraction hybridizes with the same bands of salivary gland chromosomes as the SAT-fraction obtained from diploid non-polytene DNA by centrifugation. These experiments in polytene DNA reveal the decisive importance of methodology. To date, the method of differential melting has not been used to find AT-rich fractions in polytene DNA of *Drosophila* species in general.

We assume that the differences between polytene DNA of *Chironomus melanotus* and *Glyptotendipes* on one side, and *Drosophila melanogaster* and *D. virilis* on the other side, are caused by the existence of chromocenters of condensed heterochromatic chromosomal segments in the latter species, but only by band-like kinetochores in the former species, resulting in different conditions concerning the solubility of polytene DNA. The results of Steinemann (1978), Schmidt (1980), and Schmidt et al. (1980) clearly demonstrate that the absence of SAT-DNA is no general characteristic property of polytene chromosomes themselves. In large salivary gland nuclei of two *Chironomidae* species Walter (1973) finds exact doublings of DNA quantities by cytophotometric measurements. But nothing is known about the growth of the nuclei during larval development respecting measurement values of small polytene nuclei or their relationship to the 4 C value.

Methodological Details

Discrepancies between the biochemical results from non-polytene and polytene DNA could also be due to methodological difficulties as in earlier cytophotometric measurements of DNA in Feulgen-stained nuclei (refs.). Dickson et al. (1971), Lee and Thomas (1973), and Spear (1977) extract DNA with phenol, although the experiments of Skinner and Triplett (1967) show that phenol specifically removes AT-sequences, i.e. especially the light satellite DNA. In contrast to this, Gall et al. (1971), Hennig (1972), and Steinemann (1978), avoiding phenol, do not completely deny the existence of small bumps that could be satellite DNA. Using $NaClO_4$ for DNA isolation, it has been found that the light satellites are also attacked (Brutlag et al. 1977a), but the method is used by Botchan et al. (1971) and Kram et al. (1972) to isolate diploid non-polytene DNA, and by Gall et al. (1971) who isolate polytene DNA.

Gall et al. (1971) assume that the variability in the buoyant density pattern of diploid and polytene DNA is mainly due to their different extractibilities, i.e. is due to methodological difficulties during DNA isolation and is not at all caused by fundamental differences in the DNA species themselves. Special attention must be attached to the finding

of variable quantities of the satellite fractions in different DNA preparations of the same tissue, ranging from less than 1% to about 10% (Laird and McCarthy 1968b). Wollenzien et al. (1977) also report quantitative differences in preparations of diploid DNA from nuclei of the same karyotype, but they exclude methodological inadequacies in the performed procedures.

In any biochemical experiment all DNA fractions must first be quantitatively dissolved. The disagreements between results probably arise because this pre-condition is not fulfilled. Additionally, the results are often influenced by the use of phenol and perchloric acid during DNA isolation. Kram et al. (1972) report that the quantity of SAT-fractions varies between 8–16%, depending on the method of extracting the adult diploid DNA. Woodcock and Sibatani (1975) describe a severe loss of DNA in each biochemical extraction procedure of larval or adult DNA, independent of the use of phenol. Schmidt et al. (1980) report of a loss of even 70–80% of the total larval DNA during isolation, i.e. they perform the experiments with only 20–30% of the original DNA quantity. G. Dührssen/Gießen (personal communication) also reports a loss of diploid DNA of *Daucus carota,* under certain conditions amounting to 20–30%. It is not known whether all fractions are damaged or only specific ones. In addition to this Helmsing and van Eupen (1973) report that the method of obtaining a nuclear pellet improves the yield only about 50%. As long as the problem of complete or incomplete isolation of all DNA fractions is not convincingly resolved, any quantitative results on DNA fractions are flawed.

With the view to the different behavior of eu- and heterochromatin during hydrolysis before Feulgen reaction (review: Dennhöfer, in press), it seems possible that different solubilities of the DNA fractions during extraction result in a lack of SAT-DNA in a gradient loaded with polytene DNA. The larger the polytene nuclei, the more heterochromatin exists. In *Drosophila melanogaster,* in *D. virilis,* and in *D. hydei* most of the heterochromatin is fused forming a chromocenter. An increase of material is not automatically correlated with a proportional increase of its surface. Polytene chromosomes are specialized interphase chromosomes, where the euchromatin is extremely uncoiled, and the heterochromatin is completely condensed. The molecular density of heterochromatic DNA is higher than that of euchromatic DNA. This is shown by different quantities of incorporated cRNA (Cohen and Bowman 1979). In contrast to the small heterochromatic portions of diploid DNA, the heterochromatic block within the polytene nuclei cannot be as easily removed by the more recent methods of DNA isolation. In comparison with non-polytene DNA, the corresponding extractibility has to be tested in both chromatin fractions of polytene chromosomes with ^{3}H-labeled nuclei in order to decide whether the DNA is completely removed or whether a labeled residue remains.

Apart from these chemical aspects, an unwitting selection of the nuclei will increase the effect of unproportional quantities of SAT-fractions: in arbitrarily chosen tissues most of the nuclei are in interphase. A constant ratio between eu- and heterochromatin, which is a pre-requisite for accurate quantitative DNA determinations, is only obtained provided all nuclei are in the same physiological state. Generally DNA of nuclei from embryos, pupae or adults is fractionated. Therefore the DNA is taken from a non-homogeneous population of nuclei originating from all possible phases of the replication cycle. In contrast to this the DNA from salivary glands originates from only a very small population of nuclei. For example, Gall et al. (1971) isolate polytene DNA from 10–12 pairs of glands, i.e. from about 2200–3400 nuclei. Since there is synchronous stepwise growth of these chromosomes, it is clear that the nuclei within a gland belong to the same physiological state of the replication process. As yet in *Drosophila melanogaster,* all polytene DNA isolations are done with salivary glands from mature third instar larvae ("migration larvae"). In this developmental stage the salivary gland nuclei have just started a duplication cycle (Rodman 1967). About 77–99% of the nuclei of those larvae incorporate ^{3}H-thymidine. A considerable number of unlabeled nuclei is at first typical for the early prepupal period (Dennhöfer, in preparation). Only in *D. virilis* has polytene DNA of tissues other than salivary glands been tested: Endow and Gall (1975) analyse polytene DNA of adult Malpighian tubules, which originate from larvae and persist up to the imaginal stage. Nothing is known about the process of replication in these nuclei.

DNA isolation from populations containing only growing polytene nuclei, results in a clear disproportion of the quantities of eu- and heterochromatin. For example, in nuclei which have completed a replication cycle, the relationship between euchromatic DNA (slow annealing DNA) and heterochromatic DNA (fast annealing DNA of repetitive sequences) may come to 80:20 in *Drosophila melanogaster* (Peacock et al. 1978). When, however, at the moment of DNA isolation the euchromatic DNA has already duplicated, and the heterochromatic DNA has not, the relationship changes to 160:20. Furthermore although no difference is usually made between polytene DNA from female and male glands, the proportions of heterochromatin are quite different; also no polytene DNA from larvae with aberrant karyotypes like X/O or XX/Y has ever been analysed.

The few experiments dealing with polytene DNA of *Drosophila* species are not satisfactory. For example, the possibility of differences in the solubility of heterochromatin in non-polytene and polytene nuclei has not been tested. Furthermore nobody has yet tried to separate satellite fractions by centrifugation in gradients other than CsCl or by differential melting. In non-polytene embryonic DNA the satellites are best found in a $CsSO_4$-$HgCl_2$-gradient (Blumenfeld and Forrest 1971; Endow et al. 1975) or in an actinomycin-D-gradient (Peacock et al. 1973). The existence of repetitive sequences in polytene DNA of *Drosophila melanogaster, D. virilis,* and *D. hydei* is generally supported by the finding of ring formations in re-associated DNA (Lee and Thomas 1973). In *D. hydei* Hennig (1972) finds ring formations in re-associated adult DNA making up about 35%, but in polytene DNA these ring formations amount to about 15%. Schachat and Hogness (1973), however, dealing with diploid DNA, do not agree with the assumption that ring formations are only characteristic for repetitive sequences of SAT-DNA, but are also formed by sequences of the main-band DNA. To sum up the above findings, it is gathered that SAT fractions do exist in polytene DNA of *Drosophila* species, but they have neither been found or quantitatively determined by standard biochemical methods of DNA isolation and fractionation.

SAT-DNA and Histone Fractions

Further information about the existence and the quantity of SAT-DNA in polytene nuclei is given by results dealing with histone fractions. Blumenfeld et al. (1978) demonstrate the relationship between histone fractions and individual SAT-fractions in DNA of *Drosophila virilis.* The authors show that the phosphorylated subfractions of H1 are small, but the cor-

responding non-phosphorylated fractions are larger in polytene than in non-polytene nuclei. The authors conclude that phosphorylated H1 histones bind and compact SAT-DNA resulting in heterochromatic configurations, confirming the conclusions of Yumis and Yasmineh (1971). In hybridization experiments Pardue et al. (1972) locate histone mRNA of *Psammechinus milaris* in the bands of the region 39 E–40 A in salivary gland chromosomes of *Drosophila melanogaster.* Moreover it is known that synthesis of histones and DNA replication are synchronized throughout the cell cycle (Elgin and Weintraub 1975) and that the proportion of phosphorylated H1 histone initiates chromosome condensation (Matthews and Bradbury 1978); its special interaction with DNA is not completely clear, but is described as a cross-linking effect with DNA. Gurley et al. (1978) analyse the histone fractions of interphase nuclei of two mice species with different heterochromatic contents. The rate of phosphorylated H1 is the same in both interphase chromatin types, but the fraction of phosphorylated H2a is larger in the heterochromatin-rich DNA. The authors conclude that H1 is essential in some molecular level of organization, but H2a is involved in some way or other with interphase heterochromatin structures. In *Drosophila melanogaster,* Cohen & Gotchel (1971) find five identical histone fractions both in nonpolytene and polytene DNA. The relative quantities of histone fractions of different chromatin types of *Drosophila melanogaster, D. hydei,* and *D. virilis* are described by Holmgren (1980). The quantity of H1 is larger in polytene than in embryonic nuclei, but the phosphorylation rate is lower in the latter. The same applies to histone H2a: the phosphorylated subfraction is small compared to that of embryonic nuclei. Holmgren (1980) concludes that these differences are caused by underreplication of heterochromatin in polytene nuclei. However the author, like Blumenfeld et al. (1978), compares histone fractions of polytene interphase nuclei with histone fractions of embryonic, mixed nucleus' populations, containing mostly mitotically active chromosome sets.

Polyploid Nuclei

Some results of polyploid nuclei have to be reported in addition. In publications often no difference is made between polyploid and polytene nuclei. Polyploid, as opposed to polytene, chromosome sets contain the duplicated number of individualized chromosomes, which have failed to segregate in a previous anaphase. Such nuclei are able to pass through further mitotic cycles. In contrast, polytene chromosomes are specialized interphases chromosomes, which contain duplicated numbers of chromatids and do not divide, except in special cases. In polyploid nuclei duplicates of chromosomes cannot be distinguished by cytological methods from their corresponding homologues in the diploid nuclei. However, even when a nucleus is cytologically polyploid, it is possible that only partial replication has occurred and there is less than a polyploid amount of DNA.

Based on cytophotometric DNA measurements of interphase nuclei, Fox (1970) reports DNA quantities indicating non-duplication in somatic polyploid tissues of locusts. But the author does not know whether these nuclei replicate at the moment of dissection or not. In contrast to this, Gage (1974) demonstrates by biochemical methods that in polyploid nuclei of the silk gland in *Bombyx mori* during polyploidization all DNA fractions are replicated to the same extent. This result is verified by the extensive investigations of Perdrix-Gillot (1979). A heterochromatic portion of female nuclei grows significantly during larval development, incorporating as much ^{3}H-thymidine as expected for complete doublings.

Biochemical investigations have been done with polyploid DNA of different tissues of *Drosophila virilis* (Endow and Gall 1975). The authors report different proportions of SAT-fractions even within one tissue (adult ovaries), which decrease with age. Phenol was used during DNA isolation. Renkawitz-Pohl and Kunz (1975) report a proportionally smaller quantity of SAT-DNA fractions in polyploid ovarian tissue of *D. virilis,* compared with diploid DNA in the buoyant density pattern in a neutral CsCl-gradient. During the DNA isolation procedure, the polyploid DNA is treated four times with phenol, but the diploid DNA only three times. Other experiments on polyploid DNA containing one or several nucleolus organizers (Spear 1977) show that the proportion of rDNA is not correlated with the number of nucleolus organizers. In tetraploid thoracic muscle cells of *D. hydei,* the rDNA is not underreplicated, although the amount in diploid nuclei is not reached. In two different diploid karyotypes the level of rDNA is even dissimilar, showing gene compensation in a diploid tissue (Grimm and Kunz 1980).

In *Drosophila hydei,* the number of repetitive 5S RNA genes, located within an euchromatic segment, is almost the same in diploid, polyploid, and polytene nuclei. In the latter replication of these genes is slower than in the diploid nuclei (Renkawitz-Pohl 1977, 1978). The same has been found for repeated genes in polyploid ovaries of *Drosophila melanogaster* (Renkawitz-Pohl 1979).

As in the case of polytene DNA, a general difference between the solubility of eu- and heterochromatin in polyploid DNA is possible. Only the repetitive euchromatic genes exist in proportional numbers. However DNA quantities of heterochromatic sequences

cannot be found to be proportionally related to euchromatic sequences. Till further experimental evidence is presented, it is suspected that the difference between polyploid and diploid DNA is also caused by different conditions concerning the solubility of the eu- and heterochromatic fractions.

General Conclusions

The assumption of Heitz (1934) that α-heterochromatin fails to grow, is confirmed by Lakhotia (1974) demonstrating that α-heterochromatin within the chromocenter of a polytene chromosome set does not incorporate any ^{3}H-thymidine during larval polytenization. Evidence exists that replication of rDNA of the nucleolus organizer is controlled, dependent on the number of nucleolus organizers in the genome, in another way than the other satellite fractions. It is suspected that the control in diploid nuclei is different from the control in polytene chromosomes (Ritossa and Spiegelmann 1965; Hennig and Meer 1971; Spear and Gall 1973; Spear 1974; Zuchowski and Harford 1976). However Lakhotia and Roy (1979) demonstrate by autoradiographic experiments while rDNA replicates slower compared to euchromatic DNA, it nevertheless mainly replicates together with the latter.

There is no evidence for a general underreplication of all heterochromatic parts of the chromosomes during polytenization. For the case of *Drosophila melanogaster* the cytophotometric measurements of the DNA content in Feulgen stained, ^{3}H-thymidine non-incorporating salivary gland nuclei contradict the hypothesis of underreplication (Dennhöfer 1981, in press). The data of unlabeled polytene nuclei fall exclusively into the ranges of the theoretically expected 4C doublings. The number of such nuclei within a gland only depends on the state of the replication process (Dennhöfer, in preparation). These results are found in different karyotypes which are characterized by a deficient or an additional Y-chromosome. The DNA of heterochromatic chromosomes thus appears to replicate to the same extent as DNA of euchromatic chromosomes during polytenization.

The fact that the detection of SAT-DNA depends on methodological details, is demonstrated by different experiments in *Chironomus th. thummi* and *Ch. th. piger*. Results of cytophotometric DNA determinations are interpreted as a verification of the hypothesis of underreplication during polytenization (Keyl 1965). Schmidt et al. (1980) cannot detect SAT-fractions by centrifugation in a gradient, but an AT-rich fraction is exclusively obtained by differential melting of DNA.

It is surmised that the absence of satellite fractions after centrifugation of polytene DNA from *Drosophila* species is caused by methodological inadequancies, similar to those resulting in cytophotometric underreplication. In order to clarify this point it is necessary to reinvestigate the biochemical centrifugation experiments of polytene DNA with respect to the different solubilities of eu- and heterochromatin. Further gradients other than CsCl should be used, and the method of differential melting should be applied. Moreover attention should be paid to different proportions of heterochromatin, i.e. a difference should be made between polytene salivary gland nuclei from female and male larvae of *Drosophila*. Last but not least the glands must be dissected from larvae of a distinct developmental stage in order to obtain comparable results, and the state of the replication process has to be tested in each gland by autoradiographic treatment.

Some authors (Rudkin 1969, 1972; Nagl 1976) assume that the centromer respectively the chromocenter of the polytene chromosome set does not replicate, because it has no function in the non-dividing nuclei. This assumption is not conclusive, because some publications demonstrate that during metamorphosis polytene nuclei of some larval tissues undergo so-called somatic reduction steps until the diploid number of chromosomes per nucleus is restored (Berger 1938; Risler 1959, 1961). Due to the general consensus of cytological facts in eukaryotic organisms it is unlikely that this somatic reduction in polytene chromosomes is only a curiosity in some mosquito species; therefore investigations on the cytological processes during metamorphosis are necessary.

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