Conservation of Epigenetic Regulation, ORC Binding and Developmental Timing of DNA Replication Origins in the Genus *Drosophila*

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ABSTRACT

There is much interest in how DNA replication origins are regulated so that the genome is completely duplicated each cell division cycle and in how the division of cells is spatially and temporally integrated with development. In the *Drosophila melanogaster* ovary, the cell cycle of somatic follicle cells is modified at precise times in oogenesis. Follicle cells first proliferate via a canonical mitotic division cycle and then enter an endocycle, resulting in their polyploidization. They subsequently enter a specialized amplification phase during which only a few, select origins repeatedly initiate DNA replication, resulting in gene copy number increases at several loci important for eggshell synthesis. Here we investigate the importance of these modified cell cycles for oogenesis by determining whether they have been conserved in evolution. We find that their developmental timing has been strictly conserved among *Drosophila* species that have been separate for ~40 million years of evolution and provide evidence that additional gene loci may be amplified in some species. Further, we find that the acetylation of nucleosomes and Orc2 protein binding at active amplification origins is conserved. Conservation of DNA subsequences within amplification origins from the 12 recently sequenced *Drosophila* species genomes implicates members of a Myb protein complex in recruiting acetylases to the origin. Our findings suggest that conserved developmental mechanisms integrate egg chamber morphogenesis with cell cycle modifications and the epigenetic regulation of origins.

THE genome must be completely and accurately duplicated in each cell division cycle to ensure that daughter cells inherit a euploid gene complement. To accomplish this, DNA replication initiates from numerous origins whose activity is regulated during the cell cycle. The division of cells must also be spatially and temporally integrated with developmental processes to ensure the proper size, patterning, and functioning of organs. In some cases, developmental integration results in a change in replication origin usage or in a fundamentally modified cell division cycle. For example, in a variety of organisms, including humans, certain differentiating cells become polyploid by entering an endocycle, which is comprised of alternating G and S phases without mitosis (EDGAR and ORR-WEAVER 2001 for review).

Oogenesis in *Drosophila melanogaster* is a model genetic system for understanding how cell cycle programs and origin regulation are modified in coordination with development (SPRADLING 1993; LILLY and DURONIO 2005; SWANHART *et al.* 2005 for reviews). The cell cycles of both germline and somatic cells are precisely coordinated with the maturation of the egg chamber as it migrates down an ovariole (Figure 1, A and B). Both the

germline and somatic cell precursors originate from stem cells in the germarium at the anterior tip of the ovariole and initially divide by a mitotic cell cycle (LIN and SPRADLING 1993; MARGOLIS and SPRADLING 1995). Germline cells divide synchronously four times with incomplete cytokinesis resulting in 16 interconnected cells. One of these cells becomes an oocyte and enters meiosis, while its 15 sister cells adopt the nurse cell fate and enter an endocycle (DE CUEVAS *et al.* 1997; HUYNH and ST JOHNSTON 2004 for reviews). Through repeated endocycles, nurse cells ultimately become highly polyploid by the end of oogenesis (PAINTER and REINDORP 1939; LILLY and SPRADLING 1996; DEJ and SPRADLING 1999). Somatic follicle cells proliferate and then surround the 16-cell germline cyst as it buds off from the germarium, forming a stage 1 egg chamber. While nurse cells have entered the endocycle by this point, the follicle cells continue to mitotically proliferate until stage 6, after which they too enter an endocycle (Figure 1B) (MAHOWALD *et al.* 1979). The developmental timing of this follicle cell mitotic-to-endocycle transition at stage 6 is coordinated by the Notch signaling pathway (DENG *et al.* 2001; LOPEZ-SCHIER and ST JOHNSTON 2001; SHCHERBATA *et al.* 2004). Follicle cells then complete three endocycles from stage 7 to stage 10A, achieving a final ploidy value of 16C (LILLY and SPRADLING 1996; CALVI *et al.* 1998).

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FIGURE 1.—Follicle cell cycles in the *D. melanogaster* ovary. (A) A longitudinal section through a stage 10 egg chamber. Somatic follicle cells (red) form an epithelial sheet around the nurse cells and oocyte. At this stage most follicle cells have migrated to posterior positions around the oocyte (to the right), but a few remain surrounding the nurse cells. Anterior is to the left. (B) Follicle cell cycle modifications during oogenesis. A typical single ovariole is shown with stages of oogenesis denoted below and follicle cell cycles above (KING 1970; CALVI *et al.* 1998). Egg chambers emerge from the germarium and move down the ovariole (to the right) as they mature. During egg chamber maturation, follicle cells mitotically proliferate until stage 6, endocycle from stage 7 to stage 10A, and then enter a synchronized amplification phase in stage 10B.

Beginning precisely in stage 10B, all follicle cells surrounding the oocyte enter a specialized cell cycle phase during which only a few, select origins repeatedly initiate replication in the absence of other genomic replication (Figure 1B) (CALVI *et al.* 1998; CLAYCOMB and ORR-WEAVER 2005; CALVI 2006). This “amplification phase” results in a local increase in the DNA copy number of genes required for rapid eggshell synthesis, with the two most highly amplified loci being those on the X and third chromosomes that encode structural proteins of the eggshell (chorion), while two other loci at cytogenetic positions 30B and 62D amplify to a lesser extent (SPRADLING 1981; CLAYCOMB *et al.* 2004). Replication from these amplification origins can be seen as distinct nuclear foci of different intensities after labeling with BrdU (CALVI *et al.* 1998). This highly selective site-specific replication represents an extreme form of origin developmental specificity, but it is currently unknown what coordinates this amplification phase with the development of the egg chamber.

Genetic and molecular analyses have indicated that the regulation of amplification origins resembles that of other origins in that they are controlled by cell cycle kinases and are licensed by the binding of a pre-replicative complex (pre-RC) (LANDIS *et al.* 1997; CALVI *et al.* 1998; ASANO and WHARTON 1999; AUSTIN *et al.* 1999; LANDIS and TOWER 1999; ROYZMAN *et al.* 1999; LOEBEL *et al.* 2000; YAMAMOTO *et al.* 2000; SCHWED *et al.* 2002). For many of these proteins, their specific localization to amplification origins and forks can be visualized by immunofluorescent microscopy, for example,

the pre-RC protein Orc2 (ROYZMAN *et al.* 1999). The analysis of amplification has also led to the discovery of new proteins and mechanisms that control origins. For example, a large complex called Myb–MuvB binds to the amplification origins and is essential for their activity. This complex contains the fly orthologs of the human Myb proto-oncogene, Rb tumor-suppressor proteins, and other proteins (BOSCO *et al.* 2001; BEALL *et al.* 2002, 2004; KORENIAK *et al.* 2004; LEWIS *et al.* 2004).

Analysis of amplification origins, and other origins, have identified *cis* sequences important for origin function, but a DNA consensus sequence for origins of DNA replication in multicellular eukaryotes has not been identified (ALADJEM *et al.* 2006 for review). Emerging evidence from a variety of organisms suggests that epigenetic modification plays a major role in origin activity. The modification of chromatin can alter the time when origins initiate replication during S phase and which origins initiate replication in different cells in development (MECHALI 2001; VOGELAUER *et al.* 2002; LIN *et al.* 2003; DANIS *et al.* 2004). In *D. melanogaster*, the nucleosomes at active amplification origins are hyper-acetylated, suggesting that epigenetic regulation contributes to the differential activity of origins in stage 10B follicle cells (AGGARWAL and CALVI 2004; HARTL *et al.* 2007). It is not known, however, which histone acetyl transferase enzyme is responsible for this acetylation, nor how this enzyme is recruited specifically to the amplification origins.

It has been previously shown that follicle cells become polyploid and chorion genes amplify in ovaries of several *Drosophila* species and the Mediterranean fruit fly *Ceratitis capitata* (MARTINEZ-CRUZADO *et al.* 1988; SWIMMER *et al.* 1990; VLACHOU *et al.* 1997; VLACHOU and KOMITOPOULOU 2001). Here we evaluate the importance of cell cycle developmental timing and the epigenetic regulation of origins by determining whether these processes have been conserved in evolution. We find that the developmental timing, epigenetic regulation, and Orc2 protein binding have been strictly conserved over 40 million years of evolution. We also present evidence that additional loci may be amplified in some species. Conservation of DNA subsequences in the chorion origin among 12 *Drosophila* species suggests that a Myb complex may recruit acetylases to the origin. Our evidence suggests that the timing of endocycles and amplification are important for oogenesis and that epigenetic regulation is a conserved mechanism that contributes to the developmental specificity of amplification origins.

MATERIALS AND METHODS

Fly husbandry: Fly strains were obtained from the Tucson *Drosophila* Stock Center and were raised on standard cornmeal molasses media except for *D. persimilis*, *D. pseudoobscura*, *D. mojavensis*, and *D. guttifera*, which were raised on Banana–Opuntia media, and *D. grimshawi*, which was raised on

TABLE 1
Summary of species analyzed and results for follicle cells

Species analyzed (abbreviation) ^a	Mitotic cycles	Endocycles	BrdU foci stage 10B: no. of large:medium:small ^b	Acetylated histone foci stage 10B	Orc2 foci stage 10B
<i>D. melanogaster</i> (<i>Dmel</i>)	Gerarium stage 6	Stages 7–10A	1:1:2	+	+
<i>D. simulans</i> (<i>Dsim</i>)	Gerarium stage 6	Stages 7–10A	1:1–2:4–6	+	+
<i>D. sechellia</i> (<i>Dsec</i>)	ND	Up to stage 10A	+	ND	+
<i>D. yakuba</i> (<i>Dyak</i>)	ND	Up to stage 10A	+	ND	ND
<i>D. erecta</i> (<i>Dere</i>)	ND	Up to stage 10A	+	ND	ND
<i>D. ananassae</i> (<i>Dana</i>)	ND	Up to stage 10A	+	ND	ND
<i>D. pseudoobscura</i> (<i>Dpse</i>)	ND	Up to stage 10A	+	ND	ND
<i>D. persimilis</i> (<i>Dper</i>)	ND	Up to stage 10A	+	ND	ND
<i>D. guttifera</i> (<i>Dgut</i>)	Gerarium stage 6	Stages 7–10A	1:1:3–8	+	ND
<i>D. willistoni</i> (<i>Dwil</i>)	ND	Up to stage 10A	1:2–3:3–4	ND	ND
<i>D. mojavensis</i> (<i>Dmoj</i>)	Gerarium stage 6	Stages 7–10A	1:1:2–3	+	+
<i>D. hydei</i> (<i>Dhyd</i>)	Gerarium stage 6	Stages 7–10A	1:1:2–4	+	ND
<i>D. virilis</i> (<i>Dvir</i>)	Gerarium stage 6	Stages 7–10A	1:1:4–6	+	ND
<i>D. grimshawi</i> (<i>Dgri</i>)	ND	To stage 10A	+	ND	ND

ND, not determined.

^a Abbreviations as recommended by the AAA genome consortium.

^b A plus indicates that foci were observed but not counted.

Wheeler–Clayton media (see <http://stockcenter.arl.arizona.edu> for strains, recipes, and advice on rearing).

Ovary antibody labeling and analysis: Dissection of ovaries, antibody labeling, and confocal analysis were as previously described (CALVI *et al.* 1998; AGGARWAL and CALVI 2004; CALVI and LILLY 2004). Antibodies and dilutions used were rabbit antiphospho-histone H3 1:500 (Upstate), mouse monoclonal anti-BrdU 1:20 (Becton Dickinson), rabbit polyclonal anti-acetylated histone H3 1:200 (Upstate), rabbit polyclonal anti-acetylated lysine 8 histone H4 1:200 (Upstate), and rabbit polyclonal anti-Orc2 antibody 1:200 (provided by M. Botchan).

Sequence analysis: Sequence analysis of the third chorion origin began by identifying the orthologs of *D. melanogaster* chorion protein genes in other species by tblastn of genomic scaffolds from the Consortium for Assembly, Alignment, and Annotation (AAA) of 12 *Drosophila* genomes (<http://rana.lbl.gov/drosophila/>) using the FlyBase species blast server (<http://flybase.bio.indiana.edu/blast/>). Genomic sequence surrounding the *cp18* orthologs was then downloaded from the Gbrowse web server (<http://flybase.bio.indiana.edu/cgi-bin/gbrowse/dmel/>), and subregions were aligned using ClustalW. Regions conserved among chorion loci in *Drosophila* species were also identified by a blastn search of genomic scaffolds.

RESULTS

The developmental timing of endocycle entry is conserved in *Drosophila* species: In *D. melanogaster*, nurse cells complete mitotic divisions and enter the endocycle by stage 1, whereas follicle cells enter an endocycle after stage 6. To evaluate the importance of this timing, we asked whether it was conserved in the ovaries from other *Drosophila* species (see Table 1 for species analyzed). These species represent several phylogenetic subgroups, including 11 other species for which whole-genome sequence was recently determined, with the most distant sequenced species being *D. mojavensis*

and *D. virilis*, which diverged from *D. melanogaster* at least 40 million years ago. The morphological changes associated with specific stages of egg chamber maturation are similar among these *Drosophila* species, permitting us to directly compare the timing of modified cell cycles in oogenesis (KING 1970; BUNING 1994).

Earlier investigations that employed histological DNA stains and radioactive nucleotide labeling have shown that follicle cells become polyploid in a number of insect species (MAHOWALD *et al.* 1979; BUNING 1994 for review). We evaluated the timing of mitotic cycle cessation and endocycle entry with high resolution using modern fluorescent probes and confocal microscopy. Ovaries were incubated with anti-phospho-histone antibody (PH3), which labels condensed chromosomes in mitosis (HENDZEL *et al.* 1997). In all species tested, mitotic divisions of germline cells were restricted to the gerarium (Figure 2 and data not shown). Somatic follicle cells in mitosis labeled with anti-PH3 in early stage egg chambers up to approximately stage 6, but not thereafter (Figure 2 and Table 1). Rarely, however, a single mitotic follicle cell was detected in the posterior of stage 7 egg chambers (Figure 2, B and E). These results indicated that, as in *D. melanogaster*, most follicle cells complete mitotic divisions before stage 7.

To determine if follicle cells continue to cycle after mitotic divisions cease, we incubated ovaries in BrdU, which detects cells in S phase (CALVI and LILLY 2004). Unlike anti-PH3, this resulted in mosaic labeling of nurse cells and follicle cells in both early and late stage egg chambers, suggesting that after completion of mitotic cycles in stage 6 these cells continue to periodically duplicate their genome during endocycles (Figure 3, A and C, and data not shown). Consistent with repeated

FIGURE 2.—The developmental timing of the follicle cell mitotic to endocycle transition is conserved. PH3 labeling of ovarioles indicates that germine mitosis is restricted to the germarium while follicle cell mitosis continues until approximately stage 6 (bright green cells). Rarely, a follicle cell in the posterior of the egg chamber was observed in stage 7 (arrowheads in B and E). (A) *D. melanogaster*. (B) *D. simulans*. (C) *D. guttifer*. (D) *D. mojavensis*. (E) *D. hydei*. (F) *D. virilis*. Images are projections of overlapping confocal sections through the entire ovariole. Bar, 50 μ m for all images.

endocycles, the nuclear volume and DNA fluorescence of both germine and follicle cells increased during these postmitotic stages (Figure 3, A and C, and data not shown). These results indicate that the developmental timing of the mitotic-to-endocycle transition is evolutionarily conserved in both germine and somatic cells.

The timing of endocycle completion is conserved: We next asked whether the developmental timing of endocycle exit is conserved in follicle cells. In *D. melanogaster*, follicle cells undergo three endocycles from stage 7 to stage 10A of oogenesis. Because endocycles are not synchronized among follicle cells in an egg chamber, follicle cells complete their third and final endocycle and arrest at different times during stage 9 to stage 10A. During this time, most follicle cells migrate to the posterior around the oocyte, while only a few remain in the anterior surrounding the nurse cells (Figure 3, A and B). In all other species analyzed, BrdU incorporated into S-phase follicle cell nuclei up to stage 10A of oogenesis (Figure 3C and data not shown). Incorporation was mosaic among follicle cells in an egg chamber, and progressively fewer cells labeled with BrdU from stage 9 to late stage 10A, indicating that follicle cell

endocycles are not synchronized with one another and arrest at different developmental times. Despite different times of endocycle completion among cells, a nuclear flow-sorting analysis indicates that, like *D. melanogaster*, most follicle cells in these other species achieve a final DNA content of 16C (G. Bosco, personal communication; Bosco *et al.* 2007). These results reveal that the developmental timing of endocycles and endocycle exit is conserved among *Drosophila* species.

Conserved developmental timing and cell cycle synchronization of the amplification phase: Subsequent to endocycles in *D. melanogaster*, the amplification of select loci can be detected by BrdU incorporation, which appears as four distinct foci of different intensities in follicle cell nuclei beginning in stage 10B of oogenesis (Figures 3B and 4A) (CALVI *et al.* 1998). Because the homologous polytene chromosomes are paired in follicle cells, each amplified locus is represented by a single focus of BrdU; the large- and medium-size focus corresponding to the highly amplified chorion genes on the third and X chromosome, respectively, while the two smaller foci represent the amplicons at cytogenetic positions 30B and 62D, which amplify to lower levels (Figure

FIGURE 3.—The timing of endocycle exit and synchronous amplification onset is conserved. Ovaries from *D. melanogaster* (A and B) and *D. mojavensis* (C and D) labeled with BrdU (red) and DNA stain TOTO-3 (blue). (A and C) BrdU incorporation during genomic replication indicates that mitotic cycles and endocycles are not synchronized within an egg chamber. (A) *D. melanogaster*, stages 8 and 9/10A. (C) *D. mojavensis*, germarium to stage 10A. (B and D) In stage 10B, BrdU foci appear simultaneously in the nuclei of all follicle cells around the oocyte, but not of the few follicle cells that surround the nurse cells (arrowheads). Bar, 50 μ m for all images.

FIGURE 4.—The number of amplification foci differs among species. BrdU incorporation (red) into stage 10B follicle cell nuclei (TOTO-3 blue) of the indicated species. (A) *D. melanogaster*. (B) *D. simulans*. (C) *D. guttifera*. (D) *D. mojavensis*. (E) *D. hydei*. (F) *D. willistoni* (see also Table 1). Bar, 10 μ m for all images.

4A) (CALVI *et al.* 1998; CLAYCOMB *et al.* 2004). Although the mitotic cycles and endocycles are not synchronized among follicle cells in an egg chamber, the onset of the amplification phase occurs synchronously, which is revealed by the simultaneous appearance of focal BrdU incorporation in all follicle cells around the oocyte in stage 10B (CALVI *et al.* 1998).

We detected several nuclear BrdU foci in post-endocycle follicle cells in all other species analyzed, including the distant Hawaiian species *D. grimshawi* (Figure 3, B and D, Table 1, and data not shown). The developmental timing and cell cycle synchronization of amplification was conserved, with BrdU foci appearing simultaneously in follicle cells in stage 10B (Figure 3, B and D, and data not shown). The spatial patterning of amplification also resembled *D. melanogaster* in that BrdU foci appeared in the follicle cells around the oocyte, but not in the anterior follicle cells that surround the nurse cells (Figure 3, B and D). These results suggest that a conserved mechanism coordinates the developmental timing, patterning, and cell cycle regulation of a specialized amplification phase.

A high-magnification analysis of the amplification foci revealed a further similarity with *D. melanogaster* in that they were of different sizes, typically one large, one medium, and two or more small foci (Figure 4, Table 1, and data not shown). It was previously shown that the orthologs of the *D. melanogaster* chorion genes on the X and third chromosome amplify in *D. subobscura*, *D. virilis*, and *D. grimshawi*. Therefore, the large- and medium-size foci seen in these and other species likely correspond to the highly amplified orthologs of the *D. melanogaster* chorion genes. The appearance of smaller foci suggests that orthologs of *D. melanogaster* genes at 30B and 62D may also amplify in these species (Figure 4, B–F, and Table 1). In some species, a greater number of medium- to small-size foci were seen (Table 1). The most extreme example was *D. willistoni*, which had up to eight foci ranging from large to small sizes (Figure 4F). This suggests that additional, unknown gene loci may amplify in some species.

Acetylation of origin nucleosomes and Orc2 localization is conserved: The nucleosomes at active amplification origins in *D. melanogaster* are hyper-acetylated relative to other genomic regions, which in part explains why they are active while other origins in the same nucleus are not (AGGARWAL and CALVI 2004; HARTL *et al.* 2007). To determine if this epigenetic regulation of amplification origins is conserved, we labeled ovaries from the other species with antibodies raised against poly-acetylated histone H3 (anti-AcH3) and histone H4 acetylated on lysine 8 (anti-H4K8). In all species, these antibodies labeled both germline and somatic cell nuclei in all stages of oogenesis. Beginning in stage 10B, however, one or more prominent foci could be seen in the nuclei of follicle cells surrounding the oocyte, but not in the follicle cells associated with the nurse cells (Figure 5, A–F, and data not shown). Reminiscent of the BrdU foci, the sizes of these acetylation foci differed, although the efficacy of labeling and the number of foci detected differed among samples and species. The spatial and temporal restriction of acetylation foci to follicle cells around the oocyte beginning in stage 10B suggests that they represent amplifying loci.

Acetylation foci in *D. melanogaster* have a bar shape because hyper-acetylation is restricted to nucleosomes near the origin, and fluorescent labeling detects only a narrow cross section through the aligned amplified DNA fibers (Figure 5A inset) (CALVI and SPRADLING 2001; AGGARWAL and CALVI 2004). This is distinct from the focal morphology after labeling for BrdU or fork proteins, which appear as two bars representing replication forks emanating bidirectionally from the origin (LOEBEL *et al.* 2000; CALVI and SPRADLING 2001; CLAYCOMB *et al.* 2002; AGGARWAL and CALVI 2004). In all other species, acetylation foci often had a single bar morphology, (Figure 5F inset). Double labeling for AcH4K8 and BrdU confirmed that acetylation foci corresponded to the amplicons (Figure 6A and data not

FIGURE 5.—Amplification origins are acetylated in other species. Ovaries labeled with antibodies against histone H4 antibody acetylated on lysine 8 (anti-H4K8 green) had one or more prominent foci that were restricted to follicle cell nuclei around the oocyte in stage 10B to stage 12. (A) *D. melanogaster*. (B) *D. simulans*. (C) *D. guttifera*. (D) *D. mojavensis*. (E) *D. hydei*. (F) *D. virilis*. Insets in A and F show an enlarged image of a single AcH4K8 focus with bar-shape morphology in *D. melanogaster* (A) and *D. virilis* (F). Bar, 10 μ m for all images.

shown). This also showed that the single acetylation bar was centrally located between the double bars of replication forks, similar to the pattern seen in *D. melanogaster*, suggesting hyper-acetylation occurs on origin proximal nucleosomes (Figure 6A and data not shown) (AGGARWAL and CALVI 2004; HARTL *et al.* 2007).

In *D. melanogaster*, coincident with hyper-acetylation in stage 10B-11, proteins of the origin recognition complex (ORC), and other pre-RC proteins, preferentially bind amplification origins, which can be seen as foci after antibody labeling for these proteins. To determine if ORC is preferentially localized to amplification origins in other species, we labeled ovaries of *D. simulans*, *D. sechellia*, and *D. mojavensis* with antibody raised against *D. melanogaster* Orc2 (provided by M. Botchan). This antibody labeled bar-shaped foci in stage 10B follicle cell nuclei in all three species analyzed (Figure 6B and data not shown). Together, our results suggest that epigenetic regulation and preferential ORC binding to

FIGURE 6.—Acetylation and Orc2 localization at active amplicons. (A) Double label with BrdU (red) and AcH4K8 (green) in *D. hydei* indicates that acetylation is located at the center of the actively replicating amplicon between the bidirectional forks labeled with BrdU, suggesting that it represents origin proximal nucleosomes. (B) Orc2 antibody labeling (green) in *D. simulans* detects bar-shaped foci, suggesting that ORC is enriched at the amplification origin, as it is in *D. melanogaster* (AUSTIN *et al.* 1999). Bar for A and B, 2 μ m.

amplification origins in stage 10 egg chambers have been maintained over at least 40 million years of evolution.

Conservation of DNA subsequences at the origin implicates the Myb-MuvB complex in epigenetic regulation: The evolutionary conservation of nucleosome hyper-acetylation supports previous findings that it plays an important role in origin regulation. It is not known, however, which acetylase is responsible for modifying origin nucleosomes or which proteins recruit this acetylase to the origins. To achieve locus specificity, the proteins responsible for recruiting the acetylase likely bind to DNA sites within origin regions that are essential for origin function. Previous analysis had revealed similarity between amplification origins in *D. melanogaster* and several other flies (SWIMMER *et al.* 1990; VLACHOU and KOMITOPOULOU 2001). However, with the recent availability of genome sequences for 12 *Drosophila* species, we felt that a new bioinformatics analysis was warranted.

We focused on the origin at the third chromosome chorion locus because it is the best defined genetically and molecularly. At this locus, the two most important regions are the 320-bp ACE3 and the 840-bp Ori- β , which are upstream and downstream of the *chorion protein 18* (*cp18*) gene transcription unit, respectively (Figure 7A) (ORR-WEAVER and SPRADLING 1986; DELIDAKIS and KAFATOS 1987, 1989; AUSTIN *et al.* 1999). Although most replication initiates in Ori- β , both ACE3 and Ori- β are required for high-level amplification, are hyper-acetylated, and are bound by the ORC (DELIDAKIS and KAFATOS 1989; ORR-WEAVER *et al.* 1989; HECK and SPRADLING 1990; AUSTIN *et al.* 1999; LU *et al.* 2001; AGGARWAL and CALVI 2004; HARTL *et al.* 2007).

We found that the four tandem transcription units at the third chorion locus were syntenic in all *Drosophila* species, enabling us to anchor the alignment of ACE3 and Ori- β among the genomes by their position relative

FIGURE 7.—Subsequences are highly conserved within ACE3 of the third chromosome chorion origin. (A) The organization of the third chromosome chorion locus in *D. melanogaster*. The four major chorion protein transcription units are indicated by arrows below. The two most important origin elements, ACE3 and Ori- β , are designated by gray boxes. The ACE3 region is shown enlarged below, with red and blue boxes indicating binding sites for the Mip120 and Myb proteins, respectively. (B) ClustalW alignment of the region from -669 to -276 upstream of the *D. melanogaster* (*Dmel*) *cp18* gene transcription start site with 11 other *Drosophila* species (see Table 1 for other abbreviations). Sequences are arranged in descending order according to their evolutionary distance from *D. melanogaster* on the basis of the whole-genome sequence. Gray backgrounds enclose nucleotides that are identical among more than half of the species. The more thickly outlined box demarcates the ACE3 region. The blue and red lines above the sequence denote the Myb- and Mip120-binding sites, respectively. The highly conserved region corresponds to the most 3' Mip120 site and part of the adjacent Myb site.

to *cp18* orthologs (Figure 7A and data not shown) (SWIMMER *et al.* 1990). A blast and clustalW alignment of these sequences revealed subregions that are extensively conserved in both ACE3 and Ori- β , consistent with findings of previous sequence analyses (Figure 7B and data not shown) (SWIMMER *et al.* 1990). Most notable was a 71-nucleotide sequence in ACE3 that is highly conserved among all species. Importantly, this highly conserved region corresponds to a DNase footprint site for the Mip120 protein and part of an adjacent site protected by Myb protein, two proteins of the large Myb–MuvB complex (BEALL *et al.* 2002). Although part of the Myb footprint region is conserved, the two consensus Myb-binding sites in this region are not, nor are the other two Mip120 sites in the 5'-end of ACE3 (Figure 7, A and B) (BEALL *et al.* 2004). It has been previously shown that the Myb–MuvB complex is genetically required for amplification in *D. melanogaster* and that deletion of the Mip120- and Myb-binding sites in ACE3 cripples the activity of the origin (SWIMMER *et al.* 1989; BEALL *et al.* 2004; LEWIS *et al.* 2004; ZHANG and TOWER 2004). The strong conservation of these sites, together with our observation that the hyper-acetylation of origin nucleosomes is conserved, suggests that Mip120 and its orthologs may be responsible for recruiting acetylation to the origin.

DISCUSSION

Our results indicate that the developmental timing for distinct types of cell cycles in the *Drosophila* ovary has been strictly conserved over at least 40 million years of evolution. This suggests that this timing is important for egg chamber morphogenesis. It further suggests that the developmental mechanisms that coordinate these cell cycles with oogenesis are also likely conserved. This includes a synchronous onset of a specialized amplification phase in stage 10B follicle cells. The presence of numerous amplification foci in some species suggests that additional, unidentified loci are amplified. The acetylation and preferential ORC binding at these amplification origins are significant because they support the model that epigenetic regulation contributes to origin activity and developmental timing. Retention of the DNA-binding site for Mip120 protein in the third chromosome origin in all species implies that a Myb-like complex may recruit acetylases to the origin.

Conserved developmental timing of cell cycle programs: In all species that we examined, follicle cells completed mitotic divisions and entered endocycles in mid-oogenesis before stage 7. In *D. melanogaster*, this transition is controlled by the Notch-signaling pathway, which has several targets for preventing mitosis and promoting endocycles (DENG *et al.* 2001; LOPEZ-SCHIER and ST JOHNSTON 2001; SHCHERBATA *et al.* 2004). Our results predict that Notch pathway orthologs also control the timing of endocycle entry in these other species.

The strict conservation of timing for endocycle entry suggests that it is important for egg chamber maturation. One purpose may be to increase the biosynthetic capability of the follicle cells. In addition to their role in developmental patterning and eggshell production, follicle cells also supply yolk and other proteins to the oocyte. The onset of endocycles in stage 7 corresponds to the beginning of vitellogenesis when the oocyte begins to accumulate yolk in earnest. Perhaps more importantly, follicle cell growth in the absence of mitotic division may be more conducive to egg chamber morphogenesis. From stage 6 onward, the egg chamber changes shape as the oocyte grows, and, during stage 9, follicle cells rapidly migrate and change shape, processes that would likely be disrupted by the cell shape changes and cytokinesis associated with mitotic divisions. Indeed, cell division and migration have been shown to have an inimical relationship during gastrulation in *Drosophila* and *Xenopus* (GROSSHANS and WIESCHAUS 2000; MATA *et al.* 2000; SEHER and LEPTIN 2000; LEISE and MUELLER 2004).

We also found that the developmental timing of endocycle completion is maintained. Different cells completed endocycles at different times during late stage 9 to late stage 10A, consistent with their asynchronous entry into endocycles during stage 6 to stage 7. The timing of this endocycle arrest likely sets the stage for amplification, permitting the cell to dedicate its DNA replication machinery to rapid reduplication of only a few genomic regions.

In all species, stage 10B marked the onset of a synchronized amplification phase. Amplification foci appeared simultaneously in all follicle cells around the oocyte, but not in those around the nurse cells. We could not determine whether some amplification occurs before the amplification phase during endocycle S phases because nucleus-wide incorporation of BrdU would obscure detection of amplification foci. Indeed, earlier results suggested that the origin at the third chromosome chorion locus in *D. melanogaster* initiates more than once during the last endocycle S phase (CALVI *et al.* 1998). Nonetheless, our results suggest that a conserved developmental timer synchronizes follicle cell entry into a specialized amplification cell cycle phase.

It remains unclear, however, what coordinates morphogenesis of the egg chamber with the transition from endocycles into the amplification phase. In *D. melanogaster*, this coordination acts through the S-phase regulator cyclin E/Cdk2. Cyclin E levels oscillate with asynchronous follicle cell endocycle S phases, but then rise to high levels synchronously in all follicle cells at the onset of amplification (CALVI *et al.* 1998). Since follicle cells in *D. melanogaster* complete exactly three endocycles at different developmental times, some aspect of this coordination likely entails a cell-autonomous cell-cycle-counting mechanism. However, the strict spatial and temporal coordination of amplification onset with

egg chamber morphogenesis suggests that developmental signaling pathways also contribute to this cell cycle transition. Our results suggest that both cell-autonomous and cell-nonautonomous mechanisms have been maintained during *Drosophila* evolution.

Evolution of developmental gene amplification: Previous quantitative genomic Southern blots had indicated that chorion genes amplify in several other *Drosophila* species and in the medfly *C. capitata* (MARTINEZ-CRUZADO *et al.* 1988; SWIMMER *et al.* 1990; VLACHOU *et al.* 1997; VLACHOU and KOMITOPOULOU 2001). We extend these results and show that the developmental coordination of this amplification is also conserved. Moreover, the appearance of more than two amplification foci in these other species suggests that, in addition to the major chorion protein genes, genes at other loci are amplified. Some of these loci may encode orthologs of the *D. melanogaster* genes at amplicons 30B and 62D. However, the appearance of more than four foci in some species suggests that additional, unknown loci are also amplified.

An alternative explanation for the extra foci is that the polyploid follicle cells in some species are not strictly polytene. However, we disfavor this interpretation for several reasons. First, polyteny is the default state for all polyploid cells in *D. melanogaster*, with only the nurse cells having an active mechanism to partially disperse polytene structure. Second, the bar-shaped morphology in these other species was comparable with *D. melanogaster*, suggesting that homologs and sister chromatids remain synapsed.

It remains possible that the extra amplicons that we detected in some species do amplify in *D. melanogaster*, but that a species-specific difference in the timing or level of their amplification makes them more apparent after BrdU labeling. Indeed, the size and intensity of the BrdU foci differed among species, suggesting that orthologs may amplify to different copy numbers. Future identification of these loci should provide insight into origin structure, regulation, and evolution and may identify new genes whose amplification is important for oogenesis.

Origin structure, epigenetic regulation, and ORC binding: Despite a continued molecular and genetic dissection of origins in metazoa, unifying rules for origin anatomy remain largely unknown (ALADJEM *et al.* 2006 for review). The alignment of DNA sequences from 12 *Drosophila* genomes revealed islands of high-level conservation within the amplification origin at the third chromosome chorion locus. The most extensive conservation was a 71-nucleotide sequence in ACE3, which is also conserved upstream of the amplified *cp18* ortholog in the medfly *C. capitata* (VLACHOU and KOMITOPOULOU 2001). This conservation is functionally significant because deletion of this sequence impairs amplification and the similar sequence from *D. grimshawi* can direct amplification in *D. melanogaster* (SWIMMER *et al.* 1990;

BEALL *et al.* 2002; ZHANG and TOWER 2004). A DNA fragment from ACE3 that contains the conserved region was shown to bind ORC, further stressing its importance, but the specific DNA residues that ORC contacts are unknown (AUSTIN *et al.* 1999). The 71-nucleotide sequence resembles those found at other origins in that it is highly AT rich; otherwise, strict rules for a DNA consensus sequence at metazoan origins still do not apply.

Mounting evidence in a variety of organisms suggests that the epigenetic modification of chromatin is an important determinant of origin activity (MECHALI 2001 for review). The site-specific replication in stage 10B follicle cells represents an extreme example of origin developmental specificity. The evidence for conservation of nucleosome acetylation is consistent with a model that it plays an important role in origin function (AGGARWAL and CALVI 2004). This conserved acetylation is associated spatially and temporally with preferential ORC binding, consistent with the idea that these origins are, in part, epigenetically specified. Taken together, our results further suggest that this epigenetic modification may regulate origin developmental timing.

The most highly conserved sequences within ACE3 correspond to footprint sites for the Mip120 and Myb protein of the Myb–MuvB complex (BEALL *et al.* 2002). Previous studies in *D. melanogaster* and humans suggested that the Myb–MuvB complex activates and represses promoters and may have similar activities at origins (BEALL *et al.* 2004; KORENJAK *et al.* 2004; LEWIS *et al.* 2004). In *D. melanogaster*, mutation of the *Myb* gene, or deletion of the Myb- and Mip120-binding sites in ACE3, severely impairs amplification of the third chromosome locus (BEALL *et al.* 2002). Mutations in another protein of the Myb–MuvB complex, Mip130, resulted in inappropriate genomic replication in stage 10B follicle cells, suggesting that Myb–MuvB represses genomic origins (BEALL *et al.* 2004). Indeed, the Myb–MuvB complex contains the histone deacetylase enzyme Rpd3, whose mutation also results in genomic replication in stage 10B follicle cells (AGGARWAL and CALVI 2004; LEWIS *et al.* 2004). The conservation of DNA sequence in ACE3 suggests that Mip120 and Myb orthologs may regulate amplification origins in these other species. However, the entirety of the Myb site and the other two Mip120 sites in ACE3 are not well conserved. The difference in conservation between Myb- and Mip120-binding sites implies that, at least in some species, Mip120 binding plays the more prominent role in origin function. Alternatively, Myb may contribute, but the functional constraints on sequence and/or spacing for Myb DNA-binding sites may be relaxed and therefore not strictly conserved. Orthologs of the *D. melanogaster* Mip120, Myb, and other Myb–MuvB complex members are present in the other 11 sequenced *Drosophila* genomes (data not shown), but whether they bind and regulate amplification origins, and perhaps other origins, in these species awaits further investigation.

An important remaining question is what recruits acetylase activity to amplification origins. The conservation of origin acetylation, together with the high conservation of the Mip120 binding site, makes it tempting to speculate that the Myb-MuvB complex recruits acetylases to the origin. A cogent model is that developmental signaling pathways in follicle cells around the oocyte converge on the Myb complex to recruit acetylases and epigenetically regulate the amplification origins (LEWIS *et al.* 2004). Myb complexes are known to recruit acetylases to promoters, including those in human cancer cells (DAI *et al.* 1996; JOHNSON *et al.* 2002). The *Drosophila* orthologs of the retinoblastoma tumor suppressor (Rb) are also part of the Myb-MuvB complex at amplification origins and evidence supports that they may locally repress origins in follicle cells (BOSCO *et al.* 2001; BEALL *et al.* 2004). In human cells, Rb recruits histone deacetylases to repress promoters and has been shown to bind near some origins (KENNEDY *et al.* 2000; AVNI *et al.* 2003; FROLOV and DYSON 2004). Given the evidence for Rb and Myb in origin regulation, it may be that perturbed epigenetic regulation of origins contributes to genome instability in these cancers. A continued analysis that employs the genetics of *D. melanogaster* with the genomic information and tools now available for 11 sibling species should contribute to a deeper understanding of origin structure and regulation in metazoa.

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