Multiple Replication Origins Are Used during *Drosophila* Chorion Gene Amplification

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Abstract. DNA from *Drosophila* egg chambers undergoing chorion gene amplification was analyzed using the two-dimensional gel technique of Brewer and Fangman. At stage 10, 34% of DNA molecules from the maximally amplified region of the third chromosome chorion gene cluster contained replication forks or bubbles. These nonlinear forms were intermediates in the process of amplification; they were confined to follicle cells, and were found only within the replicating region during the time of amplification. Multiple origins gave rise to these intermediates, since three separate regions of the third chromosome chorion locus contained replication bubbles. However, initiation was nonrandom; the majority of initiations appeared to occur near the Bgl II site located between the s18 and s15 chorion genes. The P[S6.9] chorion transposon also contained abundant replication intermediates in follicle cells from a transformed line. Initiation within P[S6.9] occurred near two previously defined *cis*-regulatory elements, one near the same Bgl II site (in the AER-d region) and one near the ACE3 element.

EUKARYOTIC chromosomes replicate in a highly ordered process from multiple initiation points (reviewed by Hand, 1976). Both the number of initiations and the rate of fork elongation are modulated during development (Callan, 1972; Blumenthal et al., 1973; Cordeiro and Menghenini, 1975). Accumulating evidence supports the idea that specific origins are used during chromosomal replication, particularly in yeast (reviewed by Umek et al., 1989). Autonomously replicating sequences (ARS)[1] conferring the ability on yeast plasmids to be maintained extrachromosomally are dispersed throughout yeast chromosomal DNA, and in at least some cases function as replication origins on plasmids. It remains to be established to what extent initiation at such ARS elements resembles pathways used in prokaryotic genomes or in animal viruses (reviewed by Campbell, 1986; McMacken et al., 1987; Kelly, et al., 1988). Whether similar elements function in chromosomes from higher eukaryotes is also unknown, although evidence for specific initiation in the amplified CHO dehydrofolate reductase domain has been reported (Leu and Hamlin, 1989; Anachkova and Hamlin, 1989). In principle, regulating initiation at specific origins could modulate chromosome replication during both the cell cycle and throughout development.

Drosophila chorion genes provide an exceptional opportunity to study the replication of specific chromosome regions in a higher eukaryote. During the last 16 h of oogenesis, eggshell genes clustered at two chromosomal sites differentially replicate in the ovarian follicle cells, and then abundantly produce eggshell structural proteins by transcription of the amplified templates (reviewed in Mahowald and Kambysellis, 1980; Spradling and Orr-Weaver, 1987). The third chromosome cluster amplifies >60-fold by initiating multiple rounds of replication in the vicinity of four tandemly arranged genes. Each newly initiated fork progresses along the chromosome, duplicating both the gene region and up to 50 kb of flanking chromosomal DNA. The combination of frequent initiation (2.5 h doubling time) and slow fork elongation (50–100 bp/min) produces a bell-shaped gradient of amplification centered at the site of the gene cluster (Spradling, 1981; Spradling and Leys, 1988).

The genetic regulation of amplification has been extensively studied using P element-mediated transformation (reviewed in Kafatos et al., 1985; Spradling and Orr-Weaver, 1987). Segments of genomic DNA containing the two 5′ genes in the third chromosome cluster, s18 and s15, can induce follicle cell–specific amplification in transformed flies. However, the level of amplification is usually lower than normal and depends strongly on the site of insertion (deCicco and Spradling, 1984). Larger transposons containing three or all four clustered genes amplify to higher levels on average, but are still subject to position effects (Delidakis and Kafatos, 1987). Deletion experiments have localized a series of *cis*-regulatory elements within the gene cluster. The strongest element (called "amplification control element/third chromosome" or ACE3), lies just upstream of the s18 gene (Orr-Weaver and Spradling, 1986). Several additional elements (called "amplification-enhancing regions" or AERs) within the cluster appear to stimulate the average level of am-

1. *Abbreviations used in this paper*: ACE3, amplification control element/third chromosome; AER, amplification enhancing regions; ARS, autonomously replicating sequences.

A
B
C
D
E
F
G
H
O
X
Y
Z
Eco R1
3.9 kb
Eco R1
Sal 1
ACE
s18
β
s15
Sal 1
s19
Eco R1
(Eco R1)
s16
4.2 kb
7.7 kb

plification in transformants (Orr-Weaver and Spradling, 1986; Delidakis and Kafatos, 1987; Delidakis and Kafatos, 1989).

The development of sensitive two-dimensional gel techniques that separate replicating from nonreplicating molecules has facilitated replication origin mapping in yeast (Brewer and Fangman, 1987; Nawotka and Huberman, 1988). By analyzing the structure of replicating plasmid molecules cleaved with restriction enzymes, a specific replication origin was mapped coincident with the ARS element of the yeast 2-μm circle, and with the ARS1 element (Brewer and Fangman, 1987; Huberman et al., 1987). Recently, the replication of *S. cerevisiae* chromosomal rDNA has been analyzed by these methods (Linskens and Huberman, 1988; Brewer and Fangman, 1988). Although initiation events were confined to an ARS-containing site within the nontranscribed spacer, fewer than one in five ARS elements functioned during a single cell cycle. Furthermore, replication was largely unidirectional, probably because forks could not progress through an active gene in a direction opposite to that of transcription. In this report we have used two-dimensional gels to map replication initiation sites within the third chromosome chorion domain during amplification.

Materials and Methods

Preparation of DNA

For each digest, DNA was purified from 100–400 hand-isolated staged egg chambers by lysis at 37°C in 0.1 M NaCl, 10 mM EDTA, 50 mM Tris, pH 8.0, 0.5% SDS containing 500 μg/ml proteinase K. DNA samples were extracted with phenol/chloroform/isoamyl alcohol, and precipitated with ethanol as described previously (Spradling, 1981). A population cage of strain Oregon R (P2) was used except as indicated. Nuclei from embryos (Oregon R) and K_c cells were lysed, washed extensively with TE, and digested in an agarose insert before electrophoresis as described by Schwartz (1985). The pattern of replicative intermediates was not noticeably influenced by variations in the conditions used to deproteinize the DNA, the duration of the restriction digestion, or by extended storage at −20°C. Digestions with restriction enzyme were carried out in the presence of 10 μg/ml RNAase at 37°C for 1–4 h. Reactions were terminated by the addition of 10 mM EDTA and 0.5% SDS, and then loaded directly on the first-dimension gel.

Two-dimensional Gel Electrophoresis

DNA was resolved on two-dimensional agarose gels as described by Brewer and Fangman (1987). Up to 4 μg of DNA was loaded on each lane of a 0.4% agarose gel in TBE buffer, and subjected to electrophoresis at 0.75–1.0 V/cm for 20–36 h, depending on the size range of interest. The gel was stained with 0.3 μg/ml ethidium bromide (EtBr) and photographed to calibrate a size scale for the first dimension based on the mobility of lambda DNA restriction fragments run in parallel lanes. Individual lanes were excised and a second-dimension gel containing 1% agarose and 0.3 μg/ml EtBr in TBE was poured at a right angle to the first dimension. Electrophoresis in the second dimension was carried out at 4–5 V/cm for 6–14 h (depending on the size range of interest) in the same buffer at 4°C. The buffer was either recirculated or substituted once during the course of the separation. After electrophoresis, each gel was photographed to establish the position of the linear molecules.

The first-dimension gel separates primarily by molecular weight, whereas conformation and molecular weight determine mobility in the second dimension. Linear molecules migrate on a diagonal that is readily identified by staining with EtBr. Fig. 7 illustrates the structures of common families of replicative intermediates and their mobilities relative to linear molecules of equal mass, by showing the expected behavior of a hypothetical 3-kb restriction fragment. Molecules that are Y-shaped, due to the presence of a replication fork, vary from one to two times (3 to 6 kb) in mass as determined by their mobility in the first dimension (Fig. 7 *A*). They migrate just above linear molecules of equal mass when the fork is located near either end of the fragment, but are greatly retarded in the second dimension when the three arms are nearly equal in length. Molecules containing a centrally located bidirectional origin also vary between 3 and 6 kb in mass. Unlike Y-containing molecules, however, these bubble-containing forms do not approach a linear structure as the forks progress, but diverge progressively from the diagonal of linear DNAs (Fig. 7 *B*). These molecules exhibit a minimum mobility in the second dimension somewhat before replication is complete for reasons that are not clear. χ-shaped molecules are all exactly 6 kb in molecular weight, but differ in structure depending on the location of the crossover (Holliday junction). When near either end, the molecules migrate nearly as linears, but when the junction is closer to the center, they are retarded in the second dimension (Fig. 7 *C*). The slight backward angle of the χ-arc is due to slowing of nonlinear molecules in the first dimension. A discontinuous pattern results when an origin is asymmetrically located within a DNA fragment (Fig. 7 *D*). At early times after initiation the molecules migrate as bubbles. However, after one fork leaves the fragment, simple-Y's result that are partially replicated. The position of the transition between bubbles and Y's reveals the location of the origin. Further details and evidence supporting these interpretations, including direct visualization by EM of molecules isolated from specific gel regions, can be found in Brewer and Fangman (1987) and Brewer et al. (1988).

Blotting and Hybridization

DNA was transferred to nitrocellulose and hybridized as previously described (Spradling, 1981). Alternatively, transfer to nylon membranes and hybridization was carried out as described by Church and Gilbert (1984). Usually, probe DNAs were digested free of vector sequences, isolated after electrophoresis in an agarose gel, and labeled by nick translation or random priming. In some cases, fragments subcloned in pBR322 or SP64 vectors were labeled without first isolating the *Drosophila* sequences. The signal-to-noise ratio was noticeably better when isolated DNA was used. A laser scanning densitometer (Molecular Dynamics, Sunnyvale, CA) was used for densitometric scanning of autoradiograph films.

Results

Replicative Intermediates Are Present at High Levels during Amplification

The third chromosome domain contains four clustered chorion genes that increase 60-fold in copy number between stages 9 and 14 of oogenesis (Fig. 1). The generally accepted model of chorion gene amplification predicts that replication forks should occur frequently throughout the amplified regions. Because the level of amplification increases exponentially with a doubling time of ~2.5 h, and forks elongate at

Figure 1. The third chromosome chorion gene cluster contains specific chorion replicative intermediates during amplification. DNA was extracted from the indicated tissues, digested with Eco RI (or in *G*, Sal I), and resolved by two-dimensional electrophoresis. Hybridization was with the 7.7-kb Eco RI fragment as described in Materials and Methods, except in *G*, the 8.2-kb Sal I genomic fragment containing rosy was used as probe. In *A–F*, the strongly labeled spot corresponds to linear 7.7-kb molecules. Arcs representing Y-shaped molecules (*Y*), bubble-containing molecules (*O*), and χ-shaped molecules (*X*) are labeled. (*A*) DNA from ovarioles containing stage 1–8 egg chambers. (*B*) DNA from stage 9–10 egg chambers. (*C*) DNA from stage 11–12 egg chambers. (*D*) DNA from stage 13–14 egg chambers. (*E*) DNA from K_c tissue culture cell nuclei. (*F*) DNA from nuclei of 2–4 h *Drosophila* embryos. (*G*) DNA from stage 10 egg chambers. (*H*) EtBr stain of two-dimensional gel (Eco RI digest) of stage 10 egg chamber DNA (a lower magnification is shown to illustrate the entire gel). The exposure times varied between 16 h and 7 d to correct for differences in signal strength due to the amplification of the 7.7-kb fragment in stage 9–14 egg chambers. At the bottom is a map showing the third chromosome gene cluster, including the transcription units of the four tandem chorion genes s18, s15, s19, and s16. The locations of the *cis*-regulatory sequence ACE3 and of the β region are shown.

no more than 100 bp/min, a fork should be present on average every 15 kb (2.5 h × 60 min/h × 0.1 kb/min). Thus, a very large fraction of the restriction fragments throughout the amplified domain are expected to contain forks. The 7.7- and 4.2-kb gene-containing Eco RI fragments amplify maximally, whereas adjacent regions replicate less extensively. Therefore, most replication forks must initiate within this 11.9-kb region, a conclusion supported by EM (Osheim and Miller, 1983; Osheim et al., 1988). Replication bubbles, indicative of origin activity, are therefore expected in this region.

We used the two-dimensional gel technique of Brewer and Fangman to look for replication intermediates in follicle cells. DNA was prepared from 100–400 egg chambers at stages before and during amplification. After Eco RI digestion, the DNA was resolved on two-dimensional gels, transferred to membranes, and probed with the central 7.7-kb Eco RI fragment (Fig. 1). As controls, DNA purified from tissue culture (K_c) cells in logarithmic phase, and from postcleavage-stage *Drosophila* embryos were also studied. In Fig. 1, the exposure times were normalized so that the strength of the hybridization to the 7.7-kb linear molecules is approximately equal in DNA from these sources. Replicating molecules were identified based on their pattern of migration as summarized in Materials and Methods (Fig. 7). The behavior of molecules containing single replication forks (Ys), single replication bubbles (Os), two converging forks (double Ys), and single recombination joints (χs) has been described previously (see Brewer and Fangman, 1987; Brewer et al., 1988).

As expected, nonlinear molecules were abundant in DNA from amplifying egg chambers but were not detected in the other, nonamplifying, DNAs. DNA from early egg chambers before the time of chorion gene amplification had none of the usual replicative intermediates associated with it (Fig. 1 *A*). A prominent arc of Y-shaped molecules (Fig. 1 *B*, *Y*), as well as bubble-containing molecules (Fig. 1 *B*, *O*), were observed in the 7.7-kb third chromosome region DNA from egg chambers throughout the period of amplification (Fig. 1, *B–D*). In contrast, this same genomic fragment from K_c cells (Fig. 1 *E*) and 2–4 h embryos (Fig. 1 *F*) migrated almost entirely as linear molecules. A genomic region that does not amplify, the 8.2-kb Sal I fragment containing the *Drosophila* rosy gene, contained only linear molecules in amplifying chambers (Fig. 1 *G*). We concluded that the nonlinear molecules in egg chamber DNA were replicative intermediates in the process of amplification.

Two additional classes of molecules complementary to the 7.7-kb probe were also observed. A streak of molecules extended upward from the 15.4-kb position characteristic of fully replicated linear molecules (Fig. 1 *B*, *X*, and subsequent figures). This is the behavior expected of χ-shaped molecules resulting from homologous recombination between two 7.7-kb monomers (Brewer et al., 1988). All such molecules have a molecular mass of 15.4 kb, but differ in structure depending on the location of the crossover point (Holliday junction). They were specifically associated with amplifying DNA (compare Fig. 1, *B–D* with *E–G*). A second type of nonlinear molecule (Fig. 1 *B*, *Z*) migrated in a steep diagonal extending above the diagonal of linears into the high molecular weight region. Unlike the other forms, molecules with the mobilities of these Z-forms have not been characterized previously. Their appearance in early egg chamber stages (Fig. 1 *A*) before or at the onset of amplification distinguished them from the other replicative intermediates.

Our experimental conditions efficiently recovered and resolved amplifying molecules. Because the previous calculations predicted a fork once every 15 kb, 25–50% (7.7/15 kb) of the 7.7-kb fragments should migrate nonlinearly. (Bidirectional replication from a central location would result in the lower value of 25%, since two forks would duplicate the fragment.) To test this, regions of the filters corresponding to the various types of molecules in Fig. 1 were excised and counted (Fig. 2). Close to expectation, 34% of the signal was present as Y-, O-, or χ-shaped molecules during stage 10. The amounts of these forms decreased during later stages of amplification (stages 11–14). This decrease implied that the rate of initiation slowed late in oogenesis. The abundance and efficient recovery of replicating molecules during stage 10 suggested that it should be possible to map major sites of fork initiation within the gene cluster.

Mapping Replication Origins

The distribution of replicative intermediates within the amplified region should reveal the location of specific replication origins if amplification begins at one or more specific sites. Restriction fragments that lack an origin, for example those lying outside the 11.9-kb central region, will be duplicated by replication forks which enter from one side and pass entirely through. Consequently only Y-shaped molecules are expected when such fragments are used to probe two-dimensional gels of egg chamber DNA, and the full molecular weight range of the Y-arc should be present. In contrast, origin-containing regions should contain both bubbles and Y-shaped molecules. If all replication results from a single origin within the fragment, only portions of the bubble- and Y-arcs will be represented, depending on the distance of the origin from the center of the fragment (see Materials and Methods). These "discontinuous" patterns allow the location of a specific origin to be mapped because the transition point between bubble- and Y-forms corresponds to the point at which one of the oppositely traveling forks leaves the end of the fragment. Origin position can be inferred, and then verified by analyzing multiple digests in which the origin is

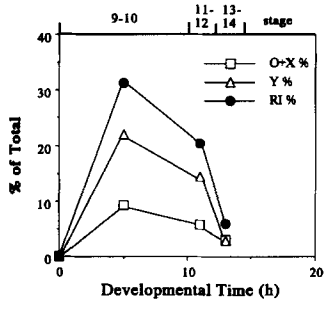


Figure 2. Developmental changes in replicative intermediates during oogenesis. The labeled regions were excised from the filters shown in Fig. 1, *A–D* and counted in aqueous scintillation fluid. The fraction of 7.7-kb chorion region genomic DNA molecules containing the indicated replicative intermediates is plotted during stages 9–10, 11–12, and 13–14. The midpoint of each stage is plotted on a time scale where 0-h represents the beginning of stage 9. *Y%* = percent of total counts per minute in Y-arc, *O + X%* = percent of total counts per minute in bubble- and χ-arcs, *RI%* = percent of total counts per minute in Ys, Xs, and Os. Total counts per minute varied between 350 (stage 1–8) and 2,500 (stage 13–14).

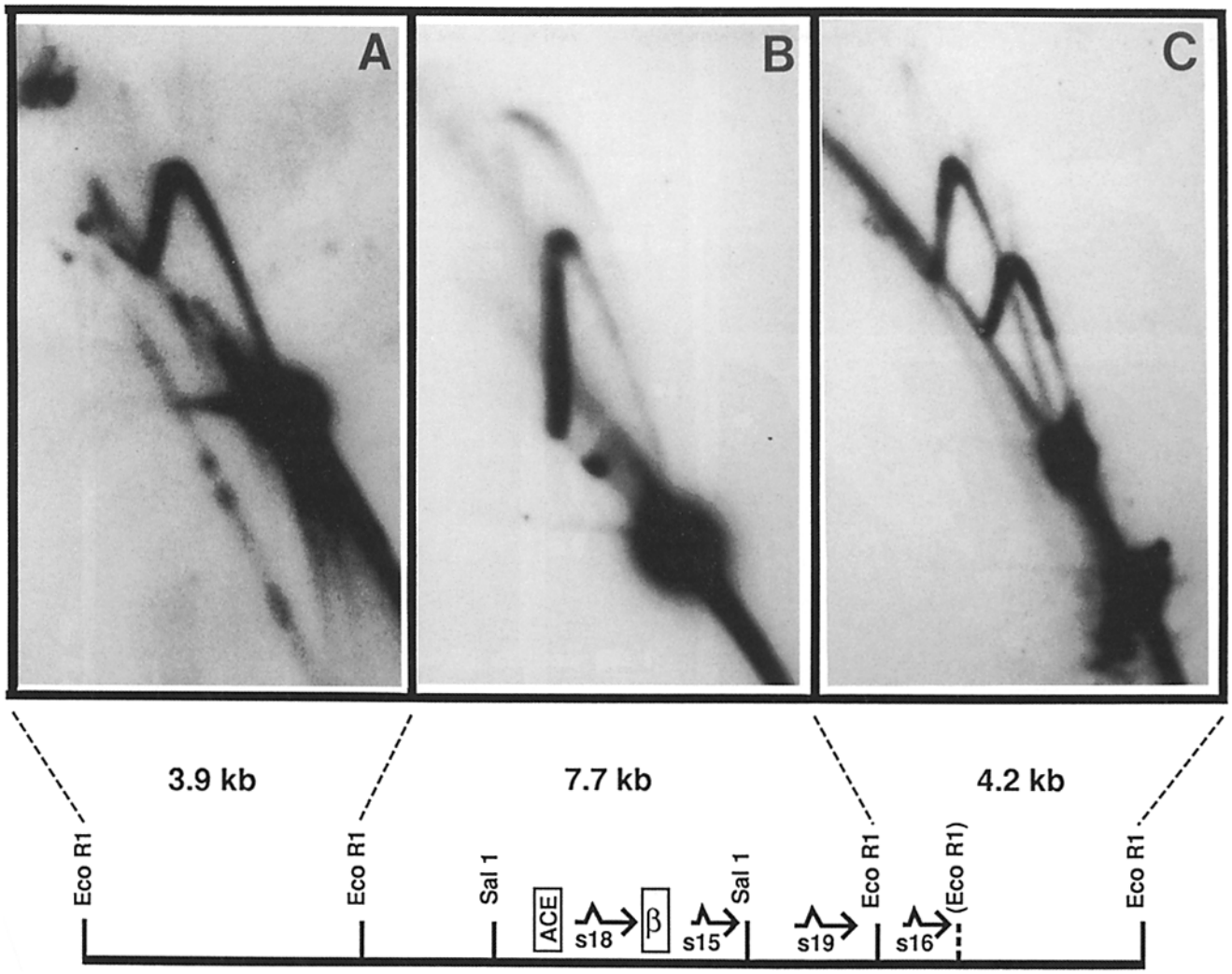


Figure 3. Survey of stage 10 replicative intermediates in Eco RI fragments of the third chromosome chorion cluster. The map illustrates the location of the Eco RI fragment used to probe the filters. The dotted line indicates the location of a polymorphic Eco RI site. (*A*) Oregon R DNA probed with the 3.9-kb Eco RI fragment. (*B*) Oregon R DNA probed with the 7.7-kb Eco RI fragment. (*C*) Oregon R DNA, probed with the 4.2-kb Eco RI fragment.

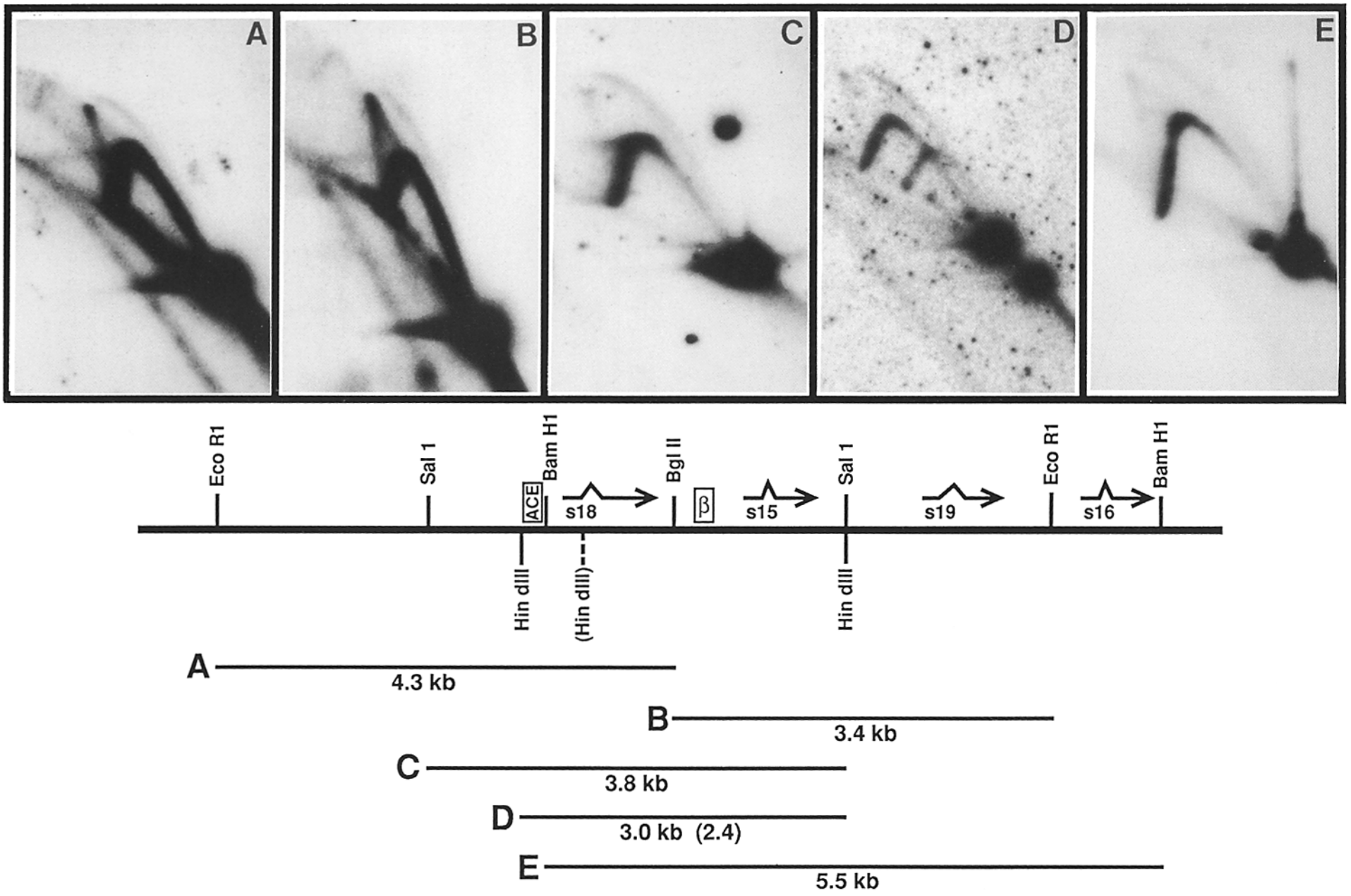
located at different distances from the center of the fragment (Brewer and Fangman, 1987).

More complex behavior may also be deduced from the pattern of replicative intermediates. Pause sites slowing fork progression should produce regions of the Y-arc with abnormally strong intensity (Brewer and Fangman, 1988). Unidirectional and bidirectional replication predict distinguishable patterns of intermediates within a series of overlapping fragments containing the origin. Multiple origins firing on the same molecule will generate "double-Y" forms, as adjacent replication forks approach each other. Finally, if initiation is random within a region, then all sub-fragments will contain the same distinctive pattern of nonuniform bubble- and Y-arcs.

A survey of intermediates in stage-10 egg chamber DNA provided an overview of the amplification process (Fig. 3). A fragment outside the central region, the 3.9kb Eco RI fragment upstream of the chorion genes, showed only Y-shaped intermediates (Fig. 3 *A*). Furthermore the distribution of molecules in the Y-arc was uniform, as expected if forks entered exclusively from the right and elongated through the fragment at a constant rate. A prominent bubble-arc (and Y-arc) was labeled by the central 7.7-kb fragment, suggesting that one or more origins were located within (Fig. 3 *B*). The downstream 4.2-kb Eco RI fragment labeled two Y-arcs due to the presence of an Eco RI polymorphism in about half the flies (Fig. 3 *C*). Both were nearly uniform and complete. However, a very weak bubble arc was visible above the 4.2-kb molecules. Thus, a small number of forks must have initiated within the 4.2-kb fragment, but the majority of initiations were within the 7.7-kb region.

Forks Initiate at Multiple Sites within the 7.7-kb Region

To localize initiation sites within the 7.7-kb Eco RI fragment, additional digests of stage 10 egg chamber DNA were analyzed with probes from this region. Digestion with both Eco RI and Bgl II separates the 7.7-kb fragment into a 4.3-kb segment containing the s18 gene and upstream DNA (Fig. 4 *A*),


A
B
C
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Eco R1
Sal 1
Bam H1
ACE
s18
Bgl II
β
s15
Sal 1
s19
Eco R1
s16
Bam H1
Hin dIII
(Hin dIII)
Hin dIII
A
4.3 kb
B
3.4 kb
C
3.8 kb
D
3.0 kb (2.4)
E
5.5 kb

and a 3.4-kb segment containing s15, s19, and their intergenic regions (Fig. 4 *B*). Long exposures revealed that both regions contained bubbles and hence origins, in addition to strong uniform Y-arcs. Densitometry showed that approximately twice as many bubble-containing molecules relative to the number of linear molecules were present downstream of the Bgl II site as upstream. The Sal I fragment (Fig. 4 *C*), the Hind III fragment (Fig. 4 *D*), and the Bam HI fragment (Fig. 4 *E*) also contained both bubbles and Y-shaped intermediates. However, in these digests the Y-arcs were nonuniform; molecules that had just begun to replicate were underrepresented. If two or more bidirectional origins fired within a single chromosome, double-Y molecules with converging replication forks are expected (Brewer and Fangman, 1988). Some labeling was observed in the position expected for double-Ys in Fig. 4 *B*, however these molecules were not observed in other digests spanning the same region (Fig. 4, *C–E*). Therefore in most molecules, newly initiated forks must elongate and exit the gene cluster before a subsequent initiation at the same or a nearby origin.

These observations showed that replication forks initiated on both sides of the Bgl II site between s18 and s15. Two points argue that initiation is not random within this maximally amplified domain. First, as the distribution of bubble-arcs was asymmetric, it appeared that replication initiated twice as frequently downstream of the Bgl II site as upstream. Second, the distribution of fork-containing molecules differed between subfragments, a result inconsistent with random initiation within the 7.7-kb fragment. In particular, the uniform Y-arcs in Fig. 4, *A* and *B* suggested that a majority of the replication forks were generated near the Bgl II site. In contrast, the Y-arcs observed in the other digests of this region were nonuniform (Fig. 4, *C–E*). It is important to note that the chorion genes within this cluster are not transcriptionally active until stages 13 and 14; thus replication fork movement is probably not being influenced by RNA polymerase activity. Nonetheless, the observed patterns did not allow the location of specific origins to be deduced unambiguously.

Mapping Origins within the Chorion Transposon P[S6.9]

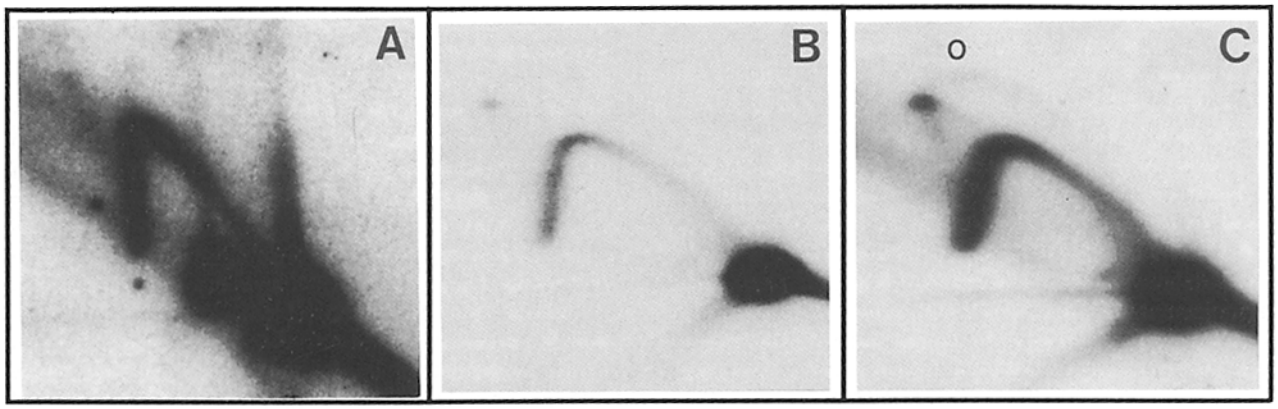
To investigate a simpler case, we examined the amplification of an integrated P element transposon containing only part of the endogenous gene cluster. We studied the amplification-competent transposon P[S6.9] containing the 3.8-kb Sal I fragment bearing genes s18 and s15 (deCicco and Spradling, 1984; Wakimoto et al., 1986). In P[S6.9] the s15 gene is fused to the *Escherichia coli* lacZ gene; in addition, the transposon contains a rosy marker (which does not amplify in its normal chromosomal position, see Fig. 1 *G*). Both of these sequences provide probes specific for the transposon as they will not hybridize to endogenous chorion sequences (Fig. 5). We studied P[S6.9] amplification in line 5 (S6.9-5), where the insertion was shown previously to amplify at nearly wild-type levels (deCicco and Spradling, 1984).

Initiations during P[S6.9] amplification did not occur outside of the chorion sequences. The Hind III rosy fragment from the left end of the transposon lacked bubbles and contained only Y-shaped intermediates, even in very long exposures (Fig. 5 *A*). Likewise, only a complete uniform Y-arc was observed in the 6.6-kb Eco RV fragment extending from within the lacZ gene into the DNA flanking the right side of the insertion (Fig. 5, *F* and *G*).

Both the presence of bubble-containing molecules and the structure of discontinuous Y-arcs pointed to a region a few hundred basepairs downstream of the Bgl II site between s18 and s15 as containing an origin (results are shown in the lower half of Fig. 5). The pattern of intermediates seen in Eco RV digests was particularly informative (Fig. 5, *F* and *G*). The complete Y-arc associated with the 6.6-kb fragment served as an internal control for the very short Y-arc arising from the 5.8-kb fragment (thus the small Y-forms were not preferentially lost during the experiment). This pattern suggested the presence of an origin of replication close to, but not at, the center of the 5.8-kb Eco RV fragment. The bubble-arc from this fragment, a more direct demonstration of a replication origin, is apparent in the more sensitive experiment of Fig. 5 *G*. Forks must initiate near ACE3, left of center, the β region which lies right of center, or at both sites, to produce this pattern. The presence of bubbles in the Hind III fragment (Fig. 5 *E*, *O*), which lacks the left of center site, and the nonuniform appearance of Y-arcs from both the Sca I fragment (Fig. 5 *D*) and the Hind III fragment (Fig. 5 *E*) argued that a substantial number of these initiations must occur to the right of center.

We have used the position of the discontinuity in the Y-arcs to map the origin within this region more precisely. In the Eco RV fragment, the pattern of large Ys more than 70% replicated is the result expected for bidirectional synchronous replication from a site 2.0 kb from the right end of the fragment ([0.5 × 70%] × 5.8 kb = 2.0 kb). The sequence of P[S6.9] indicated that this site would lie ~390 bp upstream of the s15 transcription start site. Likewise, the discontinuity of the Hind III Y-arc at 35% replication points to an origin 1.1 kb from the left end of this fragment ([0.5 × 35%] × 6.1 kb = 1.1 kb), or about 570 bp upstream of s15. These values fall within the β, or AER-d, *cis*-regulatory region, which lies 390–810 bp upstream of s15. Our interpretation was confirmed by the analysis of the Sca I and Kpn I fragments (Fig. 5 *H*). An origin located 390 bp upstream of s15 predicts that the Sca I fragment Y-arc should be discontinuous at 20% replication, ([0.5 × 20%] × 4.7 kb = 470 bp from the left end of the fragment). Likewise, the predicted value for a discontinuity in the Kpn I fragment Y-arc given bidirectional replication would be 44%. Although biological

Figure 4. Analysis of replicative intermediates from the third chromosome gene cluster. A restriction map of the region containing the four chorion genes is shown at the bottom. (*A*) Eco RI/Bgl II digest, probed with the 4.3-kb fragment diagrammed below. (*B*) Eco RI/Bgl II digest, probed with two smaller fragments comprising this region. (*C*) Sal I digest, probed with the 3.8-kb Sal I fragment. (*D*) Hind III digest, probed with the 3.0-kb Hind III fragment. The Oregon R (P2) DNA used contains a polymorphic Hind III site 0.6-kb from the left end within the s18 gene as indicated by the dashed line. (*E*) Bam HI digest, probed with the 7.7-kb Eco RI fragment. In addition to the major 5.5-kb fragment, the expected fragments of 1.4 and 1.0 kb were observed (not shown).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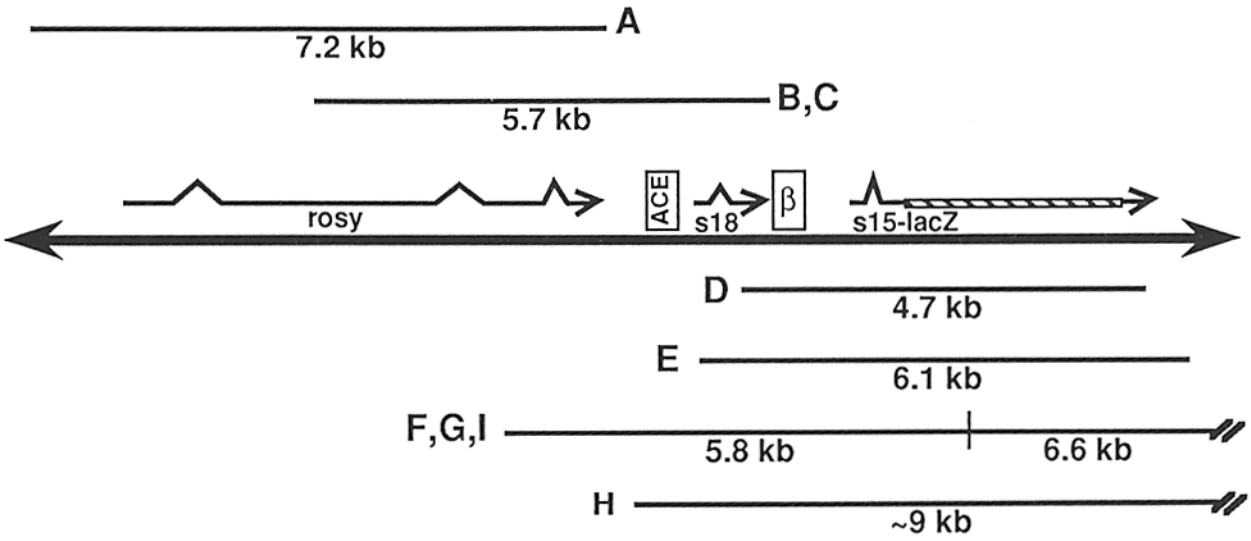
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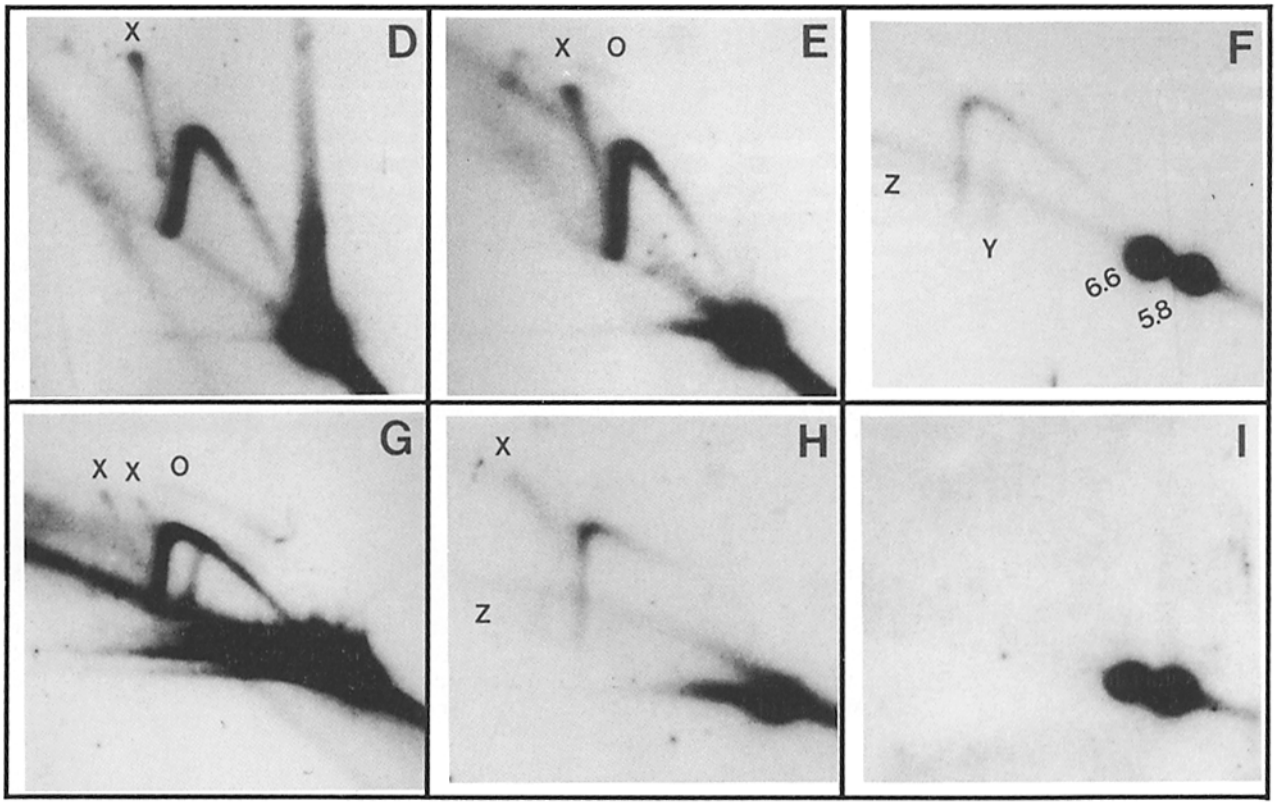
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variations in the rate and synchrony of fork movement are expected to blur Y-arc discontinuities somewhat, the observed patterns are consistent with these expectations. The uncertainty in our mapping of the origin near β was estimated from the Eco RV Y-arc. If the uncertainty in the Eco RV Y-arc transition is taken as 60–80% replication, then the origin location is 390 ± 300 bp upstream of s15.

Additional study of P[S6.9] pointed to a second origin upstream of the Bgl II site. The Bgl II fragment shown in Fig. 5, *B* and *C* extends from within the rosy gene rightward into chorion sequences, including ACE3 but not β. It contained a weak bubble-arc detectable on long exposures, implying that at least some initiations occurred upstream of the β region (Fig. 5, *C*, *O*). The Y-arc was continuous, but molecules less than about 40% replicated appear to be underrepresented (Fig. 5 *B*). This pattern could result from a superposition of complete Ys produced by initiation within β, and discontinuous Ys produced by an origin within the Bgl II fragment. Since the Hind III rosy fragment contained a complete Y-arc, the origin must lie in the right end of the Bgl II fragment (Fig. 5 *A*). These results and the Eco RV Y-arc discontinuity at 70% replication are both consistent with an origin in the vicinity of the ACE3 region 400 bp upstream of s18 that initiates replication less frequently than the β origin. However these experiments could not precisely map the upstream origin or rule out the existence of additional weak origins.

If the origins upstream of s18 and s15 both fired on the same molecule, double-Y molecules would be expected within fragments containing both regions. Double-Ys might correspond to the Z-forms, seen in fragments containing both ACE3 and β (e.g., Fig. 5, *F* and *H*). However the fast mobility of the Z-arc in the second dimension and its presence at molecular weights greater than two times the fragment length are inconsistent with previously characterized double-Y molecules (Brewer and Fangman, 1987). The Z-forms may therefore correspond to a series of novel structures, arising through some interaction between the two origins.

As in the case of the endogenous gene cluster, χ-forms were observed (Fig. 5, *D*, *E*, *G*, and *H*; *X*). The presence of χ-forms in association with both the 6.6- and the 5.8-kb Eco RV fragments indicated that Holliday junctions initiated in or branch migrated outside of chorion DNA sequences. All the nonlinear forms resolved in these experiments were associated with amplification, since Eco RV-digested DNA from a nonamplifying P[S6.9] transformant showed no intermediates (Fig. 5 *I*).

Discussion

Replicative Intermediates Can Be Identified in Drosophila DNA

Our experiments demonstrated that replicative intermediates from a specific chromosomal region can be detected in *Drosophila melanogaster* egg chamber DNA. Fork- and bubble-containing molecules were only observed within the chorion domain and only late in oogenesis when this domain of the chromosome replicates rapidly. Based on known parameters of amplification, replicative intermediates constituted close to the expected fraction of the DNA. We have therefore analyzed molecules responsible for producing the majority of the amplified genes. The ability to detect and map intermediates associated with an integrated chorion transposon was particularly important. The role in chromosomal amplification played by specific sequences can now be tested after transformation of transposons that have been mutated in vitro. Comparing the amplification of transposons integrated at different chromosomal sites may also reveal how position effects alter amplification. Recently, Delidakis and Kafatos (1989) have also analyzed intermediates in third chromosome chorion amplification.

The distribution of replicative intermediates observed probably reflected the situation in vivo before cell disruption. If specific DNA structures were unstable during purification, digestion, or electrophoresis, our ability to deduce patterns of replication would be complicated. This was chiefly of concern in the case of bubble-containing molecules, which constituted a small fraction of the total intermediates in some experiments. Because the expected fraction of bubbles depends on origin location, it was difficult to be sure that loss was occurring. Bubbles and χ-forms constituted 10% of the intermediates in the 7.7-kb Eco RI fragment (Fig. 2), but represented a much smaller fraction of intermediates within the Eco RI-Bgl II fragments (Fig. 4) and in P[S6.9]. Purified bubble-containing DNA can undergo fork extrusion, especially at elevated temperature, unless constrained on supercoiled molecules (Zannis-Hadjopoulous et al., 1981). However, the salt concentration and temperature we used for DNA extraction and restriction digestion should have minimized the loss of bubble-containing molecules based on measured rates of extrusion. In addition, the patterns we have observed were highly reproducible, indicative of an inherent stability after lysis in SDS. Although it will be important in the future to discover methods that improve the recovery of bubble-containing intermediates, the possible loss of some bubbles did not interfere with our analysis.

Specific Origins Are Used during Amplification

Transposon P[S6.9] initiated replication in the β region, and also at an upstream site. Although these experiments analyzed an integrated chorion transposon, it is likely that the normal locus also uses one or both of these sites. A model in which usually a single origin fires per strand, and in which the β origin is preferred 70–80% of the time can explain most of the observed replicative intermediates in Figs. 1–4. The replicative intermediates probably also reflect the existence of initiations within the 4.2-kb fragment, and the possibility that origin utilization may be coordinated in some manner.

Figure 5. Amplification of transposon P[S6.9] in stage 10 DNA. A map of transposon P[S6.9] is shown in the center of the figure. The restriction fragments analyzed are indicated by the solid lines. (*A–C*) (*top*) were probed with a 4.7-kb fragment from the 3′ end of the rosy gene. (*D–I*) (*bottom*) were probed with a 3.0-kb lacZ fragment. (*A*) Hind III digest. The linear molecules migrating slower than the expected 7.2-kb fragment represent the endogenous rosy locus in the ry^{506} host. (*B*) Bgl II digest (*C*) Fourfold longer exposure of *B*. (*D*) Sca I digest. (*E*) Hind III digest. (*F*) Eco RV digest, experiment 1. (*G*) Eco RV digest, experiment 2. (*H*) Kpn I digest. (*I*) Eco RV digest of DNA from the nonamplifying line S6.9-3. Where appropriate, bubble- (*O*) and χ-arcs (*X*) are labeled.

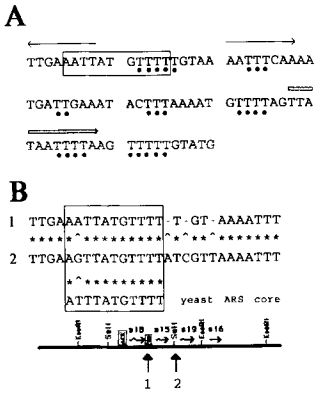

Figure 6. Putative origin sequences. (*A*) An 80-bp sequence within the β region beginning 348 bp from the s18 poly(A) site is shown (Levine and Spradling, 1985). A sequence resembling the yeast ARS core is boxed. Sequences downstream are A/T-rich and display runs of T residues (indicated by dots) with an ~10 bp periodicity on both strands. An 8-bp inverted repeat and an 11-bp repeat shared with the ACE3 region are indicated by arrows. (*B*) Sequence similarity between a portion of the region shown in *A* (1) and residues beginning 360 bp downstream of the s15 poly(A) site (2). Sequence 2 lies within a 414 bp region that stimulates transposon amplification (Delidakis and Kafatos, 1989). These sequence data are available from EMBL/Genbank/DDBJ under accession number X02497.

The existence of replication origins in the β region and within an upstream 2.3-kb region containing ACE3 is consistent with previous EM and biochemical data. Rarely, amplified chorion DNA can be recognized in Miller spreads by virtue of transcript morphology and spacing (Osheim and Miller, 1983; Osheim et al., 1988). Based on the structure of two amplifying molecules, these authors localized an origin within a 4-kb region containing both the β region and ACE3. Our results also agree with previous attempts to locate the peak of the amplification gradient surrounding the gene cluster. Analyzing differences in band intensity in Southern blots suggested that the chorion region upstream of s18 amplified slightly more than sequences downstream of s16 in several chorion transposons (Delidakis and Kafatos, 1987).

We examined the DNA sequence in the β region (Levine and Spradling, 1985; Wong et al., 1985) for motifs that might be related to origin function. Yeast replication origins contain a core ARS consensus sequence as well as adjacent regions having quantitative effects (reviewed in Umek et al., 1989). In some cases the flanking DNA is A/T-rich and has been suggested to facilitate strand separation. Sequences that may share these properties were present within the Bgl II-Xba I fragment (Fig. 6 *A*). The 80-bp sequence shown begins 530 nt upstream of the s15 transcription start site. It contains a 10/11 match to the yeast ARS core, followed by a long AT-rich region characterized by runs of As and Ts having a 10-bp periodicity. Near the right end lies an 11-bp sequence TTPuTAATTTTA. This sequence is also present as a tandem repeat within ACE3, where upstream forks may have initiated. At present it is unknown whether any of these sequences is important for origin function, however.

Regulation of Amplification

Previously, *cis*-regulatory sequences important for differential replication were mapped based on changes in the average level of amplification undergone by chorion transposons inserted at various genomic sites. Our finding that a replication origin lies near the regulatory element AER-d (β), and possibly also near ACE3, suggested that the deleterious effects of deleting these regions might have resulted simply from origin loss. However, ACE3's strength in controlling amplification may not be explicable solely in terms of its ability to function as a replication origin. More than twice as many bubble-containing molecules were detected downstream of the Bgl II site as upstream in the endogenous locus, and the majority of initiations also appeared to occur in the β region in P[S6.9]. These results suggest that ACE3 might function as a developmental control element, stimulating replication at β and possibly from downstream origins.

Both genetic and biochemical data suggested that additional origin sequences exist downstream of s15. An additional quantitative element lies 190–620 bp downstream of the s15 poly(A) site (Delidakis and Kafatos, 1987, 1989). In some transposons, the β region is functionally redundant in the presence of this element. For example, only one of these elements was deleted in two constructs which amplified at high levels, whereas both were removed from two constructs exhibiting low levels of amplification (Delidakis and Kafatos, 1989). Interestingly, these two elements share significant similarity in a 25-bp region that includes a near match to the yeast ARS consensus (Fig. 6 *B*). Several additional sequences showing partial homology to the yeast ARS consensus were previously identified within other regions influencing amplification levels (Delidakis and Kafatos, 1987).

Recently, it has been shown that not all yeast rDNA repeats initiate replication in a single cell cycle (Linskens and Huberman, 1988; Brewer and Fangman, 1988). If each repeat contains a potentially functional origin, then some process must activate only a subset of these sites during each S phase. Our results suggested that in the third chromosome chorion cluster, only one of several possible origins is activated during each step in the amplification process. The ability to detect sites of initiation within transposons containing various combinations of these regulatory elements should allow progress in understanding in general how specific sites of initiation are selected and activated.

Novel DNA Forms May Reveal Unexpected Mechanisms in Follicle Cells

We have characterized replicative intermediates during amplification, yet this information alone provides little insight into initiation mechanisms. It is unlikely that any intermediate occurring before elongation, such as a partially denatured region, would be resolved on the gels as a discrete structure in deproteinized DNA. More clues concerning initiation mechanisms may result from identifying the novel Z-form structures. These molecules appeared in chorion DNA during earlier stages of oogenesis than other intermediates, suggesting a possible role in the initiation of amplification. Molecules with a similar mobility were observed in some P[S6.9] digests. These structures were only observed in fragments containing both ACE3 and β.

The presence of molecules with the properties expected of χ-forms suggested that homologous recombination may occur during amplification. Opportunities for homologous recombination are great during amplification. Follicle cells become approximately 16-ploid prior to the onset of amplification (Mahowald et al., 1979; Hammond and Laird, 1985; Bohrman et al., 1987). Although polytene chromosomes are not visible, electron microscopy and in situ hybridization ex-

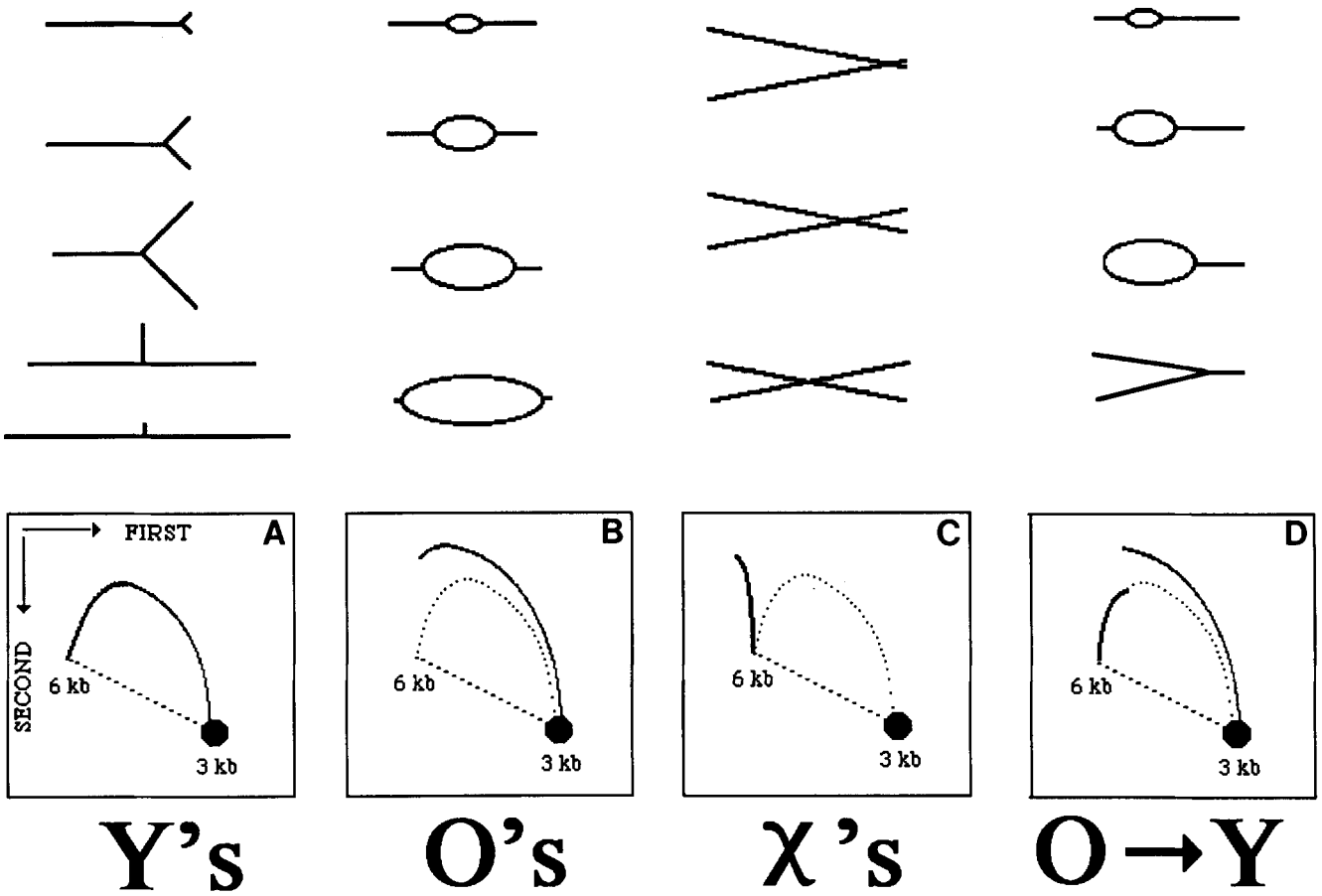

Figure 7. Patterns of replicative intermediates detected by two-dimensional electrophoresis. Replicative intermediates containing simple-Ys (*A*), bubbles (*B*), recombination intermediates (χ-s) (*C*), will produce arcs in the locations indicated on a two-dimensional gel. The pattern of intermediates produced by an asymmetric origin is illustrated in *D*.

periments revealed that the multiple sister strands remain close to each other in follicle cell nuclei (Osheim and Miller, 1983; Hammond and Laird, 1985). Furthermore, the slow progression of replication forks must constrain daughter strands to remain in proximity to each other for a substantial period of time. Further study will be required to determine if the χ-forms arise during DNA preparation, or result from recombination events in vivo.

Developmental Regulation of Replication

Chromosome replication during certain other times in development could probably also be analyzed using these methods. Cleavage-stage embryos undergo synchronous nuclear divisions every 10 min. Based on an elongation rate of 2.6 kb/min (Blumenthal et al., 1973), a fork would require 3 min to pass through the 7.7-kb chorion fragment (7.7/2.6), so that ~30% of these molecules should contain forks (3/10). (Note: for simplicity, we have not corrected for the exponential growth of the nuclei in this estimate. Internal initiations would reduce the values by up to one-half.) Thus, cleavage-stage embryos provide material as favorable for study as amplifying egg chambers, except that proportionately more DNA would be required to compensate for the absence of amplification. In contrast, for a tissue culture cell doubling every 20 h using forks elongating at 2.6 bp/min, only ~0.25% of the 7.7-kb fragments will contain forks (7.7 kb/2.6 kb/min/1,200 min). This >100-fold lower frequency is presumably why replicative intermediates were not detected in DNA from K_c tissue culture cells. Nonetheless, it should be possible to analyze intermediates present at this level by enriching replicative intermediates using BND-cellulose, and by increasing both the amount of DNA loaded on each gel and the probe-specific activity. Such studies will provide an opportunity to understand in detail how chromosome replication is modulated during the cell cycle and throughout development.

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