Chorion Gene Amplification in *Drosophila*: A Model for Metazoan Origins of DNA Replication and S-Phase Control

Brian R. Calvi and Allan C. Spradling

Howard Hughes Medical Institute Research Laboratories, Carnegie Institution of Washington, Baltimore, Maryland 21210

The mechanisms controlling duplication of the metazoan genome are only beginning to be understood. It is still unclear what organization of DNA sequences constitutes a chromosomal origin of DNA replication, and the regulation of origin activity during the cell cycle has not been fully revealed. We review recent results that indicate that chorion gene amplification in follicle cells of the *Drosophila* ovary is a model for investigating metazoan replication. Evaluation of cis sequence organization and function suggests that chorion loci share attributes with other replicons and provides insights into metazoan origin structure. Moreover, recent results indicate that chorion origins respond to S-phase control, but escape mechanisms that inhibit other origins from firing more than once in a cell cycle. Several identified genes that mediate amplification are critical for the cell cycle control of replication initiation. It is likely that further genetic screens for mutations that disrupt amplification will identify the cadre of proteins associated with origins and the regulatory pathways that control their activity. Furthermore, the recent development of methods to detect amplification in situ has uncovered new aspects of its developmental control. Examining this control will reveal links between developmental pathways and the cell cycle machinery. Visualization of amplifying chorion genes with high resolution also represents an opportunity to evaluate the influence of nuclear and chromosome structure on origin activity. The study of chorion amplification in *Drosophila*, therefore, provides great potential for the genetic and molecular dissection of metazoan replication.

Key Words: amplification; chorion; DNA replication; *Drosophila*; oogenesis.

In recent years there have been significant advances in understanding how the eukaryotic cell controls duplication of chromosomes during the cell cycle. Genomic DNA replication is best understood in yeast. The question remains, however, how similar replication origins and S-phase control mechanisms in multicellular organisms are to those in yeast. The recent identification in Metazoa of homologs to critical yeast replication proteins suggests that some aspects of replication control are conserved among all eukaryotes. Regulation of DNA replication in Metazoa is likely more specialized, however, because it must be integrated with the cell cycle molecules and developmental processes specific to multicellular organisms. Moreover, it is still unclear to what extent the cis sequence organization of replication origins resembles that defined in yeast. Development of assays that allow evaluation of origin activity and regulation within the context of development is critical to the study of cell cycle control of DNA replication in Metazoa. Recently, a number of genes involved in controlling DNA replication have been identified in *Drosophila melanogaster*. *Drosophila* genetics permits evaluation of the function of these genes, and provides the potential for identification of other genes critical for DNA replication. In this review we discuss results that show that chorion gene amplification during *Drosophila* oogenesis provides an in vivo assay for origin function and a genetic system for identifying genes critical to the cell cycle control of DNA replication.

Amplification of the genes encoding the major eggshell, or chorion, proteins occurs in somatic follicle cells in the latter part of *Drosophila* oogenesis (Fig. 1). This selective increase in DNA copy number occurs for two clusters of chorion genes. The X-chromosome cluster is amplified ~16- to 20-fold, and the third-chromosome cluster ~60- to 100-fold, above the remainder of the follicle cell genome (hereafter X chorion and third cho-
rion). By the end of oogenesis, each gene cluster lies at the peak of a gradient of copy number that extends approximately 40–50 kb in both directions (1), leading to the suggestion that amplification occurs through repeated replication. Observation of replication forks centered about the chorion locus in electron microscopic images of lysed nuclei (2, 3) and the detection of replication intermediates by two-dimensional gel electrophoresis (4, 5) confirmed the replication-based mechanism for amplification. By measuring the progressive increase in DNA copy number at different points along the gradient, it was determined that the gradient of copy number results from the migration of successively initiated replication forks that, by the end of oogenesis, travel outward different distances from the chorion locus (6). Chorion gene amplification is a developmentally regulated process, in contrast to amplification at certain vertebrate loci that occurs during oncogenic transformation, or in response to prolonged drug challenge, which involves DNA rearrangement [see (7) for review]. During the last hours of oogenesis, the different chorion genes are transcribed to high levels from the amplified templates in a temporally specific pattern, and are a model system for the study of developmentally specific transcription, which has been reviewed elsewhere (8). The robust replication that initiates from a discrete region of the genome has defined chorion amplification as one of the most tractable systems for the study of metazoan DNA replication.

CHORION LOCI AS METAZOAN REPLICONS

Growing evidence now suggests that replication initiates in defined regions of metazoan chromosomes, but

![Diagram](Image)

**FIG. 1.** Two clusters of chorion genes are amplified in somatic follicle cells surrounding the oocyte. (A) A cluster of chorion genes located at cytogenetic position 7F on the X chromosome amplifies 16- to 20-fold and a cluster located at cytogenetic position 66D on the third chromosome amplifies 60- to 100-fold. (B) An epithelial sheet of somatic follicle cells surround the developing egg chamber. Chorion gene amplification occurs in the follicle cells that surround the oocyte, but not those over the nurse cells. During choriogenesis these follicle cells secrete the chorion proteins between themselves and the oocyte and subsequently are lost from the mature egg. Shown is a schematic of a longitudinal section through a stage 10 egg chamber.

**FIG. 2.** Organization of the third-chromosome chorion locus. Genetic mapping indicates that there are five regions that are important for amplification (gray boxes) interspersed among the four chorion transcription units (s18, s15, s19, s16 shown below). Two-dimensional gel mapping indicates that replication initiates most frequently in Ori-$\beta$ (black box), but occasionally replication begins at other regions (dashed line). There are two sequences within AER-d and AER-c that match the ARS consensus sequence (ACS) from yeast at 10 of 11 positions (arrows above).
Chorion gene amplification is extremely sensitive to genomic position effect. Chorion P elements at some genomic positions would amplify highly, whereas, when resident at many other insertion sites they would amplify little, if at all. This required analysis of multiple insertions to control for this variability and made genetic mapping difficult. A partial solution to this problem was the creation of deletions within a P element inserted at a single genomic location using P-element transposase (5). The extreme position effect on smaller elements and redundant nature of amplification control regions, however, made the results of fine mapping of subsequences mediating amplification equivocal.

In addition to genetic functional mapping, the location within the third-chorion cluster from which replication forks initiate was examined by two-dimensional (D) gel electrophoresis (4, 5). This analysis indicated that replication begins most often within a discrete 1-kb region, subsequently named Ori-β, but that sometimes replication initiates at other sites within the chorion locus (Fig. 2). Located within the Ori-β region is AER-d, which suggests that the importance of this genetically mapped element resides in its function as a site of replication initiation (Fig. 2). AER-d is located 1.5 kb from ACE3. P elements containing only ACE3 and AER-d that amplify highly also initiate replication primarily at Ori-β (4). These results suggested that the importance of ACE3 may be due to its action as a replication enhancer on Ori-β (4, 5). Similar to transcriptional enhancers, ACE3 can stimulate amplification when inverted or displaced 3 kb from Ori-β (14). In addition, analysis of the gradient of copy number for P elements containing only ACE3 suggested that it directs replication initiation outside of itself in the flanking marker gene (18). Although transcriptional enhancers can influence the activity of eukaryotic viral and yeast chromosomal origins [see (23) for review], the sequences within ACE3 that direct transcription are functionally separable from those required for amplification (14). Moreover, unlike enhancers, multimers of ACE3 alone did not greatly increase amplification level (18), and fusion of chorion sequences to heterologous transcriptional enhancers failed to induce amplification in other tissues (14). Thus, although AER-d appears to correspond to Ori-β, it has not been fully determined whether the importance of other genetically mapped regions resides in their function as enhancers of replication or sites of initiation. Chorion loci have yet to be subjected to the newer origin mapping techniques that depend on detecting newly replicated DNA at sites of initiation (9, 24, 25).

A recent advance for genetic mapping was the finding that chorion amplification can be protected from genomic position effects by suppressor of hairy wing protein binding sites [su(Hw)BS] (26). These DNA elements had been previously shown to buffer promoters from position effects on transcription, which requires binding by wild-type su(Hw) protein (27). When su(Hw)BS were placed on both ends of a P element containing ACE3 and Ori-β, amplification was observed in all genomic positions tested. Thirty-one of thirty-one insertions amplified greater than 10-fold, whereas the same chorion sequences flanked by single su(Hw)BS amplified above 10-fold in only 7 of 21 positions tested (26). This buffering required su(Hw) protein; in su(Hw) mutant background amplification of many of the buffered elements was reduced. Interestingly, although su(Hw)BS efficiently blocked negative effects on replication initiation, they did not impede the passage of replication forks into flanking genomic DNA. The precise mechanism by which su(Hw)BS insulate sequences from position effects is unknown [see (28) for review].

Within buffered P elements, it is now feasible to test smaller subsequences for their ability to direct amplification and discern if they have distinct functions. Using this approach, preliminary results suggest that Ori-β alone does not direct high-level amplification, but does so if ACE3 is also included in the P element (J. Tower, personal communication). Moreover, it appears that su(Hw)BS placed between ACE3 and Ori-β reduces amplification. Amplification is restored when these blocking su(Hw)BS are removed in vivo by site-specific recombination. This is consistent with the proposal that the primary role of ACE3 is to stimulate initiation of replication at Ori-β. Whether other regions of the chorion cluster must act in concert can be tested by interposing su(Hw)BS between them. It is anticipated that the near future will see rapid advances in our understanding of replicon structure at the chorion locus.

The picture that is emerging from investigations into other metazoan replicons is that they have properties first described for the chorion locus. Mapping of chromosomal origins by molecular techniques suggests that one primary origin of bidirectional replication (OBR) resides within a broad “initiation zone” where frequently replication initiates at other sites (28a) [see (9) for review]. Further reminiscent of chorion replicons, many origins appear to have a modular organization. It has been shown that deletion of sequences distant from a physically mapped origin can affect origin activity (29). Even in yeast there is evidence for modularity. Most origins analyzed to date have multiple copies of an essential ARS consensus sequence (ACS) and variable numbers of auxiliary sequences (29a) [see (30) for review]. The deletion of auxiliary subsequences has only modest effects on origin activity, but the deletion of two auxiliary subsequences has severe effects (31). As more is learned about metazoan origin structure, therefore, it may be found that they share a common
AMPLIFICATION AS A MODEL FOR S-PHASE INITIATION

Although early work defined chorion loci as one of the best understood metazoan replication origins, it was unclear whether amplification was a valid model for cell cycle control of genomic replication. This uncertainty was largely resolved when the relationship of amplification to the control of follicle cell cycles was examined (32). Follicle cells initially proliferate through mitotic cycles, and then become polyploid by entering an endocycle characterized by alternating G and S phases (Fig. 3) [see (33) for review]. Bromodeoxyuridine (BrdU) labeling of dissected ovaries allowed an assessment of when follicle cell endoreplications ceased, and revealed that amplification could be detected in individual nuclei. This analysis indicated that endocycles are complete by stage 10A of oogenesis. Immediately thereafter, in stage 10B, all follicle cells surrounding the oocyte simultaneously incorporate BrdU into four subnuclear foci of different intensities (Fig. 4). Double labeling by BrdU and fluorescence in situ hybridization (FISH) using chorion probes confirmed that the most intense incorporation corresponds to amplification at the third-chorion locus, the less intense to the lower-amplifying X-chorion locus, and two fainter spots to unknown, low-amplifying loci. In agreement with previous measurements of amplification, punctate BrdU labeling at chorion loci was continuously observed through stage 13 of oogenesis and did not appear to cycle. These results, combined with previous quantitative Southern analyses, indicate that the majority of amplification occurs after endocycles when the remainder of the genome does not replicate.

A technique that allows ovarian nuclei to be flow sorted by DNA content indicated that follicle cells undergo three endocycles and achieve a final ploidy of 16C (32, 34). When flow-sorted nuclei were collected and analyzed by quantitative Southern blotting, it was found that third-chorion genes are amplified 2-fold in 8C endocycling follicle cells, but the X-chorion locus is not. After the final endocycle, the third locus was found to be 4-fold amplified, but again the X is not. Therefore, although most amplification occurs after endocycles have ceased, amplification for the third occurs in two phases, during and after endocycles (Fig. 3). The early amplification is apparently restricted to endocycle S phases, since BrdU incorporation at the third-chorion locus was not observed during G phase. This suggested that the absence of X-chorion amplification during endocycles may result from a slower initiation rate and the inability to reinitiate replication during the time constraint of a single S phase. The observation of periodic amplification of third-chorion loci during S phase is significant because it suggested chorion origins respond to cell cycle regulators that induce genomic replication.

In support of this, the major S-phase regulator, cyclin E (CycE), was found to be critical for amplification (32). CycE is highly conserved among metazoans and, with its kinase partner cdk2, is required for S phase in both mitotic and endocycles of Drosophila (34–38). Levels of CycE in follicle cell nuclei are tightly correlated with amplification (32). Immunofluorescence microscopy indicated that CycE levels rise commensurate with periodic S phases of follicle cell endocycles when the third-chorion genes amplify. Importantly, oscillations of CycE are modified during the later, continuous phase of amplification. In stage 10B, when subnuclear BrdU labeling at chorion loci first appears, CycE levels rise simultaneously in all follicle cells over the oocyte. Unlike previous endocycles, CycE persists and fails to cycle throughout the remainder of late amplification. CycE appears to be required, as overexpression of an inhibitor of CycE/cdk2 complexes, Dacapo (39, 40), severely reduced amplification (32). Chorion origins, therefore, are linked to mechanisms that regulate genomic replication. The requirement for CycE sug-
gests chorion amplification involves other proteins required for cell cycle regulation of S-phase initiation.

**GENETIC DISSECTION OF REPLICATION INITIATION**

If the process of amplification employs pathways that mediate normal S phase, identification of mutations that disrupt amplification should recover genes essential for genomic replication. This is supported by the finding that a female sterile mutation that reduces chorion amplification levels (41) is a lesion in *Drosophila* ORC2 (the k43 gene) (42–44). In yeast, ORC2 is part of the six-member origin recognition complex (ORC) which binds DNA at origins during most of the cell cycle and is indispensable for replication (45–47). During G1, other proteins associate with the ORC to form a prereplicative complex (pre-RC), which prepares origins for initiation during the subsequent S phase (48–51, 51a) [see (52) for review]. More severe alleles of the k43 gene result in cell cycle defects and lethality, suggesting it performs essential functions at other replication origins in *Drosophila* (42). The recent identification of other yeast pre-RC homologs in *Drosophila* (43, 53–55) and other higher eukaryotes [see (56–58) for review], combined with the evidence from Xenopus egg extracts (59–63), indicates that many aspects of pre-RC assembly and function first described in yeast have been conserved within the Eukaryota [see (64) for review]. The finding that ORC2 is required for amplification suggests that other proteins known to comprise pre-RCs assemble onto chorion origins.

Genetic screens for female sterility by several groups recovered mutations in six other genes that resulted in thin eggshells and that were subsequently shown to reduce chorion gene amplification to ~3–13% of wild-type levels (Table 1). Where known, more severe alleles of these genes result in lethality, suggesting they, too, encode proteins critical for normal S phase. Preliminary results indicate two of these essential genes encode proteins with similarity to those in yeast intimately associated with S-phase initiation. The chiffon gene has limited similarity to the yeast Dbf4 protein (J.

**FIG. 4.** Detection of amplifying loci in late-stage follicle cell nuclei. Shown here is a reverse monochrome image of immunofluorescence detection of BrdU incorporation in follicle cells surrounding the oocyte of a stage 10B egg chamber. BrdU incorporation reveals that there are four loci that amplify during the postendocycle period when the majority of the follicle cell genome does not replicate. The most intense spot is the third-chorion locus, the intermediate spot is the X-chorion locus, and the two fainter spots are unidentified loci that amplify to low levels. Inset: Higher magnification of a single nucleus.
Tower, personal communication), the regulatory subunit of Cdc7 kinase (65, 66), which directly contacts yeast pre-RCs (67), and is essential for origin firing (68, 69). Another mutation that impairs amplification is an allele of the mus101 gene (70, 71), which resembles the Rad4/Cut5 protein from Schizosaccharomyces pombe and Dpb11 from Saccharomyces cerevisiae (71a). In yeast, these proteins are required for both normal S phase and cell cycle checkpoints that monitor DNA damage or incomplete replication (72), and may associate with pre-RCs during initiation. Interestingly, some viable mutations in mus101 impart sensitivity to chemical mutagens (70), suggesting it is involved in DNA repair pathways in Drosophila. The similarity of mus101 and chiffon to proteins in yeast, and their developmental requirement, suggests they are involved in replication initiation. Two other genes that were previously known to be critical for S phase in Drosophila have recently been implicated in amplification (Table 1). Hypomorphic, viable mutations in Drosophila E2f and DP genes are female sterile (73) and disrupt amplification (T. Orr-Weaver, personal communication). These transcription factors are in the CycE pathway and induce expression of genes required for S phase [see (74, 75) for reviews].

The genetic analysis of trans-acting mutants suggests amplification is more sensitive than normal S phase to slight defects in replication proteins. Hypomorphic lesions in essential genes result in viable, but sterile, females. It is not surprising that amplification is more sensitive to small changes in replication initiation frequency. Chorion must repeatedly initiate replication during the last hours of oogenesis. Modest reductions in initiation rate would result in the absence of several rounds of initiation and the drastic reductions in final copy number observed in the mutants. During other cell cycles in development, elongation from multiple origins, combined with regulation by checkpoint mechanisms that monitor incomplete replication, likely compensate for reductions in initiation rate in the mutant strains. Thus, thin eggshells resulting from amplification defects constitute an especially favorable phenotype for identifying genes involved in replication initiation.

The fact that amplification requires many proteins involved in initiation at other origins opens up further possibilities for identification of cis sequences critical for amplification and, perhaps, all replication. For example, it is now possible to ask what regions of the third-chorion cluster interact with the Drosophila ORC. Within yeast origins, the ORC has been shown to bind conserved DNA sequences that are essential for replication (45, 76, 77). Initial in vitro binding studies suggest that the Drosophila ORC, assembled from subunits expressed in cell culture, preferentially interacts with sequences in the region of ACE3 and Ori-β (M. Botchan, personal communication). It had been noted previously that sequences within the third-chorion locus resemble sequences from yeast origins (4, 17). Within Ori-β and AER-c there are 10 of 11 base pair matches to the essential ACS, which contributes to the ORC binding site in yeast (4). These sites may be important for ORC binding at the chorion locus. Implicating chorion subsequences by virtue of their interaction with replication proteins avoids the problem of functional redundancy which confounded earlier genetic tests. Once implicated, however, their importance can be tested by mutating multiple sites and measuring amplification levels within buffered P elements. By merging biochemical and genetic approaches, the sequences important for determining origin activity can be identified.

AMPLIFICATION AS AN ESCAPE FROM REREPLICATION CONTROL

Although sharing attributes with normal replication initiation, chorion amplification differs from replica-

<table>
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<tr>
<th>Gene</th>
<th>Essential function?</th>
<th>Gene product</th>
<th>Proposed or known function</th>
<th>References</th>
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<tr>
<td>I(3)k43</td>
<td>Yes</td>
<td>ORC2</td>
<td>Binds origin DNA</td>
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<td>cyclin E</td>
<td>Yes</td>
<td>Cyclin E</td>
<td>Activates cdk2 kinase</td>
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<td>chiffon</td>
<td>Yes</td>
<td>Dbf4-like</td>
<td>Activates cdc7 kinase</td>
<td>(110; J. Tower personal communication)</td>
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<td>mus101</td>
<td>Yes</td>
<td>cut5/rad4-like</td>
<td>Replication/repair</td>
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<td>E2f</td>
<td>Yes</td>
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<td>S-phase transcription factor</td>
<td>(73, 112, 113; T. Orr-Weaver, personal communication)</td>
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<td>DP</td>
<td>Yes</td>
<td>DP</td>
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<td>(73, 114; T. Orr-Weaver, personal communication)</td>
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<td>fs(3)272-9</td>
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<td>Unknown</td>
<td>Unknown</td>
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<td>Unknown</td>
<td>Unknown</td>
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<tr>
<td>fs(1)K1214</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
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<td>fs(1)575</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
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tion of other sequences in one significant respect. Chorion amplification is an exception to the rule of replicating only once per cell cycle. It should be noted that with each periodic endoreplication, copy number of other sequences does not increase by more than a factor of 2 (78, 79). Thus, endocycling cells appear to have mechanisms that restrict duplication of sequences to no more than once per cell cycle. The ability of third-chorion genes to rereplicate during endocycles indicates it somehow can escape this rereplication control.

Considerable evidence suggests that CDKs are responsible for restricting replication to once per cycle by inhibiting assembly of pre-RCs onto origins in S, G2, and M (50, 80–82). It is only in G1 when cyclin levels are low that pre-RCs can form, preparing origins for the subsequent S phase. CycE/cdk2 is the only known oscillating cyclin complex in endocycling cells of Drosophila (34, 37), and has been shown to inhibit replication in vertebrate nuclei within frog extracts (81). It was therefore proposed that CycE inhibits rereplication during a single endcycle (32, 34, 83). Evidence that CycE can inhibit rereplication in follicle cells comes from the observation that misexpressing it during the entire endcycle does not allow more than one S phase, presumably because a period in G phase when CycE is low is required for origins to reset (32). Moreover, during late amplification, when naturally occurring levels of CycE do not cycle, follicle cell genomic replication does not occur. Thus, most origins may not reassemble pre-RCs in the presence of continually high CycE/cdk2 activity. The rereplication of chorion genes during late amplification and endocycles suggests chorion origins locally escape inhibition by CycE. To explain this local escape, it was proposed that chorion origins assemble special amplification complexes that, in addition to proteins found at other origins, contain unique amplification factor(s) that impart resistance to Cycl-dependent inhibition (32). Given the central role played by Cdc6/18 family members for pre-RC assembly (49, 62, 84) and their ability to induce rereplication when misexpressed or altered (85–87), it is likely that an unidentified Drosophila cdc6/18 homolog will be involved in amplification. Unlike known properties of cdc6/18, however, some factor must interpret primary sequence information at chorion origins to engender rereplication specifically to these loci. By studying amplification as an exception to once-per-cycle replication, insights should be gleaned into the mechanisms of rereplication control operative in all cycles.

**DEVELOPMENTAL CONTROL OF AMPLIFICATION**

Metazoan cell cycles must be integrated with development. Chorion gene amplification is specific for ovarian follicle cells, but it is unknown what instructs this developmental specificity. Despite all the different modifications and genomic positions of chorion sequences tested, amplification has not been observed in any other tissue or at any other time. Amplification, and the associated modification of follicle cell cycles, represents an opportunity to investigate intersections between developmental pathways and cell cycle control mechanisms.

It is unknown what mediates the transition from endocycles to late amplification. During this transition, there is a synchronization of follicle cell behavior (32). Follicle cell endocycles are not synchronized relative to one another within a developing egg chamber, but, at the onset of late amplification, BrdU foci appear in every follicle cell over the oocyte at the same time. This transition is tightly coordinated with egg chamber development when most follicle cells migrate posteriorly to cover the growing oocyte. Late amplification occurs after migrations only in those follicle cells that surround the oocyte, but not in those that remain over the nurse cells. The correlation of follicle cell position with the simultaneous onset of late amplification suggests there may be an inductive signal emanating from the oocyte. It is less clear to what extent the cessation of endocycles is controlled by extrinsic signals before this time. Current evidence is consistent with a mechanism whereby the transition from endocycles to late amplification involves compromised CycE degradation (32). How this is coordinated with egg chamber development is an interesting subject for future investigations.

Additional hints that germ line signaling pathways may impact amplification come from the observation that BrdU labeling at chorion loci was greatest in dorsal–anterior follicle cells of the egg chamber (32). These cells synthesize specialized chorion structures called the dorsal appendages and participate in dorsal–ventral signaling pathways (88). Previous to the period of increased BrdU labeling, the dorsal axis of the Drosophila egg is specified by the position of the oocyte nucleus when it migrates, anteriorly to one edge of the oocyte. This results in localized production in the oocyte of the transforming growth factor (TGF-α)-like molecule gurken (GRK), which signals to nearby follicle cells via the Drosophila epidermal growth factor receptor (EGFR). Thus it is possible that signals that specify the dorsal axis also increase rates of amplification to meet the demand for chorion dorsal appendage formation. In addition, production of the TGF-β-like protein DPP in anterior follicle cells influences which cells along the anteroposterior axis produce the dorsal appendages (89). The possible influence of DPP on amplification levels is made more compelling by the observation that TGF-β is known to influence the decision to progress into S phase in vertebrate cells (90). Formation of eggshells that are of normal thickness in
dorsal–ventral patterning mutants, however, suggests that the signals that increase amplification in dorsal–anterior follicle cells may differ from the general signal that induces the synchronized onset of late amplification in all follicle cells over the oocyte.

Expression of the broad complex (BR-C) in dorsal appendage follicle cells is influenced by GRK and DPP signaling (91). This locus encodes several transcription factors (92) and is known to mediate signaling by the steroid molecule ecdysone in other tissues during larval metamorphosis (93–95). Hypomorphic BR-C mutants result in smaller chorion dorsal appendages (91, 96), and have been reported to reduce levels of chorion gene amplification (97), although the 50% reduction is modest compared with those of other amplification mutants. BR-C is also expressed in all stage 6 follicle cells (91). In stage 7, follicle cell endocycles begin and egg chamber development is sensitive to circulating steroid molecules that control the decision to enter vitellogenesis [see (98, 99) for reviews]. It is tempting to speculate that hormonal signals may act through BR-C to induce production of an amplification factor in endocycling follicle cells and that BR-C expression later in dorsal–anterior follicle cells increases amplification. Current evidence is not sufficient, however, to conclude whether BR-C plays any role in amplification.

In sciarid flies, amplification of cocoon protein genes in endocycling cells of larval salivary glands is induced by the steroid hormone ecdysone [see (100) for review]. The hint that hormonal signals may mediate chorion amplification makes it tempting to speculate that evolution has arrived at similar solutions for induction of rereplication in salivary glands and ovaries. In fact, steroid hormone receptors are known to bind chorion promoters (101), and it is known that ecdysone induces transcription of the II/9A in S. coprophila (102). It is interesting to ask, therefore, whether sciarid sequences function in Drosophila. Although ecdysone signaling in D. melanogaster salivary glands induces the promoter of the S. coprophila II/9A gene (103), fragments containing the physically mapped amplification origin from II/9A are not detectably amplified in salivary gland cells or follicle cells of Drosophila (B. Calvi, F. Urnov, A. Spradling, and S. Gerbi, unpublished). Because the transformation and genetics possible in D. melanogaster have not been developed in sciarids, it is not known whether this subfragment is sufficient for amplification in those flies, and therefore this result should be viewed as preliminary. If larger fragments of the II/9A locus contained within buffered P elements are able to amplify in D. melanogaster, new avenues of investigation into the developmental control of amplification will be possible using the genetic and molecular techniques available in this organism. Examining the developmental control of amplification may reveal the intersection of developmental signaling pathways with the cell cycle machinery.

**DOES HIGHER-ORDER STRUCTURE AFFECT AMPLIFICATION ACTIVITY?**

A variety of evidence suggests that higher-order structure within the metazoan nucleus contributes to replication origin usage [see (104) for review]. The extreme effect of genomic position on the amplification of chorion P elements may reflect the influence of nuclear or chromosome structure on origin activity. The ability to detect chorion amplification using BrdU and FISH allows an evaluation of the effects of higher-order structure on replication. Preliminary results indicate that amplification does not require targeting to a distinct nuclear subdomain (B. Calvi and A. Spradling, unpublished). The endogenous third- and X-chorion clusters amplify in different nuclear locations in different nuclei. Moreover, chorion P elements amplify in subnuclear locations distinct from the endogenous loci. It has also been proposed that as DNA replicates it is spooled through replication factories fixed to a nuclear matrix [see (105, 106) for reviews]. The robust amplification of chorion loci permits a direct examination of chromatin structure of a defined origin during replication. Because the polyploid follicle cells are already 16C, by the end of amplification there are approximately 1024 copies (64 × 16C) of the third-chorion genes and 256 copies (16 × 16C) of the X genes. FISH using different chorion probes showed no evidence for loops attached to a nuclear matrix, contrary to the replication factory model (B. Calvi and A. Spradling, unpublished). Although su(Hw)BS are able to buffer negative position effects on amplification (26), it is unclear by what mechanism they do so. It has been proposed that the association of two su(Hw)BS with the nuclear scaffold delimits a permissive chromatin domain [see (28) for review] (107), which may target sequences to a permissive subnuclear compartment near the nuclear envelope (108). Whether su(Hw)BS buffer position effect through changes in chromatin structure or nuclear location can be assessed by examining the P elements containing chorion sequences and su(Hw)BS in the BrdU and FISH assays. Continued examination of spatial and structural attributes of amplification will allow direct tests of models that invoke chromosome and nuclear organization in determining origin usage.

**THE FUTURE**

We are currently in the midst of a period of rapid advance in our understanding of metazoan origins and
S-phase control. The identification of highly conserved homologs and the application of basic lessons learned in yeast have jump-started investigations in higher eukaryotes. The challenge is to understand how the cell cycle control of replication is specialized within the Metazoa. Investigations into the cell cycle control of replication is specialized within the eukaryotes. The challenge is to understand how the in yeast have jump-started investigations in higher cycles. The identification of highly conserved well-defined origin in all Metazoa and currently the only well-defined origin in Drosophila. Origin mapping indicates that chorion amplification resembles other metazoan replicons where one major site of DNA initiation resides among others used less frequently. As more is learned about metazoan origin structure, it may be found that they share a modular organization similar to that at chorion loci. Moreover, recent results indicate that initiation of replication at chorion origins entails mechanisms that control S phase in other cell cycles. Therefore, all evidence indicates it is valid to consider chorion replicons as models for metazoan replication.

The continued genetic identification of trans-acting loci required for amplification will likely recover genes critical for S-phase initiation. These genes can be initially identified by screening for female sterility resulting from thin eggshells. Detection of amplification in individual cells by BrdU and FISH represents a facile assay for further evaluating whether these trans-acting mutations disrupt the amplification process. These screens should identify molecules that constitute pre-RCs. Although some pre-RC proteins have been identified in Drosophila, recovery of mutations in these genes will allow genetic dissection of function and further gene identification in enhancer and suppressor screens. Genetic screens also may reveal the unknown links between CycE/cdk2 and origin firing and may identify other cell cycle and developmental pathways that control S phase. Some of the genes recovered may be required only for amplification, and will be candidates for amplification factors that allow chorion origins to escape rereplication control. Investigation into this escape will provide insight into the normal mechanism by which origin firing is restricted to once per cell cycle.

The protection of chorion sequences from position effect by su(Hw)BS will greatly facilitate the genetic dissection of cis sequence function. The combination of these genetic tests with trans factor binding studies should delimit important sequence elements within the chorion clusters. The requirement for ORC2, and binding by the ORC, suggests other proteins known to constitute yeast pre-RCs will be associated with chorion origins. It will be interesting to see if DNA footprinting at the chorion locus reveals amplification-associated changes analogous to those that first characterized pre-RC assembly in yeast (48). One important remaining question is whether origins used during amplification initiate replication in all cell cycles of the fly. It is likely that this will be the case given its interaction with the ORC. If chorion origin recognition is changed during S phase of other cycles, uncovering the mechanism of this change will be illustrative for understanding origin designation in general.

Chorion gene amplification also allows a direct test of the spatial correlates observed for replication in the metazoa. The detection of this highly robust differential replication at defined loci represents an opportunity to evaluate the role of chromosome and nuclear structure in determining replication origin activity. The ability to visualize replicating chromatin at chorion loci with high resolution also represents the potential to implicate proteins in various aspects of replication by immunofluorescence. In this way, proteins may be found to be primarily near origins of elongating forks or as components of nuclear structure associated with amplifying replicons. The study of amplification may expose aspects of replication control in higher eukaryotes that differ from the process in yeast. It is expected that in the coming years chorion gene amplification will play an important part in revealing the mysteries of metazoan replication.

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