Sequence requirements for function of the *Drosophila* chorion gene locus ACE3 replicator and ori-β origin elements

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Summary

The developmentally regulated amplification of the *Drosophila* third chromosome chorion gene locus requires multiple chromosomal elements. Amplification control element third chromosome (ACE3) appears to function as a replicator, in that it is required in cis for the activity of nearby DNA replication origin(s). Ori-β is the major origin in the locus, and is a sequence-specific element that is sufficient for high-level amplification in combination with ACE3. Sequence requirements for amplification were examined using a transgenic construct that was buffered from chromosomal position effects by flanking insulator elements. The parent construct supported 18- to 20-fold amplification, and contained the 320 bp ACE3, the ~1.2 kb *S18* chorion gene and the 840 bp ori-β. Deletion mapping of ACE3 revealed that an evolutionarily conserved 142 bp core sequence functions in amplification in this context. Several deletions had quantitative effects, suggesting that multiple, partially redundant elements comprise ACE3. *S. cerevisiae* ARS1 origin sequences could not substitute for ori-β, thereby confirming the sequence specificity of ori-β. Deletion mapping of ori-β identified two required components: a 140 bp 5′ element and a 226 bp A/T-rich 3′ element called the β-region that has significant homology to ACE3. Antibody to the origin recognition complex subunit 2 (Orc2) eliminated the ORC2 foci. By contrast, with a null mutation of *chiffon (dbf4-like)* that eliminates amplification, diffuse ORC2 staining was still present, but failed to localize into foci. The data suggest a novel function for the Dbf4-like chiffon protein in ORC localization. Chromosomal position effects that eliminated amplification of transgenic constructs also eliminated foci formation. However, use of the buffered vector allowed amplification of transgenic constructs to occur in the absence of detectable foci formation. Taken together, the data suggest a model in which ACE3 and ori-β nucleate the formation of a ORC2-containing chromatin structure that spreads along the chromosome in a mechanism dependent upon chiffon.

Key words: Dbf4, DNA replication, Chiffon, ORC, Amplification, *Drosophila*

Introduction

*Drosophila* chorion gene amplification provides a genetically tractable model for the study of metazoan DNA replication (Calvi and Spradling, 1999; Royzman and Orr-Weaver, 1998). In addition to providing entrée to the basic DNA replication machinery, amplification represents a dramatic example of how DNA replication can be regulated in response to the developmental program. The *Drosophila* oocyte is surrounded by a somatically derived epithelium called the follicle cells. The follicle cells lay down protective coverings around the oocyte, including the proteinaceous eggshell, or chorion. The genes encoding the major chorion proteins are arranged in two clusters in the genome, one on the X chromosome and one on the third. To facilitate the rapid production of copious protein, the follicle cells amplify the chorion gene clusters up to ~60 fold, thereby providing an increased copy number of chorion gene templates for transcription. Amplification occurs through the repeated firing of DNA replication origins located within each chorion gene cluster. Bi-directional replication forks proceed unusually slowly away from the origins, creating an onionskin-like DNA structure (Claycomb et al., 2002; Osheim et al., 1998; Spradling and Leys, 1988). After synthesizing the chorion, the follicle cells are destroyed prior to egg-laying, and therefore the mitotic apparatus needs never deal with this unusual DNA onionskin.

If amplification is prevented by a trans-acting mutation or a chromosomal rearrangement that moves the origin away from the gene cluster, the resultant under-production of chorion proteins causes female sterility characterized by thin eggshells and nonviable eggs (Orr et al., 1984; Spradling and Mahowald, 1981). This thin eggshell/female sterile phenotype has allowed for identification of numerous trans-acting genes that regulate amplification. The first of these trans-acting genes to be cloned, *k43*, was found to encode the second largest subunit of the origin recognition complex (ORC) (Gossen et al., 1995; Landis et al., 1997). The six-protein ORC was originally discovered in yeast and is required for DNA replication and other aspects of chromosome function in all eukaryotes (Beall et al., 2002; Bell, 2002; Chesnokov et al., 1999). ORC binds at origins and is required for recruitment of numerous additional proteins that
insulators can block the interaction of enhancers with promoters when they are placed in between, and can also inhibit amplification, with the majority of initiations occurring near the β region. While no initiations could be detected at ACE3. Taken together, the data suggest that ACE3 functions as a ‘replicator’ and interacts with ori-β in cis to allow DNA replication initiation at ori-β. The buffered vector system was used to analyze the sequence requirements for ACE3 and ORC function in greater detail, including the formation of ORC2 foci.

Materials and methods

DNA constructs

All ACE3 and ori-β mutation constructs are derivatives of the ‘Big Parent’ (BP) construct (Fig. 2A) (Lu et al., 2001). The 320 bp ACE3 and 840 bp ori-β in the BP construct were used as the templates for all PCR reactions.

**pACE3mt-1**

pBS*K is a derivative of pBluescript KS+ (Stratagene) in which the KpnI site has been destroyed. A 2.8 kb fragment containing one su(Hw)BS, ACE3, S18 and ori-β was liberated from the BP construct by digestion with NotI and Xhol. The fragment was cloned into the NotI to Xhol sites of pBS*K to generate pBS*K-2.8. A subregion of the 320 bp ACE3 (ACE3mt-1) was amplified by PCR using primer set 1, digested at the KpnI and BamHI sites engineered into the primers, cloned into the KpnI to BamHI sites of pBS*K-2.8 to create an intermediate construct. Then a fragment containing su(Hw)BS, ACE3mt-1, S18 and ori-β was liberated from the intermediate construct by digestion with NotI and Xhol, and cloned into the NotI to Xhol sites of the BP construct to generate pACE3mt-1.

**pACE3mt-2, pACE3mt-3, pACE3mt-4 and pACE3mt-5**

Subregions of the 320 bp ACE3 fragment in BP construct were amplified by PCR using primer sets 2, 3, 4 and 5, respectively. The PCR products were digested at KpnI and BamHI sites engineered into the primers, and subcloned into pBS*K-2.8 to create intermediate constructs. Then a fragment containing su(Hw)BS, ACE3mt, S18 and ori-β was liberated from the intermediate construct by digestion with NotI and Xhol, and cloned into the NotI to Xhol sites of the BP construct to generate the final constructs.

**pOrIf** mt-1 through **pOrIf** mt-9

The 840 bp ori-β fragment in the BP construct was amplified by PCR using primer sets 6-14, respectively. The PCR products were digested at the BglII and Xhol sites engineered into the primers, and then cloned into the BglII to Xhol sites of the BP constructs to create the series of ori-β deletions (Fig. 4, Fig. 5).

**pBP-ARS1**

A plasmid called pARS/WTA was provided by Bruce Stillman (Marahrens and Stillman, 1992). The wild-type yeast ARS1 sequence from 737 bp to 926 bp was amplified by PCR using primer set 15, digested at the BamHI and Xhol sites engineered into the primers, and then cloned into the BglII to Xhol sites of the BP construct.

**pBP-B2**

The set 16 oligonucleotides represent the two DNA strands of the B2 element of yeast ARS1 from 793 bp to 812 bp plus added BglII...
and XhoI half-sites. The oligos were annealed and the fragment cloned into the BglII to XhoI sites in the BP construct to generate pBP-B2.

pMini-1
This construct contains one ACE3 mt-3 and one ori-β mt-2 with no intervening sequences. The 840 bp ori-β fragment in the BP construct was amplified by PCR using primer set 7, digested at BglII and XhoI sites engineered into the primers, and cloned into the BglII to XhoI sites in pBS*K-2.8 (described above) to generate pBS*K-2.6. The 320 bp ACE3 fragment in the BP construct was amplified by PCR using primer set 3, digested at KpnI and BamHI sites, and cloned into the KpnI to BglII sites in pBS*K-2.6. A fragment containing one ACE3 mt-3 and one ori-β mt-2 was liberated by digestion with NotI and XhoI, and cloned into the NotI to XhoI sites in the BP construct to generate pMini-1.

pMini-2
This construct contains three copies of ACE3 mt-3 and one ori-β mt-2 without the intervening sequences. The 320 bp ACE3 fragment in the BP construct was amplified by PCR using primer sets 17, 18 and 19, respectively. The PCR products were digested with KpnI and EcoRI, EcoRI and Nhel, and Nhel and BamHI, respectively. The three fragments were cloned into the KpnI to BglII sites in pBS*K-2.6 (described above) in the same reaction. A fragment containing three ACE3 mt-3 and one ori-β mt-2 was then liberated by digestion with NotI and XhoI, and cloned into the NotI to XhoI sites in the BP construct to generate pMini-2.

pMini-C
This construct contains one ori-β mt-2 without ACE3 and the intervening sequences. pBS*K-2.6 (described above) was digested with KpnI and BglII. The larger fragment containing su(Hw)I/BS, plasmid vector and ori-β mt-2 was end-filled by T4 DNA polymerase, and then ligated to circularize. A fragment containing one ori-β mt-2 and su(Hw)I/Bs was liberated by digestion with NotI and XhoI, and cloned into the NotI to XhoI sites in the BP construct to generate pMini-C.

Primer sequences

| SET1: 5’ AGCTGGTACC KpnI CTGAGCCTGGCAACACTAA 3’ | 3’ AGCTGGATCC BamHI GAGCTGGACACCGATTTTCG 3’ |
| SET2: 5’ AGCTGGTACC KpnI GAAAGTGGAACGGTTGTGTTTA 3’ | 3’ AGCTGGATCC BamHI GCAATGGTTCACGATTTTCCG 3’ |
| SET3: 5’ AGCTGGTACC KpnI GAAAGTGGAACGGTTGTGTTTA 3’ | 3’ AGCTGGATCC BamHI GAGCTGGACACCGATTTTCCG 3’ |
| SET4: 5’ AGCTGGATCC KpnI GAAAGTGGAACGGTTGTGTTTA 3’ | 3’ AGCTGGATCC BamHI GCAATGGTTCACGATTTTCCG 3’ |
| SET5: 5’ AGCTGGTACC KpnI CTACCAAGCGCAAGTATTTCTT 3’ | 5’ AGCTGGATCC BamHI GAGCTGGACACCGATTTTCCG 3’ |
| SET6: 5’ AGCTGGATCC BglII GCATATCTTAGCTGA 3’ | 5’ AGCTGGATCC BglII GCATATCTTAGCTGA 3’ |
| SET7: 5’ AGCTGGATCC BglII GAACTTGGCTTGTCTAAGTGA 3’ | 5’ AGCTGGATCC BglII GAACTTGGCTTGTCTAAGTGA 3’ |
| SET8: 5’ AGCTGGATCC BglII AACGCGTTTATTTTCGAATACAC 3’ | 5’ AGCTGGATCC BglII AACGCGTTTATTTTCGAATACAC 3’ |
| SET9: 5’ AGCTGGATCC BglII GTTTGGGGTAATCAATCATTTG 3’ | 5’ AGCTGGATCC BglII GTTTGGGGTAATCAATCATTTG 3’ |
| SET10: 5’ AGCTGGATCC BglII GCCATCTTTAGCTGA 3’ | 5’ AGCTGGATCC BglII GCCATCTTTAGCTGA 3’ |
| SET11: 5’ AGCTGGATCC BglII GCATATCTTAGCTGA 3’ | 5’ AGCTGGATCC BglII GCATATCTTAGCTGA 3’ |
| SET12: 5’ AGCTGGATCC BglII GCCATCTTTAGCTGA 3’ | 5’ AGCTGGATCC BglII GCCATCTTTAGCTGA 3’ |

Results
The organization of the third chromosome chorion gene cluster and the sequences involved in amplification are diagrammed (Fig. 1). The parent construct (called Big Parent or BP) for
Fig. 1. Organization of the third chromosome chorion gene cluster and amplification regulatory elements. The chorion genes are indicated by arrows. Stimulatory regions (‘amplification enhancing regions’ or AERs) are indicated by hatched boxes. The ACE3 and ori-β elements, which are necessary and sufficient for amplification, are indicated by black boxes. Evolutionarily conserved sequences within ACE3, regions of homology between ACE3 and ori-β, and the location of the α and β sequence elements are indicated. All numbering is relative to the published sequence for the 3.8 kb SalI fragment of the third chromosome chorion gene locus (Levine and Spradling, 1985).

Fig. 2. Sequence requirements for ACE3 function in amplification. (A) The BP construct and ACE3 deletion mutants. For convenience, ACE3 (751 to 1071 in Fig. 1) is numbered from 1 to 320. The shaded regions are conserved among four Drosophila species. The α region was identified by sequence analysis and has homology to the β region in ori-β. The two Myb consensus binding sites are indicated by stars with one of them outside the conserved shaded regions. The three p120-binding regions are indicated by black bars, the sizes of which are not to scale (Beall et al., 2002).

(B) Southern blot analysis of amplification levels for representative independent transgenic lines. BP and ACE3 total deletion (AD) lines were analyzed as controls in the same experiment. rDNA was used as the loading control. (C) Quantitation of three independent assays for each independent transgenic line according to the measurement of amplification levels in Materials and methods. Average and standard deviation are presented in the bar graph. The average fold amplification level is given below the name of the construct. P values are presented for a comparison of each construct to BP using unpaired, two-sided t-tests.
from sub-optimal spacing cannot be ruled out; however, the data suggest that the majority of such negative effects will be due to loss of specific sequence elements.

Five deletion mutants of ACE3 were generated to determine if the conserved sequences would be sufficient for ACE3 function (Fig. 2A). Five or more transgenic lines were generated for each construct and assayed for amplification activity using the Southern blot assay (Fig. 2B). DNA was isolated from stage 13 egg chambers where amplification is maximal, as well as from males as a non-amplifying control. Amplification of the ACE3 deletion mutants was compared with the activity of BP and to a complete deletion of ACE3 (AD). Each construct was assayed in three independent experiments and the results are presented in the bar graphs as average±s.d., and the average amplification for each mutant was compared with BP using unpaired, two-sided t-tests (Fig. 2C). BP supported ~19 fold amplification and the complete deletion of ACE3 (AD) reduced amplification to fourfold. No amplification yields a value of 1 meaning that deletion of ACE3 almost but not completely eliminates amplification in this context. Deletion of the non-conserved 5’ or 3’ ACE3 sequences in mutants 1 and 2 had quantitative effects, and reduced amplification to average ~14 fold and ~15 fold, respectively. Mutant 3 containing only the conserved sequences supported average ~11-fold amplification, demonstrating that the evolutionarily conserved sequences are sufficient for partial ACE3 activity. Deletion of some 3’ conserved sequences in mutant 4 further reduced activity, and deletion of a significant part of 5’ conserved sequences in mutant 5 reduced activity close to the extent of a deletion of all of ACE3. The data suggest that multiple, partially redundant elements comprise ACE3. The same conclusion was obtained in a previous deletion analysis of ACE3 in unbuffered vectors (Orr-Weaver et al., 1989). In addition, the data presented here indicate that the evolutionarily conserved sequences are sufficient for most of ACE3 activity.

Previously, deletion of all of ori-β was found to eliminate amplification, indicating that ori-β was also a sequence specific element required for amplification. To begin to analyze the sequence requirements for ori-β function, constructs were generated to determine if the equally A/T-rich S. cerevisiae ARS1 origin sequences could substitute for ori-β. The complete 193 bp ARS1 and the 20 bp B2 DNA unwinding element from ARS1 were substituted for ori-β in constructs BP-ARS1 and BP-B2, respectively (Fig. 3A). The BP construct supported an average ~19-fold amplification, while a complete deletion of ori-β reduced amplification to ~3 fold. The yeast sequences were found to have no detectable activity in amplification, thereby confirming the sequence specificity of ori-β (Fig. 3B).

Deletion analysis was undertaken to determine the sequence requirements for ori-β function. Ori-β contains an A/T-rich section called the β region that has significant homology to the α region in ACE3, and is indicated by shading (Fig. 4A). Deletions were generated in the context of the BP construct containing the starting 840 bp ori-β. BP supported ~20 fold amplification while a complete deletion of ori-β (OD) reduced amplification to ~4 fold. Deletion of the 3’ 140 bp in mutant 1 had a quantitative effect, reducing amplification to ~12 fold (Fig. 4B). More extensive 3’ deletions in mutant 2 and mutant 9 did not further reduce amplification (Fig. 4 for ori-β mutant 2, and Fig. 5, see below, for ori-β mutant 9). The data indicate that, with the possible exception of a stimulatory effect of the 3’ most 140 bp, the 3’ half of the starting 840 bp ori-β is not essential. By contrast, deletion of the 5’ 140 bp in mutant 3 or more extensive 5’ deletions in mutants 4 and 5 reduced activity to the extent of a deletion of all of ori-β (Fig. 4B), indicating that the 5’ 140 bp are essential for ori-β activity. It is unlikely that deletion of the 140 bp at the 5’ end of ori-β is simply causing a suboptimal spacing between ACE3 and ori-β, as deletion of the entire ~1.2 kb S/I′ gene in SP construct (Lu et al., 2001), or deletion of the 3’ 52 bp of ACE3 in ACE3 mt-1 (Fig. 2) have much smaller negative effects.

An additional series of 3’ deletions were generated to determine if the A/T-rich β region was also required for ori-β function (Fig. 5A). The 5’ 140 bp found to be essential above were not sufficient for ori-β activity in mutant 6. Addition of the 5’ half of the β region in mutant 7 resulted in a very slight increase in activity, while inclusion of the entire β region in mutant 8 supported amplification of average ~14 fold (Fig. 5B). Therefore, a 366 bp fragment containing a 5’ 140 bp element and the 226 bp A/T-rich β-region was sufficient to function as ori-β.

It was of interest to determine if the smaller ACE3 and ori-β fragments found to function in the context of the BP construct would be sufficient to support amplification in combination with each other. Previously, the starting 320 bp ACE3 element and the starting 840 bp ori-β element in the SP construct were found to be sufficient to support moderate levels of amplification, ranging from 4 to 14 fold. By contrast, the
and trans requirements for foci formation were analyzed in further detail. The k43fs393 mutation almost eliminates amplification (Landis et al., 1997), and was found to eliminate all ORC2 antibody staining (Whittaker et al., 2000) (Fig. 7F), indicating that the mutation either disrupts the epitope or that the mutant ORC2 protein is unstable. The satin gene mutation satin^{Sc46} causes female sterility and thin eggshells (Schupbach and Wieschaus, 1991). satin^{Sc46} was found to greatly reduce chorion gene amplification, as detected by Southern blot assay (data not shown), and to also eliminate ORC2 foci formation (Fig. 7G). The chiffon gene is related to the S. cerevisiae dbf4 regulator of DNA replication and cell cycle (Landis and Tower, 1999). chiffon null mutations are viable and completely eliminate chorion gene amplification. In chiffon-null mutant follicle cells, diffuse nuclear staining of ORC2 was still as in wild type, but it failed to localize into foci at stage 10A (Fig. 7H,I). The analysis of these trans regulators therefore confirms the intimate association between amplification and the localization of ORC to the chorion gene loci. However, they also suggest a surprising role for chiffon (Dbf4-like) in ORC localization.

In wild-type follicle cells, faint and diffuse nuclear ORC2 staining and genomic endoreplication persists through stage 9 of oogenesis. Endoreplication ceases by stage 10A, and coincidentally the diffuse nuclear ORC2 staining disappears. At this time the ORC staining relocalizes to the chorion gene loci coincident with the initiation of amplification (Royzman et al., 1999) (Fig. 7A). An alternative explanation for the chiffon phenotype might be that in chiffon mutants genomic endoreplication persists, thereby preventing or masking the relocalization of ORC2 to the chorion gene loci. However, BrdU labeling of wild-type and chiffon mutant follicle cells shows that genomic endoreplication does not persist in the vast majority of chiffon mutant follicle cells (Fig. 7J-L). Therefore, chiffon does appear to be genuinely required for ORC localization and the formation of the dramatic foci. Interestingly, faint and patchy BrdU labeling was seen to persist in rare, isolated, chiffon mutant follicle cells (Fig. 7K-L). It is not clear if this faint labeling represents genomic endoreplication or low level chorion gene amplification, or both.

Transgenic chorion gene constructs can create an extra focus of ORC staining in follicle cell nuclei (Austin et al., 1999). However, previous experiments indicated that extra ORC foci could not be detected for the BP construct (Lu et al., 2001). This result might have been due to the modest amplification level for BP (~20 fold), or to the different sequence content of BP, and experiments were undertaken to try to distinguish between these two possibilities. Multiple transgenic lines for three different constructs were assayed for the presence of additional foci of ORC2 localization (Fig. 7; additional data
not shown), and selected lines were re-assayed side-by-side to confirm their relative amplification levels (Fig. 8). Two transgenic lines of BP supported 18- and 26-fold amplification, respectively (Fig. 8B), with no detectable extra ORC2 foci formation (Fig. 7B). However extra foci of BrdU incorporation were readily observed (data not shown). By contrast, three similarly active lines of the buffered Yes-3.8S construct, containing more extensive chorion gene locus sequences, exhibited extra dramatic ORC2 localization (Fig. 7C). The data demonstrate that the lack of foci formation observed for the BP construct is not simply due to a low amplification level. Construct Caryos-3.8S contains the same sequences as Yes-3.8S, but is not buffered by flanking insulator elements (Fig. 8A), so amplification occurs at some chromosomal locations but not others due to negative chromosomal position effects (Fig. 8B). With Caryos-3.8S, foci formation correlated with amplification level, in that foci formation was observed only for highly amplifying insertion sites (Fig. 7D,E; additional data not shown). The data suggest that negative chromosomal position effects that reduce amplification also reduce foci formation, but that use of the buffered vector allows amplification to occur in the absence of (visible) foci formation.

The fact that the insulator elements allowed amplification to occur in the absence of visible foci formation suggested the possibility that the insulators had reduced or eliminated the requirement for ORC2 activity in amplification of such buffered transgenic constructs. To test this possibility, three buffered transgenic inserts were crossed into a k43fs293 mutant background and assayed for amplification (Fig. 8C). In all three cases, amplification of the buffered transgenic constructs was eliminated by the k43fs293 mutation, demonstrating that amplification of buffered constructs still requires ORC2 activity.

Discussion

The use of insulator elements, the suppressor of Hairy-wing protein binding sites [su(Hw)BSs], protects transgenic chorion gene constructs from chromosomal position effects (Lu and Tower, 1997), and allows for detailed analysis of sequence requirements for amplification. Previously the ACE3 replicator and ori-β origin elements were found to be necessary for efficient amplification (Lu et al., 2001). A construct containing only the 320 bp ACE3 and the 840 bp ori-β (‘Small Parent’ or SP) demonstrated that these elements are also sufficient for amplification; however, the levels of amplification were moderate and were subject to significant chromosomal position effects even in the presence of the flanking insulator elements. In the BP construct, the 320 bp ACE3 and the 840 bp ori-β formation (Fig. 7B). However extra foci of BrdU incorporation were readily observed (data not shown). By contrast, three similarly active lines of the buffered Yes-3.8S construct, containing more extensive chorion gene locus sequences, exhibited extra dramatic ORC2 localization (Fig. 7C). The data demonstrate that the lack of foci formation observed for the BP construct is not simply due to a low amplification level. Construct Caryos-3.8S contains the same sequences as Yes-3.8S, but is not buffered by flanking insulator elements (Fig. 8A), so amplification occurs at some chromosomal locations but not others due to negative chromosomal position effects (Fig. 8B). With Caryos-3.8S, foci formation correlated with amplification level, in that foci formation was observed only for highly amplifying insertion sites (Fig. 7D,E; additional data not shown). The data suggest that negative chromosomal position effects that reduce amplification also reduce foci formation, but that use of the buffered vector allows amplification to occur in the absence of (visible) foci formation.

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were in their normal context, i.e. spaced by the ~1.2 kb S18 chorion gene, and these sequences supported efficient amplification (~20 fold) with minimal position effects. For this reason, the BP construct was chosen for detailed analysis of ACE3 and ori-β sequence requirements. Evolutionarily conserved core sequences were found to be sufficient for the majority of ACE3 activity. Deletion of the less conserved 5’ and 3’ flanking sequences within ACE3 had quantitative effects, suggesting that multiple, partially redundant elements comprise ACE3. These results and conclusions are analogous to those from a previous study of ACE3 sequence requirements done in the context of a larger, unbuffered construct (Orr-Weaver et al., 1989). In that study, no deletion of a subset of ACE3 sequences reduced amplification to the extent of a deletion of all of ACE3. The sequence requirements for ACE3 function in amplification defined here correlate well with the sequence requirements previously defined for ORC binding in vitro (Austin et al., 1999). The central region of ACE3, corresponding to the evolutionarily conserved sequences, was most crucial for ORC binding, while the 5’ and 3’ flanking regions within ACE3 stimulated ORC binding. Taken together, the data suggest that the multiple, partially redundant elements that comprise ACE3 are ORC binding sites, and that one crucial function of ACE3 in amplification is to bind ORC.

Recently a protein complex containing Drosophila Myb, p120 and three other proteins was found to bind to both ACE3 and ori-β sequences, and Myb was found to be required in trans for amplification (Beall et al., 2002). Both Myb and p120 are capable of DNA binding on their own, and have binding sites that overlap with the essential core region of ACE3. There are two Myb consensus binding sites (121 to 127 and 137 to 142 in Fig. 2A) and three p120 binding regions (27 to 56, 89 to 105 and 184 to 216 in Fig. 2A) in ACE3 element. Small (30-40 bp) deletions that removed one of Myb consensus binding sites or one of the p120-binding sites in the core region of ACE3 had negative effects on amplification in the context of the BP construct. Taken together, these data suggest that another function of the conserved core region sequences of ACE3 is to bind the Myb complex.

Two-dimensional gel analyses of the endogenous third chromosome chorion gene locus demonstrated that the majority (70-80%) of initiations occurred in a region containing the ori-β element (Delidakis and Kafatos, 1989; Heck and Spradling, 1990). In 2D gel analysis of the BP construct, abundant initiation events, as indicated by bubble structures, were associated with the ori-β element while no initiations could be detected for ACE3 (Lu et al., 2001). To begin to examine the sequence requirements for ori-β function, ori-β was substituted by either the entire 193 bp S. cerevisiae ARS1 origin sequence, or the 20 bp B2 element from ARS1, which is a putative DNA unwinding element. No activity in supporting amplification was detected for either fragment, indicating that ori-β is not simply an A/T-rich or easily unwound sequence. Deletion mapping suggests two sub-components of ori-β: an essential 5’ 140 bp region that is not particularly A/T-rich, and the 226 bp A/T-rich β region. The 366 bp fragment containing both regions was sufficient for the majority of ori-β activity. In addition the 3’ most 140 bp of the starting 840 bp ori-β fragment may have a small stimulatory
effect. The portion of the ori-II/9A pupal case genes, resulting in chromosomal DNA ‘puffs’. The salivary gland cells amplify several loci containing putative developmentally regulated origin in another dipteran fly, *Sciaradiplasma* function of the hypothesize that, like the sequences in ACE3, one required can bind ORC in vitro (Austin et al., 1999). Therefore, we in ori-

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**Fig. 8.** Comparison of amplification levels among different transgenic lines. (A) BP: pYes-3.8S and pCaryos-3.8S constructs. (B) Quantitation of amplification level for representative transgenic lines. (C) Amplification levels of endogenous third chromosome chorion gene locus and transgenic constructs in wild-type and *k43* mutant backgrounds. All transgenic constructs are homozygous. Y8, Yes-3.8S construct transgenic line 8; k43/TM6B, heterozygous (non-mutant) background; k43/k43, mutant background k43/k43, mutant background k43/k43, mutant background k43/k43, mutant background k43/k43; E, endogenous amplification level; T, transgene amplification level. Average fold amplification levels are presented below the graph.

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the nucleotide level and has similarities to the yeast ARS. *Drosophila ORC* has been shown to bind to an 80 bp region adjacent to this replication start site (Bielinsky and Gerbi, 2001).

Analysis of trans-acting gene mutations confirmed the intimate association between amplification initiation and the formation of a large focus of ORC2 localization at amplifying chromosomal loci. Mutations in *k43* (Orc2) itself, or the newly identified trans-regulatory gene *satin*, eliminated ORC2 antibody staining and focus formation. Null mutations of *chiffon*, a *dhp4*-like gene, completely eliminate amplification (Landis and Tower, 1999). In *chiffon*-null mutant follicle cells, diffuse ORC2 staining was still present in the nucleus, but it failed to localize into foci at stage 10A. A similar phenotype had previously been observed for mutations in the amplification trans-regulators *dhp* (a subunit of E2F) and *Rh* (Bosco et al., 2001; Royzman et al., 1999).

A role for *chiffon* in ORC localization was surprising given the well-characterized order of events known for other organisms. In *S. cerevisiae* and *Xenopus* in vitro systems, ORC is bound at origins and is required for the subsequent binding of Dbf4 and its catalytic subunit CDC7, which is one of the last events before origin firing (Bell, 2002; Bell and Dutta, 2002). The data suggest two possible models for the role of *chiffon* in ORC2 focus formation during amplification. In the first model, *chiffon* protein would bind first to the chorion gene sequences, either directly or more likely via an interaction with another DNA-binding protein, as the *chiffon* sequence suggests no obvious DNA-binding motifs. Chiffon would then recruit *Drosophila ORC2* to the DNA. This model seems unlikely given the opposite order of events observed in yeast and in *Xenopus* in vitro systems. In the second and favored model, a relatively small amount of ORC binds first to the chorion gene loci, most probably to the conserved core sequences in ACE3 and the β region in ori-β. Chiffon protein would then interact with the ORC complex(es) and catalyze the further binding of large amounts of ORC to generate the dramatic foci observed upon staining with ORC2 antibody. We envision a mechanism in which the α and β regions nucleate ORC binding, and then through a process dependent upon *chiffon*, an ORC-containing chromatin structure spreads along the chromosome to form the dramatic foci. This model is appealing in that it provides a way for ACE3 and ori-β to interact and form a chromosomal domain activated for DNA initiation events. Previous data
indicated that ACE3 and ori-β interact during amplification in a way that can be blocked by an intervening insulator element (Lu et al., 2001). Moreover, analysis of the endogenous locus indicates that ACE3 is required for the activation of multiple origins spread throughout a chromosomal domain containing the chorion gene cluster. This model is testable in that it predicts that the insulators would form a boundary for this ORC-containing chromatin structure.

The possibility cannot be ruled out that chiffon is not the true Dbf4 homolog in Drosophila, but this appears unlikely. Chiffon shows conservation with Dbf4 homologs from all other species in the key ORC-binding domain (called CDDN2) and the CDC7-binding domain (called CDDN1) (Landis and Tower, 1999). Moreover, there is no other gene in the Drosophila genome with detectable homology to Dbf4. However, chiffon contains an additional large C-terminal protein domain present only in Dbf4 homologs from closely related species, such as Medfly and mosquito. We speculate that this C-terminal domain may play a specific role in chorion gene amplification. Further experiments will be required to determine if a role in ORC localization is a characteristic of all Dbf4 family members, or whether this represents a function unique to the large chiffon protein.

Consistent with the correlation between ORC2 focus formation and amplification initiation, dramatic ORC2 foci can form at the sites of amplifying transgenic chorion gene constructs. It was therefore surprising that in no cases were foci observed at the sites of actively amplifying BP constructs. This is despite the fact that amplification was readily observed at these sites by BrdU incorporation. One possible explanation might be the moderate amplification level of BP (18-20-fold). However, the YES-3.8S construct amplifies to similar levels as BP, and an extra ORC2 focus was observed for every line. In addition multimers of ACE3 with very low amplification levels are capable of creating additional ORC2 foci (Austin et al., 1999). Therefore, the lack of focus formation with BP is not simply due to its moderate amplification level, but must reflect the specific sequence content or arrangement in BP. The lack of focus formation in BP is also not simply due to the presence of flanking insulator elements, as the YES-3.8S construct contains the same flanking insulator elements. The data suggest two non-exclusive possibilities. The first is that the difference is due to the fact that BP contains less extensive chorion gene sequences than YES-3.8S. Although deletion of these sequences has no significant effect on amplification level, it may be that redundant ORC binding sites have been deleted, thereby dramatically reducing visible focus formation. The second possibility is that the relevant difference is the amount of sequence present inside the insulators. BP contains only 2.4 kb between the insulators, whereas Yes-3.8 contains 9 kb. If the insulators limit the size of the domain in which an ORC containing chromatin structure can spread from ACE3 and/or ori-β, then the small size of this domain in BP may not create a visible focus. In this model, the insulators would have two significant effects on amplification: they would prevent the spread of negative chromatin structures into the bounded region and thereby prevent negative chromosomal position effects; and they would limit the ORC containing chromatin structure and initiation activity to the bounded region. These models should be testable in the future by CHIP analysis of chromatin structures associated with chorion gene sequences and transgenic constructs (Austin et al., 1999). It will be of interest in the future to determine if su(Hw)BS insulators or other types of insulators are involved in organizing the endogenous chorion gene locus and the rest of the genome into domains of DNA replication activity.

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