

# Sequence requirements for function of the *Drosophila* chorion gene locus ACE3 replicator and ori- $\beta$ origin elements

Hongjun Zhang and John Tower\*

Molecular and Computational Biology Program, Department of Biological Sciences, University of Southern California, Los Angeles, CA 90089-1340, USA

\*Author for correspondence (e-mail: jtower@USC.edu)

Accepted 8 January 2004

Development 131, 2089-2099  
Published by The Company of Biologists 2004  
doi:10.1242/dev.01064

## 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- $\beta$  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- $\beta$ . 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- $\beta$ , thereby confirming the sequence specificity of ori- $\beta$ . Deletion mapping of ori- $\beta$  identified two required components: a 140 bp 5' element and a 226 bp A/T-rich 3'

element called the  $\beta$ -region that has significant homology to ACE3. Antibody to the origin recognition complex subunit 2 (ORC2) recognizes large foci that localize to the endogenous chorion gene loci and to active transgenic constructs at the beginning of amplification. Mutations in *Orc2* itself, or the amplification trans regulator *satin* 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- $\beta$  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 entré 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., 1988; Spradling and Leys, 1988). After synthesizing the chorion, the follicle cells are destroyed prior to egg-laying, and therefore the mitotic apparatus 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

participate in replication initiation. Data from yeasts and *Xenopus* in vitro systems indicate that among the last proteins to bind prior to origin firing are the CDC7 protein kinase and its regulatory subunit Dbf4. The *Drosophila* gene *chiffon* was found to be required for chorion gene amplification and to be related to yeast *dbf4* (Landis and Tower, 1999). Additional conserved DNA replication factor genes required for chorion gene amplification include those encoding Cyclin E (Calvi et al., 1998), E2F (Royzman et al., 1999), DP (Royzman et al., 1999), RBF (Bosco et al., 2001), CDT1 (Whittaker et al., 2000), geminin (Quinn et al., 2001) and MCM6 (Schwed et al., 2002). Antibody to ORC1 or ORC2 reveals that, coincident with the initiation of amplification, ORC moves from a diffuse nuclear distribution into dramatic foci localized at the chorion gene loci (Asano and Wharton, 1999; Calvi et al., 1998; Royzman et al., 1999). The data demonstrate that *Drosophila* chorion gene amplification uses evolutionarily conserved machinery for initiation; however, some mechanism must exist to uniquely mark the chorion gene loci origins for activation during amplification, when overall genomic replication has ceased.

Large cloned fragments of the chorion gene loci can be re-introduced into the *Drosophila* genome via P element-mediated germline transformation, and these transgenic constructs can amplify with the correct tissue and temporal specificity (deCicco and Spradling, 1984). However, amplification is highly sensitive to chromosomal position effects and only ~1/3 of inserts will be active. The third chromosome locus has been studied in the greatest detail, and a 3.8 kb *SalI* fragment was capable of high level amplification at some sites. This fragment was found to contain two striking sequence elements: The 'α region' upstream of the *S18* chorion gene and the related 'β region' downstream were A/T-rich, internally repetitive and had similarities to the yeast origin consensus sequence, suggesting that they might function in regulating amplification (Levine and Spradling, 1985). Deletion mapping of transgenic constructs identified a required 320 bp element that contained the most of α region and was called ACE3 (Orr-Weaver et al., 1989). The function of ACE3 in amplification and several ACE3 sequence elements are conserved among multiple *Drosophila* species (Swimmer et al., 1990), and deletion analysis indicated that ACE3 is composed of multiple, partially-redundant elements (Orr-Weaver et al., 1989). Regions between the chorion genes were found to be stimulatory for amplification (Delidakis and Kafatos, 1989). Two-dimensional gel analysis of DNA replication intermediates revealed that multiple origins functioned in amplification, with the majority of initiations (70-80%) occurring near the β region downstream of *S18* (Delidakis and Kafatos, 1989; Heck and Spradling, 1990).

Insulator elements are DNA sequences first identified in studies of transcription (Gerasimova and Corces, 2001). Insulators can block the interaction of enhancers with promoters when they are placed in between, and can also protect transgenes from chromosomal position effects on transcription when they are placed flanking the transgene. The *suppressor of Hairy-wing* protein binding site insulator [su(Hw)BS] was found to be able to protect chorion gene locus constructs from position effects on amplification (Lu and Tower, 1997). This provided a more sensitive and convenient assay for amplification sequence requirements. Using such a

buffered vector an 840 bp sequence-specific origin element called ori-β was identified (Lu et al., 2001). Ori-β is located downstream of the *S18* chorion gene and contains the β region. Ori-β was necessary and sufficient for amplification in combination with ACE3. An insulator placed between ACE3 and ori-β inhibited amplification, indicating that ACE3 and ori-β interact in cis. Two-dimensional gel analysis of a construct containing ACE3, *S18* and ori-β identified initiations occurring at ori-β, while no initiations could be detected at ACE3. Taken together, the data suggest that ACE3 functions as a 'replicator' (Stillman, 1993) 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 ori-β 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 *XhoI*. The fragment was cloned into the *NotI* to *XhoI* 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 *XhoI*, and cloned into the *NotI* to *XhoI* 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 *XhoI*, and cloned into the *NotI* to *XhoI* sites of BP to generate the final constructs.

### pOriβ mt-1 through pOriβ 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 *XhoI* sites engineered into the primers, and then cloned into the *BglII* to *XhoI* 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 *XhoI* sites engineered into the primers, and cloned into the *BglII* to *XhoI* sites in 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 *BglIII* to *XhoI* sites in the BP construct to generate pBP-B2.

#### pMini-1

This construct contains one ACE3 mt-3 and one ori- $\beta$  mt-2 with no intervening sequences. The 840 bp ori- $\beta$  fragment in the BP construct was amplified by PCR using primer set 7, digested at *BglIII* and *XhoI* sites engineered into the primers, and cloned into the *BglIII* 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 *BglIII* sites in pBS\*K-2.6. A fragment containing one ACE3 mt-3 and one ori- $\beta$  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- $\beta$  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 *NheI*, and *NheI* and *BamHI*, respectively. The three fragments were cloned into the *KpnI* to *BglIII* sites in pBS\*K-2.6 (described above) in the same reaction. A fragment containing three ACE3 mt-3 and one ori- $\beta$  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- $\beta$  mt-2 without ACE3 and the intervening sequences. pBS\*K-2.6 (described above) was digested with *KpnI* and *BglIII*. The larger fragment containing su(Hw)BS, plasmid vector and ori- $\beta$  mt-2 was end-filled by T4 DNA polymerase, and then ligated to circularize. A fragment containing one ori- $\beta$  mt-2 and su(Hw)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* CTGAGCCTGGCCAACATCTAA 3'; 5' AGCTGGATCC *BamHI* GAGCTTGACACCGATTTTTTCAG 3'  
 SET2: 5' AGCTGGTACC *KpnI* GAAAGTGGAAACGGTTGTGTTTA 3'; 5' AGCTGGATCC *BamHI* GCATAGTTTCGATCA 3'  
 SET3: 5' AGCTGGTACC *KpnI* GAAAGTGGAAACGGTTGTGTTTA 3'; 5' AGCTGGATCC *BamHI* GAGCTTGACACCGATTTTTTCAG 3'  
 SET4: 5' AGCTGGTACC *KpnI* GAAAGTGGAAACGGTTGTGTTTA 3'; 5' AGCTGGATCC *BamHI* AAGGCAGTGGCCTGAAAATTC 3'  
 SET5: 5' AGCTGGTACC *KpnI* CTACCAACGCAGCAGAATTTTC 3'; 5' AGCTGGATCC *BamHI* GAGCTTGACACCGATTTTTTCAG 3'  
 SET6: 5' AGCTGAGATCT *BglIII* GCATATCTTAGCTGA 3'; 5' AGCTCTCGAG *XhoI* GGCTGGAATATACTCACATTTG 3'  
 SET7: 5' AGCTGAGATCT *BglIII* GCATATCTTAGCTGA 3'; 5' AGCTCTCGAG *XhoI* AACCGCTTTATTTTCGAATACAC 3'  
 SET8: 5' AGCTAGATCT *BglIII* AATGAAGCTGCAAAGCT-AAAATA 3'; 5' AGCTCTCGAG *XhoI* GTTTGGGGTAATCAATCAAATA 3'  
 SET9: 5' AGCTAGATCT *BglIII* TTGCTTGAAATTATGTTTTTGTAAAA 3'; 5' AGCTCTCGAG *XhoI* GTTTGGGGTAATCAATCAAATA 3'  
 SET10: 5' AGCTAGATCT *BglIII* AATGAAGCTGCAAAGCT-AAAATA 3'; 5' AGCTCTCGAG *XhoI* CAGCCGGTTTTTCTGATAAAAC 3'  
 SET11: 5' AGCTGAGATCT *BglIII* GCATATCTTAGCTGA 3'; 5' AGCTCTCGAG *XhoI* TCATTCGCCATGACAATTATTC 3'  
 SET12: 5' AGCTGAGATCT *BglIII* GCATATCTTAGCTGA 3'; 5' AGCTCTCGAG *XhoI* ATCGCGTTTTATGTAATAGATTC 3'

SET13: 5' AGCTGAGATCT *BglIII* GCATATCTTAGCTGA 3'; 5' AGCTCTCGAG *XhoI* AGTCACATACAAAACCTTAAAATA 3'  
 SET14: 5' AGCTGAGATCT *BglIII* GCATATCTTAGCTGA 3'; 5' AGCTCTCGAG *XhoI* GAACTTGGCTTGTCTAAGTGA 3'  
 SET15: 5' AGCTGGATCC *BamHI* CTAACAAAATAGCAAA-TTTCG 3'; 5' AGCTCTCGAG *XhoI* ACAATCAATCAAAAAG-CCAAA 3'  
 SET16: 5' GATCT *BglIII* TATTTATTTAAGTATTGTTTC 3'; 5' TCGAG *XhoI* AAACAATACTTAAATAAATAA 3'  
 SET17: 5' AGCTGGTACC *KpnI* GAAAGTGGAAACGGTTGTGTTTA 3'; 5' AGCTGAATTC *EcoRI* GAGCTTGACACCGA-TTTTTTCAG 3'  
 SET18: 5' AGCTGAATTC *EcoRI* GAAAGTGGAAACGGTTGTGTTTA 3'; 5' AGCTGCTAGC *NheI* GAGCTTGACACCGA-TTTTTTCAG 3'  
 SET19: 5' AGCTGCTAGC *NheI* GAAAGTGGAAACGGTTGTGTTTA 3'; 5' AGCTGGATCC *BamHI* GAGCTTGACACCG-ATTTTTTCAG 3'

#### Generation of transgenic lines

Transgenic strains were generated by P element-mediated germline transformation (Rubin and Spradling, 1982), using the *w<sup>1118</sup>* recipient strain. All inserts were made homozygous by crosses to appropriate balancer stocks, and single copy insertions were confirmed by genomic Southern blots (data not shown).

#### Measurement of amplification levels

The measurement of amplification levels for transgenic constructs was previously described (Lu and Tower, 1997). Briefly, DNA was isolated from stage 13 egg chambers (ECs), restriction digested and Southern blotted. Blots were hybridized with radiolabeled restriction fragments of the constructs, and with a ribosomal DNA probe (pDmrY22) (Dawid et al., 1978) as a control for amount of DNA loaded. Southern blot signals were quantitated by phosphorimager analysis, and the amplification level was calculated by comparing the signal for the transgene in EC DNA to the signal for the transgene in male DNA, where there is no amplification, with the following formula: fold amplification=(transgene<sup>EC</sup>/transgene<sup>Male</sup>)/(rDNA<sup>EC</sup>/rDNA<sup>Male</sup>). No amplification yields a value of 1.

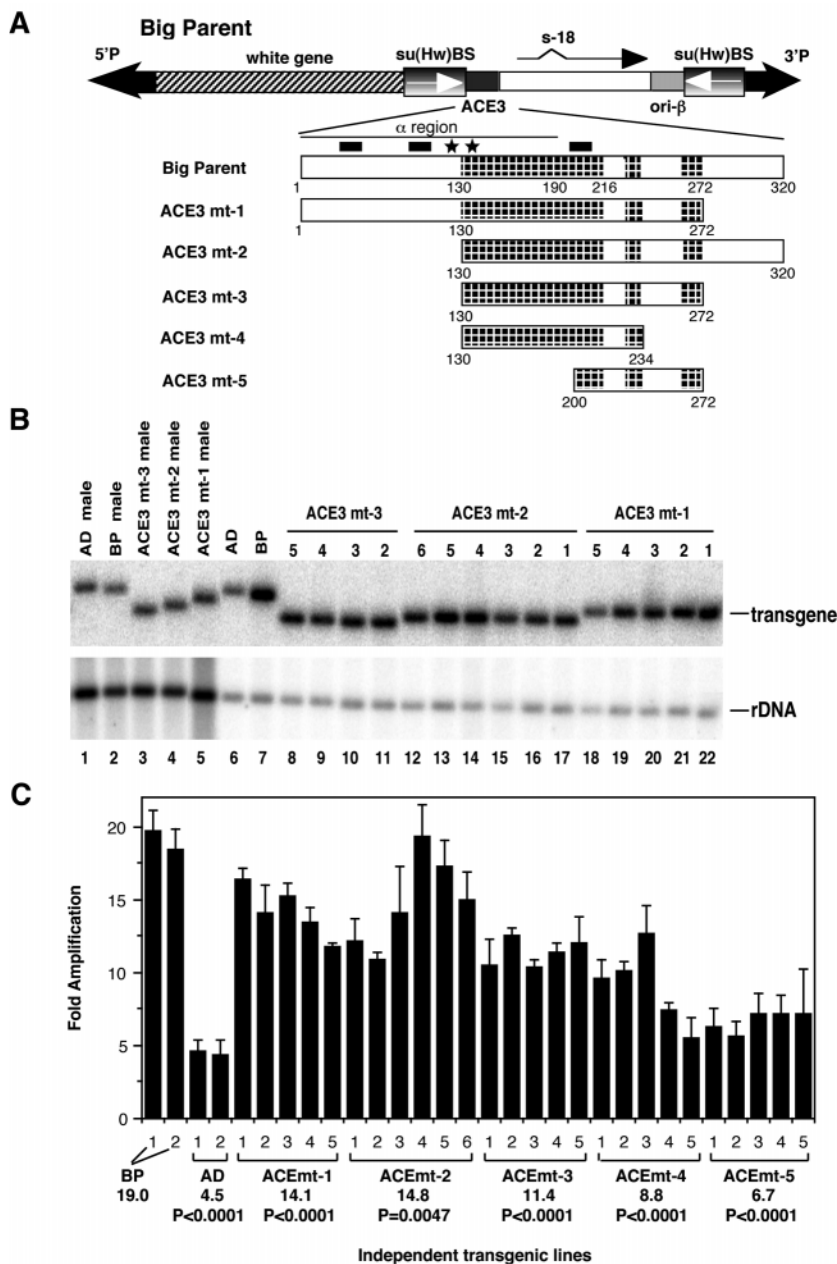
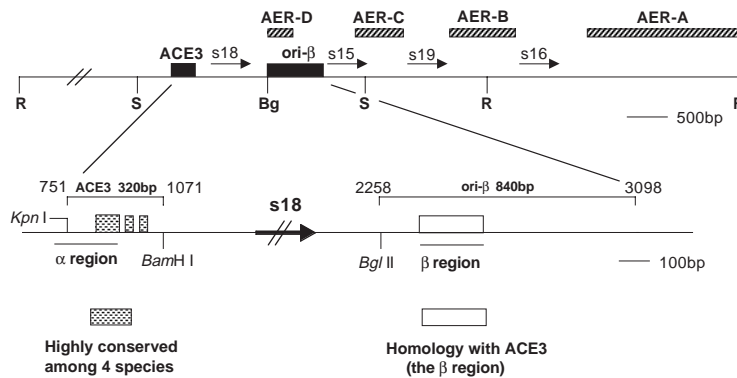
#### Antibody staining and BrdU labeling

Antibody staining procedure was provided by Bosco and Orr-Weaver (Royzman et al., 1999). Dissected ovaries were fixed by 8% formaldehyde/Buffer B solution for 5 minutes, then blocked with 2% normal donkey serum (NDS) in CHIP lysis buffer (50 mM HEPES/KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 0.1% Na-Deoxycholate). After washing, 1:2500 dilution of anti-ORC2 sera (provided by Stephen Bell) in CHIP lysis buffer plus 2% NDS was added to the ovaries, and incubated for 18-20 hours at 4°C. After washing, a 1:150 dilution of Cy3-conjugated goat anti-rabbit Ab (Jackson ImmunoResearch Laboratories) was used to visualize ORC2. DAPI staining was performed to counterstain DNA in the nuclei. Antibody staining was examined using the BioRad MRC 600 confocal microscope with a 100 $\times$  objective. BrdU labeling of ovaries was performed essentially as described (Lilly and Spradling, 1996), according to a detailed procedure provided by Calvi and Lilly (B. Calvi and M. J. Lilly, unpublished). Anti-BrdU antibody (Becton Dickinson) was used at 1:20 dilution. The labeling was visualized using a 1:150 dilution of FITC-conjugated AffiniPure goat anti-mouse antibody (Jackson Labs).

## 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- $\beta$  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- $\beta$ , and the location of the  $\alpha$  and  $\beta$  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).



deletion studies contains the 320 bp ACE3, the ~1.2 kb *S18* chorion gene and the 840 bp ori- $\beta$  element (Fig. 2A). The chorion gene locus sequences are directly flanked by su(Hw)BS insulator elements that protect the construct from negative chromosomal position effects. ACE3 contains sequences that are conserved among multiple *Drosophila* species, as indicated by shading. The exact spacing of ACE3 and ori- $\beta$  does not appear to be crucial for amplification: ACE3 and ori- $\beta$  are normally separated by ~1.2 kb, yet constructs containing only ACE3 and ori- $\beta$  immediately adjacent to each other still amplify up to 14 fold (Lu et al., 2001). The 3' deletions of ACE3 and 5' deletions of ori- $\beta$  described below will cause much smaller changes in spacing. Where these deletions have negative effects a contribution

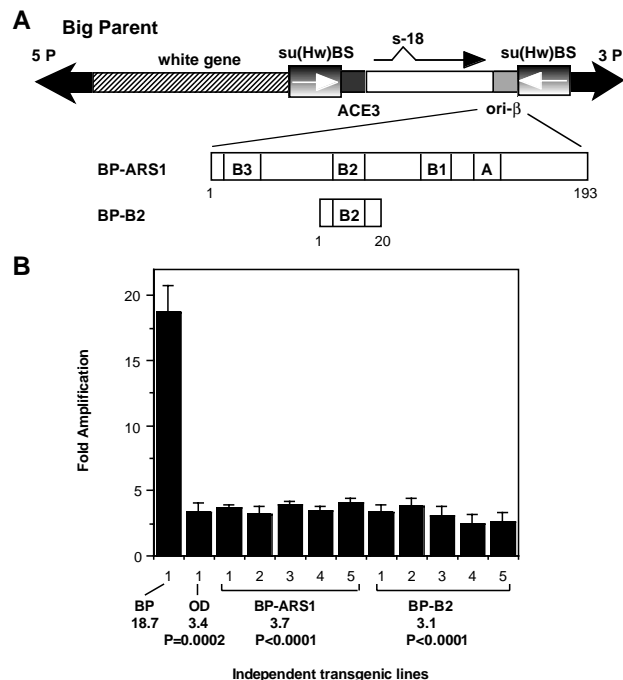
**Fig. 2.** Sequence requirements for ACE3 function in amplification. (A) The BP construct and ACE3 deletion mutants. For convenience, ACE3 (751 to 1071 in Fig. 1) is numbered from 1 to 320. The shaded regions are conserved among four *Drosophila* species. The  $\alpha$  region was identified by sequence analysis and has homology to the  $\beta$  region in ori- $\beta$ . 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  $\pm$  s.d., and the average amplification for each mutant was compared with BP using unpaired, two-sided *t*-tests (Fig. 2C). BP supported  $\sim$ 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  $\sim$ 14 fold and  $\sim$ 15 fold, respectively. Mutant 3 containing only the conserved sequences supported average  $\sim$ 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- $\beta$  was found to eliminate amplification, indicating that ori- $\beta$  was also a sequence specific element required for amplification. To begin to analyze the sequence requirements for ori- $\beta$  function, constructs were generated to determine if the equally A/T-rich *S. cerevisiae* ARS1 origin sequences could substitute for ori- $\beta$ . The complete 193 bp ARS1 and the 20 bp B2 DNA unwinding element from ARS1 were substituted for ori- $\beta$  in constructs BP-ARS1 and BP-B2, respectively (Fig. 3A). The BP construct supported an average  $\sim$ 19-fold amplification, while a complete deletion of ori- $\beta$  reduced amplification to  $\sim$ 3 fold. The yeast sequences were found to have no detectable activity in amplification, thereby confirming the sequence specificity of ori- $\beta$  (Fig. 3B).

Deletion analysis was undertaken to determine the sequence requirements for ori- $\beta$  function. Ori- $\beta$  contains an A/T-rich section called the  $\beta$  region that has significant homology to the  $\alpha$  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- $\beta$ . BP supported  $\sim$ 20 fold amplification while a complete deletion of ori- $\beta$  (OD) reduced amplification to  $\sim$ 4 fold. Deletion of the 3' 140 bp in mutant 1 had a quantitative effect, reducing amplification to  $\sim$ 12 fold (Fig. 4B). More extensive 3' deletions in mutant 2 and mutant 9 did not further reduce amplification (Fig. 4 for ori- $\beta$  mutant

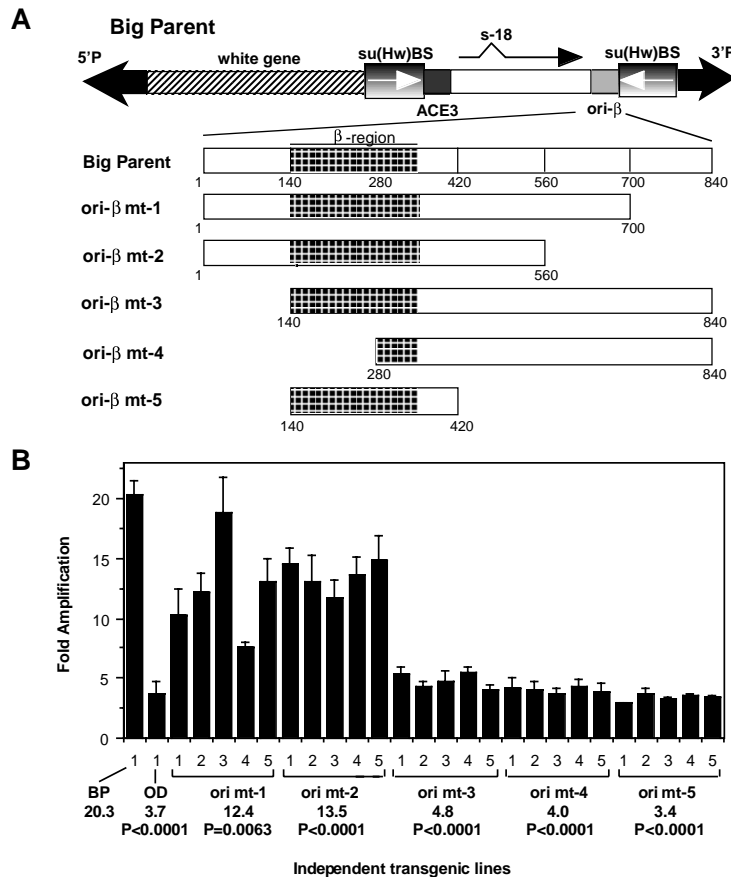


**Fig. 3.** Yeast origin ARS1 and its B2 element cannot functionally replace ori- $\beta$ . (A) Yeast origin ARS1 and its DNA unwinding element B2 were cloned into the BP construct replacing the entire ori- $\beta$  to generate constructs BP-ARS1 and BP-B2, respectively. (B) Quantitation of amplification levels for independent transgenic lines. BP, Big Parent line 3; OD, the ori- $\beta$  total deletion line 1.

2, and Fig. 5, see below, for ori- $\beta$  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- $\beta$  is not essential. By contrast, deletion of the 5' 140 bp or more extensive 5' deletions in mutants 4 and 5 reduced activity to the extent of a deletion of all of ori- $\beta$  (Fig. 4B), indicating that the 5' 140 bp are essential for ori- $\beta$  activity. It is unlikely that deletion of the 140 bp at the 5' end of ori- $\beta$  is simply causing a suboptimal spacing between ACE3 and ori- $\beta$ , as deletion of the entire  $\sim$ 1.2 kb *S18* 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  $\beta$  region was also required for ori- $\beta$  function (Fig. 5A). The 5' 140 bp found to be essential above were not sufficient for ori- $\beta$  activity in mutant 6. Addition of the 5' half of the  $\beta$  region in mutant 7 resulted in a very slight increase in activity, while inclusion of the entire  $\beta$  region in mutant 8 supported amplification of average  $\sim$ 14 fold (Fig. 5B). Therefore, a 366 bp fragment containing a 5' 140 bp element and the 226 bp A/T-rich  $\beta$ -region was sufficient to function as ori- $\beta$ .

It was of interest to determine if the smaller ACE3 and ori- $\beta$  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- $\beta$  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



**Fig. 4.** The 5' 140 bp sequence of ori- $\beta$  is required for activity. (A) BP and ori- $\beta$  mutants 1-5. For convenience, ori- $\beta$  (2258 to 3098 in Fig. 1) is numbered from 1 to 840. The  $\beta$ -region is indicated by shading. (B) Quantitation of amplification levels for independent transgenic lines.

560 bp ori- $\beta$  mutant 2 plus either one or three copies of the 142 bp ACE3 mutant 3 did not support detectable amplification at virtually all insertion sites (Fig. 6). This is probably due to the deletion of quantitative elements and/or a non-optimal spacing of the ACE3 and ori- $\beta$  sequences. The one notable exception is transgenic line 1 of the 'mini-1' construct containing one copy of the smaller ACE3 and ori- $\beta$  elements, which reproducibly amplified to ~5 fold. The data suggest that some unique aspect of this particular chromosomal insertion site stimulates amplification of an otherwise inactive construct.

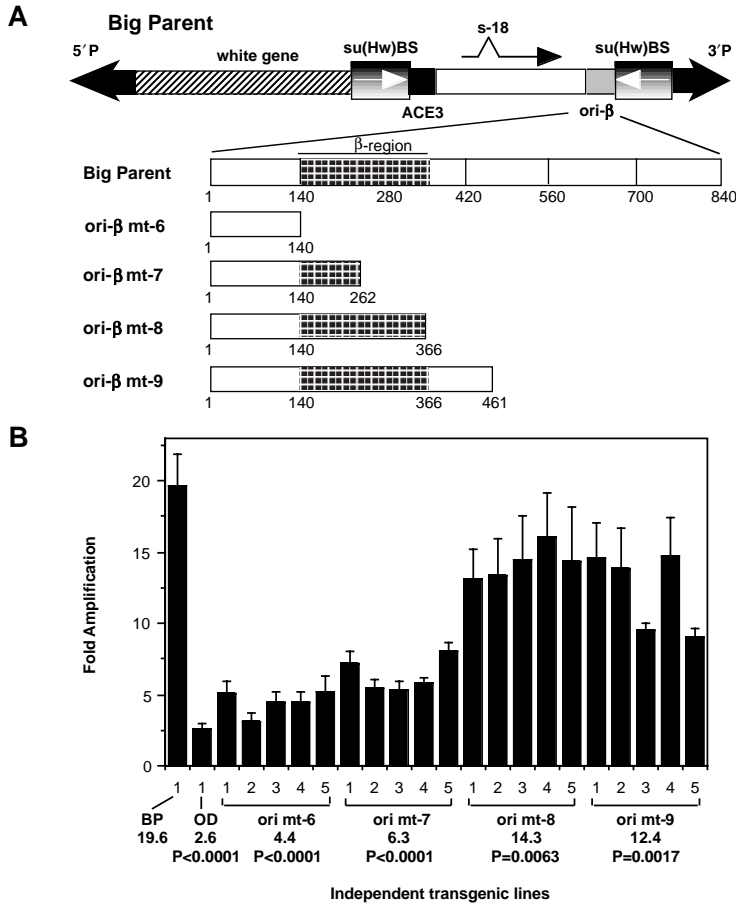
The *k43* gene is required for chorion gene amplification, and encodes the second-largest subunit of the origin recognition complex, ORC2 (Gossen et al., 1995; Landis et al., 1997). Staining early stage egg chambers with antibody specific for ORC2 reveals a diffuse nuclear distribution. At stage 10A, coincident with the initiation of amplification, the diffuse nuclear staining of ORC2 is lost and the ORC localizes into dramatic foci of staining at the chorion gene loci (Royzman et al., 1999) (Fig. 7A). DmORC2 also localizes at the site of a highly amplifying transgenic construct containing extensive third chromosome chorion locus sequences, and to the site of a poorly amplifying construct containing a large multimer of ACE3 sequences (Austin et al., 1999). Mutations that disrupt amplification either eliminate ORC2 staining or prevent its change from diffuse nuclear staining to the localized foci pattern (Whittaker et al., 2000). The data suggest that the mechanism of chorion gene amplification is intimately tied to the formation of this large ORC2-containing chromatin structure. To investigate the relationship further, both the cis

and trans requirements for foci formation were analyzed in further detail.

The *k43<sup>fs393</sup>* 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<sup>SC46</sup>* causes female sterility and thin eggshells (Schupbach and Wieschaus, 1991). *satin<sup>SC46</sup>* 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 relocates 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

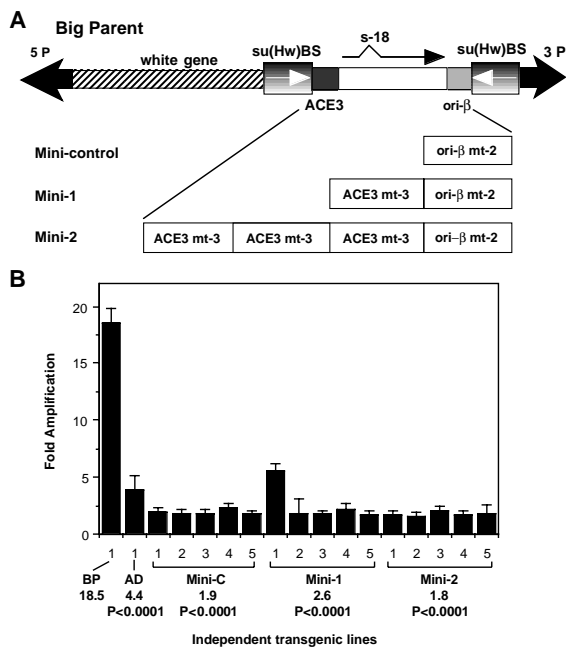


**Fig. 5.** The A/T-rich β region of ori-β is required for activity. (A) BP and ori-β mutants 6-9. (B) Quantitation of amplification levels for independent transgenic lines.

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.

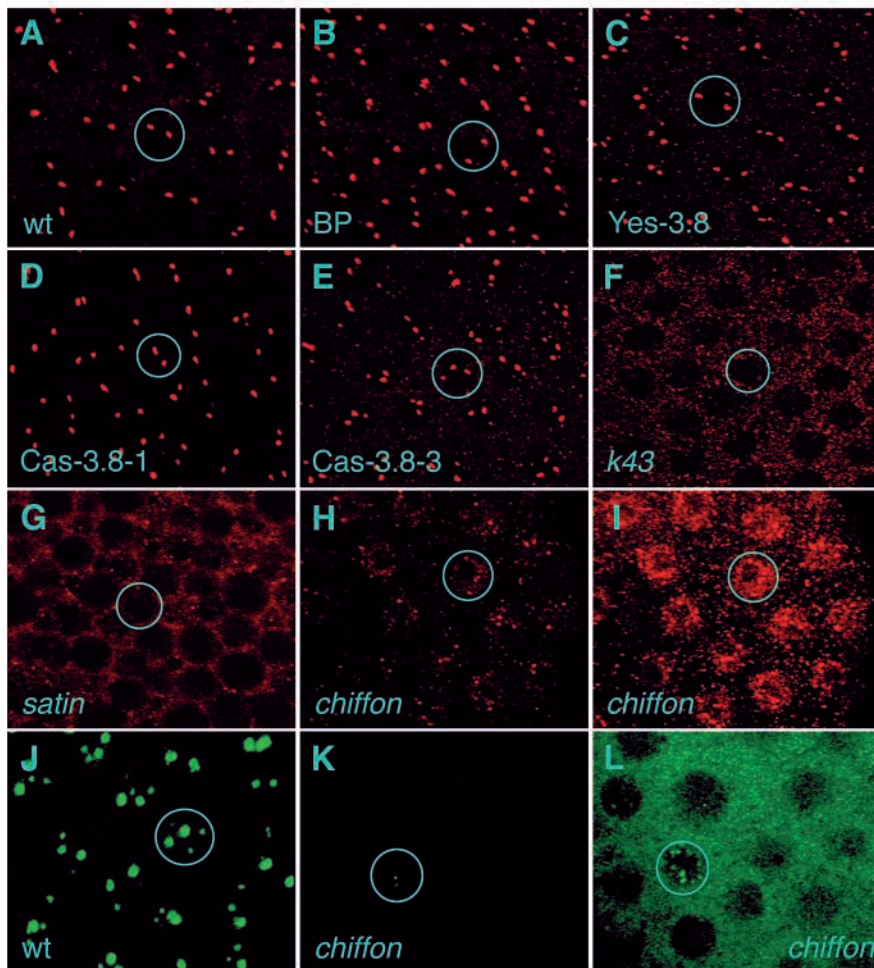
The fact that the insulator elements allowed amplification to occur in the absence of visible foci formation suggested the possibility that the insulators had reduced or eliminated the requirement for ORC2 activity in amplification of such buffered transgenic constructs. To test this possibility, three buffered transgenic inserts were crossed into a *k43<sup>fs293</sup>* mutant background and assayed for amplification (Fig. 8C). In all three cases, amplification of the buffered transgenic constructs was eliminated by the *k43<sup>fs293</sup>* 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

**Fig. 6.** The minimal replicons are not able to support amplification. (A) BP and the minimal replicon constructs. The 2.4 kb fragment containing ACE3, *S18* and ori-β in BP construct was replaced by the indicated combinations of ori-β mt-2 and/ or ACE3 mt-3. (B) Quantitation of amplification levels for independent transgenic lines.



**Fig. 7.** Anti-ORC2 antibody staining and BrdU labeling in follicle cells. All images were generated using the confocal microscope. Background was intentionally increased in images F, G, I and L to reveal presence or absence of faint staining patterns. (A) Wild-type with anti-ORC2. (B) The transgenic line for BP construct with anti-ORC2. (C) The transgenic line for Yes-3.8S construct with anti-ORC2. (D,E) The transgenic lines for pCaryos-3.8S construct with anti-ORC2, lines 1 and 3, respectively. Line 1 amplifies only to very low level due to genomic position effects, while line 3 amplifies to high level, as shown in Fig. 8. (F) *k43<sup>fs293</sup>/k43<sup>1</sup>* with anti-ORC2. (G) *satin* with anti-ORC2. (H) *chiffon* null mutant, genotype *chiffon<sup>wf24</sup>/Df(2)RA5*, with anti-ORC2. (I) *chiffon* null mutant with anti-ORC2 as in H. (J) Wild type with BrdU labeling. (K) *chiffon* null mutant, genotype *chiffon<sup>wf24</sup>/Df(2)RA5*, with BrdU labeling. (L) *chiffon* null mutant, genotype *chiffon<sup>wf24</sup>/Df(2)RA5*, with BrdU labeling as in K. The circles indicate the nuclei.

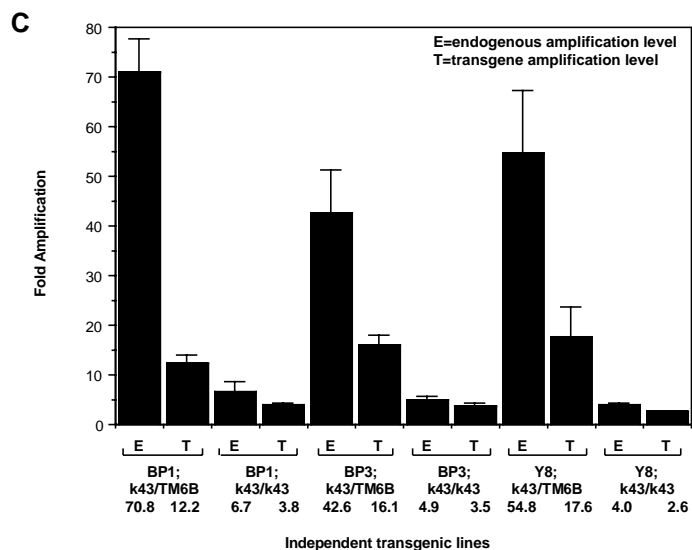
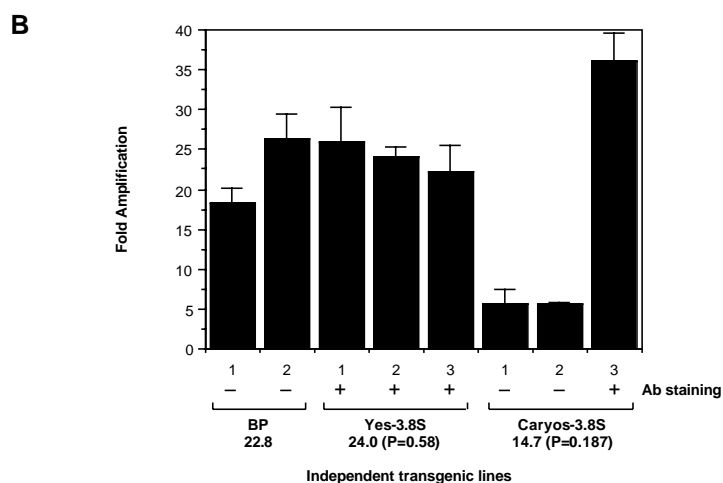
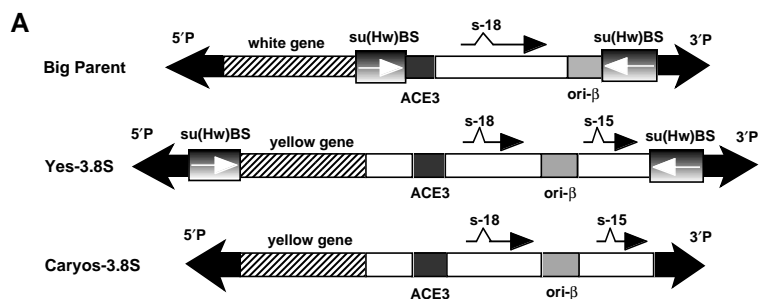
were in their normal context, i.e. spaced by the ~1.2 kb *S18* chorion gene, and these sequences supported efficient amplification (~20 fold) with minimal position effects. For this reason, the BP construct was chosen for detailed analysis of ACE3 and ori- $\beta$  sequence requirements. Evolutionarily 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- $\beta$  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 (Fig. 2A). 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- $\beta$  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- $\beta$  element while no initiations could be detected for ACE3 (Lu et al., 2001). To begin to examine the sequence requirements for ori- $\beta$  function, ori- $\beta$  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- $\beta$  is not simply an A/T-rich or easily unwound sequence. Deletion mapping suggests two sub-components of ori- $\beta$ : an essential 5' 140 bp region that is not particularly A/T-rich, and the 226 bp A/T-rich  $\beta$  region. The 366 bp fragment containing both regions was sufficient for the majority of ori- $\beta$  activity. In addition the 3' most 140 bp of the starting 840 bp ori- $\beta$  fragment may have a small stimulatory





effect. The portion of the  $\alpha$  region in ACE3 and the  $\beta$  region in ori- $\beta$  are each A/T-rich and internally repetitive, and have some sequence homology with each other (Levine and Spradling, 1985). A large fragment containing the  $\beta$ -region can bind ORC in vitro (Austin et al., 1999). Therefore, we hypothesize that, like the sequences in ACE3, one required function of the  $\beta$  region in ori- $\beta$  is to bind ORC.

A similar organization has been identified for a developmentally regulated origin in another dipteran fly, *Sciara coprophila* (Bielinsky et al., 2001). In *Sciara* larvae, the salivary gland cells amplify several loci containing putative pupal case genes, resulting in chromosomal DNA 'puffs'. The *ori II/9A* DNA replication initiation site has been mapped to

**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<sup>fs293</sup>/k43<sup>1</sup>*; E, endogenous amplification level; T, transgene amplification level. Average fold amplification levels are presented below the graph.

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 *dbf4*-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 *dDP* (a subunit of E2F) and *Rbf* (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  $\beta$  region in ori- $\beta$ . *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  $\alpha$  and  $\beta$  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- $\beta$  to interact and form a chromosomal domain activated for DNA initiation events. Previous data

indicated that ACE3 and ori- $\beta$  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- to 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 level 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- $\beta$ , 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.

We thank Lucy Lu and Gary Landis for helpful suggestions and assistance; Susan Gasser for her information and comment on *dbf4* and *chiffon* mutants; Bruce Stillman for plasmid pARS/WTA; Terry Orr-Weaver, Stephen Bell and Giovanni Bosco for providing anti-ORC2 sera and staining protocol; and Brian Calvi and Mary Lilly for BrdU labeling protocol. This research was supported by a grant from the Department of Health and Human Services (GM48449).

## References

- Asano, M. and Wharton, R. P. (1999). E2F mediates developmental and cell cycle regulation of ORC1 in *Drosophila*. *EMBO J.* **18**, 2435-2448.
- Austin, R. J., Orr-Weaver, T. L. and Bell, S. P. (1999). *Drosophila* ORC specifically binds to ACE3, an origin of DNA replication control element. *Genes Dev.* **13**, 2639-2649.
- Beall, E. L., Manak, J. R., Zhou, S., Bell, M., Lipsick, J. S. and Botchan, M. R. (2002). Role for a *Drosophila* Myb-containing protein complex in site-specific DNA replication. *Nature* **420**, 833-837.
- Bell, S. P. (2002). The origin recognition complex: from simple origins to complex functions. *Genes Dev.* **16**, 659-672.
- Bell, S. P. and Dutta, A. (2002). DNA replication in eukaryotic cells. *Annu. Rev. Biochem.* **71**, 333-374.
- Bielinsky, A. K., Blitzblau, H., Beall, E. L., Ezrokhi, M., Smith, H. S., Botchan, M. R. and Gerbi, S. A. (2001). Origin recognition complex binding to a metazoan replication origin. *Curr. Biol.* **11**, 1427-1431.
- Bielinsky, A. K. and Gerbi, S. A. (2001). Where it all starts: eukaryotic origins of DNA replication. *J. Cell Sci.* **114**, 643-651.
- Bosco, G., Du, W. and Orr-Weaver, T. L. (2001). DNA replication control through interaction of E2F-RB and the origin recognition complex. *Nat. Cell Biol.* **3**, 289-295.
- Calvi, B. R. and Spradling, A. C. (1999). Chorion gene amplification in *Drosophila*: A model for metazoan origins of DNA replication and S-phase control. *Methods* **18**, 407-417.
- Calvi, B. R., Lilly, M. A. and Spradling, A. C. (1998). Cell cycle control of chorion gene amplification. *Genes Dev.* **12**, 734-744.
- Chesnokov, I., Gossen, M., Remus, D. and Botchan, M. (1999). Assembly of functionally active *Drosophila* origin recognition complex from recombinant proteins. *Genes Dev.* **13**, 1289-1296.
- Claycomb, J. M., MacAlpine, D. M., Evans, J. G., Bell, S. P. and Orr-Weaver, T. L. (2002). Visualization of replication initiation and elongation in *Drosophila*. *J. Cell Biol.* **159**, 225-236.
- Dawid, I. B., Wellauer, P. K. and Long, E. O. (1978). Ribosomal DNA in *D. melanogaster*. I. Isolation and characterization of cloned fragments. *J. Mol. Biol.* **126**, 749-768.
- deCicco, D. V. and Spradling, A. C. (1984). Localization of a *cis*-acting element responsible for the developmentally regulated amplification of *Drosophila* chorion genes. *Cell* **38**, 45-54.
- Delidakis, C. and Kafatos, F. C. (1989). Amplification enhancers and replication origins in the autosomal chorion gene cluster of *Drosophila*. *EMBO J.* **8**, 891-901.
- Gerasimova, T. I. and Corces, V. G. (2001). Chromatin insulators and boundaries: effects on transcription and nuclear organization. *Annu. Rev. Genet.* **35**, 193-208.
- Gossen, M., Pak, D. T., Hansen, S. K., Acharya, J. K. and Botchan, M. R. (1995). A *Drosophila* homolog of the yeast origin recognition complex. *Science* **270**, 1674-1677.
- Heck, M. M. S. and Spradling, A. C. (1990). Multiple replication origins are used during *Drosophila* chorion gene amplification. *J. Cell Biol.* **110**, 903-914.
- Landis, G. and Tower, J. (1999). The *Drosophila* *chiffon* gene is required for chorion gene amplification, and is related to the yeast Dbf4 regulator of DNA replication and cell cycle. *Development* **126**, 4281-4293.
- Landis, G., Kelley, R., Spradling, A. C. and Tower, J. (1997). The k43 gene, required for chorion gene amplification and diploid cell chromosome replication, encodes the *Drosophila* homolog of yeast origin recognition complex subunit 2. *Proc. Natl. Acad. Sci. USA* **94**, 3888-3892.

- Levine, J. and Spradling, A. C.** (1985). DNA sequence of a 3.8 kilobase pair region controlling *Drosophila* chorion gene amplification. *Chromosoma* **92**, 136-142.
- Lilly, M. A. and Spradling, A. C.** (1996). The *Drosophila* endocycle is controlled by Cyclin E and lacks a checkpoint ensuring S-phase completion. *Genes Dev.* **10**, 2514-2526.
- Lu, L. and Tower, J.** (1997). A transcriptional insulator element, the su(Hw) binding site, protects a chromosomal DNA replication origin from position effects. *Mol. Cell. Biol.* **17**, 2202-2206.
- Lu, L., Zhang, H. and Tower, J.** (2001). Functionally distinct, sequence-specific replicator and origin elements are required for *Drosophila* chorion gene amplification. *Genes Dev.* **15**, 134-146.
- Marahrens, Y. and Stillman, B.** (1992). A yeast chromosomal origin of DNA replication defined by multiple functional elements. *Science* **255**, 817-823.
- Orr, W., Komitopoulou, K. and Kafatos, F. C.** (1984). Mutants suppressing in trans chorion gene amplification in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **81**, 3773-3777.
- Orr-Weaver, Y. L., Johnston, C. G. and Spradling, A. C.** (1989). The role of ACE3 in *Drosophila* chorion gene amplification. *EMBO J.* **8**, 4153-4162.
- Osheim, Y. N., Miller, O. L. and Beyer, A. L.** (1988). Visualization of *Drosophila melanogaster* chorion genes undergoing amplification. *Mol. Cell. Biol.* **8**, 2811-2821.
- Quinn, L. M., Herr, A., McGarry, T. J. and Richardson, H.** (2001). The *Drosophila* Geminin homolog: roles for Geminin in limiting DNA replication, in anaphase and in neurogenesis. *Genes Dev.* **15**, 2741-2754.
- Royzman, I. and Orr-Weaver, T. L.** (1998). S phase and differential DNA replication during *Drosophila* oogenesis. *Genes Cells* **3**, 767-776.
- Royzman, I., Austin, R. J., Bosco, G., Bell, S. P. and Orr-Weaver, T. L.** (1999). ORC localization in *Drosophila* follicle cells and the effects of mutations in dE2F and dDP. *Genes Dev.* **13**, 827-840.
- Rubin, G. M. and Spradling, A. C.** (1982). Genetic transformation of *Drosophila* with transposable element vectors. *Science* **218**, 348-353.
- Schupbach, T. and Wieschaus, E.** (1991). Female sterile mutations on the second chromosome of *Drosophila melanogaster*. II. Mutations blocking oogenesis or altering egg morphology. *Genetics* **129**, 1119-1136.
- Schwed, G., May, N., Pechersky, Y. and Calvi, B. R.** (2002). *Drosophila* minichromosome maintenance 6 is required for chorion gene amplification and genomic replication. *Mol. Biol. Cell* **13**, 607-620.
- Spradling, A. C. and Leys, E.** (1988). Slow replication fork movement during *Drosophila* chorion gene amplification. In *Cancer Cells 6/Eukaryotic DNA Replication*, pp. 305-309. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
- Spradling, A. C. and Mahowald, A. P.** (1981). A chromosome inversion alters the pattern of specific DNA replication in *Drosophila* follicle cells. *Cell* **27**, 203-209.
- Stillman, B.** (1993). Replicator renaissance. *Nature* **366**, 506-507.
- Swimmer, C., Fenerjian, M. G., Martínez-Cruzado, J. C. and Kafatos, K. C.** (1990). Evolution of the autosomal chorion gene cluster in *Drosophila* III. Comparison of the s18 gene in evolutionarily distant species and interspecific control of chorion gene amplification. *J. Mol. Biol.* **215**, 225-235.
- Whittaker, A. J., Royzman, I. and Orr-Weaver, T. L.** (2000). *Drosophila* double parked: a conserved essential replication protein that colocalizes with the origin recognition complex and links DNA replication with mitosis and the down-regulation of S phase transcripts. *Genes Dev.* **14**, 1765-1776.