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The Role of Transcription in the Activation of a Drosophila Amplification Origin

Brian L. Hua,* † Sharon Li,* and Terry L. Orr-Weaver* † †
*Whitehead Institute and †Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02142
ORCID ID: 0000-0002-7580-3399 (B.L.H.)

ABSTRACT The mechanisms that underlie metazoan DNA replication initiation, especially the connection between transcription and replication origin activation, are not well understood. To probe the role of transcription in origin activation, we exploited a specific replication origin in Drosophila melanogaster follicle cells, ori62, which coincides with the yellow-g2 transcription unit and exhibits transcription-dependent origin firing. Within a 10-kb genomic fragment that contains ori62 and is sufficient for amplification, RNA-sequencing analysis revealed that all detected RNAs mapped solely to the yellow-g2 gene. To determine whether transcription is required in cis for ori62 firing, we generated a set of tagged yellow-g2 transgenes in which we could prevent local transcription across ori62 by deletions in the yellow-g2 promoter. Surprisingly, inhibition of yellow-g2 transcription by promoter deletions did not affect ori62 firing. Our results reveal that transcription in cis is not required for ori62 firing, raising the possibility that a trans-acting factor is required specifically for the activation of ori62. This finding illustrates that a diversity of mechanisms can be used in the regulation of metazoan DNA replication initiation.

DNA replication initiation occurs at specific genomic sites called origins of replication, and proper activation of these origins is essential for the precise duplication of the genome in dividing cells. In eukaryotes, replication initiation first requires the loading of the minichromosome maintenance (MCM)2-7 replicative helicase complex to origins of replication through the cooperative activities of the origin recognition complex (ORC) and the replication initiation factors Cdt1 and Cdc6 (Costa et al. 2013). In metazoans, origins of replication are not defined by DNA sequence (Cvetic and Walter 2005), and only a relatively small number of metazoan origins have been characterized in detail. The genomic positions and temporal programs of metazoan replication origins have been determined within various cell types by genome-wide sequencing analysis (Gilbert 2010), but the mechanisms that regulate recruitment of replication initiation factors to origins of replication and how origins of replication are activated, especially in the context of development, remain poorly understood.

The Drosophila follicle cell gene amplification system provides a powerful model for the study of individual metazoan origins in vivo (Claycomb and Orr-Weaver 2005). Drosophila follicle cells are somatic cells that surround the developing oocyte in the egg chamber and secrete the components of the egg shell (Spradling 1993). During gene amplification, specific origins of replication at six distinct regions within the follicle cell genome (called Drosophila amplicons in follicle cells, or DAFCs) are repeatedly activated during follicle cell differentiation while whole-genome replication is shut off (Spradling and Mahowald 1980; Claycomb et al. 2004; Kim et al. 2011). Origin firing is followed by bidirectional fork progression, resulting in a gradient of about 100-kb of amplified DNA (Claycomb and Orr-Weaver 2005). Importantly, this gene-amplification process requires the same replication factors as used during the typical S phase (Tower 2004; Claycomb and Orr-Weaver 2005). Follicle cell gene amplification takes place during a relatively short developmental time period (7.5 hr) between stages 10B and 13 of egg chamber development. Moreover, origin firing during gene amplification is tightly coordinated with egg chamber development, allowing high temporal resolution of individual origin firing events (Claycomb et al. 2002). As we have begun to characterize individual amplification origins to delineate the parameters of origin activation, it has become apparent that metazoan origins...
of replication use a diversity of mechanisms to regulate origin firing (Orr-Weaver et al. 1989; Xie and Orr-Weaver 2008; Kim et al. 2011; Kim and Orr-Weaver 2011). In this study, we focus on the possible link between transcription and DNA replication initiation.

Genome-wide origin mapping studies reveal that most origins are influenced by an open chromatin structure and closely coincide with the transcription start sites of actively transcribed genes, suggesting a correlative link between transcription and DNA replication initiation (Lucas et al. 2007; Cadoret et al. 2008; Sequeira-Mendes et al. 2009; Hansen et al. 2010; Karnani et al. 2010; MacAlpine et al. 2010; Mesner et al. 2011; Dellino et al. 2013; Mesner et al. 2013). However, how local transcription affects origin activation remains largely unknown. Limited studies suggest that transcription may play an important local role at origins of replication in several metazoan systems, including specification of a replication origin by a local promoter element (Danis et al. 2004) and delineation of a replication origin boundary by a transcription- arrest sequence (Mesner and Hamlin 2005). In budding yeast, the transcription machinery itself plays important roles in DNA replication initiation. Yeast RNA polymerase II has been shown to anchor ORC to rDNA replication origins (Mayan 2013) and to directly interact with the MCM2-7 helicase complex (Holland et al. 2002; Gauthier et al. 2001). Taken together, these studies raise the possibility that transcription may play an important and direct role in metazoan origin activation.

Previously, we found that one specific amplicon, DAFC-62D, exhibits transcription-dependent origin activation (Xie and Orr-Weaver 2008). The DAFC-62D origin, ori62, undergoes one round of origin firing during stage 10B and a second round of firing during stage 13 of egg chamber development. ori62 maps completely within the transcriptional unit of the yellow-g2 gene, and yellow-g2 is transcribed in a strict and short developmental time window in stage 12, interspersing the two rounds of origin firing at DAFC-62D. The second round of origin firing is transcription-dependent, as addition of the RNA polymerase II inhibitor α-amanitin blocks stage 13 origin firing. Furthermore, transcription is required to localize the MCM2-7 helicase complex to ori62 to allow the second round of origin firing (Xie and Orr-Weaver 2008). The requirement of transcription is specific for DAFC-62D, because all other amplicon origins activate normally in the presence of α-amanitin (Figure 1). These findings suggested a model that transcription is cis-activating at DAFC-62D and is required locally to promote the second round of origin firing.

To test this model, we investigated the requirement of transcription specifically across ori62 for the second round of DAFC-62D origin firing. First, we identified the RNAs present in follicle cells from a 10- kb region of DAFC-62D shown to be sufficient for amplification at ectopic insertion sites. We found that yellow-g2 is the sole gene expressed in this minimal region. Therefore, we sought to delineate the role of local transcription in ori62 activation by inhibiting transcription across ori62 by yellow-g2 promoter deletion. Our findings reveal that transcription in cis is not required for stage 13 ori62 firing, suggesting the possibility of the requirement of a trans-acting factor for origin firing at DAFC-62D.

**MATERIALS AND METHODS**

**Generation of tagged genes**

All constructs used in this study were derived from the PCRA10kb plasmid, which contains the 10-kb genomic locus corresponding to the central amplified region in 62D previously described (Xie and Orr-Weaver 2008). To generate the tagged yellow-g2 transgene, an Apal/SacII fragment containing the full yellow-g2 coding sequence was subcloned into pBluescriptSK and a 21-bp tag sequence was inserted into the 3’ end of the yellow-g2 coding sequence by site-directed mutagenesis to generate pBS-Apal/SacII-tag. A BglII/PshAI fragment containing the tag was then liberated from this plasmid and used to replace the corresponding fragment in PCRA10kb to generate PCRA-10kb-tag. The NotI/AvrII fragment containing one Suppressor of Hairy-wing binding site (SHWBS) and the 10-kb locus was then liberated and cloned into the NotI and Nhel sites of the pcSpeR4-SHWBS P-element transformation vector to generate pcSpeR4-10kb-tag.

To generate the yellow-g2 promoter deletions, a NotI/Apal fragment containing a partial 5’ fragment of the yellow-g2 coding sequence and 2.5-kb of upstream sequence was subcloned into pBluescriptSK to generate pBS-NotI/Apal. Using site-directed mutagenesis, a 214-bp deletion was made to generate pBS-NotI/Apal-Δ214, and a 1226-bp deletion was made to generate pBS-NotI/Apal-Δ1226. The 214-bp deletion corresponds to the coordinates −120 to +94 relative to the transcription start site. The 1226-bp deletion corresponds to the coordinates −1132 to +94. The BbvCI/Apal fragment containing the promoter deletion was then liberated from each deletion construct to replace the corresponding fragment in PCRA-10kb-tag to generate PCRA-10kb-tag-214 and PCRA-10kb-tag-1226.

**Figure 1**  Left, Model of transcription-dependent origin activation of DAFC-62D. DAFC-62D exhibits two rounds of origin firing, the first at stage 10B and the second at stage 13 of egg chamber development, which are interspersed by transcription of the yellow-g2 gene. In the presence of the RNA polymerase II inhibitor α-amanitin, stage 13 origin firing is specifically blocked (Xie and Orr-Weaver 2008). Right, Transcription-dependent origin firing is unique to DAFC-62D, as exemplified by normal origin firing of the comparable DAFC-34B in the presence of α-amanitin (Kim and Orr-Weaver 2011).
The NotI/AvrII fragment containing one SHWB and the 10-kb locus was then liberated from each plasmid and cloned into the NotI and Nhel sites of the pCaSpeR4-SHWB P-element transformation vector to generate pCaSpeR4-Δ214 and pCaSpeR4-Δ1226.

Strains and transgenic lines
P-element transposon constructs were sent to BestGene Inc. (Chino Hills, CA) for individual injections into w1118 embryos to establish at least two independent transformation lines per construct. To examine the effects of the Suppressor of Hairy-wing (Su(Hw)) chromatin insulator, transposons on the 2nd chromosome were crossed into the su(Hw)^Sb~1/TM6, su(Hw)^5 background.

RNA isolation and RNA-seq
The RNA-seq analysis of total RNA extracted from purified 16C follicle cells has been described previously (Kim et al. 2011). In summary, RNA-seq libraries were generated using the mRNA-seq Sample Preparation kit from Illumina with the exception that RNAs were not poly (A)-selected. The RNA-seq library was size-selected for enrichment in the 200-nt range according to Illumina recommendation. This library was then subjected to Duplex-Specific Nuclease treatment to remove highly abundant RNAs such as rRNAs and tRNAs.

Transcription analysis
Total RNA was isolated from 30–50 stage 12 egg chambers using TRIzol reagent (Invitrogen). One microgram of total RNA was reverse transcribed using AMV reverse transcriptase (Promega) to generate single-stranded cDNA. Transgenic yellow-g2 transcript levels were determined by quantitative polymerase chain reaction (qPCR) using total cDNA samples and a primer set specific for the tag within the transgenic yellow-g2 transcript. In addition, a primer set that did not discriminate between the endogenous and transgenic yellow-g2 transcripts was used to measure the total amount of yellow-g2 transcript. Primer sequences are available on request. Each transcription analysis experiment was performed in biological duplicates.

Amplification assay
Genomic DNA was isolated from staged egg chambers and quantified using relative qPCR as described (Xie and Orr-Weaver 2008). A primer set specific for the transposon boundary was used to assess transposon amplification, and a primer set specific for the corresponding endogenous site was used to assess endogenous 62D amplification. Each DNA sample was internally normalized to the copy number of a nonamplified control locus at 93F. Primer sequences are available on request. Fold amplification at a given developmental stage was determined relative to preamplification stage 1–8 egg chamber DNA. Each amplification assay was performed in biological triplicates.

RESULTS AND DISCUSSION

yellow-g2 is the sole transcription unit in the 10-kb DAFC-62D amplicon
To examine the role of transcription in or62 firing, we first identified the RNAs within the 10-kb amplification-sufficient DAFC-62D region previously characterized (Xie and Orr-Weaver 2008). We isolated, sequenced, and mapped total, non-poly(A)-selected RNAs from 16C follicle cell nuclei, which are enriched for amplifying nuclei (Kim et al. 2011). Within this 10-kb region, nearly all RNAs mapped within the yellow-g2 gene. The reads that mapped outside of the yellow-g2 gene showed poor overlap in the two biological replicate experiments (Figure 2). We conclude that yellow-g2 is the sole gene expressed in this region. Because the RNA-seq libraries we analyzed were size-selected for templates in the 200-nt range, we cannot exclude the possibility that small RNAs such as microRNAs exist in this 10-kb region.

A tagged yellow-g2 transgene
To investigate the role of yellow-g2 transcription in or62 activation, we used P-element-mediated transformation to generate 10-kb DAFC-62D transposon lines in which we could modulate yellow-g2 transcription. The transposons were flanked by Suppressor of Hairy-wing (Su(Hw)) insulator binding sites (SHWB) to protect from inhibitory position effects (Figure 3A) (Lu and Tower 1997).

To distinguish between the transgenic and endogenous yellow-g2 gene, we inserted a short 21-bp tag at the 3’ end of the coding sequence of the yellow-g2 transgene. First, we assessed whether the insertion of this tag affected yellow-g2 transcription and transposon amplification. To this end, we generated a 10-kb DAFC-62D transposon that contained the full yellow-g2 locus and the inserted tag and produced transgenic lines (Figure 3B). We then assessed transcription of the yellow-g2 transgene relative to total yellow-g2 transcription by isolating RNA from stage 12 egg chambers and performing reverse transcription followed by qPCR. Insertion of the 21-bp tag did not inhibit transcription of the yellow-g2 transgene, as transgenic yellow-g2 transcript was detectable and comprised about half the total yellow-g2 transcript pool (Figure 3C). Next, genomic DNA was isolated from stage 1–8, 10B, and 13 egg chambers and genomic copy number of the transgene was measured by qPCR. Importantly, the tagged transgene amplified to normal levels and at the same developmental times as the endogenous DAFC-62D, as evidenced by twofold amplification in stage 10B egg chambers and 3- to 4-fold amplification in stage 13 egg chambers (Figure 3D). To confirm that the presence of the Su(Hw) insulator did not affect transgene transcription and amplification, we measured transcription and amplification of the yellow-g2 transgene in wild-type and Su(Hw) mutant backgrounds. We found that the yellow-g2 transgene was transcribed and amplified to comparable levels in both the wild-type and Su(Hw) mutant backgrounds (Figures 3, C and E, respectively). Taken together, insertion of the 21-bp tag into the yellow-g2 transgene did not affect transcription or amplification of transgene.

Transcription in cis is not required for or62 firing
To test whether transcription is required in cis for or62 firing, we made two deletions in the promoter of the yellow-g2 transgene to abrogate transcription across or62. We generated transposon lines harboring the 10-kb DAFC-62D region with either a 214-bp or 1226-bp deletion of the yellow-g2 promoter region (Figure 4A). Reverse-transcription qPCR analysis of RNA isolated from stage 12 egg chambers revealed that transcription of the yellow-g2 transgene was inhibited completely by both promoter deletions, as transgenic yellow-g2 transcript was not detected in either the 214-bp or the 1226-bp promoter deletion lines (Figure 4, B and C, respectively). Next, we isolated stage 1–8, 10B, and 13 egg chamber genomic DNA from each promoter deletion line to assess genomic copy number of the yellow-g2 transgene. Surprisingly, we found that stage 13 origin firing still occurred despite inhibition of yellow-g2 transcription across or62, exhibiting 3- to 4-fold amplification in both the 214bp promoter deletion line (Figure 4D) and the 1226-bp promoter deletion line (Figure 4E). We observed comparable results in the wild-type and Su(Hw) mutant backgrounds, confirming that our findings were not confounded by the presence of the Su(Hw) insulator. If transcription were required in cis for origin firing at stage 13, then inhibition of transcription of yellow-g2 should have inhibited stage 13 origin firing. Thus, from these results we conclude that transcription is not required in cis for origin activation at DAFC-62D.
How is it possible that DAFC-62D uniquely among the amplicons requires transcription for late stage origin firing, yet this transcription is not required in cis? We propose that transcription is required to produce a trans-acting factor that is required specifically for DAFC-62D origin firing in the developmental time window after stage 10B. Such an origin-specific, trans-acting regulatory factor would add to the growing list of mechanisms by which metazoan origins are regulated, emphasizing the idea that individual origins can exhibit unique and distinct mechanisms of regulation. The diversity in the molecular mechanisms that regulate metazoan origin firing is made evident by several Drosophila amplicons. These include a local replication enhancer that activates origin firing at DAFC-66D (Orr-Weaver et al. 1989), ORC-independent origin firing at DAFC-34B (Kim and Orr-Weaver 2011), and local repression of ORC binding and origin activity at DAFC-22B (Kim et al. 2011). Finally, origin-specific trans-acting regulators of origin activity could be cell-type specific, allowing an...
Figure 4  Characterization of the yellow-g2 promoter deletion transgenes. (A) Diagrams of the full-length, 214-bp promoter deletion, and 1226-bp promoter deletion yellow-g2 transgenes. The region deleted is shown in white. The 1226-bp deletion is not to scale. The extent of the full yellow-g2 promoter is not known.

Levels of transgenic yellow-g2 transcripts relative to total yellow-g2 transcripts isolated from stage 12 egg chambers were determined for the [DAFC-62D-\textsuperscript{214}] (B) and [DAFC-62D-\textsuperscript{1226}] (C) transgenic lines in either wild-type or su(Hw) mutant backgrounds. (D) Fold amplification of the endogenous 62D locus (62D Endo) and the [DAFC-62D-\textsuperscript{214}] transposon in the wild-type and the su(Hw) mutant backgrounds. (E) Fold amplification of the endogenous 62D locus (62D Endo) and the [DAFC-62D-\textsuperscript{1226}] transposon in the wild-type and the su(Hw) mutant backgrounds. Error bars represent standard deviation of the mean of the biological triplicates.
additional level of regulation of origin activation. Thus, identification of this trans-acting factor required at DAFC-62D and its mechanism of action will prove extremely valuable in our understanding of metazoan DNA replication initiation regulation.

Our results provide perspective not only on the widespread plasticity in mechanisms that control metazoan origin activation but also on the implications of replication initiation errors in genome instability and cancer progression. Misregulation of origin activation can lead to genome instability due to unreplicated or amplified chromosomal regions (Abbas et al., 2013), and genomic studies highlight the frequency of copy number changes in cancer cells (Beroukhim et al. 2010). Thus, it is crucial not only to understand at the molecular level the range of mechanisms employed to regulate origin activation in metazoan cells but also to understand to what extent these mechanisms present vulnerabilities in genome stability.

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LITERATURE CITED


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