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Control of DNA replication and spatial distribution of defined DNA sequences in salivary gland cells of *Drosophila melanogaster*

Martin P. Hammond and Charles D. Laird
Department of Zoology, NJ-15, University of Washington, Seattle, WA 98195, USA

Abstract. In dividing cells, each sequence replicates exactly once in each S-phase, but in cells with polytene chromosomes, some sequences may replicate more than once or fail to replicate during S-phase. Because of this differential replication, the control of replication in polytene cells must have some unusual features. Dennhöfer (1982a) has recently concluded that the total DNA content of the polytene cells of *Drosophila* salivary glands exactly doubles in each S-phase. This observation, along with previous studies demonstrating satellite underreplication in salivary gland cells, led us to consider the hypothesis that there is a "doubling of DNA" mechanism for the control of DNA replication in polytene cells. With this mechanism, a doubling of DNA content, rather than the replication of each sequence, would signal the end of a cycle of DNA replication. To test this hypothesis, we have reinvestigated the replication of several sequences (satellite, ribosomal, histone and telomere) in salivary gland cells using quantitative in situ hybridization. We find that underreplication of some sequences does occur. In addition we have repeated Dennhöfer's cytophotometric and labeling studies. In contrast to Dennhöfer, we find that the total DNA contents of nonreplicating nuclei do reflect this partial replication, in accord with Rudkin's (1969) result. We conclude that DNA replication in polytene cells is controlled by modifications of the mechanism operating in dividing cells, where control is sequence autonomous, and not by a "doubling of DNA" mechanism. – In situ hybridization to unbroken salivary gland nuclei reveals the distribution of specific sequences. As expected, satellite, histone and 5S sequences are usually in a single cluster. This rules out the possibility that sequences known to be underreplicated in chromosomal DNA exist as extrachromosomal copies. Telomere sequences are grouped into two to six clusters, as if the chromosome ends are partially but not completely paired in salivary gland nuclei.

Introduction

In dividing cells, each DNA sequence must replicate exactly once during the cell cycle in order to maintain the same genetic complement over successive cell generations. DNA synthesis taken place in a discrete phase of the cell cycle, called the S-phase. Initiation of replication occurs at multiple sites on each chromosome. Termination of S-phase is presumed to occur when all sequences have replicated once, preventing further initiation until the next S-phase, although the preventive mechanism remains unknown (see Harland 1981).

In cells with polytene chromosomes, discrete S-phases do exist (see Rudkin 1973), but these S-phases are not separated by nuclear or cell divisions. One of the most dramatic features of replication in polytene chromosomes is the existence of sequences that do not replicate exactly once in each S-phase. The DNA puffs of *Sciaria* are overreplicated (Crouse and Keyl 1968), while in many Diptera, heterochromatic sequences appear to be underreplicated. For *Drosophila,* there is extensive evidence for the underreplication of heterochromatic sequences in salivary gland cells. This evidence comes from cytological (Heitz 1934; Lakhotia 1974), cytophotometric (Berendes and Keyl 1967; Mulder et al. 1968) and molecular experiments (Dickson et al. 1971; Gall et al. 1971).

Moreover, Rudkin (1969) concluded that the total DNA contents of salivary gland cells correspond to the values expected if the heterochromatic 20%–30% of the genome fails to replicate during successive endoreplication cycles. In his experiments, the distribution of DNA contents of salivary gland nuclei had discrete peaks, which Rudkin assumed to correspond to nuclei that were between S-phases.

Recently, Dennhöfer (1979, 1981, 1982a, b) has challenged this interpretation. She reported that cells that are between S-phases (as determined by failure to incorporate ^{3}H-thymidine) have DNA contents that represent exact doublings of the diploid amount of DNA. If she were correct, then most of the nuclei measured by Rudkin (1969) were in S-phase. Dennhöfer's conclusion is that all sequences replicate equally during polytenization, and that the extensive previous data suggesting satellite underreplication are suspect.

Additional evidence that some polytene cells of *Drosophila* double their DNA content during endoreplication cycles comes from the work of Lamb (1982), who concluded that adult Malphigian tubule and midgut cells, which are reported to have completed DNA replication, contain exact doublings of the diploid DNA content.

These observations raise interesting questions about the control of DNA replication in polytene cells. If underreplicating and overreplicating sequences do exist, and the replication of most other sequences is controlled so as to permit only a single initiation per S-phase, as in dividing cells, then special mechanisms would be required in polytene cells to permit multiple initiations on overreplicated sequences,

and to prevent replication from initiating in, or spreading into, underreplicated sequences. An alternative possibility is that DNA replication in polytene cells is controlled in an altogether different way. For example, a doubling of the amount of DNA present at the start of S-phase could signal the cessation of DNA synthesis and the end of S-phase. In such a model, sequences with more efficient origins could replicate more than once in an S-phase, while sequences with inefficient or delayed initiation would average less than one replication per S-phase. This hypothetical combination of underreplication of satellite and overreplication of other sequences could produce the exact doublings of DNA content reported by Dennhöfer (1982a) and Lamb (1982).

We have performed experiments to help distinguish between these alternative mechanisms for the control of DNA replication in salivary gland cells. To see if sequences replicate to different extents, we measured the representation of several different sequences in salivary gland nuclei of *D. melanogaster* by quantitative in situ hybridization with labeled probes for satellite, ribosomal, histone, and telomere DNA. Quantitation by in situ hybridization avoids problems that might be caused by differential extraction of different sequences during DNA isolation, and allows us to see the range of sequence representation in individual nuclei. Because hybridization was to nuclei that were squashed without first breaking them open, we could also test the suggestion of Dennhöfer (1982b) that satellite sequences are present outside the chromocenter. Moreover, we examined salivary glands from larvae of different ages, to see when sequences fail to replicate during the growth of salivary gland cells.

Also we have repeated the experiments of Dennhöfer (1981, 1982a) by measuring both ^{3}H-thymidine incorporation and DNA content for individual salivary gland nuclei. Hence our experiments test for the existence of polytene cells with both differential replication of different sequences, and a series of total DNA contents that are exact doublings of the DNA content of the diploid genome.

Materials and methods

Preparation of slides. Drosophila melanogaster (Canton-S) were raised at 25° C on standard corneal medium under uncrowded conditions. Salivary glands were dissected in Ephrussi-Beadle Ringer's solution (130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$) from female larvae in late third instar (wandering) or early third instar. First leg discs were dissected from female white prepupae or late third instar larvae. Discs and glands were both transferred to small drops of Ringer's solution on siliconized cover slips and picked up with slides 'subbed' by dipping in 0.1% gelatin, 0.01% chrome alum. The tissues were dispersed by gentle tapping and then flattened by hard thumb pressure. Cover slips were removed after freezing in liquid nitrogen and the slides were fixed either, for Feulgen staining, overnight at 4° C in ethanol:formaldehyde:acetic acid (85:10:5) (EFA) or, for in situ hybridization, for 10 min at 22° C in ethanol:acetic acid (3:1) (EA). Following fixation, slides were rinsed in 95% ethanol and air dried.

To obtain mitotic figures, brains and ganglia from late third instar larvae were dissected in Ringer's solution and incubated in 1% trisodium citrate for 10 min and 50% acetic acid for 15 min. They were then squashed in 50% acetic acid and treated as described above.

Feulgen-DNA cytophotometry. Slides were prepared as described above. Individual slides had disc and brain cells or disc and salivary gland cells. Slides were hydrolyzed for 45 min in 5 N hydrochloric acid at 22° C, and stained in Schiff's reagent (1% Basic Fuchsin – Allied Chemical – in 0.2 N HCl, 0.1 M $K_2S_2O_5$) for 60 min at 22° C, as described in more detail by Laird et al. (1980).

Preparations were mounted in oil that matched the cytoplasm's refractive index, and photographed to permit individual nuclei to be reidentified. Nuclear densities were measured by the subtractive method (Bedi and Goldstein 1976) at 560 nm with a Vickers model M85 scanning and integrating microdensitometer. At least two measurements were made for each nucleus.

To convert machine units of integrated optical density into multiples of the DNA content of the haploid genome (C-values), we compared salivary gland cells to 4C cells from first leg discs. These disc cells were identified as 4C by being the larger class failing to incorporate ^{3}H-thymidine (see Fig. 1).

Our data are partially corrected for glare (Goldstein 1970), but not for residual distribution error (Goldstein 1971). Fully corrected values for salivary gland cells would differ from the values we present, relative to disc cells, by less than 10% in all cases, and less than 7% for all except some of the 90C cells (see Hammond 1984, for further details).

Labeling with ^{3}H-thymidine. To label cells that were synthesizing DNA with ^{3}H-thymidine, salivary glands or discs were incubated for 20 min at 22° C in a drop of Ringer's solution containing methyl-^{3}H-thymidine (New England Nuclear, 80 Ci/mmol) at 40 μCi/ml, then rinsed four times with Ringer's solution. Subsequent preparation, fixation, staining and cytophotometry of nuclei were as described above.

In control experiments, labeled, fixed preparations were treated with either ribonuclease (RNase A, Worthington, heated to 100° C for 15 min to inactivate DNase) at 20 μg/ml in 150 mM sodium chloride, 15 mM sodium citrate for 1 h at 37° C, or deoxyribonuclease (DNase I, Sigma) at 0.5 mg/ml in 20 mM Tris-HCl, 1 mM magnesium chloride, pH 7.4 for 2 h at 37° C. DNase treatment reduced labeling of nuclei to background levels, while ribonuclease treatment had no discernible effect on labeling. Hence DNA, and not RNA, is labeled in our experiments.

After cytophotometry, the mounting oil was removed in xylene and the slides were dipped in Kodak NTB-2 emulsion diluted with water 1:1. Slides were exposed for 6–8 days at 22° C and developed in Kodak D-19 for 2 min at 22° C. Grains over nuclei were counted and a correction was made for background. The background was defined as the average grain number over a tissue-free area of equal size: for disc nuclei this was <1, for salivary gland nuclei it varied from 7–30 grains depending on the size of the nucleus and on the preparation. Nuclei were classified as "unlabeled", "moderately labeled", or "heavily labeled" using the following criteria: "unlabeled" nuclei had less than twice background labeling, and "moderately labeled" nuclei had <10 grains (disc), <60 grains (small salivary gland nuclei), or <250 grains (large salivary gland nuclei).

In situ hybridization. Probes. For 28S + 18S ribosomal RNA genes, copy RNA (cRNA) was made from plasmid pDmrY22 (Dawid et al. 1978). For satellite sequences, cRNA was made from aDm 705.6 (about 320 bp of 1.705 g/cm^3 satellite in pBR322; Brutlag, personal communication). For histone genes, cRNA was made from aDm-3000-1 (a single 4.8 kb histone repeat unit from cDm500 (Lifton et al. 1978) cloned in pBR322, Hogness, personal communication). For 5S genes, cRNA was made from plasmid 12D1 (Artavanis-Tsakonas et al. 1977). For telomere sequences, cRNA was made from pDm 3.0 (the 3.0 kb repeat from cDm356 (Rubin 1978) in pBR322 (Strobel, personal communication).

Tritiated cRNA was synthesized in vitro from plasmids. Conditions were: 150 mM KCl, 40 mM Tris (pH 7.9), 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.5 mg/ml BSA, 0.3 mM rGTP, 12 μM each ^{3}H-rATP, -rCTP, -rUTP, 45 units/ml *Escherichia coli* RNA polymerase (Sigma type III), 0.1 mg/ml DNA, incubated at 37° C for 60 min. Then *E. coli* transfer RNA to 0.5 mg/ml and DNase I (Bethesda Research Laboratories) to 10 μg/ml were added and the mixture was incubated at 37° C for 30 min. The reaction mixture was then extracted with phenol-chloroform (1:1), unincorporated nucleotides were removed by chromatography on Sephadex G-50, and the cRNA was precipitated with ethanol. Specific activities were 6–8 × 10^7 dpm/μg cRNA.

Hybridization. Nuclei were prepared and fixed in 3:1 EA as described above. RNase treatment, denaturation, hybridization and removal of unbound probe were performed as described in Henikoff and Meselson (1977) except that hybridization conditions were 300 mM NaCl, 30 mM trisodium citrate, 2.5 mM Tris-HCl (pH 7), 50% formamide, 1 mg/ml *E. coli* transfer RNA, 2–8 × 10^5 dpm probe per slide, incubated at 37° C for 24 h. Hybridized slides were dipped in Kodak NTB-2 emulsion diluted with water 1:1, and developed after various exposure times (12 h–21 d). Nuclei were stained with Giemsa (Anderson Laboratories) after autoradiography.

In control experiments, preparations were treated with DNase, as described above, before hybridization. This pretreatment reduced hybridization to background levels, indicating that the probe is hybridizing to DNA.

Quantitation. To minimize differences between slides, preparations to be compared were made on the same day and hybridized as a single batch. Slides were developed before grain coincidence became significant. (In contrast, slides used to determine localization were overexposed.) Grains over nuclei were counted, and the background (the average number of grains in an equal area of tissue-free emulsion) was subtracted. The results are expressed as grains per day of exposure.

To determine the relative representation of a sequence in diploid and salivary gland cells, we compared the grain number over salivary gland nuclei to the grain number over diploid cells from first leg imaginal discs of white prepupae. This ratio, times the average C value for these disc nuclei (3.84 C; see Table 1, and Hammond 1984), is the C value of a specific DNA sequence in salivary gland nuclei. This C value can also be expressed as its percentage of the C value for total DNA, thus providing an estimate of the percentage of sequence representation relative to that expected for uniform replication.

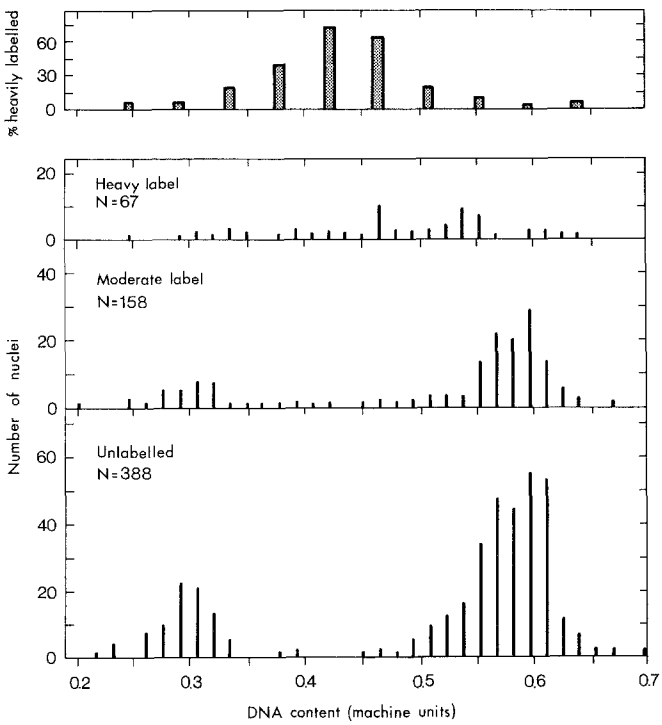

Fig. 1. DNA content and ^{3}H-thymidine incorporation in disc cells. First leg discs from female larvae in late third instar, or female white prepupae were labeled with ^{3}H-thymidine, squashed in Ringer's solution, fixed and Feulgen stained. The DNA content of each nucleus was measured cytophotometrically, and then the extent of labeling was determined by autoradiography (see Materials and methods). Data from 13 discs stained in three sessions were combined by normalizing to the mean value of unlabeled 4C cells, which had values between 0.45 mu and 0.7 mu. The top panel shows the percentage of nuclei that are heavily labeled within each 0.045 machine unit interval of DNA content. The DNA contents of heavily labeled nuclei mainly fall between the two peaks of the DNA contents from unlabeled nuclei, as expected if labeled nuclei were in S-phase

Results

Cytophotometry and labeling experiments

Disc nuclei. The distribution of DNA contents and amounts of labeling for individual nuclei from first leg discs of white prepupae and late third instar larvae is shown in Figure 1. Most of the nuclei fall into two peaks and were unlabeled. These nuclei were from 2C and 4C cells (Graves and Schubiger 1982). The moderately labeled nuclei in the peaks had presumably just entered or almost finished S-phase. Nuclei with DNA contents that fall between the peaks were labeled, usually heavily, and were therefore in S-phase. The mean of the unlabeled 4C peak is 0.586 machine units (mu) ± 0.01 (± 2 SE; N = 270). This value was taken as a standard for converting machine units of integrated densities into C-values. In a separate control experiment, the mean optical density of 4C disc cells was identical to that of prophase and metaphase mitotic figures from brain tissue (0.56 mu, ± 0.01 (± 2 SE, N = 97), and 0.56 mu, ± 0.02 (N = 28), respectively – see Hammond (1984); this confirms the 4C value of the major peak of unlabeled disc cells.

Salivary gland nuclei. Data for nuclei from salivary glands show that most nuclei were unlabeled, and fall into distinct

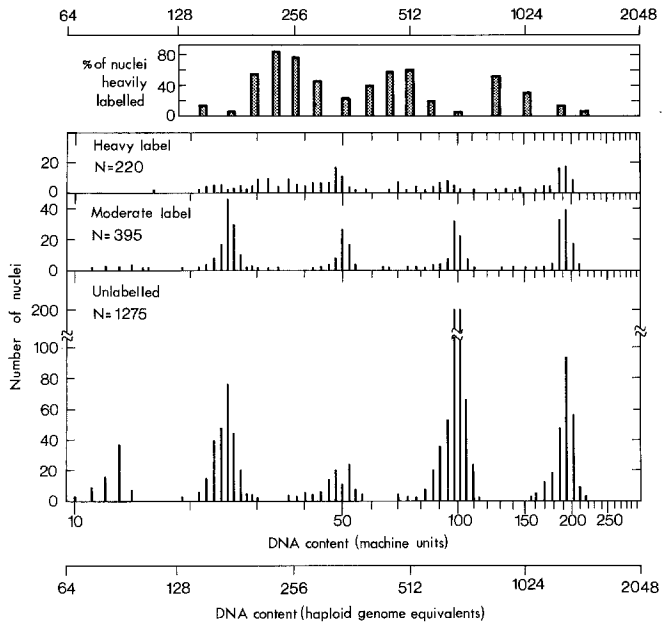

Fig. 2. DNA content and ^{3}H-thymidine incorporation in salivary gland cells from female larvae. Salivary glands were labeled with ^{3}H-thymidine, and Feulgen-stained. The DNA content of each nucleus was measured cytophotometrically, and then the extent of its labeling was determined autoradiographically. Each nucleus is plotted according to the extent of labeling and DNA content. Nuclei in the two peaks with the highest DNA content are from late third instar larvae, while nuclei in the two peaks with the lowest DNA content are from early third instar larvae. Data from 12 pairs of glands stained in two sessions were combined after normalizing to the DNA content of 4C disc cells. DNA contents in haploid genome equivalents were determined relative to 4C disc cells (see Fig. 1). The top panel shows the percentage of nuclei within successive intervals of DNA content that are heavily labeled. The heavily labeled nuclei fall predominantly between the major DNA content peaks, as expected if these nuclei were in S-phase

peaks (Fig. 2). These peaks correspond to DNA contents that are less than exact doublings of the DNA content of diploid nuclei. Most of the moderately labeled nuclei fall into the same peaks. Moderately unlabeled nuclei with DNA contents close to those of unlabeled nuclei are to be expected, because during a major period at the end of S-phase most of the genome has completed replication, but ^{3}H-thymidine is still being incorporated at a few late replicating loci (Mukherjee et al. 1980). Most of the nuclei that fall between the unlabeled and moderately labeled peaks were heavily labeled (Fig. 2), as would be expected if nuclei between those peaks were in S-phase.

In situ hybridization

The amount of hybridization of satellite, ribosomal, histone, and telomere sequences to salivary gland nuclei is shown in Figure 3. We also determined the amount of hybridization to disc nuclei. Because the average DNA contents of salivary gland and disc nuclei have been determined (see Table 1), we can compare the representation of each sequence in salivary gland cells and disc cells. The representation in salivary gland cells is expressed as a C value and as a percentage of the level expected for uniform replication. Satellite, 28S+18S rDNA, and histone sequences appear to be underreplicated during the growth of large salivary gland nuclei, in good agreement with previous studies (Table 1).

When in situ hybridization is used to compare sequence representation in different cell types, several factors must be considered. It is possible that the amount of material surrounding the target site or the chromatin structure will affect either probe access or the efficiency with which emissions are converted to silver grains. However, several studies suggest that the degree of compaction of a locus has only a limited effect on in situ hybridization results. Szabo et al. (1977) compared the efficiency of in situ hybridization

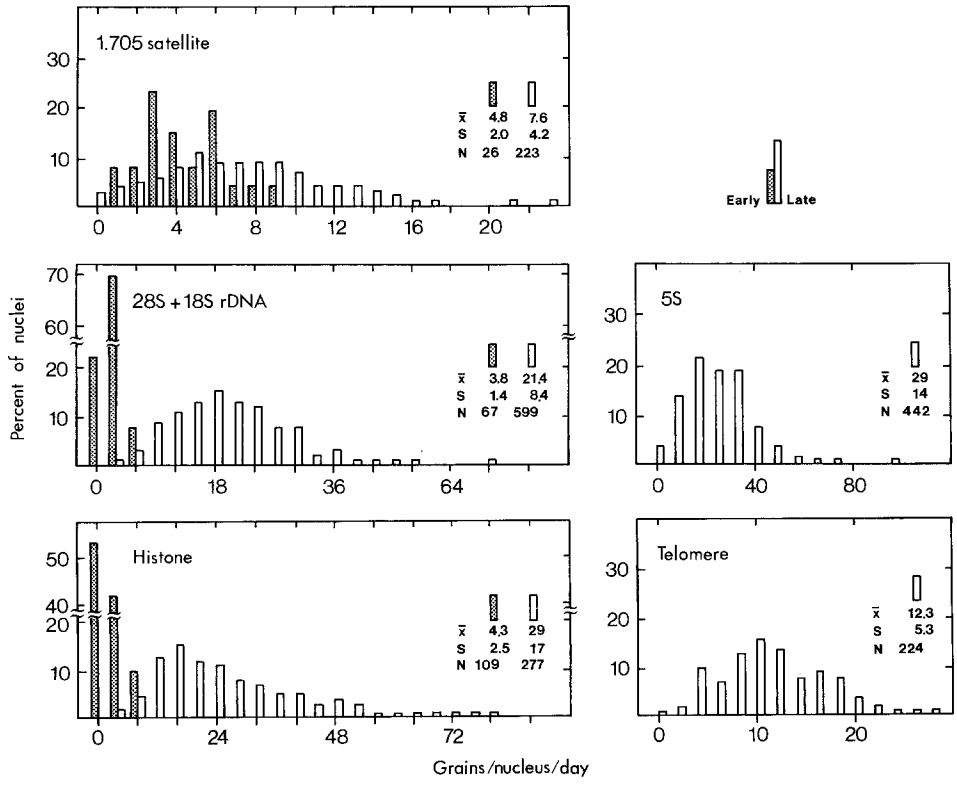

Fig. 3. In situ hybridization to salivary gland cells. Tritiated probes for satellite, 28S+18S rDNA, histone, 5S, and telomere sequences were hybridized in situ to squashed salivary gland cells. The amount of probe bound was determined autoradiographically, after various exposure times (see Materials and methods). The panels show the number of grains per nucleus per day of exposure. Separate distributions are shown, and separate calculations of the mean ($\bar{x}$) and standard deviation (s) were made for nuclei from early (stippled columns) and late third instar larvae

Table 1. Representation of total and defined DNA sequences in salivary gland cells compared to imaginal disc cells

Probe	Mean grain number/cell/day			Average DNA content		Other data[c]	Reference[d]
	Disc[a]	Salivary gland[b]		Salivary gland[b, c]			
		Early	Late	Early	Late		
Total DNA (Feulgen)	–	–	–	170C	924C	–	
Satellite (1.705)	1.90	4.8	7.6	9.6C (6%)	15.2C (1.7%)	–	
28S+18S rDNA	0.78	3.8	21.4	18.7C (11%)	105C (11%)	(17%–23%)	1, 2
Histone	0.67	4.3	29.1	24.6C (14%)	167C (18%)	(30%–50%)	3
Telomere	0.083	–	12.3	–	569C (62%)	–	
5S rDNA	0.18	–	28.7	–	612C (66%)	(100%–143%)	2, 3, 4

[a] First leg discs from female white prepupae. Of these cells 92% are 4C, 8% are 2C ($N=706$; see also Graves and Schubiger 1982). Therefore the mean DNA content is 3.84C

[b] Data from Figs. 2 and 3

[c] C values are calculated from the ratio of grain numbers for salivary gland nuclei compared with disc nuclei, times the average C value (3.84) for disc nuclei. Percentage values are calculated from the ratio of C values for the defined DNA sequence relative to total DNA. Percentage values listed under "Other data" were estimated as the percentage of sequence representation relative to that expected for uniform replication

[d] References: 1, Spear and Gall 1973; 2, Jacobs-Lorena 1980; 3, Lifschytz 1983; 4, Renkawitz-Pohl 1978 (data for *D. hydei*)

for 5S and 28S+18 rRNA binding to polytene chromosomes. Although the target sequences are in chromatin with very different appearances (band 56F and the nucleolus respectively), the efficiencies of hybridization were similar (9% and 12%). Szabo et al. (1977) also found that 5S sequences in spermatids, spermatocytes, nurse cells and salivary glands had very similar hybridization efficiencies. In *D. hydei*, however, efficiency of situ hybridization differs for pre- and postmeiotic cells (W. Hennig, personal communication). Henikoff (1981) found that the level of in situ hybridization to a heat-shock locus was the same in its normal euchromatic location and in a location next to the chromocenter (in which position it often appears heterochromatic). However, if a sequence differs in its transcriptional activity in different tissues, the efficiency of in situ hybridization may be affected (Henikoff 1981).

Our values for sequence representation in salivary glands are consistently two-fold lower than those in previous studies using other methods, perhaps due to greater self-absorption by the greater mass of material in a salivary gland nucleus, compared to a disc nucleus. However, our results confirm that the underrepresentation of satellite, histone, and rDNA is greater than would be expected if these sequences simply replicated late in each cycle. In contrast, 5S and telomere sequences are present in salivary gland nuclei at greater than 50% of the level expected from full replication.

When is the replication of satellite, 28S+18S rDNA, and histone sequences interrupted during salivary gland growth? To address this question, we hybridized these sequences in situ to salivary gland nuclei from early third instar larvae (Fig. 3). All three sequences were already underrepresented (Table 1), demonstrating that full replication is blocked early in salivary gland development. However, in all three cases, the extent of hybridization to nuclei from early third instar larvae was less than that observed to nuclei from late third instar glands. We infer that replication of each of these sequences is occurring even in the last two or three DNA replication cycles, which produce the largest salivary gland cells.

Nearly all the grains over a salivary gland nucleus which has been hybridized with the satellite probe are at a single site (or occasionally two sites); hybridization to the rest of the nucleus is not significantly above background (Fig. 4a). Thus there is no evidence for the presence of satellite sequences outside the chromocenter in these nuclei.

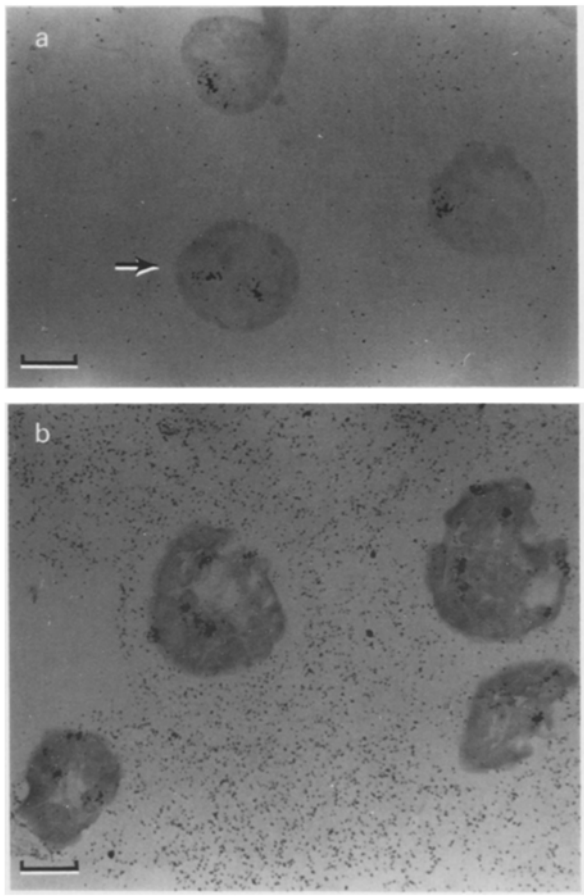

Fig. 4a, b. Localization of satellite and telomere sequences in salivary gland nuclei. Nuclei from salivary glands of late third instar larvae were hybridized in situ with ^{3}H-cRNA probes. **a** 1.705 g/cm^3 satellite sequences. Most nuclei have a single site of hybridization, but occasional nuclei have two (*arrow*). **b** Telomere sequences. The number of sites of hybridization varies. Bars represent 20 μm

Similarly, histone and 5S probes both hybridize to single sites within salivary gland nuclei, as expected from the single sites of hybridization observed when chromosomes are spread out before squashing (Lifton et al. 1978; Szabo et al. 1977). If all the telomeres were clustered in the intact nucleus, we would expect a single major site, while if all were well separated, six sites should be seen. We observed that telomere sequences hybridize to two to six sites, often well separated, in squashed nuclei (Fig. 4b).

Discussion

The mechanism of replication control

A striking feature of replication in polytene cells is the differential replication of different sequences. Such differential replication could be achieved by modifying the control mechanism used in dividing cells (see Harland 1981), or by using a different, 'doubling of DNA' mechanism, in which replication, once started, continues until the DNA content present before S-phase has doubled.

Support for the doubling of DNA hypothesis comes from several recent experiments. That cells can measure DNA content is suggested by, for example, the control of the midblastula transition in *Xenopus* embryos (Newport and Kirschner 1982). In *Drosophila*, not only are heterochromatic sequences underreplicated, but also some euchromatic sequences replicate to slightly different extents in polytene cells (Laird 1980; Lifschytz 1983). Moreover, the data of Dennhöfer (1979, 1981, 1982a, b), and Lamb (1982) appear to show that, in some polytene tissues of *D. melanogaster*, the DNA contents of cells that are not engaged in DNA synthesis can be arranged in an almost perfect doubling series. Such a result is not expected if satellite replication is blocked, and other sequences replicate only once.

To test the doubling of DNA hypothesis, we wanted to identify a population of nuclei whose DNA contents between S-phases conformed to a perfect doubling series, but which had differentially replicated sequences. We studied the salivary gland cells of *D. melanogaster*, the same cell type as studied by Dennhöfer (1981, 1982a).

Our results, and results from previous studies clearly show that satellite, 28S+18 rDNA, and histone sequences are all underreplicated during the growth of salivary gland cells (Table 1). The data on underreplication reported here expand on previous studies in several ways. Quantitative in situ hybridization eliminates problems that might be caused by differential extraction of various sequences during DNA isolation. By squashing nuclei without first breaking them open, we have eliminated the possibility that extrachromosomal sequences are lost during the nuclear lysis that accompanies the normal preparation of polytene chromosomes for in situ hybridization.

Moreover, by examining all the nuclei within a salivary gland, the existence of a sub-population of cells with full satellite replication can be ruled out. Only a few nuclei approached 30 grains/day, the grain number expected if satellite sequences have doubled four times, and none exceeded that level (Fig. 3). In contrast, determinations of total DNA content show that most sequences double eight or nine times during growth from a 4C cell to a large polytene cell. Hence it is not possible to explain a precise doubling series of the DNA content of salivary gland cells by claiming that satellite sequences are, despite previous evidence to the contrary, fully replicated (Dennhöfer 1982b).

Before concluding from these observations of satellite sequences that DNA replication is indeed controlled in a novel way, it was necessary to reexamine the issue raised by Dennhöfer (1981, 1982a) of whether or not salivary gland nuclei have, between S-phases, the total DNA contents predicted by complete doubling. We used Dennhöfer's methods (1982a), measuring both ^{3}H-thymidine incorporation and DNA content in individual nuclei.

In contrast to the results of Dennhöfer (1981, 1982a), we find that the DNA contents of unlabeled nuclei from salivary glands cluster in peaks that do not correspond to complete doublings of the 4C value. None of the peaks of distribution of unlabeled nuclei is within 20% of the values that correspond to complete doublings. Moreover, most of the nuclei with DNA contents that would represent complete doublings were heavily labeled (Fig. 2). We conclude that our data fit well with the generally accepted picture of salivary gland DNA replication: there is a discrete S-phase, during which most sequences, but not satellite sequences, replicate once. The result is that nuclei have, between S-phases, significantly less DNA than would be expected if all sequences replicated in each S-phase.

It is puzzling that our conclusions on salivary gland nuclei are so different from those of Dennhöfer, despite our attempt to duplicate her procedures. Dennhöfer (1981, 1982a) reports an unusually low proportion of nuclei that were unlabeled, compared with previous studies of labeling patterns in *D. melanogaster* (for example Rodman 1968; Mishra and Lakhotia 1982), or with our results. Dennhöfer's stringent criteria for classifying a nucleus as unlabeled may have resulted in classification of many unlabeled nuclei as labeled, because of one or two background grains. Our criteria for counting nuclei as "unlabeled" were less stringent and we may have scored some lightly labeled nuclei as unlabeled. However, we found that nuclei with no silver grains had essentially the same distribution as the rest of the nuclei we classified as unlabeled, and our criteria for labeling are therefore unlikely to have introduced errors.

Dennhöfer raised larvae at 18° C, while most of our experiments used larvae raised at 25° C. However, we obtain essentially the same results with larvae raised at 18° C or 25° C (see Hammond 1984). At lower temperature more nuclei achieve higher levels of polyteny, but mean peak values are not significantly different (see also Hartman-Goldstein and Goldstein 1979). Hence, while the lower temperature could affect the percentage of nuclei that are labeled (Mishra and Lakhotia 1982), it does not affect the DNA content of nuclei that are between S-phases.

Most authors have found that, whatever the stage of development studied, the majority of nuclei fall into distinct peaks. The mean peak values have been claimed to be exact doublings of the diploid value in some studies (Rodman 1967; Rasch 1970), but the most extensive studies indicate that these peak values result from underreplication (Rudkin 1969), as does the present study. Indeed, where peaks can be seen in Dennhöfer's data (1982a, see her Fig. 1), they also fall short of an exact doubling. If all the data in Dennhöfer's (1982a) Table 6 are plotted, however, discrete peaks are not seen. The reason for this difference is not clear.

We have also analyzed DNA replication in nurse cells (Hammond and Laird 1984). These cells are endoreplicated, but large nurse cells do not have polytene chromosomes.

As with salivary gland nuclei, the DNA contents of nurse cell nuclei that are not incorporating ^{3}H-thymidine cluster into discrete peaks, with DNA contents indicative of partial replication. Moreover, satellite sequences were found to be underrepresented using in situ hybridization. Hence, in two different endoreplicated cell types, some sequences are underreplicated, and this is reflected in total DNA contents that do not conform to exact doublings of the diploid DNA content.

It therefore appears that the control of DNA replication in the salivary gland and nurse cell populations that we have studied does not operate by ensuring a doubling of total DNA content in each S-phase. The possibility remains that DNA replication is controlled by a doubling of DNA mechanism in other cell types (for example, the adult Malphigian tubule and midgut cells studied by Lamb 1982). Differences between strains (Canton-S in our experiments, Berlin in Dennhöfer's) may also exist. An apparent series of complete doublings of DNA contents in pseudo-nurse cells of flies homozygous for the mutation *otu* (Rasch, King and Rasch 1984) also warrants further study.

Our data are consistent with the hypothesis that replication in endoreplicated cells is controlled by modifications of the same mechanism that operates in dividing cells, such that most sequences must replicate once in each S-phase, but certain sequences escape this regulation.

Continuing replication of satellite DNA

We have shown that some replication of each underreplicated sequence occurs both early and late in salivary gland growth. This result suggests that replication initiates infrequently on underreplicated sequences but that it is not blocked completely. It remains to be determined whether reduced replication results from differences in chromatin structure, or differences in initiation sequences. A clearer situation is found in *Drosophila* follicle cells, where the overreplication of chorion genes appears to require a specific initiation sequence (Spradling and Mahowald 1981). Similarly, it has been suggested that viruses of animal cells may utilize a specific initiation sequence to override normal S-phase control (Harland 1981).

Distribution of sequences in salivary gland nuclei

Our in situ hybridization experiments with salivary glands utilized nuclei that were squashed without first disrupting the nuclei. The distribution of grains over such nuclei reflects the distribution of the target sequence within an intact nucleus, and our observations are therefore relevant to the problem of chromosome arrangement within the polytene nucleus (see Agard and Sedat 1983). The histone and 5S sequences are always (>98%) clustered at a single site, suggesting that homologue asynapsis is rare in intact nuclei.

Satellite sequences are also clustered at a single site, but in 5% of the nuclei two distinct sites, usually adajcent, were distinguished (Fig. 4a). This pattern could reflect the existence of two chromocenters in some intact polytene nuclei, a phenomenon also reported by Appels et al. (1979).

Telomere sequences in squashed nuclei are clustered into two to six sites (Fig. 4b). Often, telomeres are associated with one another even after the chromosomes have been deliberately spread out (Hinton 1945; Rubin 1978). Squashing without first breaking open the nuclei should preserve associations to a greater extent, but we rarely saw a single major site, and the sites we observe are often well separated. Therefore we do not believe that all the telomeres are clustered together in the intact nucleus.

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