Functionally distinct, sequence-specific replicator and origin elements are required for *Drosophila* chorion gene amplification

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To meet the demand for the rapid synthesis of chorion (eggshell) proteins, *Drosophila* ovarian follicle cells amplify the chromosomal loci containing the chorion gene clusters up to 60-fold. Amplification occurs by repeated firing of one or more origins located within each gene cluster. Deletion analyses of transgenic constructs derived from the third chromosome cluster have identified a 320-bp amplification control element (ACE3) required for amplification, as well as several stimulatory amplification enhancing regions (AERs). Two-dimensional (2D) gel analyses have identified multiple DNA replication initiation sites (origins) that partially overlap in location with ACE3 and the AERs. To further study sequence requirements for amplification, a vector was used in which transgenic constructs are protected from chromosomal position effects by flanking insulator elements, the suppressor Hairy-wing protein binding site (SHWBS). Using the buffered vector, the 320-bp ACE3 and an 884-bp element designated ori-β were found to be necessary and sufficient for amplification. Two-dimensional gels revealed that ori-β was acting as the origin. In contrast, origin activity could not be detected for ACE3. An insulator placed between ACE3 and ori-β inhibited amplification, indicating that ACE3 activates ori-β in *cis*. The results suggest that ACE3 acts as a replicator and support and extend the replicator model for the organization of metazoan chromosomal replicons.

[*Key Words*: DNA replication; ORC; insulator; border element; suppressor Hairy-wing]

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Over 35 years ago, Jacob and Brenner proposed the replicon model for regulation of bacterial chromosome replication. A genetic element called the replicator would function as the target site for binding of an initiator protein (Jacob et al. 1963). The development of physical mapping techniques, such as two-dimensional (2D) gels has since allowed identification of origins—the locations in the DNA where DNA replication initiates (Brewer and Fangman 1987; Bielinsky and Gerbi 1999). In bacteria and yeasts, the replicator and origin are coincident. Origins have been mapped extensively in metazoans, and data indicate that sequences far from the preferred initiation sites can sometimes affect replication (DePamphilis 1999). These results have suggested a replicator model for the organization of higher eukaryotic replicons, in which replicators and origins are non-coincident (Stillman 1993). However, the lack of convenient genetic assays has hindered the analysis of higher eukaryotic replicators and has lead to some controversy as to the nature and extent of specific sequence requirements for DNA replication (Hamlin and Dijkwel 1995; DePamphilis 1999).

The CHO DHFR locus is one of the best studied of higher eukaryotic origin regions, and all of the physical mapping methods indicate that the origin(s) are located within the 55-kb spacer region between the 3′ end of the DHFR gene and the 5′ end of the next adjacent gene (Linskens and Huberman 1990; Hamlin and Dijkwel 1995; DePamphilis 1999). However, different techniques yield somewhat contradictory data regarding the exact sites of initiation, and origin activity appears to involve initiations throughout the region, with some degree of preference for initiation at three sites, called ori-β, ori-β′, and ori-γ (Burhans et al. 1990; Vaughn et al. 1990; Kalejta et al. 1996; Kobayashi et al. 1998; Wang et al. 1998). Attempts to identify DHFR replicators have so far yielded conflicting results. When a 16-kb stretch of DNA containing the DHFR ori-β was inserted at other locations in the mammalian genome, initiation was found to occur within or near the insertion, suggesting that the 16-kb ori-β region contains important *cis*-regulators (Handeli et al. 1989). However, deletion of ori-β at the endogenous locus did not alter replication of the locus,

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while deletion of the 3′ end of the DHFR gene eliminated activity of ori-β (Kalejta et al. 1998). Taken together, the results indicate that the DHFR ori-β region is not sufficient to direct initiation and that the *cis*-regulatory replicator sequences have not yet been defined.

Several labs have used physical methods to map an origin near the 5′ end of the human β-globin gene, also called ori-β. A large, naturally occurring deletion of this region in hemoglobin Lepore syndrome cells eliminates bidirectional replication, and the locus is replicated from upstream (Kitsberg et al. 1993). Hispanic thalessemia is another large, naturally occurring deletion, which removes the 5′ LCR (locus control region), as well as other sequences, but leaves ori-β intact. In this case, bidirectional replication is also eliminated, and now the locus is replicated from the 3′ direction (Aladjem et al. 1995). The LCR is an element that was first identified as being required for high-level, position-independent expression of globin transgenes. These data suggested that the LCR might be important in regulating both transcription and DNA replication in its particular chromosomal domain. However, recent genetic assays involving β-globin sequences integrated at an ectopic chromosomal position did not detect a requirement for the LCR in controlling DNA replication, whereas sequences near ori-β were found to be required (Aladjem et al. 1998). Taken together, the results with the DHFR, β-globin and other loci suggest that initiation in higher eukaryotes is controlled both by local sequences and by sequences distant from the preferred initiation sites. However, the lack of convenient genetic assays has so far precluded the systematic mapping and analysis of these potential regulatory elements, and the nature and extent of specific sequence requirements is uncertain.

Drosophila chorion gene amplification provides a model for higher eukaryotic chromosomal DNA replication that is amenable to genetic analyses (Delidakis et al. 1989; Orr-Weaver 1991; Calvi and Spradling 1999; Spradling 1999). The developing *Drosophila* oocyte is surrounded by a layer of follicle cells that synthesize the eggshell, or chorion. The genes encoding the major chorion proteins reside in two clusters in the *Drosophila* genome, one on the X chromosome and one on the 3rd. To meet the demand for the rapid synthesis of chorion proteins, the follicle cells specifically amplify the two chromosomal domains containing the chorion gene clusters, ~16-fold and ~60-fold, respectively. Amplification results from the repeated firing of a small number of replication origins interspersed within each gene cluster, producing an onionskin-type structure (Osheim et al. 1988).

The *cis*-regulatory sequences for chorion gene amplification have been studied in greatest detail for the third chromosome gene cluster. Essential and stimulatory sequences were mapped by introducing various deleted and mutated genomic constructs into the *Drosophila* germ line by P-element mediated transformation and asking whether these constructs amplified in the follicle cells of transgenic females. Amplification is highly subject to chromosomal position effects, and the majority of insertion sites for any given construct yielded little or no amplification. Previous approaches to deal with the severe positions effects were: (1) the assay and statistical analysis of large numbers of control and deletion constructs and (2) the creation of a series of deletions of one large transgenic construct by imprecise transposase-induced excision events (Delidakis and Kafatos 1989; Orr-Weaver et al. 1989). Those studies identified a 320-bp region required for high levels of amplification, called ACE3 (for amplification control element, 3rd chromosome), as well as four stimulatory regions (or amplification-enhancing regions) AER-A, AER-B, AER-C, and AER-D. Two-dimensional gel analysis of DNA replication intermediates (Brewer and Fangman 1987) isolated from the follicle cells has demonstrated that amplification uses multiple DNA replication origins that overlap in location with ACE3 and the AER regions (Delidakis and Kafatos 1989; Heck and Spradling 1990).

In amplification constructs containing a limited amount of stimulatory sequences, the ACE3 element appears essential. However, in larger constructs, low levels of amplification can be observed in the absence of ACE3, indicating some redundancy of function between different regions of the locus (Swimmer et al. 1989). ACE3 multimers were able to support very low levels of amplification, demonstrating that ACE3 is sufficient to direct low-level amplification (Carminati et al. 1992). Southern analyses indicated that the increased copy number was greatest for sequences outside the ACE3 multimer, suggesting that ACE3 might have activated cryptic origin(s) in vector or flanking sequences. However, the amplification of the multimer construct was too low to allow physical mapping studies such as 2D gels, so it is uncertain whether ACE3 and/or adjacent sequences were functioning as origins.

The suppressor of Hairy-wing protein binding site (SHWBS) from the gypsy transposon is a powerful transcriptional insulator element (Geyer and Corces 1992; Corces 1995; Mallin et al. 1998; Zorin et al. 1999). SHWBSs can block enhancer–promoter interactions when placed between the enhancer and the promoter, and they can also block the effects of certain negative regulatory elements. SHWBSs can also protect transcription from positive and negative chromosomal position effects when placed flanking a transgenic transcription unit (Roseman et al. 1993). The SHWBS insulator was found to also protect chorion gene amplification from chromosomal position effects (Lu and Tower 1997). When buffered by flanking SHWBSs, constructs amplified at all insertion sites. Amplification was equal on both sides of the SHWBSs, demonstrating that these sites do not significantly impede replication-fork passage. The data demonstrated that flanking insulators create a chromosomal domain that is permissive for the function of the chorion gene origin(s) and provided an improved assay for amplification sequence requirements. Using this improved assay, the sequence requirements for chorion gene amplification have been analyzed in further detail.

Results

Organization of the third-chromosome chorion gene cluster

The location of the ACE3 and AER *cis*-regulatory regions of the third-chromosome chorion gene cluster identified in previous deletion studies are diagrammed (Fig. 1A). One of the stimulatory regions (AER-D) partially overlaps with the region exhibiting the majority of origin activity during amplification, as determined by 2D gels (Fig. 1B; Delidakis and Kafatos 1989; Heck and Spradling 1990). Two-dimensional gels also identified a smaller number of replication forks that initiated more distally, partially overlapping the more distal AER-A, AER-B, and AER-C stimulatory regions, as well as from a more proximal region overlapping ACE3. Taken together, the previous studies indicated that amplification utilizes multiple origins, interspersed among the chorion genes, over a region of many kilobases, with a preference for the region downstream of *S18*. This organization appears similar to that of the mammalian DHFR and β-globin loci, described above. The fact that ACE3 was required but was not the primary initiation site suggested the hypothesis that ACE3 acts as a replicator element that activates nearby origins, including an origin downstream of *S18*. Sequence analysis of the locus originally identified striking, partially related A-T-rich repetitive elements in the region called α and β, and it was suggested that these elements might be involved in regulating amplification (Levine and Spradling 1985). α is located within ACE3. β is located within the 884-bp element downstream of *S18* characterized as an origin in the experiments presented below. For this reason, the 884-bp origin element is hereafter referred to as ori-β. For simplicity the sites of lower-frequency initiation are referred to here as ori-α and ori-γ, respectively (Fig. 1).

ACE3 and ori-β are sufficient for amplification

The development of vectors buffered from position effects by flanking SHWBS insulator elements facilitates mapping of *cis* sequence requirements. The 3.8-kb *Sal*I fragment from the third-chromosome chorion gene locus (Fig. 1)—containing ACE3, *S18* chorion gene, ori-β, and *S15* chorion gene—was used as the starting construct (pYES-3.8S; Fig. 2A). This sequence was previously found to support efficient amplification in both buffered and nonbuffered vectors (Lu and Tower 1997). Experiments were performed to determine if ACE3 and ori-β would be sufficient for amplification in the context of the buffered vector.

Deletion to the 5′ boundary of ACE3 and the 3′ boundary of the 884-bp ori-β region in construct pYES-2.4 (Fig. 2B) was equally as active as the parent construct containing the entire 3.8-kb *Sal*I fragment (Fig. 2D). Deletion of the *S18* gene located between ACE3 and ori-β generated construct pYES-1.2 consisting of only the 320-bp ACE3 and the 884-bp ori-β fragment. This construct was found to be capable of amplification, although the level of amplification was somewhat reduced and the construct was now more subject to position effects. The reduced activity of construct pYES-1.2 relative to construct pYES-2.4 and pYES-3.8S may be because of a specific stimulatory effect of the deleted sequences, an effect of the presence of an active gene, or merely the optimal spacing of ACE3 and ori-β. However, the results demonstrate that the 320-bp ACE3 and 884-bp ori-β fragments are sufficient to support amplification. In addition, the results demonstrate that ACE3 and ori-β can function when placed immediately adjacent to each other, a result that is important for the interpretation of additional results presented below.

An insulator (SHWBS) placed between ACE3 and ori-β reduces amplification

As discussed above, the SHWBS is an insulator element that can block activation of promoters by transcriptional enhancers (Geyer and Corces 1992) and can buffer transcription and amplification from chromosomal position effects (Roseman et al. 1993; Lu and Tower 1997). Experiments were designed to further test the hypothesis that ACE3 acts as a replicator element and activates

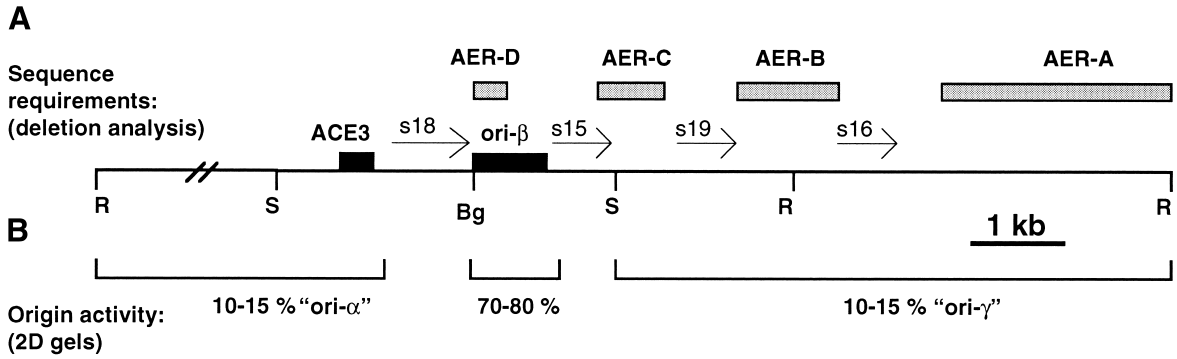

Figure 1. Organization of third-chromosome chorion gene cluster and amplification regulatory elements. (*A*) *Cis*-regulatory elements identified by deletion analyses. Solid boxes represent the essential ACE3 element and the ori-β element characterized in this study. Stippled boxes represent stimulatory sequences mapped previously (AERs). Chorion genes are indicated by arrows. (*B*) Regions of initiation activity previously identified by two-dimensional gel analyses are indicated (Delidakis and Kafatos 1989; Heck and Spradling 1990).

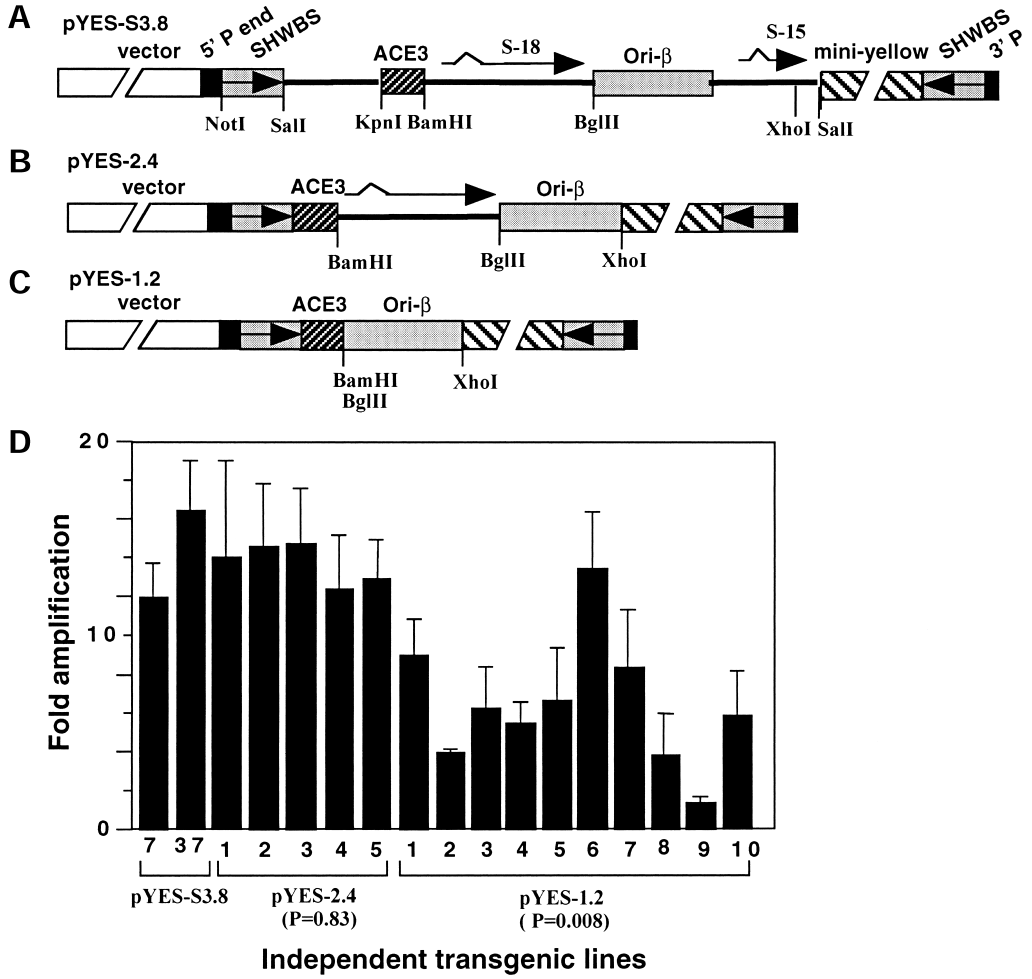

Figure 2. ACE3 and ori-β are sufficient to support amplification. (*A*) pYES-3.8S construct containing the 3.8-kb *Sal*I fragment from the third-chromosome chorion gene locus. (*B*) pYES-2.4 construct containing ACE3, *S18*, and ori-β. (*C*) pYES-1.2 construct, containing only ACE3 and ori-β. (*D*) Quantitation of amplification. In this and other figures, amplification was quantitated for multiple independent transgenic lines for each construct, as indicated. Fold amplification was calculated as described in Materials and Methods. Data are presented as the average ±S.D. of triplicate assays. The average amplification of each deleted construct (pYES-2.4 and pYES-1.2) was compared to the average amplification of the parent construct (pYES-3.8S) using unpaired, two-sided *t*-tests, and *P* values are presented below the graph.

nearby origins, primarily ori-β, in *cis* during amplification. If activation of ori-β by ACE3 is mechanistically similar to enhancer–promoter interactions, then preventing their interaction in *cis* with an intervening SHWBS might inhibit amplification. The alternate possibility is that ACE3 and ori-β do not interact in *cis* but, rather, function independently as origins, and their contribution to amplification is merely the additive amplification directed by each element. In this case, placing a SHWBS intervening between the two elements should have no effect on amplification, as DNA replication forks can pass unimpeded through a SHWBS (Lu and Tower 1997). Experiments were designed to test the ability of the SHWBS insulator to block a putative *cis*-activation step of amplification. It is known from previous experiments that the SHWBS will not impede the subsequent DNA replication forks.

A construct was generated called pYES-FRT(SHWBS) (Fig. 3A), which was buffered from position effects by flanking SHWBSs. A SHWBS was also placed intervening between ACE3 and ori-β, where it might block activation of ori-β by ACE3. The intervening SHWBS was itself flanked by FRTs, the target site for the yeast FLP recombinase. FLP recombinase expression was under the control of the HSP70 heat-inducible promoter in another transgenic construct inserted on the X chromosome (Golic and Lindquist 1989). In this way, a short heat pulse would cause production of FLP recombinase. This in turn would cause the excision of the fragment containing the intervening SHWBS (Fig. 3B) and, potentially, now restore activation of ori-β by ACE3. Chorion gene-coding region sequences (stuffer sequences in Fig. 3) were used to space the various functional elements in the construct, with the idea that this would prevent them from possibly sterically inhibiting each other. Chorion gene-coding region sequences were chosen as they appear to

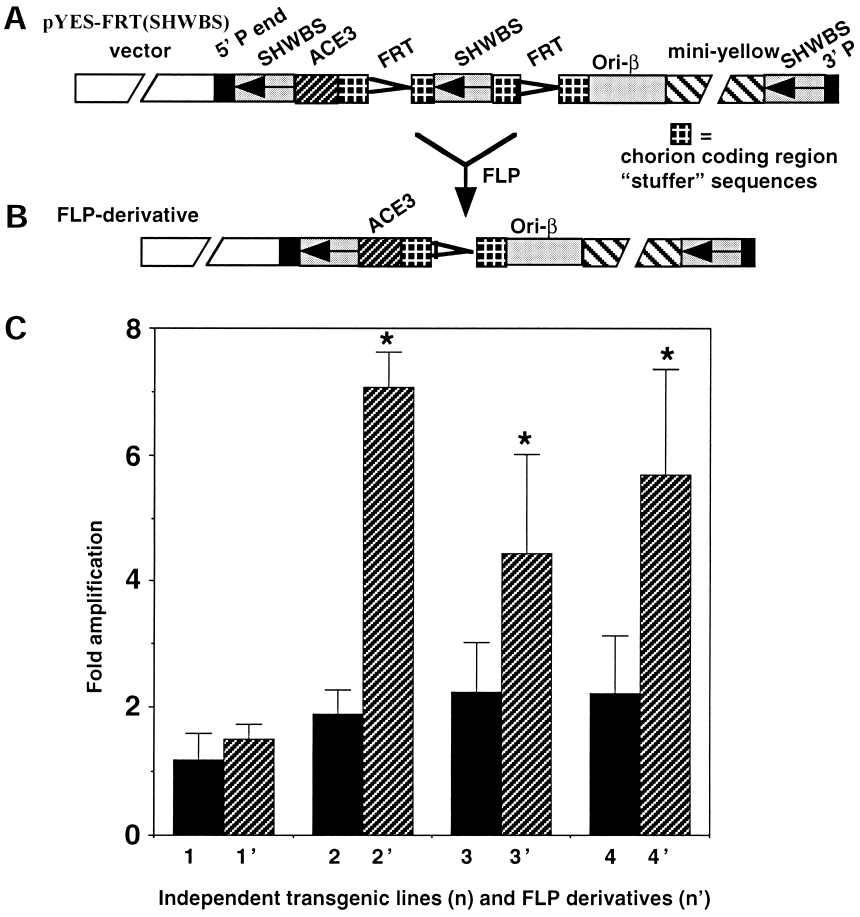

Figure 3. A SHWBS placed between ACE3 and ori-β inhibits amplification. (*A*) Construct pYES-FRT(SHWBS) containing ACE3 and ori-β with a SHWBS placed between them. The SHWBS is flanked by FRT sites, and the FRT sites are each flanked by chorion gene-coding sequences. (*B*) The expected product of FLP-mediated recombination of the pYES-FRT(SHWBS) construct. (*C*) Quantitation of amplification. Data are presented for each independent transgenic line (n) and for the average for two independent recombination derivatives for each line (n′). Derivatives were compared to the parent using unpaired, two-sided *t*-tests, and statistically significant differences ($P < 0.05$) are indicated by an asterisk.

be neutral with regard to amplification and are normally located between ACE3 and ori-β (Orr-Weaver and Spradling 1986; Delidakis and Kafatos 1989).

Four independent transgenic lines were generated for construct pYES-FRT(SHWBS), and the SHWBS between ACE3 and ori-β was found to yield little to no amplification (Fig. 3C). For each of the four lines, stable derivative lines were generated where the intervening SHWBS was excised using FLP recombinase. For three out of the four starting lines, excision of the SHWBS resulted in increased amplification, consistent with the conclusion that the intervening SHWBS reduced amplification by blocking the interaction of ACE3 and ori-β. The fact line 1 did not significantly increase in activity on excision may be because it was inserted in a location where strong negative chromosomal position effects precluded high-level amplification. Consistent with this idea is the fact that line 1 started with the lowest (undetectable) level of amplification.

An alternative explanation for the results of this experiment might be that recombination simply produced a more optimal spacing of ACE3 and ori-β. However, that possibility is ruled out by the fact that both larger and smaller distances between ACE3 and ori-β readily supported amplification (Fig. 2). The only sequences excised in addition to the SHWBS were one-half of the starting chorion gene-coding sequences and one of two FRT sites (Fig. 3A,B). The fact that the remaining chorion gene-coding sequences and single FRT allowed amplification in the recombined construct indicates that it is the SHWBS that serves to inhibit amplification in the starting construct.

A second experimental design was tried for testing the ability of the SHWBS insulator to block the interaction of ACE3 and ori-β, but a negative result was obtained that was largely uninformative. In this second experiment, the SHWBS insulator was placed between ACE3 and ori-β in the context of the 3.8-kb *Sal*I fragment, with no flanking insulators present in the construct. Multiple transgenic lines were generated, and this construct was found to yield little to no amplification (data not shown), consistent with the results of Figure 3 and an inhibitory effect of the intervening insulator. The activity of the insulator element is dependent on Su(Hw) protein binding. Therefore, if the transgenic constructs were to be crossed into a *su(Hw)* null mutant background, the insulator would become inactive and amplification should increase. Unfortunately, this experiment is impossible because *su(Hw)* gene function is required for oogenesis. In *su(Hw)* null mutants, oogenesis does not proceed to the amplification stages. The best approximation of this experiment was therefore to use a specific heteroallelic combination of hypomorphic *su(Hw)* mutant alleles, $su(Hw)^{v}/Su(Hw)^{f}$, that partially reduces *su(hw)* gene

function yet allows oogenesis to proceed (Geyer and Corces 1992). Six of the transgenic inserts were crossed in the hypomorphic *su(Hw)* mutant background, and amplification did not increase at any site (data not shown). This means either that the inhibition of amplification does not involve *su(Hw)* gene activity or that the heteroallelic combination does not reduce *su(Hw)* activity enough to restore amplification.

Taken together, the experiments demonstrate that placing a SHWBS between ACE3 and ori-β inhibits amplification. While the experiments do not demonstrate that this effect requires *su(Hw)* gene activity, they still have eliminated the possibility that the insulator is inhibiting amplification simply because of altered spacing of ACE3 and ori-β. Both larger and smaller distances between ACE3 and ori-β were shown to efficiently support amplification. Therefore, it must be the identity rather than the length of the sequences between ori-β and ACE3 that results in decreased amplification in the starting construct of Figure 3. We conclude that even in the event that Su(Hw) protein is not involved, the experiment provides evidence that ACE3 and ori-β must interact in *cis*.

ACE3 and ori-β are necessary and sufficient for amplification

The apparent ability of the SHWBS to reduce amplification when placed between ACE3 and ori-β suggested that the hypothesized ACE3 replicator may not efficiently activate an origin beyond an insulator. This result, in turn, suggested that placing insulator elements directly flanking ACE3 and ori-β might prevent the activation of cryptic origins in vector or flanking chromosomal sequences and, thereby, create constructs that are dependent on the presence of ori-β and/or other specific origins. To test this idea, two parent constructs for deletion studies were generated: the "Big Parent" construct with the 320-bp ACE3 and 884-bp ori-β with their normal spacing of the *S18* gene between them (Fig. 4A), and a "Small Parent" construct where the *S18* gene was deleted (Fig. 4D). In both contexts, efficient amplification was found to be dependent on the presence of both ACE3 and ori-β (Fig. 4G). The Big Parent construct supported high-level amplification, and deletion of ACE3 in construct Big(ACE deln) (Fig. 4B), or deletion of ori-β in construct Big(ori deln) (Fig. 4C) greatly reduced amplification. Small Parent construct supported moderate level amplification, and deletion of ACE3 in construct Small(ACE deln) (Fig. 4E) or deletion of ori-β in construct Small(ori deln) (Fig. 4F) eliminated amplification (recall that no amplification yields a value of 1).

ACE3 and ori-β are functionally distinct replicator and origin elements

Two-dimensional gels have been used by two groups to map patterns of origin activity in the third-chromosome chorion gene locus (Delidakis and Kafatos 1989; Heck and Spradling 1990). Both studies found that the majority of origin activity was associated with the ori-β (AER-D) region and that a much smaller amount was associated with the region containing ACE3 (diagrammed in Fig. 1B). Efficient amplification of the buffered Big Parent and Small Parent constructs described here was dependent on both ACE3 and ori-β. To confirm the hypothesis that ACE3 acts as a replicator, it was important to confirm that under these conditions the majority of origin activity was still associated with ori-β.

Using 2D gels, initiation events occurring within a particular DNA fragment are identified as bubble structures. While bubbles were readily identified at the endogenous third-chromosome chorion gene locus, they have been particularly difficult to observe for transgenic constructs because of position effects and the reduced levels of amplification. Only one transgenic line has been analyzed previously using 2D gels. The transgenic construct (called S6.9) included the 3.8-kb *Sal*I fragment containing ACE3, *S18*, gene and ori-β, and one particular transgenic line (5) yielded an unusually high amplification level of 59-fold, similar to the endogenous locus. Despite the high level of amplification associated with S6.9 line 5, bubble structures were near the limit of detection (Heck and Spradling 1990). Those results suggested that modifications to the 2D gel protocols would be necessary to analyze the lower amplification levels associated with the lines generated here.

A combination of several approaches was found to yield highly reproducible visualization of bubble structures. First, analyses were done with the more highly amplifying Big Parent construct. Amplification of Small Parent was too low to permit 2D gel analysis (data not shown). Second, analyses were performed with larger amounts of starting material. DNA was isolated from 100 ovaries enriched for stage 10 egg chambers, and gene amplification intermediates were purified by BND cellulose column chromatography (Liang et al. 1993). Third, analyses were done with the most highly amplifying Big Parent line (1) and a triple-insert line generated by crossing the inserts of Big Parent lines 2–4 into the same genetic background. Finally, analyses were designed so that the Southern blots involved large restriction fragments and probes (Fig. 5D).

DNA replication intermediates were purified from egg chambers from Big Parent line 1 (Fig. 5A), restriction digested, resolved on 2D gels, and transferred to Southern blots. The blots were hybridized with a probe specific for the ori-β region (Fig. 5D) that hybridizes to distinct-sized bands from the endogenous locus and from the transgenic construct. The 2D gel analysis revealed a complete fork arc and a bubble arc derived from the endogenous locus, as expected from previous studies (Fig. 5A). The bubble arc represents the initiations at ori-β in the endogenous locus, and the fork arc represents two things: the initiations at ori-β moving out of the fragment and yielding a dark, incomplete fork arc, and the forks moving into this region from the other, more minor origins of the endogenous locus, namely ori-α and ori-γ (see Fig. 1), which yields a lighter, overlapping com-

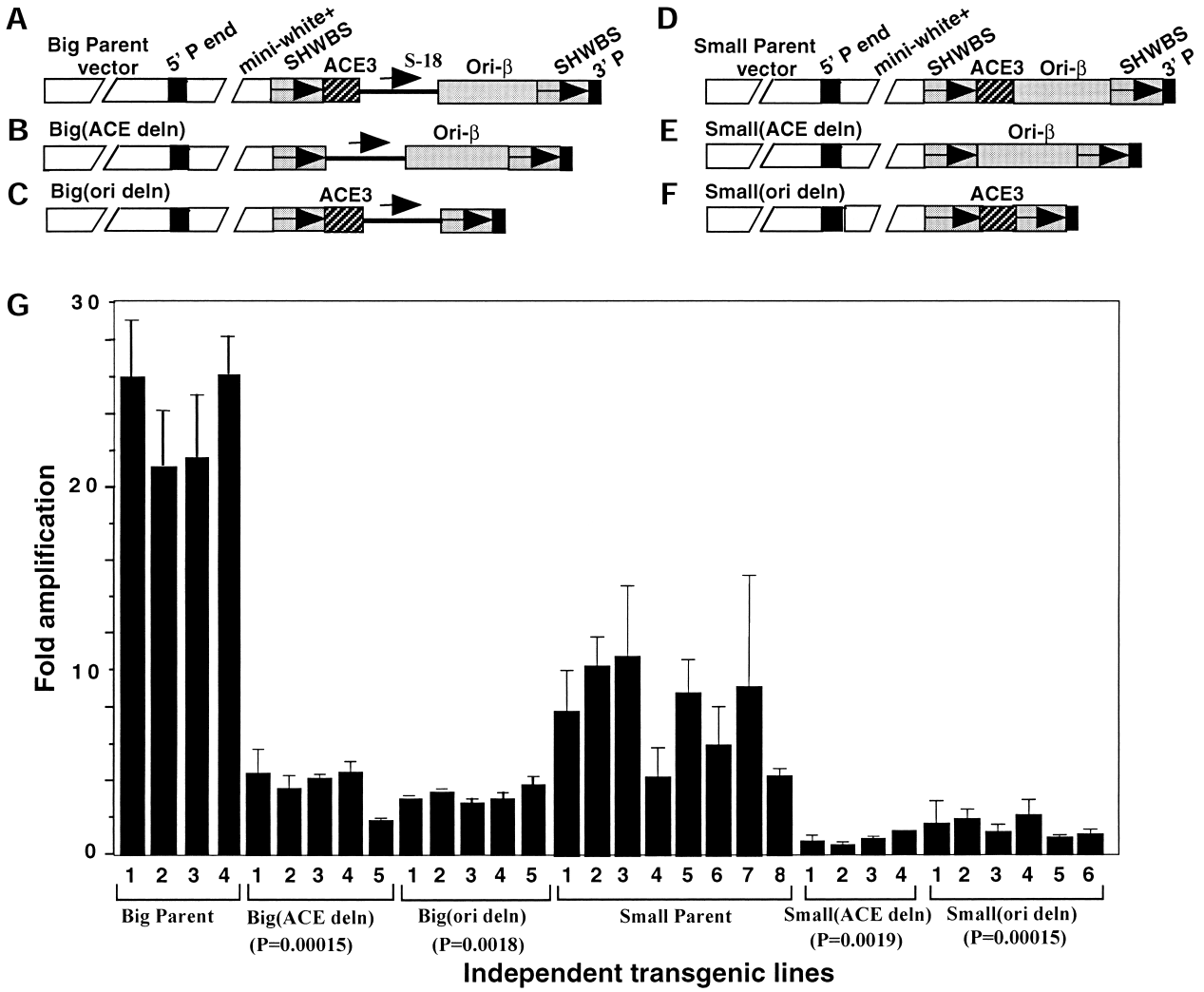

Figure 4. (*A*) Big Parent construct containing ACE3, *S18*, and ori-β immediately flanked by SHWBSs in pCaSpeR4 transformation vector. (*B*) Big(ACE deln) construct. (*C*) Big(ori deln) construct. (*D*) Small Parent construct containing ACE3 and ori-β immediately adjacent to one another and immediately flanked by SHWBSs in pCaSpeR4 transformation vector. (*E*) Small(ACE deln) construct. (*F*) Small(ori deln) construct. (*G*) Quantitation of amplification of multiple independent transgenic lines for each construct. The average amplification of each deleted construct was compared to the average amplification of the respective parent construct using unpaired, two-sided *t*-tests; *P* values are presented below the graph.

plete fork arc. The structures observed for the transgenic construct ori-β fragment consist of a bubble arc and an incomplete fork arc representing bubbles moving out of the fragment. The same experiment was done with the triple-insert line (Fig. 5B), and the signal was improved, allowing for shorter exposure times. A distinct bubble arc and an incomplete fork arc were again observed for the ori-β region. Therefore, in the transgenic construct ori-β acts as an origin.

The ACE region was analyzed using a different restriction digest and a probe specific for the transgenic ACE3 region (Fig. 5D). The experiments revealed no bubble arc, and a strong fork arc derived from the transgenic construct (Fig. 5C; data not shown). The intensity of the ACE3 region fork arc is asymmetric for two reasons: First, to distinguish the transgenic construct from the endogenous locus, it was necessary that the probe for the ACE3 region correspond to only the left (3′) half of the restriction digest fragment of the ACE3 region (Fig. 5D). This means that the probe will hybridize to the right side of the fork arc (where fork species have two copies of the probe region) twice as much as it will hybridize to the left side of the fork arc (where fork species are single copy in the probe region). Second, there is a slight pause site in the fork arc, as indicated by a spot, which will also tend to make the fork arc appear asymmetric. The pause was always observed, and based on its location in the fork arc, it appears to correspond to sequences in the *mini-white*$^+$ marker gene or the insulator. It is important to note that even though the ACE3 fork arc is uneven, the signal throughout the ACE3 fork arc is several times that of the fork arc for the ori-β region, and yet no bubble arc is detected for ACE3. The same result of strong fork arc and no bubble structures associated with ACE3 was

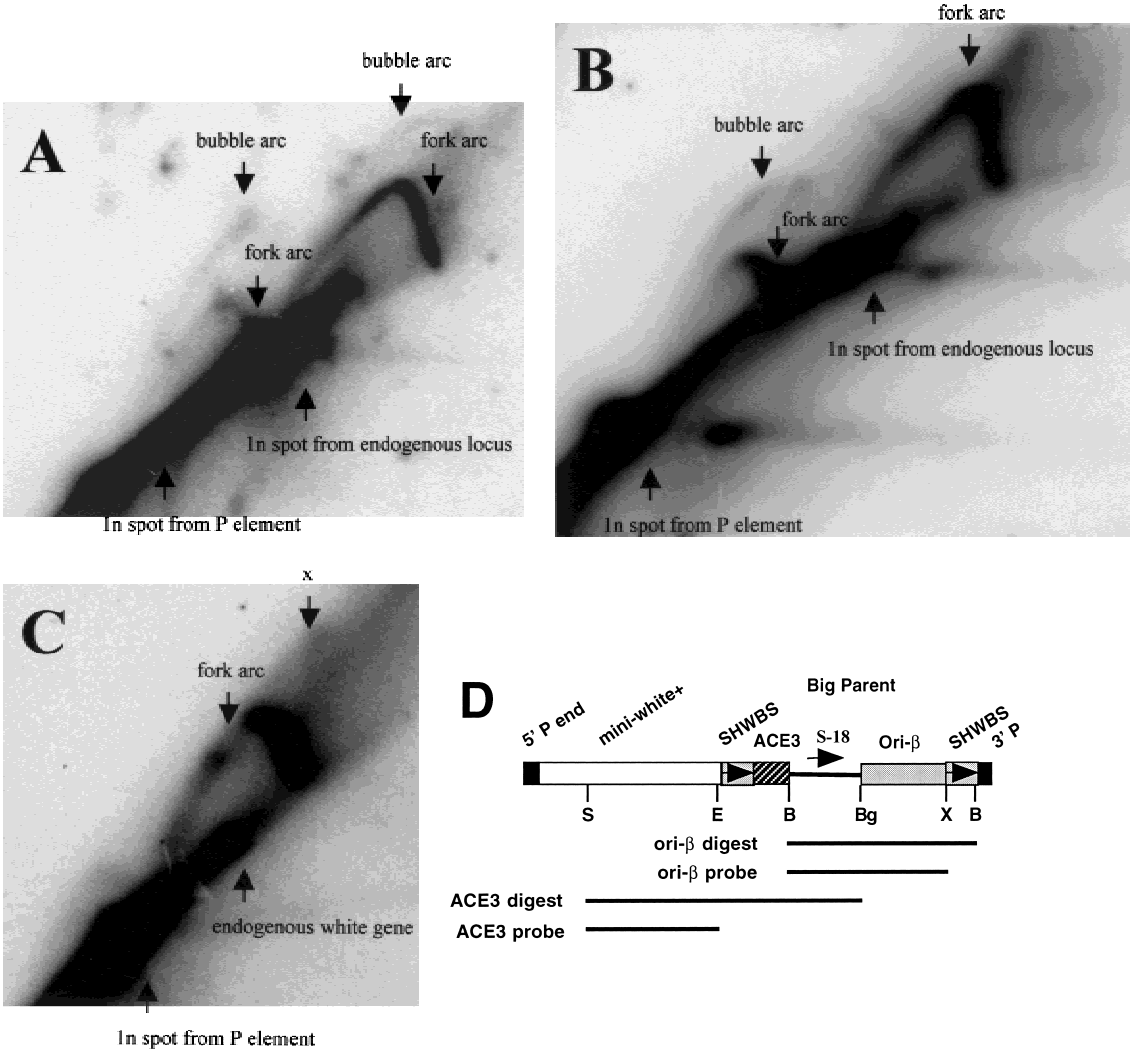

Figure 5. Two-dimensional gel analyses. Independent transgenic lines containing the Big Parent construct were analyzed by 2D gels. Linear fragments (1n spots) and replication intermediates (bubble arcs and fork arcs) are indicated with arrows and labeled. (*A*) Homozygous Big Parent transgenic line 1 containing a single copy of the Big Parent construct. The ori-β region was analyzed using the digest and probe diagrammed in *D*. (*B*) Homozygous, triple-insert Big Parent transgenic line, made by crossing the inserts of lines 2, 3, and 4 into the same background. The ori-β region was analyzed using the digest and probe diagrammed in *D*. This gel was run slightly further than in *A* to optimally resolve the species derived from the *P*-element construct. The endogenous locus bubble arc is off the top of the gel. (*C*) Homozygous, triple-insert Big Parent transgenic line. The ACE3 region was analyzed using the digest and probe diagrammed in *D*. The ACE region probe is specific for the transgenic construct to avoid hybridization to the endogenous ACE3 fragment of similar size. (*D*) Schematic of Big Parent construct and restriction digests and probes used in 2D gel analyses.

obtained with both Big Parent line 1 and the Big Parent triple-insert line (Fig. 5C; data not shown) in multiple repeated experiments for both. Therefore, no origin activity can be detected for ACE3. The data confirm that the general pattern of origin activity described for the endogenous locus is maintained in the transgenic constructs, with the majority of origin activity associated with ori-β. Because ACE3 is required for amplification but is not detected to act as an origin, the data support the conclusion that ACE3 is a replicator element. Conceivably, a more sensitive assay might detect a hypothetical origin activity associated with ACE3 in the transgenic constructs. However, any ACE3 origin activity would have to be significantly lower than that associated with ori-β, and such a result would not significantly alter the conclusions of this study.

Discussion

The 320-bp ACE3 and 884-bp ori-β elements were found to be necessary and sufficient for amplification. A SHWBS insulator was found to inhibit amplification only if located between the ACE3 and ori-β elements, suggesting that they interact in *cis* and are not merely directing amplification in an independent and additive manner. Finally, only ori-β could be detected to act as an

origin in transgenic constructs, supporting the conclusion that ACE3 functions as a replicator to activate ori-β in *cis*. The studies were made possible by creating constructs with flanking SHWBS insulators that protected the constructs from chromosomal position effects. Given their apparent ability to inhibit the activation of origin(s) by ACE3, it is likely that the flanking insulators also facilitated the experiments by preventing activation of cryptic origin sequences in vector and flanking sequences, making amplification more dependent on the chorion locus sequences in the constructs.

Taken together, the data support and extend the replicator model for the organization of amplification regulatory elements in the third-chromosome chorion gene cluster and for the organization of higher eukaryotic replicons in general (Stillman 1993). The data suggest that ACE3 is not itself an origin but, rather, is a replicator element that acts in *cis* to activate nearby origins such as ori-β. The fact that ACE3 is not strictly essential for amplification in large constructs suggests that there may be additional, more minor replicators in the locus as well. In the current model, ACE3 and any minor replicators would activate in *cis* multiple origins located in the region of initiations, with ori-β being the most active. The redundancy of origins may explain why sequence-specific origin requirements have been difficult to identify, as deletion of any one origin element would not eliminate replication. The data demonstrate that ori-β is at least to some extent sequence specific, as various other sequences could not substitute for its function, including chorion gene-coding sequences, the SHWBS site, and the equally A-T-rich ACE3 sequences. However, the sequence specificity for origin function might be quite relaxed, allowing for many origins and cryptic origins of varying strength to be located throughout the locus. Further experiments are underway to analyze the sequence requirements for origin function in detail. Notably, detailed physical mapping studies of locus *II/9A* amplification in *Sciara* salivary gland cells has localized origin activity to a fragment similar in size to *Drosophila* ori-β (Liang et al. 1993; Liang and Gerbi 1994).

These data support a replicator model that includes replicators and multiple, partially, or completely redundant origins dispersed throughout the initiation region of the replicon. This model is consistent with the observation that higher eukaryotic DNA replication involves initiations throughout a specific chromosomal domain, with a number of preferred sites for initiation. The existence of two types of elements and the redundancy of elements might help explain some of the conflicting results regarding specific sequence requirements for higher eukaryotic replicons. For example, chromosomal rearrangements and transgenic constructs might often be interacting with active or cryptic replicator or origin elements located in flanking sequences, thereby masking sequence-specific requirements.

In the yeast *Saccharomyces cerevisiae*, the replicator and origin are coincident and bind the origin recognition complex (ORC) in vivo and in vitro (Bell and Stillman 1992; Diffley and Cocker 1992; Diffley et al. 1994; Bielinsky and Gerbi 1999). The ORC is a complex of six protein subunits that associates with the origin throughout the cell cycle. Firing of the origin involves the stepwise association of additional proteins, including the Cdc7/Dbf4 protein kinase complex (Jackson et al. 1993; Dowell et al. 1994; Lei et al. 1997; Owens et al. 1997; Pasero et al. 1999; Zou and Stillman 2000). Much of this machinery appears to be conserved in function in higher eukaryotic DNA replication and in *Drosophila* chorion gene amplification. Homologs of the ORC subunits exist in *Drosophila*, and the *k43* gene encoding the *Drosophila* ORC subunit 2 (DmORC2) is required for amplification (Gossen et al. 1995; Landis et al. 1997). Similarly, the *chiffon* gene is required for amplification and encodes a protein related to Dbf4 (Landis and Tower 1999). Staining of follicle cells with an antibody specific for DmORC2 revealed that amplification correlates with a dramatic localization of ORC to the chorion gene loci (Royzman et al. 1999). In vivo, a transgenic construct containing either a 7.7-kb chorion locus fragment or nine copies of ACE3 was sufficient to cause ORC localization (Austin et al. 1999). Unfortunately, ORC localization was found to be too faint to be reliably scored with the Big Parent construct described here, which contains a much smaller chorion locus fragment in single copy (data not shown). Therefore, in vivo ORC localization could not be used as an assay for ORC binding with our constructs. In vitro, both ACE3 and ori-β (AER-D) regions have been shown previously to bind to ORC (Austin et al. 1999). As both ACE3 and ori-β are capable of ORC binding in vitro, the different functions of these elements in vivo reported here must depend on different sequence contents, a differential association with additional factors, or a difference in affinity for ORC that was not observed in vitro.

In chorion gene amplification and in higher eukaryotic replicons in general, initiations occur within a large chromosomal domain that has relatively discrete borders, beyond which initiations are not observed. The current data suggest at least three models for how such domains of initiation activity might be delimited. First, elements capable of acting as origins might be present outside the region of initiations, but the replicator elements such as ACE3 might be able to activate origins only to a limited distance on either side. This possibility seems unlikely, as one boundary for the initiation region for chorion gene amplification appears to be near the *Eco*RI site ~3 kb 5′ of ACE3 (Fig. 1; Heck and Spradling 1990) and ACE3 appears to activate ori-β, which is ~1.5 kb 3′, and possibly ori-γ, which is even more distant. Second, the domain of initiations might be defined by the location of sequence-specific origin elements like ori-β. The limits of the region of initiations might then simply represent the ends of the region containing such origin elements. However, the finding that ACE3 multimers may activate cryptic origins in flanking vector sequences tends to argue against this model, as it implies that sequences that can act as (minor) origins may be quite common (Carminati et al. 1992). Finally, the third and favored model invokes insulator elements. It was

demonstrated previously that transcriptional insulator elements create a chromosomal domain permissive for high levels of amplification by preventing negative effects of flanking DNA sequences (Lu and Tower 1997). Moreover, the present studies suggest that the ACE3 replicator may not be able to efficiently activate an origin beyond an insulator. The domains of the region of initiations observed in amplification and other higher eukaryotic replicons might therefore be generated by insulator elements that limit the region in which replicators elements are active. These hypothetical insulators might function similarly to SHWBSs or might be other sequence-specific elements.

Materials and methods

DNA constructs

All numbering is relative to the published sequence for the 3.8-kb *Sal*I fragment of the third-chromosome chorion gene locus (Levine and Spradling 1985).

pYES-1.2. pBS2N*B is a derivative of pBluescript KS+ (Stratagene) in which the *Kpn*I site is replaced by a *Not*I site and the *Bam*HI site has been destroyed. The 3.8-kb *Sal*I fragment from the third-chromosome chorion gene cluster, containing the *S18* and *S15* genes, was subcloned into the *Sal*I site of pBS2N*B to generate pBS2N*B-S3.8. An 884-bp DNA fragment (ori-β, 2212–3096) was amplified by PCR using primer set 1, digested at the *Bam*HI and *Xho*I sites engineered into the primers, and cloned into the *Bam*HI to *Xho*I sites of pBS2N*B-3.8 to generate pBS2N*B-SBaX. This construct was digested with *Kpn*I, end-filled with T4 DNA Polymerase, and digested with *Xho*I to generate a 1.2-kb fragment containing ACE3 and ori-β. This 1.2-kb fragment was then cloned into the *Sma*I to *Xho*I sites of pYES vector to generate pYES-1.2.

pYES-2.4. The 884-bp DNA fragment (ori-β) was amplified by PCR using primer set 1, digested at the *Bgl*II and *Xho*I sites engineered into the primers, and cloned into the *Bgl*II to *Xho*I sites of pBS2N*B-3.8 to generate pBS2N*B-SBgX. The 2.4-kb fragment containing ACE3, ori-β, and the intervening sequences (including the *S18* gene) was liberated by digestion with *Kpn*I, end-filled with T4 DNA polymerase, and digested with *Xho*I to generate a 2.4-kb fragment containing ACE3, *S18*, and ori-β. This fragment was cloned into the *Sma*I to *Xho*I sites of pYES vector to generate pYES-2.4.

PYES-FRT(SHWBS) A 6.5-kb *Kpn*I fragment containing FRT sites was liberated from D237 (Struhl and Basler 1993) and cloned into the *Kpn*I site of pBluescript KS+ vector to generate pBS-K6.5. A SHWBS fragment was generated by PCR using pYES vector template and primer set 3. This fragment was digested at *Nhe*I and *Bam*HI sites engineered into the primers and cloned into the *Nhe*I to *Bam*HI sites of pBS-K6.5 to generate pBS-K0.6. The 600-bp *Kpn*I fragment containing the SHWBS flanked by FRT sequences was then excised from pBS-K0.6 and cloned into the *Kpn*I to *Kpn*I sites of D237 (replacing the original 6.5-kb fragment) to generate D237-K0.6. PCR was carried out using primer set 4 and D237-K0.6 template to amplify the 600-bp sequence with added 5′ *Not*I and *Nco*I sites and added 3′ *Not*I and *Xho*I sites. This fragment was cloned into the *Not*I to *Xho*I sites of pBluescript KS+ to generate pBS-NX0.6. A 200-bp fragment of chorion gene *S18* coding sequences (1919–2119) was PCR amplified using primer set 5 and pBS2N*B-3.8 template. This fragment was digested at *Bam*HI sites engineered into the primers and cloned into the *Bam*HI site of pBS-NX0.6 to generate pBS-NX0.8. A second 200-bp fragment of chorion gene *S15* coding sequences (3105–3304) was PCR amplified with primer set 6 and pBS2N*B-3.8 as template, digested at *Nhe*I sites in the primers, and cloned into the *Nhe*I site of pBS-NX0.8 to generate pBS-NX1.0. A 500-bp fragment of chorion gene *S18* coding sequences (1412–1916) was PCR amplified from pBS2N*B-S3.8 using primer set 7, digested at *Bam*HI sites in the primers, and cloned into the *Bam*HI site of pBS2N*B-SBaX to generate pBS2N*B-SBa0.5X. The 1.0-kb *Nco*I fragment was excised from pBS-NX1.0 and inserted into the *Nco*I site of pBS2N*B-SBa0.5X. *Nsi*I digestion was carried out on the resulting plasmid, followed by T4 DNA polymerase end filling and *Xho*I digestion, to generate a 2.75-kb blunt to *Xho*I fragment that was cloned into the *Sma*I to *Xho*I sites of pYES vector to generate PYES-FRT-(SHWBS).

pBluescript-PCRA The SHWBS element was amplified from pYES template with primer set 3 and cloned into the *Eco*RV site of pBluescript KS+.

Big Parent Big Parent contains ACE3, S18, and ori-β flanked directly by SHWBSs. Construct pYES-2.4 was digested with *Not*I and *Xho*I to liberate a fragment containing ACE3, *S18*, ori-β, and one SHWBS. This fragment was cloned into the *Not*I to *Xho*I sites of pCaSpeR4 vector. A second SHWBS was isolated from pBluescript-PCRA by digestion with *Pst*I and *Xho*I and cloned into the *Pst*I to *Xho*I sites of the pCaSpeR4 construct to generate Big Parent.

Small Parent Small Parent contains ACE3 and ori-β immediately adjacent to one another and directly flanked by SHWBSs. Construct pYES-1.2 was digested with *Not*I and *Xho*I to liberate a fragment containing ACE3, ori-β, and one SHWBS, which was then cloned into the *Not*I to *Xho*I sites of pCaSpeR4 vector. A second SHWBS was isolated from pBluescript-PCRA by digestion with *Pst*I and *Xho*I and cloned into the *Pst*I to *Xho*I sites of the pCaSpeR4 construct to generate Small Parent.

Small(ACE deln) This construct is Small Parent with the 320bp ACE3 deleted. The 1.6-kb *Xho*I to *Not*I fragment containing ACE3, ori-β, and one copy of SHWBS was excised from Small Parent and cloned into the *Xho*I to *Not*I sites of pBS*K to generate pBS*K-1.2 (**1**SHWBS). The 320-bp ACE3 was deleted by *Kpn*I and *Bam*HI digestion, T4 DNA polymerase fill-in, and ligation. The resulting *Xho*I to *Not*I insert was liberated and substituted for the *Xho*I to *Not*I fragment in Small Parent to generate Small(ACE deln).

Big(ACE deln) This construct is Big Parent with the 320-bp ACE3 deleted. The 2.8-kb *Xho*I to *Not*I fragment containing ACE3, *S18*, ori-β, and one copy of SHWBS was excised from Big Parent and cloned into the *Xho*I to *Not*I sites of pBS*K to generate pBS*K-2.4(**1**SWBS). The 320-bp ACE3 was deleted by *Kpn*I and *Bam*HI digestion, T4 DNA polymerase fill-in, and ligation. The resulting *Xho*I to *Not*I insert was liberated and substituted for the *Xho*I to *Not*I fragment in Big Parent to generate Big(ACE deln).

Small(ori deln) This construct is Small Parent with the 884-bp ori-β fragment deleted. The ori-β fragment was excised from Small Parent with *Xho*I and *Bgl*II, the ends were filled in with Klenow polymerase fragment, and the construct was ligated to generate Small(ori deln).

Big(ori deln) This construct is Big Parent with the 884-bp ori-β fragment deleted. The ori-β fragment was excised from Big Parent with *Xho*I and *Bgl*II, the ends were filled in with Klenow polymerase fragment, and the construct was ligated to generate Big(ori deln).

Primer sequences SET1: 5′-AGCTGGATCC *Bam*HI TGAG TACTGTATTCTTGCTGGGT-3′, 5′-AGCTCTCGAG *Xho*I GTTTGGGGTAATCAATCAAACTATG-3′; SET2: 5′-TCGACCATGG *Nco*I AATTTTGTTGCATACCTTATCAAAA-3′, 5′-AGCTCCATGG *Nco*I ATTGGTTGTTGGTTGGCACACCACA-3′; SET3: 5′-TCGAGCTAGC *Nhe*I AATAAGTGTGCGTTGA ATTTATTCGCAA-3′, 5′-AGCTGGATCC *Bam*HI TACTGT TGCCGAGCACAATTGATCGGCT-3′; SET4: 5′-TCGAGCG GCCGC *Not*I CCATGG *Nco*I CTTACAGGATCGGTACCC GGGGATCTTG-3′, 5′-AGCTCTCGAG *Xho*I CCATGG *Nco*I GTCCTCCACCTTGCGCTTCTTCTTGGGG-3′; SET5: 5′-TC GAGGATCC *Bam*HI AGCTCGCCCTGGCCGCTCCCAGC GCTGG-3′, 5′-AGCTGGATCC *Bam*HI CTTAGTAGCTGGG CCTCTTGTAGCCCT-3′; SET6: 5′-TCGAGCTAGC *Nhe*I CT AAGCACTCACCATGAAGTACCTGGTA-3′, 5′-AGCGGC TAGC *Nhe*I ACAGGACCGTAGCCACCACCGTAGCCAC-3′; SET7: 5′-TCGAGGATCC *Bam*HI CTCAGCCTCAGAATGA TGAAGTTCATGG-3′, 5′-AGCTGGATCC *Bam*HI GCGAGG GCAATGGCCTGGGCATCGACTG-3′.

Generation of FLP recombination derivative lines

Four independent pYES-FRT(SHWBS) transgenic lines were generated and crossed to the FLP1 stock in which FLP recombinase expression is driven by the *hsp70* promoter. Larval progeny containing both constructs were subjected to heat shock at 32°C for 30 min and at 37°C for 90 min to cause recombination, as described previously (Golic and Lindquist 1989). Multiple, independent potentially recombined chromosomes were purified from each starting strain by crosses to appropriate balancer stocks. Precise deletions of the sequences flanked by FRT sites were identified by Southern analyses (data not shown), and two independent derivatives were analyzed for each starting strain.

Assay of amplification level

Assay of amplification of transgenic constructs was performed as described previously (Lu and Tower 1997). Briefly, DNA was isolated from stage 13 egg chambers (ECs) of homozygous females of each transgenic line. The DNA was restriction digested and analyzed by Southern blotting. Copy number of the construct was measured by hybridization with a probe that would yield a uniquely sized band for the transgenic construct. The probe was derived from the transformation marker gene contained in the transformation vector. Male fly DNA was used as a nonamplifying control, and the blots were hybridized to an rDNA probe as a control for loading. Signals were quantitated by PhosphorImager. Fold amplification was calculated as follows: fold amplification = (transgene^{EC}/transgene^{Male})/(rDNA^{EC}/rDNA^{Male}). No amplification yields a value of 1. For each independent transgenic line, three assays were performed using independently cultured flies, and the data are presented as the average ±SD of the three assays in bar graphs. For statistical analyses, the amplification levels of the multiple independent transgenic lines for each construct were averaged, and the average for each construct was compared to the average for its respective parent construct using unpaired, two-sided *t*-tests.

Two-dimensional gel analyses

One hundred young female flies of the indicated transgenic line were fed with wet yeast paste for 14–16 h, and ovaries were dissected into PBS. Special care was taken in timing the yeast feeding to yield the maximum possible number of stage 10 egg chambers per ovary. Total ovary DNA was extracted and digested with the indicated restriction enzymes as described previously (Heck and Spradling 1990). The digested ovary DNA was adjusted to a final concentration of 1 M NaCl and loaded onto BND cellulose columns for enrichment of replication intermediates as described previously (Dijkwel et al. 1991; Liang et al. 1993). After precipitation with isopropanol, the DNA pellet was washed with 70% ethanol, air dried, dissolved in 10–20 μL distilled water, and loaded onto the first-dimension gel. DNA was resolved on two-dimensional agarose gel as described (Heck and Spradling 1990). Electrophoresis was carried out at room temperature in 0.4% agarose gel at 0.75 V/cm for 18–21 h in the first dimension and at 4°C in 1% agarose gel plus 0.3 μg/mL EtBr at 5 V/cm for 5–7 h in the second dimension. To detect origin activity at ori-β, ovary DNA was digested with *Bam*HI. Ori-β was included in the 2.5-kb fragment, which was detected by hybridization with the 2.1-kb *Xho*I to *Bam*HI probe fragment from the Big Parent construct (Fig. 5D). To assay for origin activity at ACE3, ovary DNA was digested with *Bgl*II and *Sal*I. ACE3 was included in the resulting 3.8-kb fragment, which was detected by hybridization with the 1.9-kb *Eco*RI to *Sal*I probe fragment from the Big Parent construct (Fig. 5D).

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