The regulation of the cell cycle during *Drosophila* embryogenesis: the transition to polyteny

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Summary

The process of polytenization plays a crucial role in *Drosophila* development, and most of the larval tissues are polytene. By analyzing the pattern of DNA replication in embryos pulse-labeled with BrdU, we show that many larval tissues undergo a transition to begin becoming polytene late in embryogenesis. Our results demonstrate that in these larval tissues polyteny results from a modified cell cycle, the endo cell cycle, in which there is only an S (synthesis) phase and a G (gap) phase. A key regulator of the mitotic cell cycle, the product of the *string* gene (the *Drosophila* homologue of *cdc25*), is not required for the endo cell cycle. The developmental regulation of the endo cell cycle is striking in that tissue-specific domains undergo polytene DNA replication in a dynamic pattern at defined times in embryogenesis. During subsequent rounds of the endo cell cycle in late embryogenesis and first instar larval development, the domains are subdivided and the temporal control is not as rigid. The length of the G phase varies among different tissues. By quantifying DNA content, we show that during the early polytene S phases the genome is not fully duplicated.

Key words: cell cycle, polytene, *Drosophila* development, *string*.

Introduction

The presence of polyploid or polytene cells is widespread in nature, in fact some 300 examples have been discovered in animals, plants and protozoans (for reviews see Nagl, 1978; Brodsky and Uryvaeva, 1984). Polytene chromosomes are found in cells as diverse as the macronucleus of ciliates and the trophoblast cells that give rise to the mammalian placenta. Many tissues that become polytene or polyploid are highly metabolically active and synthesize large amounts of particular products.

Polyploid or polytene cells increase ploidy by dissociating DNA replication from mitosis, yet little is understood about how this is regulated. Polytene cells are defined by the visible synapsis of the multiple copies of the chromosomes. The term polyploid has been applied to three types of cells: those in which the endoreplicated chromosomes are not synapsed or visible, those is which cyclic condensation of the chromosomes reveals multiple distinct sets, and in some cases multinucleate cells have been referred to as polyploid also. This range of polyploid cell types suggests that there are several levels at which DNA replication can be uncoupled from mitosis: cytokinesis may be blocked, both cytokinesis and nuclear division may not occur, or chromosome segregation may be missing as well. Polytene cells are the most deviant from the normal diploid cell cycle since they retain some degree of synopsis of the replicated chromatids and exhibit none of the steps of mitosis. Polytene could result either from continuous DNA replication outside of a cell cycle, or an altered cell cycle that excludes mitosis.

Dipterans are useful organisms in which to investigate the regulation and mechanism of polytenization. They employ extensive use of polyploid and polytene tissues in their developmental strategy; all of the larval tissues except the nervous system and imaginal tissues are polytene. Most of the information on polytene DNA replication has been gained from analysis of the larval salivary gland of *Drosophila*. Endoreplication has been analyzed in the larval salivary glands by quantifying DNA content at different larval developmental times and by pulse labeling with [3H]thymidine (Rudkin, 1973; Pearson, 1974; Hammond and Laird, 1985). These studies showed that the DNA content of nuclei fell into discrete classes whose mean values differed by a factor of two, and that there were periods when [3H]thymidine was not incorporated into most of the salivary gland nuclei. This implied that during the larval periods polytene DNA replication is cyclic, rather than continuous. The quantitation also showed that during an S phase in the salivary gland cells the total genome is
not completely duplicated, and approximately 20–30% is underrepresented (Rudkin, 1969; Hammond and Laird, 1985b). Correlation of the \(^{3}H\)thymidine labeling pattern with the *Drosophila* salivary gland chromosome banding pattern by in situ analysis suggested that each S phase has an initial period of discontinuous labeling, followed by continuous labeling and finally another period of discontinuous label. This pattern is reproducible, permitting the positions of the late replication labeling to be mapped (for review see Rudkin, 1972). Thus the characterization of salivary gland replication in the late larval stages strongly suggested that replication was cyclic, demonstrated that the genome was not fully duplicated and showed a temporal order of replication of genomic regions. However, direct information about the onset of polytenization during development of the salivary gland was lacking, as was the ability to investigate the properties of a specific S phase in endoreplication.

The process of endoreplication in *Drosophila* larval tissues other than the salivary gland has not been investigated extensively, and there is no direct evidence as to whether the replication is cyclic or when, during development, polyteny initiates in these tissues. Analysis of the adult polyplody nurse cells and follicle cells indicated that DNA replication in these tissues was cyclic (Mahowald et al. 1979; Hammond and Laird, 1985a). It has been demonstrated that different polytene tissues in the larvae attain different levels of polyteny. For example the salivary gland becomes 1024–2048C (Rudkin, 1972), while Richards has estimated the fat body to be 256C (Richards, 1980). Lamb and Hochstrasser find the Malpighian tubules to be 256C (Lamb, 1982; Hochstrasser, 1987), and Lamb has reported the midgut to be 64C (Lamb, 1982). This tissue specificity in the degree of polyteny could be a consequence of slower replication, of initiating polyteny DNA replication at different points in development, or of cessation at different times.

We chose to investigate the DNA replication occurring during embryogenesis to determine when larval tissues underwent a transition to polyteny. By pulse labeling staged embryos with BrdU (bromodeoxyuridine) we were able to visualize when and where DNA replicated in whole-mount embryos. The time of endoreplication could be correlated with developmental events, and a single specific S phase could be analyzed for each tissue. Thus we were able to test directly whether DNA synthesis was cyclic in many polytenic tissues and to analyze the tissue specificity of the onset of polyteny. Our results establish that polyteny arises through cyclic DNA synthesis in what we term an endo cell cycle; here we describe the characteristics of this novel cell cycle.

**Materials and methods**

**Fly strains**

Wild-type *Drosophila melanogaster* embryos were collected from the Canton-S strain. The \(\text{sig}^{\text{mt3}}\) allele was originally isolated by Jurgens et al. (1984).

**BrdU labeling of embryos**

*Drosophila* embryos were labeled with BrdU following a modification of the protocol of Bodmer et al. (1989). After collection for 40 min at 25°C, embryos were incubated at 25°C until they had reached the appropriate developmental stage for each experiment. The embryos were dechorionated in bleach, rinsed extensively with distilled water, and once with the *Drosophila* tissue culture medium M3 (Ashburner, 1989). To allow BrdU uptake, the embryos were permeabilized by incubation in octane (Aldrich) with agitation for 5 min. Then they were transferred to M3 with 1 mg ml\(^{-1}\) BrdU (Boehringer-Mannheim) and incubated for 30 min at room temperature. In some experiments the BrdU was injected into the embryos. Prior to fixation the labeled embryos were rinsed with M3. BrdU-labeled embryos that had not yet undergone cuticle deposition were fixed in a 1:1 mixture of 3.7% formaldehyde (in 0.1 M KPO\(_4\) pH 7.5):heptane (Ashburner, 1989) and subsequently devitellinized using methanol. They were then rehydrated to 1× PBS in a methanol series. All samples were hydrolyzed and antibody labeled as described (Bodmer et al. 1989). The secondary antibody was a goat anti-mouse antibody conjugated to horseradish peroxidase (Bio-Rad). Ages given for embryos shown in the figures represent the midpoint age of the collection at the time of fixation.

To examine replication in whole-mount embryos that had secreted cuticle, multiple holes were made in the cuticle of embryos, using forceps, to allow antibody entry. To dissect tissues, embryos were transferred to double-stick tape following the labeling period and covered with *Drosophila* saline. They were hand devitellinized and dissected with glass needles. The relevant tissue was transferred to a poly-l-lysine coated slide and the tissue was fixed in 3:1 (ethanol:acetic acid) for 1 h. The tissue was rehydrated through an ethanol series. All samples were then hydrolyzed and antibody labeled as described above.

**BrdU labeling of larvae**

Larvae were labeled by feeding them BrdU using a protocol similar to that previously described (Truman and Bate, 1988). A one hour collection of embryos was allowed to incubate at 25°C (approximately 19 h) until larvae hatched. These larvae were transferred to media containing agar (0.8%), sucrose (5.0%), yeast extract (5.0%), and inactivated yeast (2.0%) and allowed to grow to the appropriate age. They were then fed on food containing 1 mg ml\(^{-1}\) BrdU (Boehringer-Mannheim) and incubated for 30 min at room temperature. In some experiments the BrdU was injected into the embryos. Prior to fixation the labeled embryos were rinsed with M3. BrdU-labeled embryos that had not yet undergone cuticle deposition were fixed in a 1:1 mixture of 3.7% formaldehyde (in 0.1 M KPO\(_4\) pH 7.5):heptane (Ashburner, 1989) and subsequently devitellinized using methanol. They were then rehydrated to 1× PBS in a methanol series. All samples were hydrolyzed and antibody labeled as described (Bodmer et al. 1989). The secondary antibody was a goat anti-mouse antibody conjugated to horseradish peroxidase (Bio-Rad). Ages given for embryos shown in the figures represent the midpoint age of the collection at the time of fixation.

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**Staining with anti-tubulin and anti-histone antibodies**

A monoclonal antibody against β-tubulin (Amersham) at a 1:2000 dilution was used to examine mitotic figures in dissected tissues from 11–13 h embryos. The signal was detected with a rhodamine-conjugated goat anti-mouse antibody (Jackson Immuno Research Laboratories).

To ensure that dissected larval tissues were accessible to antibodies they were bound to an anti-histone monoclonal
completion of the mitotic divisions. We term DNA replication that is uncoupled from mitosis in cells that polyteny occurred in embryogenesis following the cease. Thus we could determine whether a transition to pattern of mitoses during embryogenesis has been of polyteny in larval cells had not been ascertained. The development, but the developmental time of the onset levels for the chromosomes to be visible. In embryogenesis, polytene, even if the ploidy has not reached sufficient levels for the chromosomes to be visible. In embryogen-

**Microscopy**

A Zeiss Axioshot microscope equipped with Nomarski optics and fluorescence was used to observe the tissues, embryos and larvae using Plan-Neofluar 10x, 25x and 40x objectives.

**Quantification of DNA content**

Tissues to be quantified were dissected in Drosophila saline and transferred to poly-L-lysine coated slides. To control for variation in the fixation and subsequent staining, leg imaginal discs from third instar larvae were dissected onto each slide. The tissue was squashed under a coverslip to disperse the cells; the slide was then frozen in liquid nitrogen, and the coverslip removed. The tissue was fixed in 3:1 (ethanol:acetic acid) for 1 h, rehydrated in an ethanol series, and rinsed twice in 1×PBS. Staining was in 0.5 μg/ml DAPI (4,6-Diamidino-2-phenylindole, Sigma) for 5 min. The samples were then washed once in PBS, and mounted in 70% glycerol. The relative fluorescence was measured as described in Goff and Coleman (1987). A Zeiss photomicroscope interfaced to a Zeiss microspectrofluorometer and a Zeiss Plan-Neofluar 25x objective were used. A measuring diaphragm of 0.4 mm was used because it just circumscribed the nuclei being measured.

The values of fluorescence were measured in relative machine units that are not directly comparable from experiment to experiment. For each slide the average value of the 2C peak of imaginal cells was used as the standard to determine the ploidy of the endoreplicated cells. The statistical significance was tested using the Wilcoxon-Mann-Whitney rank test which tests whether two distributions have different means (Lindren et al. 1978).

The quantification of DAPI fluorescence is very reproducible in that when a specific nucleus was measured repeatedly the range in fluorescence measured differed only by 5%. In addition, results obtained in different experiments showed comparable levels of replication, indicating the reproducibility of these measurements. For both the salivary gland and the hindgut, the peaks are not made broad by the presence of replicating cells, since the tissues were dissected from embryos at a stage when these tissues were no longer endoreplicating. Replicating cells would also not make a significant contribution to peak width in the imaginal disc measurements because only 10% of leg disc cells are in S phase at any given time (Graves and Schubiger, 1982).

**Results**

The transition to polyteny during embryogenesis

Most cells undergo endoreplication during larval development, but the developmental time of the onset of polyteny in larval cells had not been ascertained. The pattern of mitoses during embryogenesis has been described in detail (Campos-Ortega and Hartenstein, 1985; Foe, 1989), and it is known when mitotic divisions cease. Thus we could determine whether a transition to polyteny occurred in embryogenesis following the completion of the mitotic divisions. We term DNA replication that is uncoupled from mitosis in cells that later in development contain polyteny chromosomes as 'polytene', even if the ploidy has not reached sufficient levels for the chromosomes to be visible. In embryogen-

esis there are 13 rapid cleavage divisions that occur in a syncytium; following cellularization most cells that will give rise to tissues in the larva undergo only three additional mitoses that are completed by the sixth hour of embryogenesis. The neural and imaginal tissues are the sole tissues that continue to grow mitotically during embryonic and larval development. To determine if polytene DNA replication was initiated during embryogenesis, we tested whether BrdU incorporation could be detected in cells at a developmental time after the completion of their mitotic divisions. Staged embryos of the appropriate age were collected, permeabilized, labeled with BrdU and the incorporated label was visualized by antibody staining.

These experiments revealed polytene DNA replication in larval precursor cells after the last mitosis of embryogenesis. In the period between 8 and 15 h after fertilization, we observed DNA replication in the salivary gland, midgut, hindgut, Malpighian tubules and a group of cells on the dorsal side of the embryo (Fig. 1). The pattern of polyteny replication was striking in that it occurred in tissue-specific domains that replicated at specific developmental times, and the replication was not continuous (Fig. 2). For example, all of the cells of the salivary gland replicated during a 1 h period beginning at 8.5 h after fertilization (Fig. 1A,B). Next, cells at the anterior and posterior of the midgut underwent replication. This replication initiated in a group of cells in the anterior and the posterior midgut and spread in a wave anteriorly and posteriorly, respectively (Fig. 1B–D). Replication also occurred in a cluster of large, flat cells located between the anterior and posterior midgut (Fig. 1C). From 10 to 12 h of embryogenesis the hindgut replicated; this was followed by replication of the Malpighian tubules (Fig. 1C,D). Skaer had previously described replication in the Malpighian tubules at this time after the completion of mitosis (Skaer, 1989). In the two hours immediately preceding cuticle deposition, we observed a second round of replication in the midgut (Fig. 1E,F). This replication initiated in a band of cells that corresponded to the site of the first constriction of the midgut, and it spread anteriorly and posteriorly so that cells throughout the midgut replicated. The second and third constrictions of the midgut were not preceded by replication in a band of cells. Immediately prior to cuticle deposition BrdU was incorporated into a group of cells on the dorsal side of the embryo (Fig. 1F). The DNA replication pattern is shown only prior to cuticle deposition in this figure. The replication occurring during later embryogenesis and early larval stages is described below.

The replication observed in the salivary gland, gut and Malpighian tubules is endoreplication leading to polyteny because periods of replication (see below for a description of later rounds of replication) are not separated by mitosis (Fig. 3). These tissues were dissected from 11–13 h embryos and examined for mitotic figures by staining them with a monoclonal antibody against β-tubulin. In agreement with the previous results of others (Campos-Ortega and Harten-
Fig. 1. Endoreplication during embryogenesis. Wild-type embryos were labeled with BrdU for 30 min, fixed and stained with an antibody using the HRP reaction. The darkly staining cells are those that incorporated BrdU during the pulse. All of the embryos in this figure are oriented with their anterior side to the left and the dorsal side up. (A) An 8.5 h old embryo at the germ band extended stage (stage 11), showing replication in the salivary gland (sg). The other replicating cells in this embryo are part of the neural tissues which are dividing mitotically. (B) A 9.25 h embryo (stage 12) undergoing germ band retraction and endoreplication in the developing anterior midgut (am), posterior midgut (pm), and the salivary gland (sg). (C) By 9.75 h (stage 13) the hindgut (hg) is replicating, while replication persists in the anterior and posterior midgut. (D) A 10.5 h embryo (stage 14) in which replication has initiated in the Malpighian tubules (mt), as well as continuing in the anterior and posterior midgut, and hindgut (hg). (E) A 12 h embryo (stage 15) with a stripe of cells replicating in the midgut. (F) A 13.75 h embryo (stage 16) with more extensive labeling throughout the midgut and in a group of cells on the dorsal side of the embryo. Magnification ×190.

stein, 1985), we failed to detect mitotic figures in these tissues (data not shown). The sensitivity of detection of BrdU label appears to be such that DNA replication but not DNA repair was detected. If DNA repair was observed, we would have predicted more homogeneous labeling of the embryos rather than the dynamic pattern of domains observed. The domains of DNA replication are not a consequence of impermeability to BrdU since labeling both by permeabilization with octane and injection of the nucleotide analogue gave the same pattern of replication domains.

In addition to demonstrating that larval precursor cells undergo a transition to polyteny during the latter part of embryogenesis, our results directly show that the yolk nuclei are polyploid or polytene and are the first nuclei to switch from a mitotic cell cycle to polyteny. After the first nine syncytial divisions the majority of the nuclei migrate to the surface of the embryo (Foe and Alberts, 1983). Approximately 50 nuclei are left behind, referred to as the yolk nuclei, and go through two more mitotic divisions. Based on the increasing size of the yolk nuclei following their last mitosis, it has been assumed that they become polyplid. We confirmed this by demonstrating that after cleavage cycle 11 the yolk nuclei underwent DNA replication that was uncoupled from mitosis, thus increasing their ploidy (data not shown).
Fig. 2. The spatial patterning of endoreplication during embryogenesis. The endoreplication domains are shown for the embryonic stages between the onset of polyteny and cuticle deposition. The developing gut and salivary gland are shown in grey at the times when these tissues are not observed to replicate. (A) As the germ band begins to retract (stage 11) the salivary gland (green) is the first tissue in which endoreplication is observed; (B) During germ band retraction (stage 12) the anterior and posterior midgut (red) begin to endoreplicate. The small red circles represent the large replicating nuclei seen between the two parts of the developing midgut; (C) During the dorsal closure stage of embryogenesis (stage 13) the hindgut (yellow) endoreplicates. Shortly thereafter replication begins in the Malpighian tubules (purple); (D) At head involution (stage 14) replication persists in the developing midgut, hindgut and the Malpighian tubules. The replication in the midgut proceeds anteriorly in the anterior midgut and posteriorly in the posterior midgut. As the replication ceases in the anterior and posterior midgut, there is a stripe of replication near the middle of the sac-like midgut (blue); (E) The replication seen at the middle of the midgut is coincident with the first constriction of the developing midgut (stage 15). As the constrictions appear, the stripe of labeling observed in the midgut expands anteriorly and posteriorly. Replication is next observed in a group of cells that extend along the posterior part of the dorsal side of the embryo (magenta); (F) As the midgut becomes more convoluted (stage 16) replication is observed to extend through the entire midgut and replication persists in the dorsal cells.
The endo cell cycle and polyteny

Fig. 3. Regulation of the cell cycle during Drosophila embryogenesis. A time line of embryogenesis until cuticle deposition is shown with morphogenetic events indicated by arrows on the top. For the tissues examined in this study the mitotic divisions are shown below the line. Thirteen cleavage divisions occur prior to cellular blastoderm formation; the post-blastoderm divisions are indicated by black boxes. (data of Campos-Ortega and Hartenstein, 1985 and Foe, 1989). Skaer cites the last division of the Malpighian tubules as being slightly later, between 6–10 h (Skaer, 1989). The periods during which endoreplication occurs (as assayed by BrdU incorporation) are shown by hatched boxes for each of the tissues labeled on the right. The length of replication in the midgut is shown as long due to the changing pattern of labeling observed within this tissue (see Fig. 1). Because the origin of the dorsal cells is not known, the post-blastoderm mitoses for this tissue are not illustrated.

Post-blastoderm divisions

endo replication

midgut

hindgut

salivary gland

malpighian tubules

dorsal cells

Developmental fates underwent endoreplication together as a domain (summarized in Fig. 2). The time of onset of polytene DNA replication relative to the last mitotic division for each of these lineages varied (Fig. 3). There were three hours between the last mitotic division in the salivary gland precursor cells and endoreplication, while four and a half hours lapsed in the Malpighian tubules. In the salivary gland and Malpighian tubules it was possible to identify unambiguously all of the cells of the tissue, and the vast majority replicated synchronously (Fig. 1). However, while some of the polytene domains included every cell of a tissue, this was not always the case. For example, a group of cells in the posterior of the hindgut, adjacent to the anal plate, did not replicate with the other hindgut cells during the first period of replication (Fig. 1). The replication domains are reminiscent of the domains characterized for the post-blastoderm mitotic divisions (Foe, 1989). Foe observed that the 14th mitosis occurs in a temporally and spatially regulated pattern in which cells undergoing a similar developmental fate divide together as a domain.

Although we identified the domains of the salivary gland, gut and Malpighian tubules, it is possible that we did not identify cells of other tissues that were replicating individually. In particular it was difficult to identify unambiguously the cells of the fat body since it is spread throughout the embryo. In addition, we have been unable to assign the replicating dorsal cells to a previously defined tissue. Nevertheless, it is clear that a transition to polyteny is made in the latter half of embryogenesis and that this transition is developmentally regulated.

The nature of the endo cell cycle

Having shown that polytene replication began in embryogenesis, we investigated the regulation of its onset and the relationship of polytenization to the mitotic cell cycle. Our results indicated that polyteny results from a modified cell cycle as opposed to continuous DNA replication. Each domain underwent replication for a specific period of time and replication was not observed during other times (Figs 2, 3). Since replication occurred in a discrete period rather than continuously, polyteny appears to be a consequence of an altered cell cycle with an S(synthesis) phase followed by a G(gap) phase, the endo cell cycle.

The BrdU labeling patterns provided a qualitative description of the periods of polytene replication. However we wished to quantify the actual extent of replication during each of these periods to address two aspects of the endo cell cycle. First, we wanted to determine whether the genome is replicated only once during each period of labeling (the presumed S phase of the endo cell cycle). Second, since in the larval stages the salivary gland chromosomes have regions that are underrepresented and the entire genome is not endo-replicated (Rudkin, 1969), we also wanted to test whether there was underreplication of the chromosomes during the first round of polytene DNA synthesis.

To measure the relative DNA content of endoreplicated cells free from interference of surrounding tissues, it was necessary to dissect the tissues from the embryo. The desired tissue was dissected from staged embryos and the cells were dispersed into a monolayer on a slide, and the DNA content was quantified by
Fig. 4. Quantitation of hindgut DNA content. Imaginal discs from third instar larvae and hindgut cells from 13 h embryos were dissected onto slides, flattened to a monolayer, stained with DAPI, and the relative fluorescence was measured on a Zeiss photomicroscope equipped with a Zeiss microdensitometer. The ploidy of the endoreplicated hindgut cells was calculated from the ratio of the mean of this peak to the mean of the 2C peak of the imaginal discs. The data were pooled from an experiment measuring the hind guts of three animals. Data from a second experiment showed comparable replication in the hindgut.

Fig. 5. Quantitation of salivary gland DNA content. Imaginal discs from third instar larvae and salivary glands from 13 h embryos were dissected and the DNA content quantified as in Fig. 4. The data were pooled from an experiment measuring the salivary glands of two animals. Data from two other experiments showed comparable replication in the salivary gland.

(Wilcoxon-Mann-Whitney statistic = 4.22, \( P < 0.001 \)). This would correspond to underreplication of 30% of the genome. DAPI shows a preference in binding A–T rich DNA, and therefore if the unrepresented DNA was heterochromatic satellite DNA, our data may overestimate the percent underreplication. There are differing results as to the extent of the bias of DAPI binding, but even in the most extreme preference reported, our data would still show a 3.6C value for the hindgut cells, a significant underrepresentation of 20% (Lin et al. 1977; Ashburner, 1989; Daxhelet et al. 1989). The 2C peak presumably was composed of regions of the hindgut that were unreplicated, such as cells adjacent to the anal plate (Fig. 1) and imaginal cells. Our results further imply that the endoreplicating hindgut cells had a ploidy of 2C prior to endoreplication and that these cells entered the endo cell cycle from G1.

The quantitation of salivary gland DNA content at 13 h showed two populations of cells; one with a ploidy value of 2C and another with a peak at 6.2C (Fig. 5). The 6.2C peak was made up of cells that definitely had endoreplicated since their DNA content exceeded the 4C value. The 2C cells are most likely the cells of the imaginal ring of the salivary gland because they are located near the anterior tip of the gland in our
squashes (data not shown). In contrast to the hindgut, the salivary gland achieved a ploidy higher than the 4C peak of imaginal disks. This was surprising because in mapping replication domains we only observed one round of endoreplication in the salivary gland. There are three possible explanations for these results: (1) the endoreplication in the salivary gland starts from G2 of the mitotic cell cycle; (2) there is an earlier round of replication before involution of the salivary gland; or (3) the genome replicates twice during the observed period of replication. We favor the first explanation because the precursors of the salivary gland divide only twice after blastoderm formation, and it has been shown that during these two mitotic divisions cells enter S phase immediately after the completion of mitosis without an intervening G1 phase (Campos-Ortega and Hartenstein, 1985; Edgar and O'Farrell, 1990). Thus it is likely the cells of the salivary gland enter the endo cell cycle with a 4C value. However, since we are unable to isolate and quantify the precursor cells for the salivary gland prior to its involution, we cannot rule out the other two alternatives. The 6.2C value observed does not correspond to an integral doubling of the genomic content. The percent underreplication that this represents would depend on whether two rounds of endoreplication occurred or whether the salivary gland cells entered the endo cell cycle from G2.

The regulation of the endo cell cycle
Since our results showed that polyteny in many tissues arises from a cycle of alternating G and S phases and that the transition to the endo cell cycle occurs in embryogenesis, we wanted to determine whether the endo cell cycle is affected by regulators of the diploid, mitotic cell cycle. Several Drosophila genes involved in regulating the entry into mitosis have been identified (Edgar and O’Farrell, 1989; Lehner and O’Farrell, 1989; Jimenez et al. 1990; Lehner and O’Farrell, 1990a; Whitfield et al. 1990). In particular, string has been shown to be a key regulator that drives the post blastoderm divisions (Edgar and O’Farrell, 1989); string is a Drosophila homolog of the Schizosaccharomyces pombe cdc25 gene, a positive regulator of the G2–M transition (Russell and Nurse, 1986; Edgar and O’Farrell, 1989). Even though mitosis is not occurring in the endo cell cycle, genes that trigger the G2–M transition could also drive the endo cell cycle if the mitotic machinery was uncoupled from the cell cycle at a point past the transition step. If string drives the endo cell cycle then we would not expect to observe endoreplication in string mutant embryos.

To test whether string regulates the endo cell cycle we determined whether polytene DNA replication could be observed in string mutant embryos. Staged string embryos were pulse-labeled with BrdU, and the one quarter of the embryos that were string mutants were identified by their altered morphology. The midgut underwent DNA replication at times when endoreplication occurs in wild-type embryos (Fig. 6). To be sure that these replicating cells were not yolk nuclei, we mapped the position of yolk nuclei in string mutants by using an enhancer trap line that labels yolk nuclei (data not shown). Replication was also observed in other tissues that become polytene, such as the hindgut and Malpighian tubules. A group of cells at the position of the salivary gland replicated at the correct developmental time for salivary gland replication. Moreover, the replication observed in string embryos showed a spatial and temporal pattern similar to the pattern in wild-type embryos, and the midgut appeared to go through two rounds of endoreplication prior to cuticle deposition.

Our experiment clearly shows that endoreplication occurs in the absence of string product. This result is consistent with the observations of Edgar and O’Farrell that the string gene is no longer expressed in wild-type embryos when these cells are undergoing an endo cell cycle. Two other genes essential for the G2–M transition, cyclinA and cyclinB, also are not expressed in these tissues as they become polytene (Lehner and O’Farrell, 1989; Lehner and O’Farrell, 1990b; Whitfield et al. 1990).

An additional conclusion arose from our analysis of endoreplication in string mutant embryos. Although the 14th through 16th mitotic divisions are not completed in string mutants, we still observed the onset of the endo cell cycle in the gut at the appropriate stage of development. Thus the completion of all 16 mitotic

Fig. 6. BrdU labeling of a string mutant embryo. Replicating cells can be seen in the gut of this homozygous stg8653 mutant embryo at the same developmental time at which the midgut replicates in string mutant embryos. Magnification ×330.
divisions is not a requirement for the establishment of the endo cell cycle, and it is not necessary to titrate out mitotic factors to enable the transition to endoreplication to occur.

The endo cell cycle in late embryonic and early larval development

We wanted to determine the length of the G phase of the endo cell cycle, and to derive this information we needed to know when subsequent rounds of replication occurred in the endoreplicating tissues. In addition, the full characterization of subsequent rounds of replication would reveal if endoreplication persisted in domains. The only tissue in which we saw a second period of replication prior to cuticle deposition was the midgut, in which some cells reinitiated replication by 12.5 h of embryogenesis. For the specific set of midgut cells that began a second round of endoreplication the G phase was 3 h, and this second round initiated within a specific subdomain of the midgut and then spread throughout the midgut.

To analyze the next round of endoreplication in additional tissues necessitated examining embryos after cuticle deposition. We took two approaches to overcome the problem of limited access of the antibody: (1) we dissected out tissues from BrdU-labeled animals and performed the antibody incubation on the isolated tissue; and (2) we opened the cuticle at multiple sites in whole animals to allow antibody entry. To test that any variation in BrdU labeling was not due to differential accessibility to the antibody, dissected first instar larval tissues were labeled with an anti-histone antibody. All the cells within the tissues were observed to stain with this control antibody (data not shown).

We did not observe another period of replication in the salivary gland during embryogenesis in either dissected tissue or in whole-mount animals (Fig. 7). This was confirmed by quantification of the DNA content of the salivary gland at hatching; the DNA content was identical to that of salivary glands from 15 h embryos. However, the fat body cells next to the salivary gland did endoreplicate during late embryogenesis (Fig. 7). The salivary gland replicated again in the first instar larva, 7 h after hatching. Thus there was a gap phase in the salivary gland of 18 h, a gap much longer than that observed for cells in the midgut. Therefore, the endo cell cycle differs from tissue to tissue in the length of the G phase.

Endoreplication continued in developmentally regulated domains in the gut after cuticle deposition in the embryo and during the first larval instar, but these domains were smaller subdivisions of the first endo cell cycle domains. Examples of such subdomains in the midgut are shown in Fig. 8. The hindgut endoreplicated at all times of first instar development that we examined; the subdomains consisted of anterior-posterior stripes of cells (Fig. 8A). In addition to these subdomains, isolated cells replicated in the midgut, hindgut and gastric caeca (Fig. 8A,B). In some tissues, most notably the gastric caeca, isolated cells were seen to replicate prior to larger numbers of cells replicating in domains. The most extreme difference from the early embryonic replication domains occurred in the cells of the Malpighian tubules, where single cells replicated independently both in late embryogenesis and the first instar larval stage (Fig. 8A,B, and data not shown). Later in larval development replication was observed in the gastric caeca as a domain, as well as in other subdomains of the midgut (Fig. 8C). The subdomains observed in these later developmental stages comprised the majority of cells within a region, but not always all of the cells. Subdivision of domains as tissues differentiate has been observed for the post-blastoderm mitotic domains as well (Foe, 1989).

The striking difference between the early endoreplication domains in the embryo and those in the larva was that the larval domains did not exhibit the same degree...
Fig. 8. Endoreplication in larval tissues. (A) This dissected gut tract from a 3.5 h old larva shows subdomains of replication in the midgut (mg). Isolated replicating cells are seen in the gastric caeca (gc), hindgut (hg), and Malpighian tubules (mt); (B) The posterior portion of the gut tract from a 7.5 h larva shows extensive replication in the hindgut and Malpighian tubules, as well as subdomains of replication in the midgut; (C) The anterior midgut of an 8.5 h larva shows replication in subdomains of the midgut and extensive replication in the gastric caeca. The salivary gland (sg) and adherent fat body (fb) of the same animal are undergoing endoreplication. Magnification ×95.

of rigid developmental control. Therefore, while we were able to draw general conclusions about the later replication domains, we were unable to characterize the developmental pattern in fine detail. For example, although for all times examined subdomains of the larval midgut underwent replication, the exact number of labeled regions and the relative positioning varied from animal to animal. Although there was variability between animals, we were able to detect developmental regulation of the onset of S phase in different tissues. Except for isolated cells, no subdomains of replication were seen in the anterior portion of the midgut (the ventriculus) or in the gastric caeca before the 5th hour post hatching. Cells at the base of the Malpighian tubules were not observed to replicate until the 5th hour after hatching. The portion of the hindgut where the
Malpighian tubules attach was not observed to replicate until the 7th hour after hatching, while the salivary gland initiated replication as a domain at approximately the same time. After this time the amount of replication in these areas was observed to increase and reach a maximum 9h after hatching. The variability between animals that we observed may result partially from the difficulty in staging development after cuticle formation due to the absence of morphological criteria to ascertain independently developmental age. However, we think this is not the sole explanation for the variance because the asynchrony is observed so soon after the unambiguous developmental landmark of hatching. Environmental factors could well influence replication; such effects have been documented for salivary gland replication in the later instars (Rudkin, 1973).

**Discussion**

The process of polytenization plays a significant role in *Drosophila* development. Previous work had established that most larval tissues are polytene and indicated that polytene DNA replication in the salivary gland was cyclic. Our results show that the cells that will constitute many polytene larval tissues begin becoming polytene midway through embryogenesis. Polytene DNA replication occurs in domains that are spatially and temporally regulated. Moreover, the polytene replication was cyclic in all of the tissues that we observed. Thus the transition to polytene involves a change from a diploid cell cycle with a G1, S, G2 and M phase to an endo cell cycle, in which the S phase of DNA synthesis alternates with a G phase.

The developmental specificity of the transition to polyteny was very striking. The endo cell cycle initiates in domains that are regulated developmentally in a dynamic pattern, and after the initial rounds of endoreplication the domains are subdivided. Although we cannot be certain that the subdomains observed after cuticle deposition correspond to regions of specific function, it seems likely that they reflect further differentiation of these tissues. For example, one subdomain that we observed in the midgut is a band of cells at which the first constriction of the gut appears. The tissue specificity and temporal regulation of these replication domains raises the question of whether endoreplication is a prerequisite for or a consequence of cell differentiation. While our data do not address this directly, we argue for the latter, based on recent data on the post-blastoderm mitotic domains. The three post-blastoderm mitotic divisions occur in domains in which cells that are differentiating into the same tissues undergo mitosis together as a domain (Foe, 1989). When the *string* gene is placed under the control of the *hsp70* promoter and induced by heat shock, the mitotic domains are overridden and mitosis is induced synchronously throughout the embryo. In this situation, the embryo nevertheless develops normally, indicating that the mitotic domains reflect the developmental fate of cells rather than directing it (Edgar and O'Farrell, 1990).

We showed that during the initial S phases of the endo cell cycle in the hindgut and salivary gland the genome is not fully duplicated. It had been demonstrated previously that during the larval stages, the salivary gland DNA is not fully represented; under-representation also occurs in several other polyplaid or polytene tissues (for review see Spradling and Orr-Weaver, 1987). If the underrepresented DNA is due to underreplication, it most likely results from the differential activation of replication origins in particular genomic regions, in the case of the salivary gland, in the centric heterochromatin and euchromatin. The mechanism by which these replication origins are shut off with the first cycles of endoreplication remains to be elucidated. However, it has been proposed recently that the underrepresented DNA in polytene tissues has been somatically eliminated rather than differentially replicated (Karpen and Spradling, 1990). If so, our results show that this elimination would have to occur during a brief interval in embryogenesis. In our experiments, we directly measured the extent of DNA representation after the first round of endoreplication. In experiments in *Drosophila nasuoides* Zacharias measured DNA content at multiple points during larval development and by quantifying the rate of increase was able to extrapolate that the hindgut began underreplication in the first endo cell cycle, the salivary gland in the second, and the Malpighian tubules in the first or the second endo cell cycle (Zacharias, 1986).

Polyteny arises from a cell cycle with solely an S and a G phase. Is this endo cell cycle actively regulated, or is it a default consequence of failure to complete mitosis properly? A precedent for the latter idea is the observation that a mutant with defective mitotic spindles in *Drosophila* does not arrest the cell cycle and produces polyploid cells (Ripoll *et al.* 1985). The precise developmental regulation of the endo cell cycle argues for its active regulation. Moreover, the observation that endoreplication occurs with proper developmental timing in *string* mutants shows that the endo cell cycle is independent of G2–M regulation and indicates that it cannot be solely a consequence of defective mitosis or a cytoskeletal alteration. Certainly cytoskeletal changes must accompany the transition to the endo cell cycle, but we assert that these changes are downstream from the regulatory steps as opposed to triggering the events. A predicted key cytoskeletal alteration would be dissociation of the centrosomes from the nuclear envelope. The centrosomes have been well characterized in the polyploid follicle cells of the *Drosophila* adult, and they have been observed to disappear, but not until several rounds of endoreplication have occurred. However, several changes in the morphology of the centrosomes are coincident with the onset of the endo cell cycle, including a closer association with the plasma membrane than with the nuclear envelope, and a randomization of the orientation of the two centrioles within each follicle cell (Mahowald *et al.* 1979).

It is possible that the endo cell cycle may be controlled by critical regulatory steps of the mitotic cell cycle. The two key regulatory points of the mitotic cell
cycle are the G1–S and G2–M transitions. The genes that trigger either of these cell cycle decision points could be responsible for the cyclic nature of polytene DNA replication, provided mitosis was subsequently uncoupled from S phase. A key regulator of both the G1–S and G2–M transitions is the kinase encoded by the cdc2 gene, initially identified in *S. pombe* (Nurse and Bissett, 1981; Simanis and Nurse, 1986). The specificity is conferred by G2–M specific and G1–S specific cyclins (Hagan et al. 1988; Richardson et al. 1989; Wittenberg et al. 1990). Our results show directly that the key regulator of the G2–M transition in *Drosophila*, the product of the *string* gene and an activator of cdc2, is not involved in the regulation of the endo cell cycle. Moreover, both of the G2 cyclins, *cyclin A* and *cyclin B*, are not expressed in cells undergoing polyteny, and it is therefore unlikely that they play a regulatory role in this cycle (Lehner and O’Farrell, 1990b; Whitfield et al. 1990). Recent evidence suggests that cdc2 is also not involved in the endo cell cycle. A functional homologue of cdc2 that is able to complement the *S. pombe* and *S. cerevisiae* mutations has been cloned from *Drosophila* (Jimenez et al. 1990; Lehner and O’Farrell, 1990a). There is an additional gene in the *Drosophila* genome that has 53% homology to cdc2 (Lehner and O’Farrell, 1990a). Interestingly, it has been reported that neither of these cdc2 homologues is transcribed during embryogenesis in polytene cells (Jimenez et al. 1990; Lehner and O’Farrell, 1990a).

Two alternatives remain. A novel set of regulatory genes unique to the endo cell cycle may exist, or additional, as yet unidentified, regulators of the mitotic cell cycle may act on both this cell cycle and the endo cell cycle. The phenotype of *string* mutant embryos, arrested at mitosis of the first post-blastoderm division, indicates strongly that it is the limiting trigger responsible for entry into mitosis in *Drosophila* (Edgar and O’Farrell, 1989). The genetics of the G1–S transition has not been investigated in *Drosophila*, so there exists the possibility of regulators for this transition in addition to cdc2 and the G1–S cyclins. In either case, the isolation of mutations affecting the endo cell cycle will be critical to elucidating its regulation. Our description of the developmental timing of the onset of the endo cell cycle will be useful in screening for such mutations.

In addition to the overall change in the cell cycle during polytenization, feedback controls that serve as checkpoints in the diploid cell cycle must be altered. In particular, the controls that ensure that another round of DNA replication is not initiated prior to mitosis must be circumvented in the endo cell cycle. Blow and Laskey have shown that nuclear envelope permeabilization is sufficient to permit reinitiation of DNA replication in a cell-free system in *Xenopus* (Blow and Laskey, 1988). Thus one can hypothesize that the normal block to reinitiation could be overcome in the endo cell cycle by cyclic alterations in the nuclear envelope. It is interesting that the pore complexes of the nuclear envelope of yolk nuclei go through cyclic changes synchronously with the blastoderm nuclei, even when the yolk nuclei are becoming polyploid (Staafstrom and Staehelin, 1984). Clearly further investigation of the structure of the nuclear envelope during the endo cell cycle is warranted.

While the employment of polyteny as a developmental strategy is most pronounced in the Diptera, many organisms have polytene or polyploid cells. It is likely that the novel cell cycle that we have characterized in *Drosophila* also functions in these other organisms. The insights gained from characterization of the endo cell cycle that leads to polyteny will provide a framework for understanding the cell cycles that regulate polyploidy. Moreover, the identification of regulators for the endo cell cycle in *Drosophila* will provide candidates for the regulation of polyteny and polyploidy in other organisms.

We thank Mike Bate, Volker Hartenstein, Jose Campos-Ortega and Norbert Perrimon for many stimulating discussions and for instruction on embryo dissection and anatomy. Annette Coleman provided invaluable assistance in microdensitometry and access to her equipment. We acknowledge the advice of Rolf Bodmer on embryo permeabilization and BrdU labeling. We are grateful to Walter Olbricht for an illuminating discussion on statistics. We thank Bruce Edgar and Pat O’Farrell for communication of results prior to publication and Allan Spradling, Ruth Lehmann, Bob Horvitz, Sima Misra and members of this laboratory for helpful comments on the manuscript. A.S. was supported by NIH predoctoral training grant GM07287 and by a grant from the Lucille P. Markey Charitable Trust. T.O-W. was supported by NIH grant GM39341, the Searle Scholars Program/The Chicago Community Trust, and by a grant from the Lucille P. Markey Charitable Trust.

### References


