Isolation of a Drosophila amplification origin developmentally activated by transcription

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We exploited the Drosophila Amplicon in Follicle Cells, DAFC-62D, to identify a new metazoan amplification origin, ori62. In addition to the origin, DAFC-62D contains two other developmental stage-specific binding regions for the Origin Recognition Complex (ORC) and the replicative helicase MCM2–7. All three of these regions are required for proper amplification. There are two rounds of amplification initiation at ori62, and the second round is preceded by transcription across ori62. We show by α-amanitin inhibition that RNA polymerase II (RNAPII) transcription is required to localize MCM2–7 (but not ORC) to permit the second round of origin firing. This role for transcription appears unique to DAFC-62D, because neither other DAFCs nor ectopic transposons with the DAFC-62D replication elements bounded by functional chromatin insulators are affected by α-amanitin. By sequential chromatin immunoprecipitation, we show that the MCM complex and RNAPII are bound to the same 100–500 bp pieces of chromatin during late origin firing. These results raise the possibility that RNAPII may recruit MCM2–7 to some metazoan replication origins.

DNA replication | gene amplification | MCM | ORC | RNA polymerase II

Proper regulation of the initiation of DNA replication is crucial for cell division in eukaryotes. The first step of initiation is the selection of origins by the prereplicative complex (pre-RC), composed of the six-subunit origin recognition complex (ORC), Cdc6, Cdt1, and MCM2–7 (1). Although these protein factors are highly conserved, the DNA sequences that define origin activity in different organisms are not (2). With recent advances in DNA microarray technology, genome-wide mapping of replication origins has begun to establish the spatial and temporal program of replication initiation (3). However, the mechanisms of origin selection, especially in response to developmental cues in metazoans, remain poorly understood. Only a handful of model metazoan replication origins have been studied in detail (2, 4). Furthermore, few observations of cell-type specific or developmental regulation of replication origins have been reported (5, 6).

Developmental gene amplification in the ovarian follicle cells of Drosophila provides a powerful system for the analysis of metazoan DNA replication and developmental regulation of origin firing (7, 8). Amplification occurs by repeated rounds of origin firing and bidirectional movement of replication forks from these origins to produce 100 kb gradients of amplified DNA (7). This process depends on the same replication initiation and elongation proteins necessary for genomic replication (7, 8). P-element mediated transformation experiments, facilitated by the use of insulators to buffer transposons from chromosomal position effects (9), have allowed dissection of cis regulatory elements required for amplification. In the well characterized third chromosome chorion amplicon, DAFC-66D, both an origin of replication (oriB) and a replication enhancer (ACE3) have been defined [for review see (8)]. ACE3 stimulates replication from proximal origins and provides the developmental specificity for amplification by acting to load ORC, which appears to localize broadly across the amplicon, rather than strictly to the origin. ORC activity and origin firing at DAFC-66D are regulated by the transcription factors E2F1/RBF (10, 11) and the Myb protein complex (12, 13).

A newly identified amplicon, DAFC-62D, differs in its developmental timing from the other DAFCs (Fig. 1A), providing the opportunity to decipher how origin firing is influenced by differentiation events (14). The most abundantly amplified amplicon, DAFC-66D, undergoes approximately five rounds of origin activation, restricted to stages 10B and 11, to give an amplification level of 30–40 fold at the origin (15). There are no initiation events during subsequent stages of follicle cell development, but only the elongation of previously formed replication forks continues (Fig. 1A). At DAFC-62D, amplification initiates only once in stage 10B, but in stage 13 there is an additional increase in copy number at a very precise region (14). We, therefore, investigated mechanisms that control the unique timing of DAFC-62D origin activation.

Results and Discussion

Identification of the Replication Origin and Pre-RC Binding Sites in DAFC-62D. To determine the site at which DNA synthesis initiates during amplification at DAFC-62D, nascent strand analysis was performed as described (16, 17) on replicative intermediates isolated from stage 10B or 13 egg chambers. We observed a 1 kb region that was highly enriched in the 0.5–1 kb (Fig. 1B) and 1–1.6 kb (data not shown) fractions of nascent DNA, thus containing origin activity in both stage 10B and 13. We have designated this region as ori62. As a control for the α-exonuclease digestion and uniform efficiency of PCR, DNA of size 5 kb and above that was not expected to contain nascent strands displayed uniformly low levels across DAFC-62D (Fig. 1B). As a positive control, we found that the known origin oriB of DAFC-66D was enriched in the 0.5–1 kb fraction ~14-fold over a locus 5 kb away (data not shown).

We used quantitative chromatin immunoprecipitation (ChIP) with antibodies against the ORC2 subunit (18) to test whether ORC was present at ori62 and/or additional sequences, quantifying the amounts present by real-time PCR. ORC2 has been localized to DAFCs by immunofluorescence (10, 15) and also shown to be necessary for amplification, because a hypomorphic, female-sterile mutation in the Drosophila orc2 gene causes reduced levels of amplification (19). In DAFC-62D, we detected significant localization of ORC to ori62 by ChIP and real-time PCR quantification (Fig. 1D). In contrast to ACE3 (Fig. 1C), ORC binding remained present in stages 12 and 13 at ori62, paralleling the fact that an additional round of amplification takes place at DAFC-62D in stage 13 (14).

In addition to ori62, ChIP on stage 10A, 12, and 13 egg
chamber DNA showed localization of ORC approximately 3 kb away (~3.0) from ori62 (Fig. 1D). ORC also localized 3.5 kb away on the opposite side of ori62 (+3.5), but only in stage 10A (Fig. 1D). Therefore, ORC differentially localized to three zones at DAFC-62D, remaining associated with two of them (ori62 and ~3.0) from stage 10A on (Fig. 2A).

We also observed by ChIP that the MCM complex was broadly localized around ori62 in stage 10A (Fig. 1F), reflecting its dual role in replication initiation and elongation. In stage 12, MCM2–7 disassociated from the origin (Fig. 1F), although ORC remained bound (Fig. 1D). Strikingly, the MCM complex was reloaded to ori62 and ~3.0 in stage 13 (Fig. 1F). In contrast, at DAFC-66D, MCM2–7 associated with ACE3 and oriβ in stage 10A but not afterward (Fig. 1E), paralleling the binding pattern of ORC (Fig. 1C). This result suggested that at DAFC-62D there was developmentally regulated pre-RC binding that used different cis-acting elements to direct origin firing in different stages [Fig. 2A and supporting information (SI) Fig. S1]. The differential control of pre-RC binding that we observed may be due to specification of cis elements and/or trans factors such as transcription proteins that could affect ORC binding (10, 13).

ORC-Binding Sequences Are Required for Amplification. We used P-element mediated transformation to test the function of the cis elements that associated with the pre-RC in vivo, exploiting the Suppressor of Hairy-wing binding sites (SHWBS) insulator to protect the transposon from inhibitory position effects (9) (Fig. 2B). We first tested ori62 alone, but found that in two of two transformation lines, the transposons did not amplify (Fig. 2D), indicating the requirement for additional sequences such as enhancer-like ACE3 elements.

The amplification properties of a 10 kb fragment spanning the maximally amplified region of DAFC-62D were tested in P-element transformation lines by FISH/BrdU double labeling and real-time PCR quantification (Fig. 2B–D). In three of three transformation lines tested, the 10 kb fragment amplified at the same levels and developmental times as the endogenous ampiclon.

We tested whether the ORC binding zones were required for amplification and found that multiple elements were essential. When either ori62 (origin) or ~3.0 (control element) was deleted from the 10 kb transposon, the remaining sequences did not support detectable amplification, as demonstrated by real-time PCR quantification (Fig. 2C).
amplified region (data not shown). This origin localization is striking, contrasting with the fact that both $ACE3$ and $ori\beta$ are intergenic and upstream of chorion genes (21–23), and, thus, we determined when the yg2 gene is transcribed relative to the two rounds of origin firing. Nascent yg2 transcripts were detected as a specific focus in the nucleus by RNA FISH (24, 25), and observed in a narrow time window of early stage 12 (Fig. S1A). Slightly later, cytoplasmic yg2 message began to accumulate, and nuclear staining became undetectable (data not shown). Thus, yg2 transcription occurs in stage 12, between the two rounds of amplification origin firing.

$\alpha$-Amanitin Specifically Inhibits DAFC-62D Stage 13 Amplification. To investigate potential functional links between transcription and amplification at DAFC-62D, we used $\alpha$-amanitin, an RNAPII inhibitor (26), to block RNAPII elongation. Dissected ovaries were incubated in $\alpha$-amanitin and allowed to develop in vitro for 5 h, the time window that spans stage 10B through 13 under physiological conditions (Fig. 1A) (11). The toxin did not affect the developmental programs in general, because the relative abundance of each developmental stage was not significantly changed, and there was apparent progression in development compared with dissected egg chambers that did not undergo in vitro culturing (Fig. S2). Such treatment strongly diminished mRNA signals of the chorion gene $Cp38$ detected by in situ hybridization experiments (data not shown) and completely eliminated the stage 12 FISH spot of nascent yg2 transcripts (Fig. 3F). It also affected the nuclear distribution of RNAPII (SI Text and Fig. S1).

We used real-time PCR to measure quantitatively the effects of $\alpha$-amanitin and found that the stage 13 round of initiation at DAFC-62D was specifically inhibited (Fig. 3C), whereas initiation in stage 10B was unchanged (Fig. 3B). The effect of blocking transcription elongation was specific for the late firing at DAFC-62D. The treatment did not change the cumulative amplification levels of DAFC-66D in stage 13 (Fig. 3A). Furthermore, origin firing in stage 12 at a newly identified amplicon was not affected by $\alpha$-amanitin (J. Kim, F. Xie, and T. Orr-Weaver, unpublished results). These results suggested that transcription was required specifically for origin activation in stage 13 at DAFC-62D.

We examined the effect of $\alpha$-amanitin on DAFC-62D transposons as a further test of whether the requirement for transcription could be a cis effect rather than the need to transcribe a gene whose product is required for initiation. Unexpectedly, we observed that three independent transposon insertions carrying the 10 kb fragment from DAFC-62D underwent a normal round of amplification in stage 13 in the presence of the toxin (Fig. 3D). This result indicated strongly that the inhibition of amplification at DAFC-62D was not due to a general block of all amplification initiation in stage 13 imposed by $\alpha$-amanitin, but rather revealed a specific role of transcription for replication at the endogenous DAFC-62D site.

Because all transposons were buffered from position effects by SHWBS, we investigated whether the presence of insulators made amplification of these transposons independent of transcription and, therefore, resistant to $\alpha$-amanitin. SHWBS recruits the Su(Hw) (Suppressor of Hairy-wing) (27, 28) and additional proteins to form insulator bodies that are not influenced by either positive or negative position effects (29). The $su(Hw)$ allele combination eliminates insulator activity (30) and can reduce the amplification level of transposons buffered by SHWBS, if they are inserted at sites subject to negative position effects (9). Transposons containing the 10 kb DAFC-62D fragment were crossed into the $su(Hw)^{y}$ background, and two independent transformation lines displayed proper transposon amplification as determined by real-time PCR analyses (Fig. 3E), most likely because their insertion sites were permissive for amplification. One line failed to amplify...
in this background (data not shown). Strikingly, in the absence of Su(Hw) insulator function, both transposons became sensitive to α-amanitin, and the stage 13 round of amplification was specifically inhibited (Fig. 3E).

We also analyzed transcription of the yg2 gene on the transposon by RNA FISH. The ectopic copy of yg2 carried by the transposon was actively transcribed with proper developmental timing, as shown by the appearance of an additional locus of yg2 nascent transcripts in stage 12 (Fig. 3F). After α-amanitin treatment, only one spot of yg2 transcripts was detectable, presumably from the transposon, because endogenous transcription of yg2 was completely abolished by α-amanitin in non-transformants (Fig. 3F). When the Su(Hw) protein was mutated, transcription in the transposon was blocked by α-amanitin (Fig. 3F). These experiments show that neither transcription nor amplification of transposons was inhibited by α-amanitin when buffered by insulators.

The strict correlation between transcription elongation through yg2 and origin firing in stage 13 raises the possibility that RNAPII elongation is required in cis for replication initiation. The alternative is that α-amanitin blocks the transcription of a gene with a product that is essential for stage 13 origin firing at DAFC-62D, but not other amplicons. In addition, such a factor would have to be dispensable for activation of ori-62D when present on an insulated transposon.

It remains to be determined how RNAPII elongation within an insulated domain occurs even in the presence of α-amanitin. Because functional Su(Hw) protein is required for resistance to α-amanitin, it is likely a consequence of a unique chromatin structure within the “insulator bodies” (29). The inhibition or slowing down of RNAPII by α-amanitin (31) may be minimized by the chromatin environment to allow transcription of yg2 and the following round of amplification in the presence of the toxin.

Inhibition of Transcription Affects MCM2–7 Localization. ChIP analysis of RNAPII confirmed that α-amanitin treatment affected its distribution across DAFC-62D. In untreated follicle cells, RNA-PPII localized upstream of yg2, and after stage 10, also appeared at ori62 within the coding region of yg2 (Fig. 4A and Fig. S4A). The toxin prevented this redistribution into ori62 from stage 10 to 13, consistent with the finding that it blocks translocation/elongation of RNAPII across yg2 (Fig. 4A and Fig. S4A). To investigate the mechanisms by which RNAPII transcription could affect replication, we also analyzed the association of pre-RC components with DAFC-62D in the presence of α-amanitin. The binding of ORC2 in stage 10A through 13 was unchanged by the treatment (Fig. S3). The loading of MCM2–7 in stage 13, however, was completely abrogated by α-amanitin, whereas it was unaffected in stage 10 (Fig. 4B and Fig. S4A). We next performed sequential ChIP against MCM2–7 and RNAPII to examine whether they co-occupy the same sheared DNA fragments. In stage 13, αMCM2–7 immunocomplexes containing MCM-binding DNA were specifically pulled down by αRNAPII antibody, because in the final ChIP products, there was enrichment of ori62 and +3.5 (Fig. 4C and Fig. S4B). The αMCM2–7 supernatant did not contain any RNAPII-bound...
conclusions

DAFC-62D differs from other DAFCs by undergoing a round of amplification late in follicle cell differentiation, and this difference involves developmental control of ORC and MCM binding (Fig. S5). The late round of origin activation at DAFC-62D in stage 13 follicle cells contrasts with the other initiation events in stage 10B in that it takes place at least four hours after the cessation of previous genomic replication. This developmental delay may have created a quiescent (or even inhibitory) state of replication activation in stages 11 and 12 that has to be overcome by unique mechanisms. The analyses of DAFC-62D and ‒66D demonstrate that there are distinct mechanisms that differentially regulate amplification origins during Drosophila follicle cell development. Our findings reveal pathways to control localization of replication factors, license origins, and activate DNA replication, which provide a conceptual framework for defining how origin selection and activation are regulated by transcription in metazoan development.

Materials and Methods

Plasmid and Transformation Line Construction. All transposon constructs were individually injected into yw embryos to establish at least three independent homozygous transformation lines per each construct. At least two lines per each construct were analyzed for amplification level by real-time PCR (see below). Primers targeted transposon-specific sequences to distinguish between the endogenous DAFC-62D and the heterologous transposons. Primer sequences are available upon request. To examine the effects of Sub(Hw), transposons on either the X or 2nd chromosome were crossed into y¹sc¹w¹‡c¹‡b¹ (or 2nd chromosome), and Fig. S4B). The early round of origin activation at DAFC-62D contrasts with the other initiation events in stage 13 follicle cells (Fig. S5). The late round of origin activation at DAFC-62D in stage 13 follicle cells contrasts with the other initiation events in stage 10B in that it takes place at least four hours after the cessation of previous genomic replication. This developmental delay may have created a quiescent (or even inhibitory) state of replication activation in stages 11 and 12 that has to be overcome by unique mechanisms. The analyses of DAFC-62D and ‒66D demonstrate that there are distinct mechanisms that differentially regulate amplification origins during Drosophila follicle cell development. Our findings reveal pathways to control localization of replication factors, license origins, and activate DNA replication, which provide a conceptual framework for defining how origin selection and activation are regulated by transcription in metazoan development.

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flies and incubated in vitro in 333 μg/ml α-amanitin for 5 h at room temperature as described (11). Dissection in unsupplemented Grace’s media is important for successful in vitro development of egg chambers. Egg chambers were dissected immediately after incubation and subjected to real-time PCR analysis for amplification level in each stage. For immunofluorescence and ChIP experiments, ovaries were washed and formaldehyde fixed right after α-amanitin treatment.

Supporting Information

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**SI Text**

**Effect of α-Amanitin Treatment on RNAPII Localization.** We used antibodies against RNA polymerase II (RNAPII) to further visualize transcription during follicle cell differentiation and to examine the localization of RNAPII during amplification. In a *Sciara coprophila* amlicon, the right boundary of the initiation zone is determined by the binding of RNAPII (1), making it possible that occupancy by RNAPII affects DAFC-62D amplification. An antibody that recognizes both the phospho and nonphospho carboxyl-terminal domain of RNAPII, CTD4H8 (Upstate), stained subnuclear foci in *Drosophila* follicle cells, but RNAPII/Brdu double labeling indicated that RNAPII foci did not overlap significantly with amlicons in stage 10B [supporting information (SI) Fig. S1B]. From stage 11 on, the Brdu signal at DAFC-62D became less visible (Fig. S1B). Therefore, we used FISH/RNAPII double immunofluorescence to detect the DAFC-62D DNA. Although no colocalization between RNAPII and DAFC-62D was observed in stages 10B, 11, or 13 (Fig. S1C), in stage 12, one of the RNAPII foci clearly colocalized with DAFC-62D (Fig. S1C).

The immunostaining pattern of Brdu and RNAPII was not affected by α-amanitin in stage 10B (Fig. S1D, leftmost image). Because stage 10 is ten hours long and some egg chambers may have been in stage 10 for multiple hours before the treatment, it was possible that the 5-hour incubation was not sufficient to induce detectable defects during this developmental stage. In subsequent stages (which in total last less than four hours), RNAPII lost its concentration into subnuclear foci and showed more uniform nuclear staining (Fig. S1D). These foci of RNAPII and their elimination by α-amanitin treatment suggest that during these stages of follicle cell differentiation, transcription was localized to specific nuclear regions.

After α-amanitin treatment, the punctuate pattern of Brdu incorporation at the largest chorion amlicons remained in stages 12 and 13 (when the Brdu signal for DAFC-62D is often too small to visualize) (Fig. S1D), consistent with the absence of an effect on DAFC-66D observed by quantitative PCR analysis.

**Plasmid Construction.** To construct the transposons with the 1 kb ori62 and 10 kb central amplified region, these DNA intervals were PCR amplified from BACR22J16 using PuTuRho DNA polymerase (Stratagene), blunt-ligated into pCR-Blunt vector (Stratagene) to construct pCRBΔ-3.0, pCRB-3.0, pCRB +3.5, and pCRBΔ+3.5, respectively. The +2.0 to +6.0 fragment was isolated by NotI digestion and subcloned into pCRB +3.5 to generate pCRBΔori62. Δ-3.0, Δori62, and Δ+3.5 were excised and substituted for the NheI fragment in the ori62 transposon to generate FXA-3.0, FXΔori62, and FXΔ+3.5.

**Antibodies, Immunofluorescence, and Confocal Microscopy.** The anti-ORC2 antibodies were previously described and were obtained from Stephen Bell (3). The anti-MCM2–7 monoclonal antibody was a gift from Stephen Bell (4). The anti-RNAPII antibody (Upstate, clone CTD4H8) was used at a 1:250 dilution in double immunostaining with Brdu as described (3), with the following modifications: secondary detection of RNAPII was with rhodamine-RedX conjugated donkey anti-mouse at 1:100; rabbit anti-Brdu antiserum (Accurate Chemical) was used at 1:50; and secondary detection of Brdu was with FITC-conjugated donkey anti-rabbit at 1:100.

All images were collected on a Zeiss Axivert 100M Meta confocal microscope with LSM51 Software. A ×63 Plan Aprochromat objective was used to capture the images in Fig. 3D and a ×100 Plan Aprochromat objective was used for all others.

**Fluorescent in Situ Hybridization.** DNA FISH and Brdu double labeling was performed as described (5). The probe was prepared from the 10-kb central amplified region previously PCR cloned from BACR22J16, and 300 ng was used in a 40-μl hybridization reaction.

**RNA FISH.** To detect RNA signals by FISH (6), ovaries were dissected in nonsupplemented Grace’s medium (GIBCO-BRL) with 10 mM vanadyl ribonucleoside complex (VRC, Invitrogen) to prevent RNA degradation. Formaldehyde fixation, formamide equilibration, and prehybridization steps for RNA FISH were essentially the same as in DNA FISH except that DEPC-treated dH2O and deionized formamide (Sigma) were used whenever applicable. The probe was prepared from yg2 cDNA using the Invitrogen BioNick Labeling Kit. 100 ng of digoxigenin (DIG) labeled probe was denatured at 80°C in formamide for 10 min together with 10 μg of sonicated salmon sperm DNA, and hybridized to prehybridized egg chambers at 37°C overnight in 40 μl of buffer containing 50% formamide, 10% dextran sulfate (Sigma), 0.2% BSA (Sigma), 20 mM VRC, and 2× SSCT. Secondary detection was with goat anti-DIG FITC at 1:200 (Enzo). Samples were mounted in Vectashield (Vector Labs).

Fig. S1. Temporal and spatial correlation of transcription and amplification. (A) The position of the yellow-g2 (yg2) gene in relation to the pre-RC binding sites is shown in the diagram. In the lower images, yg2 RNA FISH detected strong nascent transcripts in early stage 12 follicle cells. Nuclei are circled. (B) BrdU (green) did not co-label significantly with RNAPII (red). The major BrdU foci corresponded to DAFC-66D and /H110027F. (C) DNA FISH to DAFC-62D (green) colocalized with RNAPII (red) in stage 12 but not in other stages. (C’) Close up of the stage 12 colocalization between 62D FISH (green) and RNAPII (red). A single follicle cell nucleus is circled. (D) BrdU (green) and RNAPII (red) immunofluorescence in egg chambers cultured with α-amanitin, in the indicated stages.
Fig. S2. Follicle cell development was not affected by α-amanitin. After 5 h of incubation in 333 μg/ml α-amanitin or medium alone, whole ovaries were dissected, and the percentage of egg chambers in each developmental stage (from stage 9 to 13, ~500 egg chambers in total) was determined. Results of three independent experiments are shown. Student’s t test shows no significant difference (P ≥ 0.97). Error bars represent standard errors. White bars represent similarly dissected ovaries that were not cultured in vitro. Higher percentage of stage 10 and lower stage 14 egg chambers in these in vivo samples relative to cultured ones indicate progression of development.
Fig. S3. ORC2 localization is not affected by α-amanitin. Real-time PCR analyses of anti-ORC2 ChIP across DAFC-62 in stage 10A-B and stage 13 showed insignificant changes in ORC association pattern or level with α-amanitin treatment. Error bars are standard deviations of triplicate PCRs.
(A) Single ChIP (anti-RNAPII or MCM2–7) across DAFC-62D. Enrichment level of ChIP DNA within the central 20 kb region of DAFC-62D is shown, with or without α-amanitin treatment. In stage 10, neither RNAPII nor MCM2–7 localization was affected by α-amanitin; RNAPII elongation was inhibited by the toxin, as was the recruitment of MCM2–7 in stage 13. (B) Sequential ChIP (anti-RNAPII and MCM2–7) across DAFC-62D. Enrichment level of ChIP DNA within the central 10 kb region of DAFC-62D is shown, in either order of antibody pull-down. RNAPII and MCM2–7 specifically associated with the same chromatin pieces in stage 13. (C) Size of sheared DNA for ChIP. 50 bp DNA ladders were used, and their sizes are shown on the right. The majority of sheared DNA was within the range of 100–500 bp.
Coordination of replication initiation and transcription. Developmental timing of origin firing events and transcription at DAFC-66D and DAFC-62D, as well as differential binding of ORC and the MCM complex at the origins. Pre-RC (possibly at higher amounts to support more rounds of firing) only associates with DAFC-66D in early stages. At DAFC-62D, ORC remains localized through stage 13, whereas the MCM complex disassociates after the first round of origin firing and is reloaded in stage 13 for the late round of initiation. This latter firing requires transcription by RNAPII, because it is inhibited by α-amanitin. One candidate mechanism is direct interaction and recruitment of MCM2–7 by RNAPII, given their co-occupation of the same small blocks of sequences.