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The endocycle controls nurse cell polytene chromosome structure during *Drosophila* oogenesis

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SUMMARY

Polytene chromosomes exhibit intricate higher order chromatin structure that is easily visualized due to their precisely aligned component strands. However, it remains unclear if the same factors determine chromatin organization in polyploid and diploid cells. We have analyzed one such factor, the cell cycle, by studying changes in *Drosophila* nurse cell chromosomes throughout the 10 to 12 endocycles of oogenesis. We find that nurse cells undergo three distinct types of endocycle whose parameters are correlated with chromosome behavior. The first four endocycles support complete DNA replication; poorly banded polytene euchromatin progressively condenses during the late S phases to produce blob-like chromosomes. During the unique fifth endocycle, an incomplete late S phase is followed by a mitosis-like state during which the 64C chromosomes dissociate into 32 chromatid pairs held together by unreplicated regions. All the subsequent endocycles lack any late S phase; during these cycles a new polytene chromosome grows from each 2C chromatid pair to generate 32-ploid polytene nuclei. These observations suggest that euchromatin begins to condense during late S phase and that nurse cell polytene chromosome structure is controlled by regulating whether events characteristic of late S and M phase are incorporated or skipped within a given endocycle.

Key words: *Drosophila*, Oogenesis, Polytene, Chromosome, Endocycle, Nurse cell

INTRODUCTION

Cellular differentiation in many plants, animals and protists involves the generation of polyploid nuclei through reiterated endocycles. While the diploid cell cycle alternates between DNA synthesis (S phase) and mitosis (M phase) each preceded by a gap phase (G phase), endocycles are modified cell cycles that contain only a G phase and S phase. Recently, considerable progress has been made in understanding how the mitotic cell cycle is controlled by cyclins and their partner cyclin-dependent kinases (reviewed in Murray and Hunt, 1993; Nurse, 1997). During *Drosophila* endocycles, polyploid cells neither express nor require the mitotic regulators: cyclin A, cyclin B, string or cdc2 kinase. Instead, S and G phases alternate under the control of cyclin E and the cdk2 homologue, cdc2c (reviewed in Orr-Weaver, 1994; Follette and O'Farrell, 1997).

Loss of mitotic regulators is a sufficient condition for polyploidy (Lehner and O'Farrell, 1989; Smith and Orr-Weaver, 1991); however, it is likely that endocycles in vivo are programmed in multiple ways. Many Dipteran polyploid cells underrepresent satellite DNAs (Gall et al., 1971), apparently because their endocycles reset before DNA synthesis is complete and before satellite DNAs have begun to replicate late in S phase (Lilly and Spradling, 1996). In other species, polyploid cells complete S phase (for example, see Steinemann, 1978). Polyploid cells from many non-Dipteran species progress into the initial stages of M phase during which chromosomes condense, but karyokinesis is absent (endomitosis; Geitler, 1938). Polyploid megakaryocytes of vertebrates advance even further into mitosis, forming aberrant mitotic spindles during endocycles (Odell et al., 1968). Thus, some endocycles include events characteristic of the late S and M phases, while others do not.

The chromatin organization within polyploid nuclei also varies widely. In most species, polyploid nuclei contain visible chromosomes only briefly (reviewed in Nagl, 1978). In contrast, in Dipterans such as *Drosophila*, the multiple chromatids within polyploid nuclei somatically pair to form permanent polytene chromosomes rich in morphological detail (reviewed by Ashburner, 1970). Dipteran polytene chromosomes differ from mitotic chromosomes in their highly banded organization, their nonhomologous association via a chromocenter and the absence of cyclic changes in the degree of condensation and chromatid association during the cell cycle. However, the polytene bands, heterochromatin content and chromosome associations also differ substantially even between Dipteran subgroups. Variations in chromosome structure may be a consequence of differences in the endocycles between particular cell types. Processes such as chromosome compaction (reviewed in Hirano, 1995; Koshland and Strunnikov, 1996) and chromatid separation (reviewed in Yanagida, 1995; Bickel and Orr-Weaver, 1996) are normally

limited to the late phases of the cell cycle, precisely the stages that vary between endocycles.

Ovarian nurse cells represent an attractive system for studying the relationship between polytene chromosome structure and the endocycle. In Dipteran oogenesis, each egg chamber contains specialized germline cells, called nurse cells, which are mitotic sisters of the developing oocyte and remain associated with the oocyte by intercellular bridges. In *Drosophila melanogaster*, 15 nurse cells synthesize most of the egg contents and transport them to the growing oocyte (reviewed in Spradling, 1993). Over a 2.5 day period corresponding to egg chamber stages 1-10, nurse cells grow enormously by undergoing 10-12 endocycles (see Fig. 1). By synthesizing future egg contents at extremely high rates, nurse cells enable oogenesis to proceed rapidly.

Although polytene chromosomes change little in appearance during the growth of most polyploid *Drosophila* tissues, nurse cell chromosomes undergo a significant programmed alteration. Nurse cell chromosomes appear polytene in stage 2-4 egg chambers but then dissociate during stages 4 and 5, after which they are no longer visible (Painter and Reindorp, 1939; Hsu and Hansen, 1953; Brun and Chevassu, 1958). Prior to breakdown, the chromosomes take on a striking, blob-like appearance (Fig. 1). However, the structure of early nurse cell chromosomes and the nature of the dispersal process have not been analyzed in detail nor has dispersal been related to the character of nurse cell endocycles.

Female sterile mutations in several genes specifically affect the appearance of nurse cell chromosomes. Nurse cell polytene chromosomes in flies mutant for *fs(2)B* (Koch and King, 1964), *ovarian tumor* (*otu*) (King et al., 1981) or *cup* (Keyes and Spradling, 1997) fail to disperse normally. Instead, polytene arms persist in highly polyploid nurse cells and display a banding pattern comparable to polytene chromosomes from the larval salivary gland (Heino, 1989, 1994). However, it is unclear how similar the structure of these giant chromosomes is to wild-type nurse cell chromosomes. Mutations in another gene, *morula*, cause mitotic-like chromosomes to form at about the time when polytene chromosomes normally disperse (Reed and Orr-Weaver, 1997).

Here we analyze the programmed changes in condensation and chromatid association that nurse cell polytene chromosomes normally undergo during oogenesis. We find that these changes occur under cell cycle control and correlate with three distinct types of nurse cell endocycle. Our observations imply that polytene chromosome structure reflects the nature of the underlying cell cycle and can be used to understand fundamental aspects of eukaryotic chromosome behavior.

MATERIALS AND METHODS

Terminology

The terms polyploid and polytene are used inconsistently in the literature. For the purposes of this paper, we employ the following definitions: Any cell with a DNA content of **nc** is **polyploid** if **n** > 2 (**c** = haploid genome content, **n** = a positive integer), regardless of chromosome structure. A polyploid cell is **polytene** only if it contains aligned, multistranded chromosomes; otherwise it is **non-polytene**. Polyploid cells with **n** identical polytene chromosome sets are called **polyploid polytene**, or **n-ploid polytene**. The individual chromosomes in polyploid polytene cells may be called **subpolytene**.

Drosophila strains

Flies were raised on standard cornmeal-based media at 25°C. For the analyses of wild-type nurse cell chromosomes, ry^{506} or $y;ry^{506}$ stocks were used. The PZ-insert line *fs(2)04443* was outcrossed to $y;ry^{506}$ to obtain heterozygous flies with a PZ-insert over a wild-type 2L homologue. The *cyclin E* genotype used for in situ hybridization experiments was $cyclinE^{1672}/Df(2L)r10$.

Nurse cell chromosome preparation

Our protocol is based upon the salivary gland chromosome squashing technique of Todd Laverty (see http://www.fruitfly.org/methods/cytogenetics.html). Newly eclosed females were dissected immediately or kept on fresh yeast with males for 1 or 3 days prior to dissection. Ovarioles or staged egg chambers were transferred to a drop of 45% acetic acid for 4 minutes, fixative (1:2:3, lactic acid:water:glacial acetic acid) for 4 to 5 minutes and then the tissue was squashed under a siliconized coverslip. Slides were submerged in liquid nitrogen, the coverslips removed and then immediately submerged in cold 100% ethanol.

FISH probe preparation

Nick-translated probes were prepared using biotin-16-2′-deoxyuridine-5′-triphosphate (bio-16-dUTP, ENZO diagnostics), fluorescein-12-2′-deoxyuridine-5′-triphosphate (fluorescein-12-dUTP, Boehringer Mannheim) or tetramethylrhodamine-6-2′-deoxyuridine-5′-triphosphate (rhodamine-6-dUTP, Boehringer Mannheim). P1s used as probes include DS00732 (at 20C3), DS05130 (at 39A3-A7), DS06524 (at 42B4-42C2), DS05705 (at 59A4), DS00046 (at 64C1-C8), DS02752 (at 69C2-C8), DS00063 (at 73E1-F4), DS01436 (at 79E1-E2), DS00453 (at 82B1-B4) and DS08057 (at 102 B1-B2; Hartl et al., 1994). Other probes include the cDNA encoding *hu li tai shao*, the cloned genomic DNAs from the *rosy*, *yellow*, *white*, *heat shock protein 83* and *ovarian tumor* loci, the 5′ portion of the PZ-element and genomic DNAs from chromosome regions 24C and 100C (provided by Lynne Schneider).

Fluorescent in situ hybridization (FISH)

Pretreatment and hybridization for biotin and directly labeled probes were performed as previously described (Zhang and Spradling, 1994). Detection of biotin-labeled probes was performed using the Oncor Chromosome In Situ Hybridization Detection System (Oncor, USA). DNA was stained with 4,6-diamino-2-phenylindole (DAPI) or propidium iodide and samples were mounted in Antifade (Oncor) or Vectashield (Vector Laboratories, Inc.). In situ hybridized nuclei were examined by epifluorescence and photographed using a Zeiss Axiophot and Elite 400 film or using a Leica TCS NT microscope and Princeton Instruments cooled CCD camera with IP Lab Spectrum software (Scanalytics).

Nuclear isolation and flow cytometry

The ovaries from newly eclosed females were dissected and nuclei were isolated and sorted by fluorescently activated cell sorting (FACS; Lilly and Spradling, 1996). The isolated populations of 16C, 32C, 64C and 128C nuclei were treated for 2 minutes in 45% acetic acid and then fixed in lactic acid:water:acetic acid (1:2:3) for 4 minutes before mounting under DAPI-glycerol. Cells were examined under fluorescence on a Leica TCS NT microscope and images were recorded using a cooled CCD camera.

Cell cycle, ploidy and condensation measurements using DAPI and BrdU

Dissected ovaries from newly eclosed females were incubated with bromodeoxyuridine (BrdU) for one hour as described (Margolis and Spradling, 1995; Lilly and Spradling, 1996). Ovarioles were then squashed and prepared as described above for FISH. BrdU was detected with anti-BrdU (Becton-Dickinson) and AffinityPure Cy3-conjugated goat anti-mouse antibodies (Jackson ImmunoResearch)

and mounted as described for FISH (Calvi et al., 1998). Measurements of DAPI intensity were determined for 2C and 4C nonincorporating follicle cells within a field and then compared to nonincorporating nurse cells to calculate the ploidy of G phase nurse cell nuclei. DAPI intensity, measurements of X chromosome length and distances between in situ signals were made on CCD images using IP Lab Spectrum software.

RESULTS

Nurse cell chromatin is organized in two distinct states

Individual ovarioles contain a series of progressively older egg chambers, each with a complement of 15 nurse cells and one oocyte (Fig. 1). Nurse cell chromosomes appear polytene and blob-like until about stage 5 (Fig. 1, inset), but they have broken down and dispersed by the end of stage 6. In order to analyze these events, we studied the structure of nurse cell chromosomes in preparations of squashed nuclei suitable for FISH. Under these conditions, nuclei with polytene (Fig. 2A), blob-like (Fig. 2C) or dispersed (Fig. 2D) chromosomes can be observed at high resolution.

During stages 2 to 4 of egg chamber development, nurse cell nuclei display polytene chromosomes of novel structure. Unlike the salivary gland nuclei, whose chromosomes are joined at a chromocenter (Fig. 2B), nurse cells contain four separate polytene elements (Fig. 2A) that correspond to individual chromosomes as shown by FISH analysis. The identity of each chromosome and chromosome arm was determined using 18 different single-copy DNA probes spanning all the chromosomes (Fig. 2G,H, and data not shown). Each single-copy probe produced one FISH signal, indicating that the arms are polytene in nature, except in some pericentric regions where paired signals indicative of homologue asynapsis were occasionally seen (Fig. 2G). DAPI-bright chromatin blocks and fine chromatin threads link the two arms of the major autosomes and correspond to centromeric heterochromatin (Fig. 2, h_2, h_{3L} and h_{3R}). The most prominent block corresponds to a large block of the 1.672 satellite DNA on chromosome 4 (Fig. 2, h_4; Lohe et al., 1993; K. J. D. and A. C. S., unpublished data).

Overall, nurse cell chromosomes are proportionately shorter and wider than previously studied somatic polytene chromosomes in *Drosophila melanogaster* (compare Fig. 2A and B). While there is some heterogeneity in DAPI staining along the length of the nurse cell chromosome arms, a distinct banding pattern is not seen. Fig. 2E and F shows the DAPI staining patterns at the tip of 2R of salivary gland and nurse cell chromosomes, respectively. As with the nurse cell chromosomes generally, this region is condensed and shows some heterogeneity in staining that is reminiscent of banding.

The absence of a chromocenter in nurse cell polytenes prompted us to examine the nature of the pericentromeric heterochromatin. To delineate the relative proportion of pericentric DNA in the nurse cell chromosomes, we used single-copy probes that flank this region on chromosome 2, at 39A3-7 and 42B4-C2, and on chromosome 3, at 79E1-2 and 82B1-4 (Fig. 2G,H, respectively). More than 25% of the total autosomal nurse cell chromosome length is occupied by centromeric heterochromatin. This value is similar to diploid mitotic chromosomes, but is much greater than the proportion found in salivary gland chromosomes. In addition, distinctive brightly staining blocks of replicated heterochromatic DNA are observed in nurse cell polytenes (see Fig. 2, K. J. D. and A. C. S., unpublished).

The blob-like polytene chromosomes (Fig. 2C) are organized in a very similar manner to those in younger nurse cells. Each chromosome remains separate, with autosomal arms joined by the same heterochromatic blocks and threads as seen in the earlier polytenes. However, these more highly condensed chromosomes show no heterogeneity in DAPI staining along their lengths. Nonetheless, all the sequences tested by FISH were still arrayed along the length of the arms in the same order and relative location as in more elongated chromosomes (data not shown).

In contrast to the polytene chromosomes visible up to stage 5, older egg chambers contain nurse cells that appear to have a dispersed chromatin organization (Fig. 2D). In general no relationship can be seen between these nuclei and the earlier polytene chromosomes by DAPI staining, except that some prominent DAPI-bright blocks, including h_4, are still recognizable.

Chromosome dispersal takes place during a specific endocycle

The transition between blob-like and dispersed chromosomes does not take place uniformly among the nurse cells within stage 5 and 6 egg chambers (see Fig. 1). Nurse cells are known to cycle asynchronously, with posterior cells undergoing more rapid endocycles than anterior cells. Since chromosome dispersal does not correspond to a precise developmental stage, we tested the possibility that it takes place during a specific nurse cell endocycle by examining the chromosome structure of DAPI-stained ovarian nuclei that had been separated by flow cytometry (Fig. 3A). Newly eclosed females containing only previtellogenic egg chambers with mitotic follicle cells were used as ovary donors in order to avoid polyploid follicle cells which are numerous after stage 6. Nuclei were collected from peaks corresponding to the G phases of endocycles 5 (32C), 6

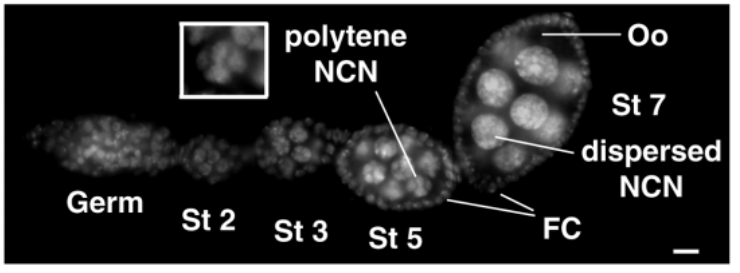

Fig. 1. A single ovariole contains a string of egg chambers that become more mature as they move away from the germarium (Germ). Egg chambers contain 15 nurse cells and one oocyte (Oo) and are categorized into stages 1-10 (stage 2, stage 3, stage 5 and stage 7 are shown). In stages 5 and earlier chambers, nurse cell nuclei contain polytene chromosomes (polytene NCN), while in stage 6 chambers and later chromatin is dispersed (dispersed NCN). The inset shows a nucleus with blob-like chromosomes from the stage 5 egg chamber. Follicle cells (FC) envelop the egg chamber by proliferating mitotically and enter endocycles at stage 6. DNA is stained with DAPI. Bar represents 10 μm.

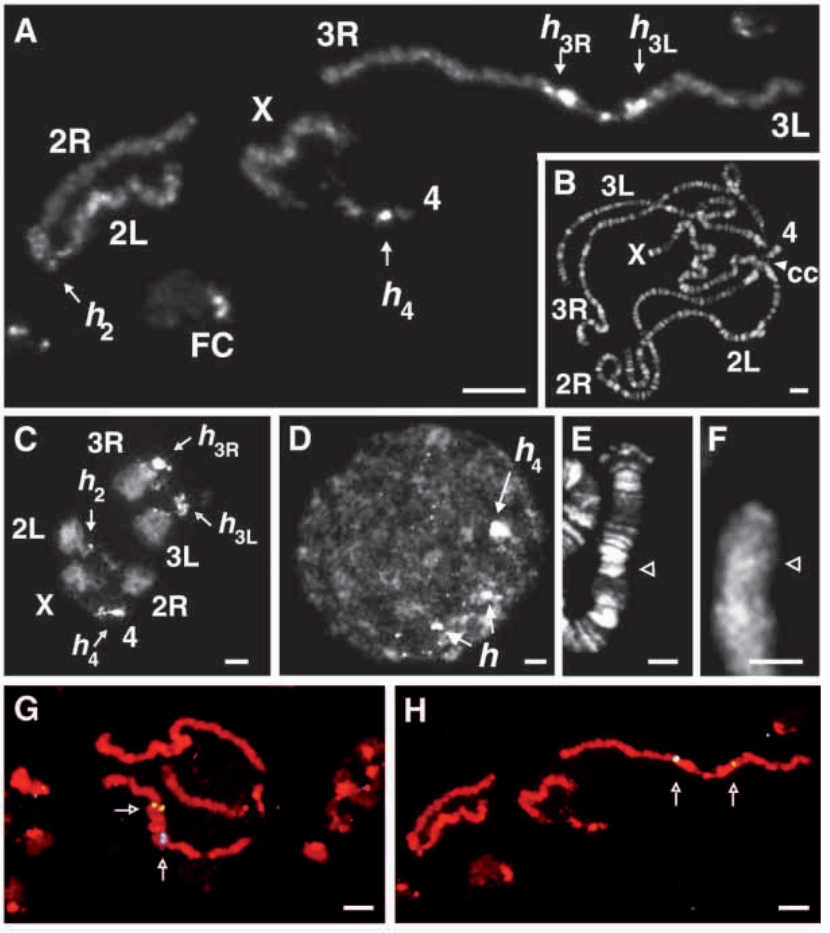

Fig. 2. Squash preparations of nurse cells reveal a novel and dynamic chromosome organization during development. (A) Five polytene chromosome arms, 2L, 2R, 3L, 3R and X, and chromosome 4 can be recognized in an early nurse cell nucleus. The centromeric heterochromatic regions associated with the euchromatic arms, h_2, h_{3L}, h_{3R} and h_4 (arrows) are also indicated. (B) For comparison, salivary gland chromosomes are organized into long, banded polytene arms and are joined at their pericentromeric regions into a chromocenter (cc). (C) A nurse cell nucleus at egg chamber stage 5 contains five blobs of chromatin that represent each of the major chromosome arms as indicated. A similar organization of the heterochromatic regions (arrows) can be seen as in A. (D) After egg chamber stage 6, the nurse cell chromatin appears dispersed. In this 256C to 512C nucleus, DAPI-bright heterochromatic blocks are still seen (*h*, arrows), including that on the 4th chromosome (h_4). (E) Tip of 2R from a salivary gland chromosome. The arrowhead indicates band 59A. (F) Tip of 2R on a nurse cell chromosome. The arrowhead indicates region 59A as determined by FISH (not shown). (G) FISH using probes specific for 39A3-7 (yellow) and 42B4-C2 (blue), arrows, that span the chromosome 2 pericentric heterochromatin. Note that the 39A3-7 signal appears as two dots, presumably due to homologue separation in this region. (H) FISH to 79E (yellow) and 82B (blue), arrows, reveals the chromatin within the pericentric region of chromosome 3 of a nurse cell nucleus. In all panels, DNA is stained with DAPI (red in G and H). In A, D, G and H, the bars represent 5 μm, in B the bar represents 10 μm and in E and F the bars represent 2 μm.

(64C) and 7 (128C), and visualized under a fluorescence microscope. In all 32C nuclei, the chromatin is organized into blob-like polytene chromosomes (Fig. 3B); however, chromatin from the 64C (Fig. 3C) and 128C nuclei (not shown) is dispersed. This shows that nurse cell polytene chromosomes undergo dispersal between the G phases of endocycles 5 and 6.

Chromosome dispersal takes place precisely at the end of endocycle 5

To determine precisely when chromosome dispersal takes place, we utilized BrdU incorporation and DNA ploidy measurements to distinguish G, early S and late S phases of endocycles 5 and 6. These studies also allowed us to investigate the nature of endocycles in more detail. We will denote endocycle phases as follows: the G phase of endocycle 5 (32C) is G_5, the S phase of endocycle 5 (32C to 64C) is S_5, etc.

In these studies, whole ovaries from newly eclosed females were again used to avoid polyploid follicle cell nuclei. The ovaries were incubated in BrdU, squashed, stained with DAPI and a monoclonal antibody against BrdU, and analyzed by fluorescence microscopy. Nuclear DNA content was determined relative to that of non-replicating (i.e. BrdU-unlabeled) 2C follicle cell nuclei by measuring the fluorescence intensity of DAPI-stained nuclei. The combination of DNA content and BrdU incorporation pattern allowed the precise endocycle phase of each nurse cell nucleus to be discerned (Fig. 4). The relative ploidy levels of the G

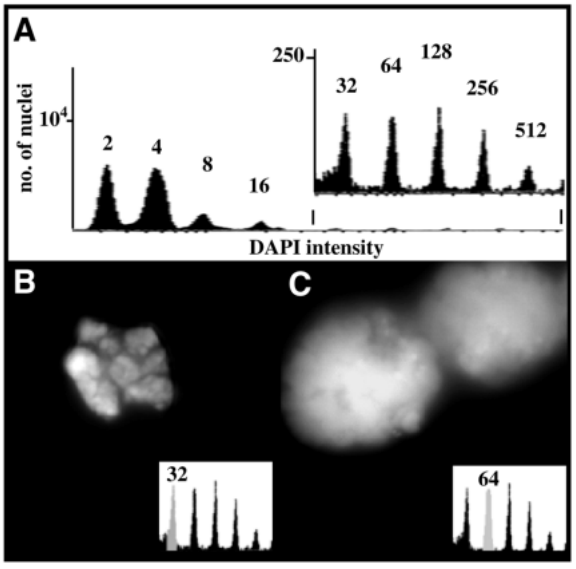

Fig. 3. Flow cytometry analysis places chromosome dissociation between G_5 and G_6. DAPI-stained nuclei from newly eclosed females were sorted by flow cytometry and their chromatin organization was examined. (A) Sorting profile of isolated nuclei from newly eclosed female ovaries. The 2C-4C peaks are predominantly follicle cell nuclei, while the 8C-512C peaks represent nurse cell nuclei. (B) Cells from the 32C peak, G_5, show a condensed, polytene organization (n=50). (C) Cells from the 64C peak, G_6, show a dispersed organization (n=50).

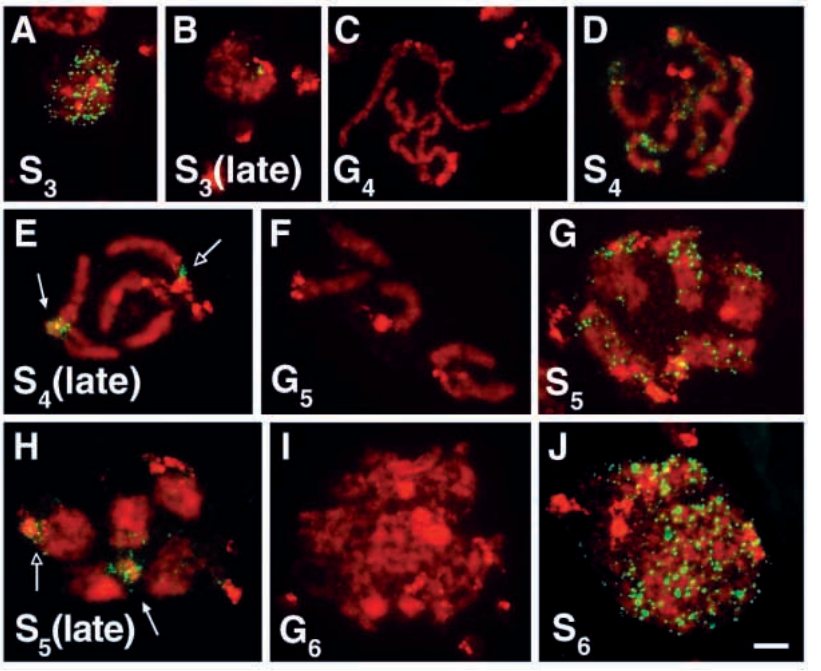

Fig. 4. Nurse cell polytene chromosomes undergo condensation and chromatid dispersal at precise points within the early endocycles. Squashed nuclei with incorporated BrdU are stained with anti-BrdU (green) and DAPI (red). (A) S_3, a replicating nucleus from endocycle 3 with an early pattern of DNA replication. (B) S_3, late replication. (C) G_4, a 16C nucleus showing polytene arms. (D) S_4, an early pattern of DNA replication in the condensed chromosomes of a nucleus. (E) S_4, late replication. (F) G_5, a 32C nucleus showing shorter and broader polytene arms. (G) S_5, early replication showing chromosome arms increasing in width. (H) S_5, late replication showing five blob organization. (I) G_6, a 64C nucleus in the dispersed state. (J) S_6, early replication of a nucleus in the dispersed state. In E and H the white arrow indicates late replication on chromosome 2 and the open arrow indicates late replication on chromosome 3. All nuclei are at the same magnification and the bar represents 5 μm.

phase (non-BrdU incorporating) nuclei (G_3, G_4, G_5) approximately doubled after each endocycle indicating that DNA replication was essentially complete during the first 4 endocycles (see Table 1). Consistent with this observation, S phase nuclei from these endocycles (S_3, S_4) showed two distinct patterns of BrdU incorporation corresponding to early (Fig. 4A,D) and late S phases (Fig. 4B,E).

After G5, the endocycle parameters begin to change. DNA content measurements of G_6 nuclei showed that DNA replication is only 90% complete during S_5 (Table 1). Although this is higher than the 76% value expected in the absence of any satellite DNA replication, it indicates that a small fraction of the DNA (equal to about 1/3 of satellite DNA) is not duplicated during S_5. Consistent with these data, late replication patterns were still observed in S_5 (Fig. 4H), but they make up a smaller fraction of all BrdU-labeled nuclei than in previous endocycles (Table 1). Even greater reductions in DNA replication occur during S_6 than during S_5. Only early incorporation patterns were observed (Fig. 4J) indicating that a late S phase does not take place. Late replication patterns were also not observed in nuclei from later endocycles. This is generally consistent with previous observations (Lilly and Spradling, 1996); however, this experiment does not exclude that some replication of satellite DNA takes place during the last endocycles in posterior nurse cells (Hammond and Laird, 1985).

The most striking cell cycle correlation follows immediately after the end of S_5. Throughout S_5, all nuclei still display blob-like polytene chromosomes. However, virtually all G_6 (64C) nuclei display dispersed chromosomes, consistent with the FACS studies (Fig. 4I). Thus, the dispersal process takes place rapidly and precisely at the junction between S_5 and G_6. Our methods would have detected even a small number of exceptional cells, indicating that the chromosome dispersal process is tightly linked to the cell cycle and is somehow associated with the termination of endocycle 5. These precise correlations with the state of the endocycle imply that the chromosomal changes described above are under cell cycle control.

Polytene chromosomes undergo condensation during late S phase

The level of chromosome condensation was also seen to depend upon the cell cycle state of the nurse cells. During the course of endocycles 4 and 5, the lengths of the polytene arms decrease dramatically. To quantify these changes and relate

Table 1. Nurse cell endocycles

Endocycle	Predicted ploidy in G phase	Observed ploidy (C) of G phase nuclei*	% in G phase‡	% in early S phase	% in late S phase	Total nuclei (N)
3	8C	8.23±0.82	76.2	14.3	9.5	42
4	16C	15.9±0.72	66.7	19.4	13.9	36
5	32C	31.2±1.1	60	28	12	50
6	64C	57.8±1.1	71.4	28.6	0	42
7	128C	106±2.8	n.d.	n.d.	n.d.	n.d.

*Nurse cell ploidy was calculated relative to 2C follicle cells by averaging the values for measurements of N cells. Both nurse cells and follicle cells used in this calculation were in G phase (not incorporating BrdU).

‡Nurse cell nuclei were categorized by endocycle phase: G phase, no BrdU incorporation, early S, general BrdU incorporation throughout nucleus and late S, BrdU incorporation only over late-replicating, DAPI-bright, heterochromatic sequences.

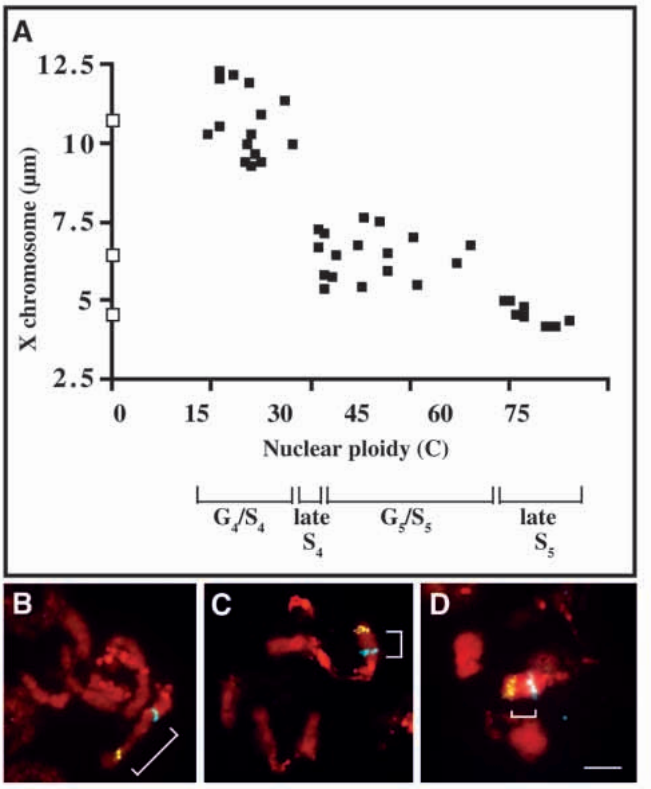

Fig. 5. Nurse cell polytene chromosomes condense during the time of late replication in S_4 and S_5. (A) The length of the euchromatic portion of the X chromosome was plotted as an indicator of condensation level versus nuclear ploidy calculated as in Fig. 3. Using the BrdU incorporation pattern to distinguish G, early S and late S, as well as ploidy, the position of nuclei within the fourth and fifth endocycles is plotted below the diagram. The points cluster in three distinct groups that correspond approximately to endocycle 4, early endocycle 5 and late endocycle 5 nuclei. The polytene X chromosome condenses twice, once in late phase of S_4 (1.7-fold) and again in the late phase of S_5 (1.4-fold). The mean value of each cluster is indicated by open squares on the y axis. A specific interval on chromosome arm 3L is identified by hybridization to 64C1-C8 (yellow) and 73E1-F4 (blue) and observed during endocycles 4 (16C)-6 (64C). DNA is stained with DAPI (red). Brackets indicate the distance between these two sites. (B) A S_4 nurse cell nucleus (<32C). (C) A G_5 nucleus shows shorter and broader arms than does the S_4 nucleus and the distance between the two probes has decreased. (D) A five-blob nucleus, in late S_5, shows evidence of minor additional condensation prior to undergoing dispersal. All nuclei are at the same magnification and the bar in D represents 5 μm.

them to the endocycle stages, we plotted the length of the X chromosome euchromatin as a function of DNA content and stage using a population of BrdU-labeled nuclei (Fig. 5A). During G_4 and early S_4, the X chromosome remains about 10.8±1.1 μm in length, whereas the width of the chromosome can be seen to increase during S phase. In contrast, late S_4 nuclei, G_5 nuclei and early S_5 nuclei were all significantly shorter (6.5±0.72 μm). Thus, condensation of euchromatin takes place rapidly during the late S phase of endocycle 4. Condensation appeared to be confined to late S, since the chromosome lengths reached during this phase did not change during subsequent cell cycle phases.

A less extensive, contraction was observed at the same time during the succeeding endocycle. Nuclei that were in the late phase of S_5 as judged by BrdU incorporation, again appeared to be shorter than previously (4.6±0.31 μm). The lesser degree of condensation during late S_5 may be due to the fact replication is not completed or due to changes associated with the onset of chromosome dissociation. We could not determine if condensation takes place during the late S phases of endocycles 1-3, because it was not possible to spread these small polytene chromosomes sufficiently to measure chromosome lengths (see Fig. 4A,B). If similar condensation did occur in these earlier cycles, it might explain why the chromosomes appear unusually short and wide even prior to endocycle 4.

To verify that X chromosome euchromatin is representative of euchromatin generally, we delineated a region of euchromatin on chromosome 3L between 64C and 73F with two FISH probes. We could not simultaneously perform BrdU incorporation and FISH, but the uniformity of the cell cycle changes made it possible to deduce the endocycle stage by measuring chromosome width. Changes in the spacing of these probes correlated with our previous measurements. Thus, in G_4 and early S_4 nuclei (Fig. 5B), the interval was quite uniform, 7.0±0.21 μm. In contrast, the probes were only 3.0±0.60 μm apart in G_5 chromosomes (Fig. 5C). The relatively small standard deviations and absence of detected intermediate values shows that the contraction takes place rapidly. A slight further reduction in length may take place in late S_5 nuclei (Fig. 5D; separation 2.8±0.18 μm) It was not possible to make corresponding measurements of autosomal heterochromatin because these regions are more susceptible to stretching during sample preparation. However, heterochromatin was not subject to an equivalent compaction during these endocycles, since the relative linear fraction of the chromosomes comprised of heterochromatin increases during endocycles 4 and 5 (see Fig. 4).

Chromosome dispersal produces 32-ploid polytene nurse cell nuclei

Organized chromosomal structure within wild-type nurse cell nuclei has not been recognized after stage 6; however, our studies revealed that chromosome dispersal leads to a novel polyploid polytene state (Fig. 6). The earliest evidence of this transition is a loosening of the association of sister chromatids late in S_5. FISH signals no longer appear as smooth bands, but resolve into a number of distinct spots of similar intensity (Fig. 6A; see also Fig. 5D), presumably as the chromatids move out of precise register. Shortly thereafter, the polytene arms fall apart into a large number of separate strands (Fig. 6B′, arrows). Fig. 6B shows a nucleus during dissolution in which individual strands from the 2nd and X chromosomes are scattered throughout a portion of the squash, while the 3L and 3R arms and their interconnecting centromeric heterochromatin remain intact. Prior to complete separation, the chromosome 2L and 2R signals separate due to homologue separation (Fig. 6C, see below). Shortly after this stage, DAPI staining no longer reveals any chromosomal substructure, but FISH demonstrates that the signals from each chromosome arm occupy specific and largely non-overlapping sectors of the nucleus (Fig. 6D).

If dispersal were complete, we would expect to observe 64 individual FISH signals in G_6 nuclei. However, counts of the

individual FISH signals using probes from a variety of sites on the euchromatic chromosome arms revealed exactly half that number, 32, through all of the following endocycles (Fig. 7A,B). This demonstrates that the 64C chromosomes do not fall apart completely, but rather into pairs of chromatids. Similar studies using probes to centromeric heterochromatin showed that dissociation is variable and less complete in these regions (data not shown). For example, the 1.672 satellite block associated with chromosome 4 fails to dissociate at all, leading to the persistence of a bright spot of DAPI staining in older nurse cells. For euchromatic regions, the 32 individual signals become progressively stronger in older nurse cells and take on the band-like appearance characteristic of polytene chromosomes (Fig. 7). In nurse cells from stage 10 egg chambers, dozens of individual polytene chromosomes with detailed banding patterns can be visualized by DAPI staining (Fig. 7C). The elongate dimensions and extensive banding structure of these subpolytenes more closely resemble salivary gland chromosomes than the short, sparsely banded polytenes of early nurse cell nuclei. These results show that *Drosophila* nurse cell chromosomes dissociate at the boundary between S_5 and G_6, and that each of the 2C chromosomes initiates polytenization. As a result, late nurse cells are not non-polytene as previously believed, but rather contain exactly 32 sets of subpolytene chromosomes. A summary of nurse cell chromosome behavior is given in Fig. 8A.

To determine the origin of the chromosome pairs, we squashed ovarioles of females heterozygous for a P-element insertion and performed FISH to the inserted element and to genomic DNA near the insertion site. If homologues remain together in the 2C pair, we would expect 32 double-labeled FISH signals. Alternatively, if two sister chromatids form the 2C pair, we would expect to see 16 hybridization sites double-labeled with both probes and 16 sites labeled only with the probe to the genomic locus (Fig. 6E). FISH at the five-blob stage shows that after chromatid dispersal, half of the labeled genomic sites cohybridize with the neighboring P-insert (Fig. 6F) and chromatids from each homologue remain clustered. Thus, the 64C chromosomes have separated into 2C pairs consisting of replicated sisters. The same results were observed in similar experiments at the *hts* locus (not shown). These experiments also confirmed that it is the homologues that separate prior to complete chromosome breakdown.

Dissociation into chromosome pairs is due to incomplete replication during S_5

Our measurements of nurse cell DNA content (Table 1) suggested a simple explanation for the fact that the 64C chromosomes dissociate into 32 sister chromatid pairs, rather than 64 individual chromatids. Nurse cell DNA levels suggest that a portion of the genome fails to duplicate completely during S_5, unlike earlier

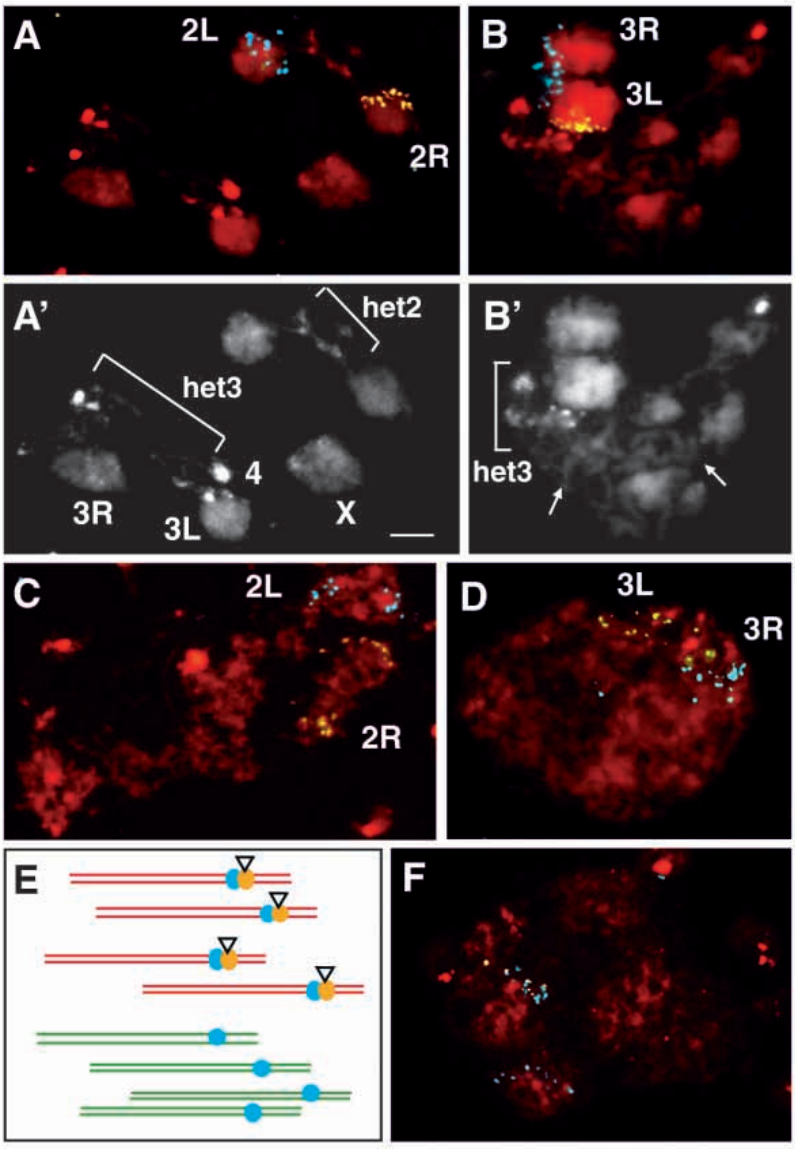

Fig. 6. Chromatid asynapsis occurs at the S_5/G_6 boundary. Chromosome 2 is identified in A and C (FISH at 39A3-A7 (2L) in blue and at 42B4-C2 (2R) in yellow) and chromosome 3 is identified in B and D (FISH at 79E (3L) in yellow and FISH at 82B (3R) in blue). In all panels, DNA is stained with DAPI and, in A-D and F, DAPI staining is shown in red. (A,A′) A nurse cell nucleus in S_5, between 32C and 64C, shows the five-blob stage. Brackets indicate the centromeric DNA. (B,B′) A nurse cell nucleus at the S_5/G_6 boundary shows dispersal of chromosome X and both arms of chromosome 2, but not of the third chromosome arms. Small chromosomes that presumably correspond to individual chromatid pairs can be seen (arrows). (C) Another nurse cell nucleus at the S_5/G_6 boundary showing homologue separation and early chromatid dispersal. (D) Diffuse G_6 nucleus showing no obvious organization; however, FISH reveals that the separated 3L and 3R chromatid pairs continue to occupy unique domains. (E) Diagrammatic representation of FISH to a strain heterozygous for a P-element insertion (*fs(2)04443*, triangle). Homologues (red and green lines), the P-element insert (yellow circle) and a neighboring genomic site (at 39A, blue circle) are indicated. (F) Results of hybridization to strain indicated in E. Location of signals from genomic DNA (blue) and the P-element (yellow) indicate that the homologues have separated. All nuclei are at the same magnification and the bar in A′ represents 5 μm.

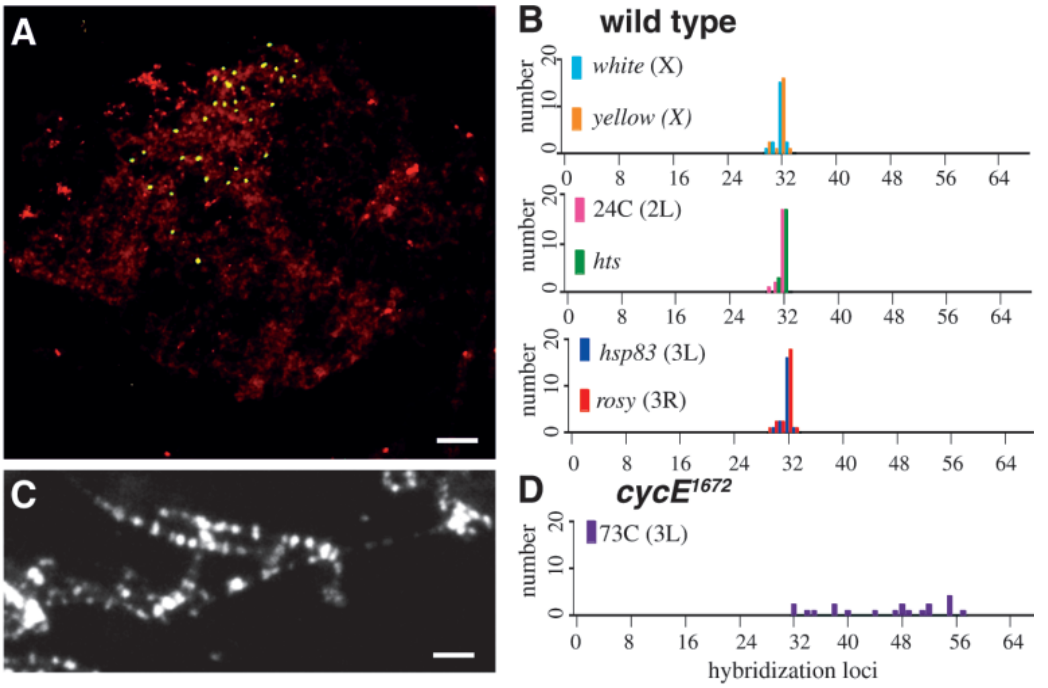

Fig. 7. Late stage nuclei show 32 subpolytenes. (A) FISH on a late stage nurse cell nucleus (about 256C) with a probe specific to 73E1-F4 reveals 32 sites of hybridization (yellow). DNA is stained with DAPI (red). (B) Histograms show that there are 32 sites of hybridization for euchromatic loci in late stage nuclei (256C to 1024C) on the X, 2nd and 3rd chromosomes. (C) In a small region from a DAPI-stained stage 10 nurse cell nucleus (>2000C), individual subpolytene chromosomes show an elongated shape and a detailed banding pattern. (D) Histogram of the number of hybridization sites on 3L (73E1-F4) in a female-sterile *cyclin E* ^{1672} */Df(2L)r10* heterozygote shows greater than 32 sites. In A, the bar represents 5 μm and in B, 10 μm.

endocycles (Table 1). Failure to complete replication would leave the sister DNA strands interconnected by unreplicated regions or their derivatives. It may be the continued presence of these interconnections that holds the two sister chromatids together in pairs after the completion of S_5 (Fig. 8B).

If incomplete replication is responsible for pairing the chromatids, mutations that allow more complete replication would be expected to increase the number of subpolytene chromosomes. The *cycE*^{1672} allele affects the nurse cell endocycle, increasing the length of S phase and partially restoring late replication (Lilly and Spradling, 1996). We examined the extent of nurse cell chromosome dissociation in females heterozygous for *cycE*^{1672} and a *cycE* deletion, *Df(2L)r10*. As predicted, rather than precisely 32 FISH signals, the number of FISH signals increased, but was always between 32 and 64 (Fig. 7D). The failure to induce complete dissociation of all 64 chromatids was not surprising since the mutation only partially restores nurse cell late DNA replication (Lilly and Spradling, 1996). In *cycE*^{1672} nurse cells, fully replicated chromatids may separate, but those that did not finish replicating still remain as 2C pairs. The increased number of signals was not due to a delay in the timing of chromosome dispersal until the subsequent endocycle, since the unusually large blob stage chromosomes that would result from such a delay were not observed.

DISCUSSION

Nurse cells develop in three phases

Our studies show that nurse cell development in *Drosophila melanogaster* takes place in three distinct phases. In the condensed polytene phase, nurse cells replicate their genomes fully and possess polytene chromosomes of a novel structure characterized by extreme condensation, lack of a chromocenter and unusually large domains of centromeric heterochromatin. During endocycle 5, the second phase, replication ceases prior to the completion of DNA synthesis and then a unique cell cycle state is induced during which the 64 chromatids rapidly disperse into 32 pairs. The ensuing final phase, comprising 5 to 7 endocycles, supports the growth of each chromatid pair into a new polytene chromosome, generating 32-ploid-polytene nuclei. Incomplete replication continues during most of the subsequent endocycles (Hammond and Laird, 1985; Lilly and Spradling, 1996). Thus, *Drosophila* nurse cells undergo three distinct endocycles during a programmed transition between a condensed polytene and a dispersed, 32-ploid-polytene phase of oogenesis (see Fig. 8A).

Cell cycle regulation of nurse cell polytene chromosome structure

We have shown that the condensation and sister chromatid association of polytene chromosomes are tightly regulated within the nurse cell endocycles. Cell cycle-linked changes have not been observed previously in *Drosophila* polytene chromosomes. In particular, we observed that euchromatin condenses during the late S phases of the last two endocycles prior to chromosome dispersal. Condensation of mitotic chromosomes is thought to be mediated by the action of SMC protein complexes following the completion of S phase (Hirano, 1995; Koshland and Strunnikov, 1996). Mitotic chromosomes then decondense again in late telophase. Our observation that nurse cell euchromatin begins condensation during late S phase, suggests that its condensation begins immediately after it finishes replicating, while heterochromatin is still duplicating. We propose that the condensation of euchromatin and heterochromatin, like their replication, take place at least in part, during separate phases of the cell cycle.

The presence of regulated condensation in young nurse cells, but apparently not in larval salivary gland cells, may reflect differences in their endocycles. Young nurse cells are unusual among polytene tissues in that they complete S phase and may

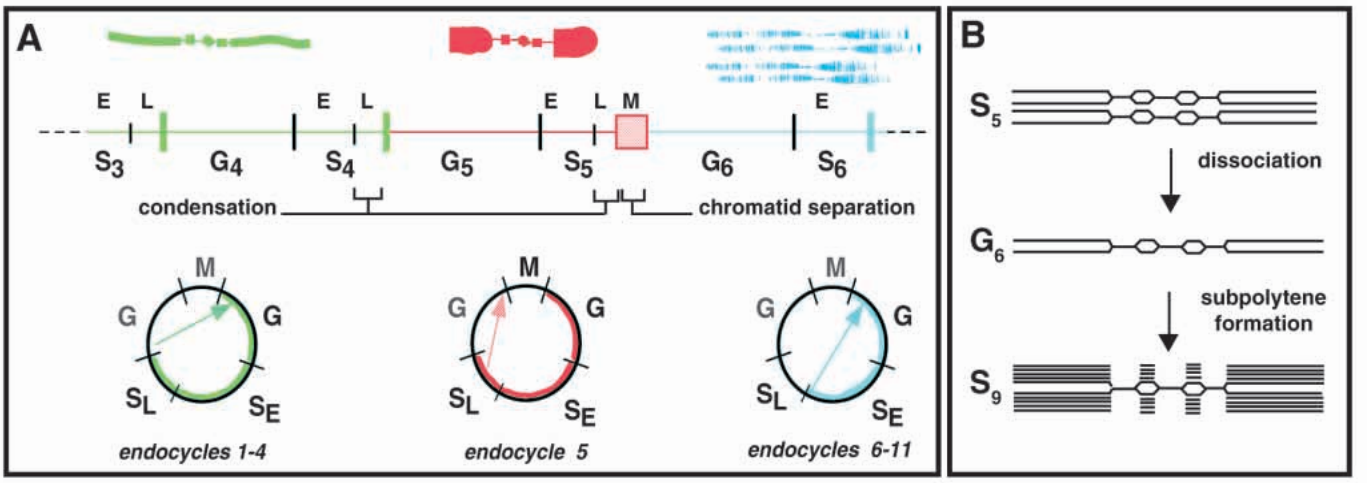

Fig. 8. (A) Changes in nurse cell endocycles and chromosome structure during *Drosophila* oogenesis. A time line of nurse cell development during endocycle 4 (green), endocycle 5 (red) and endocycle 6 (blue) is shown. (Dashes at the ends indicate that the earlier endocycles resemble endocycle 4, and later endocyles resemble endocycle 6.) Black bars separate specific phases within an endocycle (labeled as in the text), while colored bars separate successive endocycles (E = early S phase; L = late S phase). Above the line, color-coded diagrams of a polytene chromosome are shown as it would appear in the corresponding endocycle. Below the line, the times chromosomes undergo condensation are indicated with brackets, while another bracket points to the mitosis phase (M) at the end of endocycle 5 when chromatids separate. The three different endocycle types are shown on circular diagrams of a hypothetical mitotic cell cycle. The portions of the mitotic cycle postulated to occur in the indicated endocycle are indicated with a line of the appropriate color, and the position where the endocycle is reset with a colored arrow. (B) Model of sister chromatid separation at the end of endocycle 5. The chromatids within an S_5 nurse cell polytene chromosome do not fully duplicate late replicating pericentromeric heterochromatin and chromatid pairs contain unreplicated regions (top). Note that only 4 instead of 64 component chromatids are shown. Following the mitosis phase of endocycle 5, the sister chromatids separate except for those still held together by unreplicated segments (middle). During subsequent endocycles the chromatids undergo new rounds of replication in the absence of a late S phase, leading to 'subpolytene' chromosomes with the diagrammed structure (bottom).

continue into a post-S endocycle phase. The poorly banded young nurse cell polytene chromosomes may condense periodically during the late S phases of multiple early endocycles without ever decondensing, to generate the high level of compaction observed before dissociation at the termination of endocycle 5. Most of this condensation probably took place during rather than after late S phase since chromosomes that still showed late patterns of BrdU incorporation had already undergone extensive compaction in both late S_4 and late S_5 (Fig. 5). In contrast, the less condensed, highly banded salivary gland chromosomes and post-stage 6 nurse cell subpolytene chromosomes may be characteristic of endocycles which reset and enter G phase before entering late S phase. Consistent with this model, species whose polytene cells contain fully replicated satellite DNA tend to have more compact chromosomes with fewer bands than those where satellite DNA is underrepresented (see Steinemann, 1978; reviewed in Ashburner, 1970).

A unique mitosis-like phase in endocycle 5 may control chromosome breakdown

Programmed sister chromatid separation is also tightly linked to the endocycle, since it occurs precisely at the junction between endocycles 5 and 6. Several aspects of chromosome dispersal resemble events of a normal M phase (in a Dipteran with homologue pairing) or of meiosis I. These include both the order of separation (homologues before sister chromatids) and the appearance of the newly dissociated chromatids, which resemble small mitotic chromosomes (Fig. 6B′). We propose that the S phase during endocycle 5 is not truncated by resetting the endocycle back to G phase, as in the other endocycles where replication is incomplete, but by resetting to an M phase that occurs only during endocycle 5. The absence of spindle formation and cytokinesis may indicate that this M phase is truncated, as in cells undergoing endomitosis, or that nurse cells do not respond to signals that induce these aspects of mitosis in other cells. Previous studies have failed to detect evidence of mitotic cyclins in wild type nurse cells (Lilly and Spradling, 1996; Reed and Orr-Weaver, 1997). Perhaps these proteins escaped detection because they are present only briefly at the end of endocycle 5 or perhaps their role can be bypassed by other mitotic regulators.

Genetic evidence for the existence of a mitosis-like phase at the end of endocycle 5 comes from studies of the gene *morula* (Reed and Orr-Weaver, 1997). Morula is required for diploid cells to complete mitosis. Nurse cells from weak *morula* mutant females cease normal development during stages 4 to 5 and accumulate detectable levels of cyclin B. Chromosomes in the mutant nurse cells arrest as blob-like structures that are often associated with aberrant spindles. These observations show that a special requirement for Morula exists at the time of chromosome dispersal, perhaps as part of the postulated M phase at the end of endocycle 5. Our experiments provide an explanation for the stage-specificity of *morula* action, since only endocycle 5 shows evidence of an M phase. The existence of a cell cycle difference between stage 4 nurse cells and other cells was predicted by Reed and Orr-Weaver (1997).

Genetic control of nurse cell chromosome reorganization

A least three other mutations, *fs(2)B* (Koch and King, 1964), *otu* (King et al., 1981) and *cup* (Keyes and Spradling, 1996) abolish chromosome dispersal, but allow some nurse cells to continue growth. These cells develop giant polytene chromosomes whose structure can be studied in detail, particularly in the case of certain *otu* alleles (Heino, 1989; 1994; Mal'ceva and Zhimulev, 1993). The chromosomes display a banding pattern similar to that in the salivary gland chromosomes, but lack a chromocenter and ectopic fibers (Mal'ceva et al., 1995). The relationship between giant *otu* chromosomes and normal nurse cell chromosomes has never been determined. Our observations reveal that these giant chromosomes resemble early nurse cell chromosomes in their lack of a chromocenter and ectopic fibers, a situation that has not been described in any other polytene tissue from *Drosophila melanogaster*. However, the mutant nurse cell chromosomes differ in possessing long, highly banded euchromatic arms. Differences between mutant and wild-type in the nurse cell endocycles before or after the time of normal dispersal may lead to this condition.

The absence of a chromocenter and ectopic fibers in both wild-type and mutant nurse cell chromosomes may be a consequence of the fact that DNA replication is complete during the early endocycles. We propose that chromocenter attachments and visible nonhomologous ectopic associations are caused by unterminated replication forks that break and invade homologous sequences on other chromosomes. A correlation between complete replication and the absence of ectopic associations is also seen in comparisons between the salivary glands of Dipteran species that undergo complete or partial replication of satellite DNA (for example, see Steinemann, 1978).

Is polytene chromosome reorganization important for nurse cell function?

If nurse cell chromosome reorganization serves an important function, it might be conserved among other Dipteran species with polytene chromosomes. Several previous observations suggest that other Dipteran species undergo a similar transition in mid-oogenesis. In *Calliphora erythrocephala*, an initial set of 'primary' polytene chromosomes condense into blob-like structures before separating at the 32C or 64C stage (Bier, 1957). However, dissociation of component strands is less complete than in *Drosophila* since 'secondary' polytene chromosomes composed of loosely paired subpolytene components are sometimes found in later nurse cells (Bier, 1960). Secondary polytene chromosomes might arise because chromosome dissociation was incomplete, as we found in *Drosophila melanogaster* within heterochromatic regions, or because subpolytene chromosomes can homologously reassociate to form a single chromosome. In at least two other Dipteran species, *Delia antiqua* and *Delia radicum*, nurse cells follow the same general sequence of chromosome dispersal (Hartman and Southern, 1995).

What function might dispersal play during oogenesis? One possible role would be to facilitate extremely rapid ribosome synthesis. *Drosophila* nurse cells normally develop a large nucleolus that becomes dispersed throughout the nurse cell nucleus after stage 5 (Dapples and King, 1970). Nucleolar reorganization is blocked in *otu* alleles that disrupt chromosome dispersal (Khipple and King, 1976). The coincident timing of chromatid separation and nucleolar expansion suggests that programmed chromosome dissociation functions to increase ribosome production by dispersing the nucleolus organizer regions. However, polytene chromosome dispersal has also been reported in somatic cells in some species, where the need for elevated ribosome production is less clear (reviewed in Pearson, 1972). The analysis of cell cycle-regulated chromosome dissociation reported here should make it easier to test the function of nurse cell polytene chromosome dispersal.

Polytene chromosomes as indicators of normal chromosome structure and dynamics

Our analyses provide a new perspective on the nature of polytene chromosomes. Rather than highly specialized structures, our data suggest that polytene chromosomes are normal chromosomes responding to the modified endocycles to which they are exposed. In the *Drosophila* salivary gland, the endocycles each truncate sufficiently early in S phase so that little condensation occurs. In contrast, the more complete early nurse cell endocycles cause the chromosomes to shorten dramatically during their late S phases. The structure of both types of polytene chromosome may reflect the topography of chromosome compaction and higher order chromatin organization characteristic of chromosomes generally in these cell cycle states. Finally, studies of the dispersion of nurse cell chromosomes after endocycle 5 may assist in elucidating the nature and mechanism of chromatid pairing and separation. Despite many years of use simply as tools of *Drosophila* genetics, polytene chromosomes retain the potential to serve as valuable models for understanding the fundamental mechanisms of eukaryotic chromosome structure and behavior.

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