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Drosophila Chorion Gene Amplification Requires an Upstream Region Regulating *s18* Transcription

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A cluster of *Drosophila melanogaster* chorion genes at locus 66D on the third chromosome amplifies 60-fold in the ovarian follicle cells prior to the onset of gene expression. A 3.8-kilobase (kb) region of the gene cluster can induce tissue-specific amplification in transformants. Previous models postulated that amplification is activated in follicle cells by transcription of one of the two chorion genes (*s15* and *s18*) located within the 3.8-kb essential region. In this study, we showed that neither *s15* nor *s18* chorion gene transcription was required for amplification. However, a 510-bp region upstream from *s18* contained sequences essential for both amplification and *s18* transcription. No other region within the 3.8-kb fragment was required for amplification. We propose that upstream transcription control elements rather than transcription per se are involved in controlling amplification during development.

Drosophila melanogaster chorion genes located within two genomic clusters use a novel mechanism to achieve high rates of expression: the genes are amplified in the ovarian follicle cells prior to their transcription during the process of eggshell production. Amplification results when multiple rounds of replication initiate within both gene clusters and elongate bidirectionally through 30 to 40 kilobases (kb) of flanking chromosomal sequences (19, 23). *cis*-Regulatory sequences which specify this program of developmentally regulated DNA replication are located within both the X chromosome (24) and third chromosome (5) gene clusters. Each amplification control element (*ACE*) is likely to contain a specific origin of DNA replication as well as regulatory sequences that differentially activate the origin only in follicle cells during the appropriate period of oogenesis.

Amplification control elements provide attractive models for understanding the control of DNA replication during *D. melanogaster* development. Reinitiation at an origin prior to completion of S phase was not detected in cleavage-stage *D. melanogaster* embryos or cell cultures (3, 14), in contrast to amplifying follicle cells (19). An understanding of how amplification control elements allow multiple initiations may reveal mechanisms which normally prevent reinitiation. In many polyploid and polytene cells, specific chromosome regions are underreplicated (reviewed in reference 22). Control elements similar to those acting on the chorion gene clusters may limit the utilization of certain replication origins in these tissues.

Previous studies of amplification control have concentrated on the chorion gene cluster at 66D because it amplifies approximately 60-fold, about four times the amplification level of X-linked chorion genes. A 12-kb region that undergoes the full 60-fold amplification contains genes encoding eggshell proteins s19, s18, s16, and s15 in a head-to-tail tandem arrangement (9, 15, 32). An *ACE* within this region has been mapped by P-element-mediated germ line transformation (5). A 3.8-kb *Sal*I fragment containing the *s18* and *s15* genes induced amplification with proper tissue and developmental specificity at its site of reinsertion in the genome. However, P-element transposons containing the 3.8-kb region were subject to position effects that complicated efforts to further localize *ACE3* sequences. In this paper, we report the results of our studies of sequences regulating amplification within the 3.8-kb region; we used P-element transposons designed to minimize chromosomal position effects.

DNA replication and transcription are coupled in several systems. At the ColE1 origin, transcription of an RNA primer is essential in *cis* for replication initiation (11), and bacteriophage lambda replication requires transcriptional activation (8). Polyomavirus requires a transcriptional enhancer element in *cis* for origin function (7, 28). We therefore carried out studies to determine whether *s18* or *s15* gene transcription or transcriptional regulatory elements were important for amplification. In analyzing possible relationships between chorion gene transcription and replication, it is important to consider the normal timing of those events. Oogenesis is conventionally divided into 14 stages of unequal duration (18). Amplification begins in stage 9 and continues throughout the remaining 20 h prior to the death of the terminally differentiated follicle cells. Only during the last 6 h (stages 11 to 14), as the eggshell layers are secreted around the oocyte, are the chorion mRNAs and proteins encoded by the two clusters produced. Production of both *s18* and *s15* mRNA is confined to the final 4 h of oogenesis (stage 13 and 14), after many rounds of amplification have already occurred. Thus, it seemed unlikely that the major *s18* and *s15* transcripts were required to initiate amplification. However, low levels of *s15* transcription observed transiently during stages 9 and 10 were postulated to be involved in activating amplification (27).

The experiments reported in this paper demonstrate that sequences essential for amplification occur only within a 510-base-pair (bp) region upstream from the start of *s18* transcription. This region contains control signals for *s18* transcription, but transcription of the *s18* and *s15* genes in *cis* is not necessary for amplification. An origin of replication used in follicle cells during amplification is likely to be located in this region. Our results suggest a model for developmental regulation of amplification that is similar to the control of replication in several eucaryotic viral genomes.

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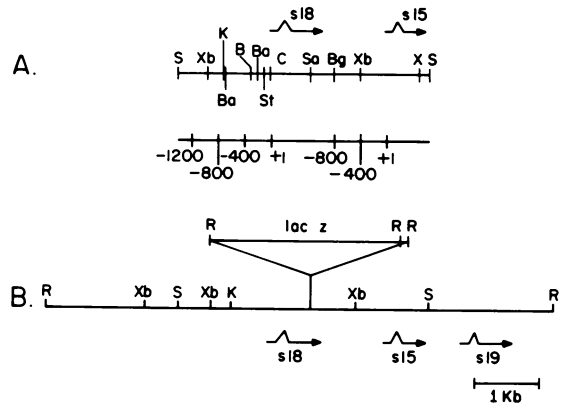

FIG. 1. Restriction map of third chromosome chorion fragments. (A) Restriction map of the 3.8-kb *Sal*I fragment, showing sites used for transposon constructions. The positions of the *s18* and *s15* chorion gene transcripts are indicated by arrows. Below the restriction map is a coordinate system for the regions upstream of *s18* and *s15*. For each of the regions, +1 designates the start site of the chorion transcript. (B) Map of restriction sites in the *Eco*RI 7.7-kb fragment used to generate $A_{26}O_9$ and $A_{27}O_1$. The position of the *lacZ* fusion in transposon $A_{10}O_{31}$ is shown. The positions of chorion gene transcripts are designated by arrows. The scale is identical in panels A and B. Abbreviations: B, *Bam*HI; Ba, *Bal*I; Bg, *Bgl*II; C, *Cla*I; K, *Kpn*I; R, *Eco*RI; S, *Sal*I; Sa, *Sac*I; St, *Stu*I; X, *Xho*I; Xb, *Xba*I.

MATERIALS AND METHODS

Plasmid constructions. The deletions 5′ to the *s15* gene were constructed in plasmid pTM9 containing the wild-type 3.8-kb *Sal*I chorion fragment inserted at the *Sal*I site in the polylinker of pUC8 (29). pTM9 also has a 3.1-kb *Sal*I fragment containing the *lacZ* gene inserted into the *Xho*I site in the 3′ untranslated region of the *s15* gene (30). The *Xba*I site upstream from the *s18* gene within pTM9 was mutated by endfilling a partial *Xba*I digest with T4 DNA polymerase (P-L Biochemicals, Inc.) to produce the derivative pA_7O_8 (Fig. 1A). This plasmid was linearized at the remaining *Xba*I site 5′ to *s15* and digested with *Bal* 31. The digestion mixture was endfilled with T4 DNA polymerase, and *Xba*I linkers (Collaborative Research, Inc.) were added. Individual *Bal* 31 deletion endpoints were determined by mapping the size of an *Xba*I-*Eco*RI fragment (normally 914 bp) and by DNA sequencing. A deletion with an endpoint at +65 in the *s15* gene was chosen for the construction of $A_{19}O_{28}$. The deleted DNA extending in the opposite direction (toward *s18*) from the *Xba*I site was replaced by substituting the *Kpn*I-*Xba*I fragment with the corresponding fragment from the undeleted 3.8-kb fragment (Fig. 1A). This deleted *Sal*I fragment in pA_7O_8 was then substituted for the normal 3.8-kb *Sal*I fragment in pR7.7. pR7.7 DNA was digested with *Sal*I, and the large fragment was isolated from a low-melting agarose gel (SeaPlaque; FMC Corp., Marine Colloids Div.). The *Sal*I fragment containing the deleted *s15-lacZ* fusion was ligated to this, and a construct with the correct orientation was isolated and designated $A_{19}O_{28}$. The deletion from −820 to −390 5′ to *s15* was produced in A_7O_8 by digestion with *Xba*I (−390) and *Bgl*II (−820) (Fig. 1A) followed by endfilling and ligation. The *Sal*I fragment containing this deletion was replaced into R7.7 as described above to produce $A_{22}O_{31}$.

The deletion in $A_{25}O_4$ was constructed from a pUC8 plasmid containing the 3.8-kb *Sal*I fragment. This plasmid was grown in a *dam*$^-$ *E. coli* strain (RK1007, from R. Kolodner, Dana Farber Cancer Institute), and the DNA was then purified and digested with *Cla*I (Fig. 1A). *Xho*I linkers (Collaborative Research) were added after endfilling. Digestion with *Xho*I removed the DNA from position +10 to the *Xho*I site at the end of the *s15* gene (Fig. 1A). The 3.8-kb *Sal*I fragment in R7.7 was then replaced with the 1.4 kb *Sal*I-*Xho*I fragment.

The translational fusion of the *lacZ* gene and the *s18* gene was produced by first inserting an *Eco*RI linker at the *Sac*I site in the *s18* structural gene (Fig. 1A). *Eco*RI linkers were added to a *Xma*I-*Sal*I fragment containing the *lacZ* gene from pMC1871 (4) that had been previously treated with *Eco*RI methylase to protect the *Eco*RI site present inside the *lacZ* gene. This *lacZ* fragment was then ligated into the newly formed *Eco*RI site in the *s18* gene. $A_{10}O_{31}$ consists of the 3.8-kb *Sal*I fragment carrying the *s18-lacZ* fusion moved into R7.7. To construct the −120-to-+10 deletion in *s18*, the plasmid with the 3.8-kb *Sal*I fragment containing the *s18-lacZ* fusion was grown in RK1007 and the DNA was partially digested with *Cla*I (there is a *Cla*I site in *lacZ*). Full-length linear molecules were isolated from a low-melting agarose gel, digested with *Stu*I, endfilled, and blunt-end ligated. This deletion was replaced into R7.7 to form $A_{18}O_5$. The −620-to-−190 deletion 5′ to *s18* lies between two *Bal*I restriction enzyme sites. The 3.8-kb *Sal*I plasmid was digested with *Bal*I and ligated. $A_{17}O_{15}$ contains this deletion with the *s18-lacZ* fusion in R7.7. The other two deletions generated 5′ to the *s18* gene were constructed in $A_{10}O_{31}$ directly (Fig. 1B). For $A_{26}O_9$, the DNA was partially digested with *Xba*I and ligated, and plasmids were screened for a deletion from the *Xba*I site 500 bp outside the *Sal*I site closest to *s18* to the *Xba*I site at −940. To construct $A_{27}O_1$, the DNA was partially digested with *Sal*I and linear molecules were isolated. These were digested with *Kpn*I, treated with T4 DNA polymerase, and blunt-end ligated. Complete degradation of the *Kpn*I ends, together with complete endfilling of the *Sal*I ends on ligation, produced a new *Sal*I site at −630.

P-element transformation. Transformation was done as described previously (21, 23a, 25). Embryos were dechorionated in bulk by treatment with bleach diluted 1:1 with water for 3 min. They were then collected on a nylon net and washed twice with 0.02% Triton X-100 and twice with water. Embryos thus dechorionated required longer desiccation prior to injection than did embryos dechlorionated manually. DNAs were injected at concentrations of 300 μg/ml for the transposon and 150 μg/ml for the helper pπ25.7wc. pπ25.7wc provides transposase activity but is itself unable to transpose, so that all transposon lines generated are stable (13). The ry^{506} stock we used had been backcrossed to a Canton-S stock by T. Hazelrigg; this gave it higher fertility and viability levels. We injected 1,000 embryos for each construct to ensure that at least 10 independent lines would be obtained. G_0 flies were individually mated to ry^{506} partners, and stocks were established from individual G_1 ry^+ progeny. An initial estimate of transposon copy number in the transformed flies was obtained by measuring the ratio of ry^- to ry^+ G_2 progeny from the G_1-ry^{506} cross. Single-copy integrants were confirmed by Southern analysis of DNA isolated from five G_2 males of each line. In situ hybridizations to polytene chromosomes were as described previously (26).

Amplification assays. For amplification assays, transformants were crossed to ry^{506} flies in the previous generation so that only flies heterozygous for the transposon insertion were dissected. DNA was prepared from staged egg chambers as described by de Cicco and Spradling (5), except that

an extra phenol-chloroform extraction and ethanol precipitation were carried out to ensure complete restriction enzyme digestion. DNA also was prepared from males and from ovarectomized females. The DNA was digested with *Bam*HI, electrophoresed on a 0.7% agarose gel, and transferred to a nylon membrane (Nytran; Schleicher & Schuell, Inc.). The blots were hybridized in 50% formamide at 42°C to nick-translated pDm2844S8.5 DNA (2), which contains the rosy gene. Washed blots were exposed on preflashed X-ray film with intensifying screens. Amplification values were calculated from areas determined from densitometer scans of autoradiograms (5). Amplification values of 1.5 or lower were scored as amplification negative; levels of 1.6 or higher were amplification positive.

Northern blots. RNA was purified from staged egg chambers as described previously (30). RNA from 100 egg chambers was electrophoresed on a 1% agarose formaldehyde gel and transferred to nitrocellulose (30). The filters were hybridized to nick-translated pMC1871 containing *lacZ* and then to a probe containing *hsp83* (33).

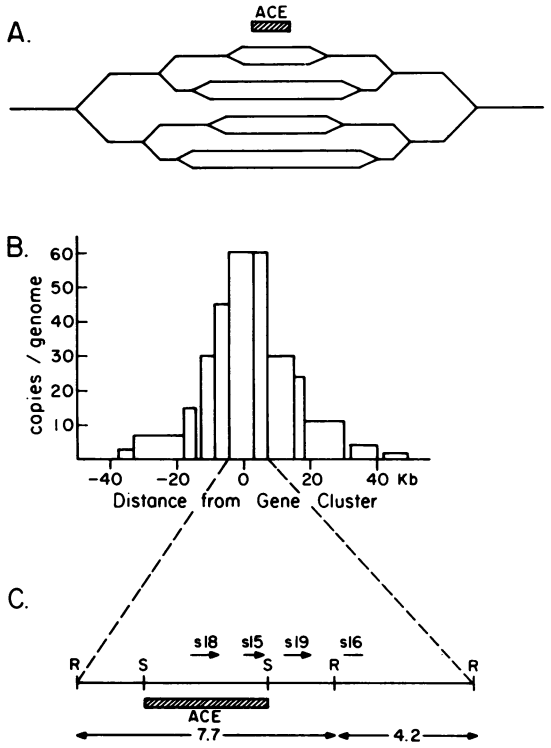

FIG. 2. Amplification of the third chromosome chorion gene cluster. (A) Model of chorion gene amplification. Amplification is postulated to result from multiple rounds of DNA replication initiating within the chorion cluster. *cis*-acting sequences which regulate this tissue-specific program of differential replication are termed an *ACE* (hatched box). (B) Chromosomal domain of approximately 100 kb that is amplified around the third chromosome chorion gene cluster. Quantitation of sequence copy number in stage 13 egg chamber DNA across this domain reveals a gradient of replication, with maximal levels in the region of the gene cluster. The zero point is defined as the 5′ end of the *s18* transcript (23). (C) Maximally amplified 11.9-kb segment, composed of two *Eco*RI fragments encoding the four major chorion protein genes. The *ACE* was mapped previously to a 3.8-kb *Sal*I fragment containing the *s18* and *s15* genes by germline transformation (5). Abbreviations: R, *Eco*RI; S, *Sal*I. The direction of transcripts is shown by arrows.

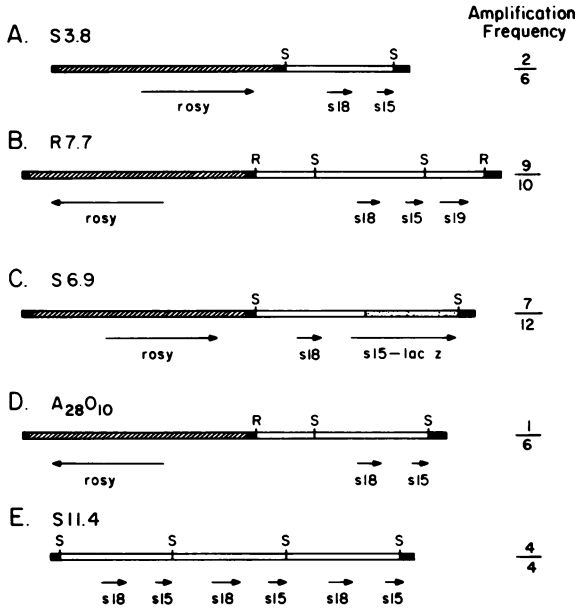

FIG. 3. Position effects on transposon amplification. Transposon structures are diagrammed on the left; the number of transformed lines in which the indicated transposon underwent amplification compared with the total number of lines tested is shown on the right. (A) S3.8, consisting of the 3.8-kb *Sal*I fragment containing *ACE3* inserted into the *Sal*I site of Car20 (5). (B) R7.7, containing the 7.7-kb *Eco*RI fragment (Fig. 2) inserted into the *Eco*RI site in the polylinker of pV11 (5). (C) S6.9, derived from S3.8 by insertion of a 3.1-kb *E. coli lacZ* DNA fragment into the 3′ untranslated region of the *s15* gene (30). (Note that the orientation of the 6.9-kb *Sal*I fragment within Car20 shown for S6.9 in reference 30 is incorrect.) (D) $A_{28}O_{10}$, equivalent to R7.7 but without the distal *Sal*I-*Eco*RI fragment. (E) S11.4, a transposon containing a tandem trimer of the 3.8-kb *Sal*I fragment (5). In all the transposons shown, the 3′ end of the P-element vector is at the left. Symbols: ▭, chorion DNA; ▬, P-element DNA; ▨, rosy DNA; ▩, *lacZ* DNA; R, *Eco*RI; S, *Sal*I. The location and direction of transcripts are shown by arrows, with the names of genes underneath.

RESULTS

Construction of an *ACE3* transposon with reduced sensitivity to position effects. The structure and amplification of the 66D chorion gene cluster and its flanking chromosomal sequences are summarized in Fig. 2. In stage 13 to 14 egg chambers, a bidirectional gradient of amplification spans a 100-kb chromosomal segment surrounding the gene cluster (at +0 kb in Fig. 2B). A schematic representation of the reinitiation model for amplification, showing three of the predicted six rounds of replication and the general location of the *ACE*, is shown in Fig. 2A. Previously, *ACE3* was mapped to a 3.8-kb *Sal*I fragment that partially overlapped the 5′ end of the gene cluster (Fig. 2C) (5).

The amplification of a transposon containing the 3.8-kb fragment and a rosy gene as a selectable marker (S3.8) varied greatly between transformant lines with insertions at different chromosomal locations (5). This variation is believed to be due to position effects rather than to mutations occurring during transformation, since structural changes in the transposons were not detected (5) and mobilization of a nonamplifying transposon into new chromosomal sites restored its ability to amplify (L. Cooley, R. Kelley, D. de Cicco, and A. Spradling, unpublished observations). We sought to decrease the position sensitivity of wild-type

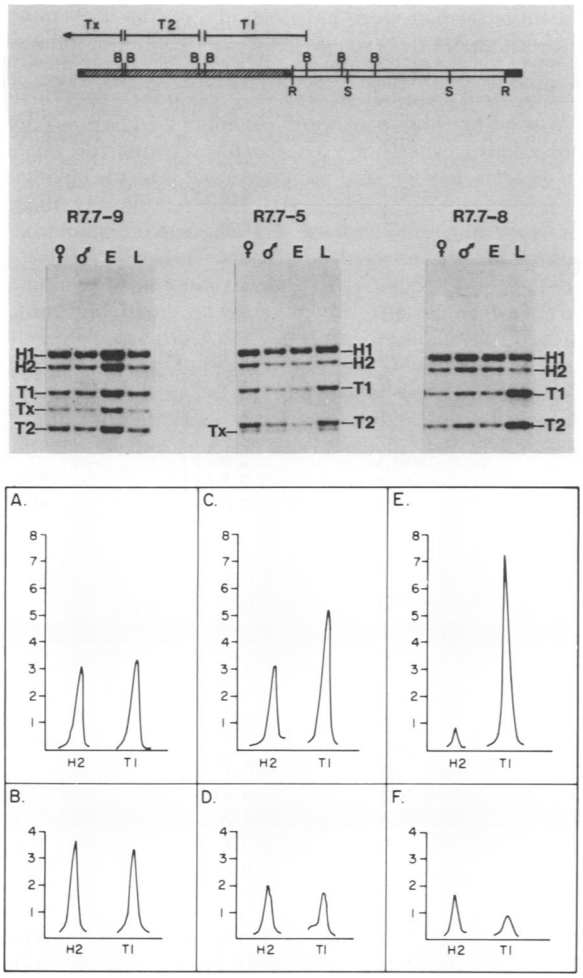

FIG. 4. Quantitation of amplification levels. Females from three transformed lines containing single-copy heterozygous inserts of transposon R7.7 were dissected. DNA was prepared from stage 1 to 8 and stage 13 egg chambers; DNA was also isolated from males and ovarectomized females. Southern transfers of the *Bam*HI-digested DNAs were hybridized with a probe homologous to the rosy gene (middle panel). As diagrammed in the top panel, *Bam*HI digestion produces three fragments from the transposon with homology to rosy: 3.2 and 2.8 kb (T1 and T2) and a third band of variable size that includes flanking DNA (Tx). Two aditional bands from the transposon were too small to be retained on the gel. The endogenous ry^{506} deletion allele gives rise to 4.7- and 4.5-kb (H1 and H2) fragments which serve as internal controls for the amount of DNA loaded on each lane. Densitometer tracings of the autoradiograms used to calculate amplification levels are shown in the lower panel. Amplification is calculated as the T1/H2 ratio in stage 13 divided by the T1/H2 ratio in early egg chambers or male DNA (Table 1). (A) Densitometer scans of stage 13 (L) lane from R7.7-9. (B) Scans of male lane from R7.7-9. (C) Stage 13 from R7.7-5. (D) Stages 1 to 8 (E) lane from R 7.7-5. (E) Stage 13 from R7.7-8. (F) Stages 1 to 8 from R7.7-8. Transposon structure designations are as in Fig. 3; B, *Bam*HI.

control elements to simplify the task of studying mutant derivatives by reducing the number of transformants required to evaluate their intrinsic ability to amplify. Initially, therefore, we investigated the effects of transposon size and structure on amplification.

The sensitivity of chorion transposons to position effects on amplification, although complex, appeared to be size dependent (Fig. 3). The 3.8-kb *Sal*I fragment is ordinarily flanked by a 2.0-kb *Eco*RI-*Sal*I fragment and a 1.9-kb *Sal*I-*Eco*RI fragment in the third chromosome chorion gene cluster (the 7.7-kb *Eco*RI fragment; Fig. 2C). The two flanking chorion fragments are unable to autonomously induce amplification (5). We compared amplification in 10 lines containing transposon R7.7, in which these normal flanking sequences are retained, with amplification of S3.8 (5), which contains only the 3.8-kb fragment (Fig. 3A and B).

An internally controlled Southern blot assay was used to measure transposon amplification. Quantitation of transposon replication in three of the R7.7 lines is illustrated in Fig. 4 (see Materials and Methods). DNA was purified from preamplification (stages 1 to 8) and postamplification (stage 13) egg chambers, from adult males, and from ovarectomized adult females that were heterozygous for the insertion. An appropriate choice of restriction enzyme and hybridization probe allowed both transposon-specific and endogenous control bands to be visualized simultaneously by Southern blotting. This provided an accurate internal control for the amount of DNA per lane. The level of transposon amplification was then calculated by comparing the relative intensities of labeled bands in the different lanes. For example, in Fig. 4, the labeling ratio of an endogenous *ry* fragment (H2) in DNA from stages 1 to 8 and stage 13 was compared with the same ratio for the transposon-specific *ry* fragment (T1). Amplification levels as low as twofold were readily detected and highly reproducible. Transposons showing between 1.0- and 1.5-fold amplification were scored as nonamplifying, because retests of such lines were invariably negative. We defined the amplification frequency of a transposon as the fraction of insertions tested that amplified. Wilcoxon's rank sum test (31) was used to assess the significance of differences in the amplification of different transposons. Alterations in developmental control of transposon amplification would be detected by changes in transposon copy number in early egg chambers or in DNA from males or ovarectomized females.

We observed significant differences in the frequency and level of amplification induced by the transposons R7.7 and S3.8 ($P = 0.05$). The R7.7 transposons amplified at 9 of 10 insertion sites, although the level of reduplication (2- to 39-fold) was still lower than normal (Table 1). Only two of six S3.8 insertions underwent amplification (5). There were several possible reasons for the increased amplification frequency of R7.7 compared with S3.8. The presence of the 2.0- and 1.9-kb chorion DNA frgments flanking the 3.8-kb *ACE* fragment might reduce position effects nonspecifically by buffering the *ACE* fragment from inhibitory sequences in novel chromosomal DNA. The inverted orientation of the rosy marker DNA could have altered an interaction between the control element and rosy sequences. Finally, a specific element important for high quantitative levels of amplification might lie within one or both of the flanking chorion DNA fragments.

Figure 3 summarizes amplification tests on several additional transposons relevant to these questions. Transposon $A_{28}O_{10}$ (Fig. 3D) differed from R7.7 only by deletion of the 1.9-kb flanking chorion DNA fragment containing the *s19* gene. $A_{28}O_{10}$ amplified in only one of six lines (Table 1). Previously, transposon S6.9 (Fig. 3C) was shown to amplify in 7 of 12 insertions, while transposon S11.4 (Fig. 3E) amplified at 4 of 4 chromosomal sites (5). The orientation of rosy did not correlate with amplification frequency. Therefore, the possibility that rosy DNA contains a short-range inhibitory sequence at one end was not supported. The inefficiency of $A_{28}O_{10}$ relative to R7.7 suggested that a

TABLE 1. Amplification levels in transposon lines

Transposon line	Cytological location	Amplification level[a]	Chorion-*lacZ* transcription[b]
R7.7			
1	68A	14[c] (+)	
2	ND[d]	3.4 (+)	
3	ND	36 (+)	
4	6C	21 (+)	
5	ND	2.0 (+)	
6	ND	39 (+)	
7	17B	4.2 (+)	
8	ND	22 (+)	
9	43E	1.4 (−)	
10	ND	14 (+)	
$A_{28}O_{10}$			
1	ND	1.3 (−)	
2	ND	3.0 (+)	
3	ND	1.3 (−)	
4	ND	1.4 (−)	
5	ND	1.2 (−)	
6	ND	0.80 (−)	
$A_{19}O_{28}$			
1	ND	14 (+)	ND
2	ND	1.9 (+)	ND
3	41-42A	18 (+)	−
4	ND	1.2 (−)	ND
5	53E	4.3 (+)	ND
6	79B,C	9.6 (+)	−
7	ND	2.6 (+)	ND
8	ND	2.7 (+)	ND
9	ND	1.8 (+)	ND
$A_{22}O_{31}$			
1	63C	4.5 (+)	+
2	ND	2.6 (+)	ND
3	ND	3.6 (+)	ND
4	ND	2.1 (+)	ND
5	ND	8.5 (+)	+
6	ND	2.2 (+)	ND
7	ND	1.3 (−)	ND
8	64D,E	2.4 (+)	ND
9	92A	1.8 (+)	ND
$A_{25}O_{4}$			
1	ND	1.4 (−)	
2	ND	1.2 (−)	
3	ND	4.6 (+)	
4	45D	0.90 (−)	
5	ND	0.78 (−)	
6	ND	1.0 (−)	
7	ND	1.8 (+)	
8	85C	6.8 (+)	
9	ND	1.1 (−)	
10	12C	4.7 (+)	
11	ND	2.9 (+)	
12	ND	1.4 (−)	
13	52A	1.9 (+)	
$A_{10}O_{31}$			
1	86B	7.7 (+)	+
2	ND	1.8 (+)	+
3	ND	1.0 (−)	+
4	ND	1.2 (−)	ND
5	ND	1.3 (−)	ND
6	36C	2.4 (+)	ND
7	ND	1.0 (−)	ND
8	21C	1.0 (−)	ND
9	34D,F	0.80 (−)	ND
10	ND	1.4 (−)	ND
11	ND	1.4 (−)	ND
12	ND	1.4 (−)	ND
13	ND	15 (+)	ND
14	ND	2.0 (+)	ND
15	ND	4.9 (+)	ND
16	ND	1.1 (−)	ND
$A_{18}O_{5}$			
1	ND	3.1 (+)	−
2	30B	1.5 (−)	ND
3	86D	15 (+)	−
4	ND	0.79 (−)	ND
5	54A	5.9 (+)	−
6	ND	1.2 (−)	ND
7	ND	1.8 (+)	−
8	36B,C	2.6 (+)	−
9	ND	2.5 (+)	ND
10	ND	2.0 (+)	ND
$A_{17}O_{15}$			
1	ND	0.92 (−)	ND
2	25D	0.87 (−)	−
3	4E,F	0.78 (−)	−
4	ND	0.70 (−)	ND
5	ND	0.60 (−)	−
6	93F	1.2 (−)	−
7	ND	0.69 (−)	−
8	ND	1.2 (−)	ND
9	21B	0.98 (−)	−
10	ND	0.93 (−)	−
11	ND	0.96 (−)	ND
12	ND	0.97 (−)	ND
13	ND	0.82 (−)	ND
14	ND	0.72 (−)	ND
15	ND	1.1 (−)	ND
16	ND	1.0 (−)	ND
17	ND	1.2 (−)	ND
18	ND	0.66 (−)	ND
19	ND	1.4 (−)	ND
$A_{26}O_{9}$			
1	51D	0.90 (−)	ND
2	ND	1.2 (−)	ND
3	ND	23 (+)	+
$A_{27}O_{1}$			
1	79F	8.1 (+)	+
2	ND	2.0 (+)	+
3	75B	1.1 (−)	ND

[a] (+), Transposon line was amplification competent; (−), transposon did not amplify.
[b] (+), Chorion-*lacZ* fusion transcript was detected on Northern blots; (−), transcript was not detected.
[c] Data for R7.7-1 from reference 5.
[d] ND, Not determined.

functional element might lie within the 1.9-kb *SalI-Eco*RI chorion fragment. However, the efficient amplification of S6.9 and S11.4 (which lack this sequence) implied that the 1.9-kb fragment reduced position effects nonspecifically, possibly by preventing inhibitory sequences postulated to exist in chromosomal DNA from lying close to the *ACE*. According to this model, in S6.9, this buffer effect would be obtained from *E. coli lacZ* DNA, while in S11.4, an additional copy of the 3.8-kb fragment would buffer the central copy.

Although the mechanism of position effects on transposon amplification remains obscure, the efficient amplification of

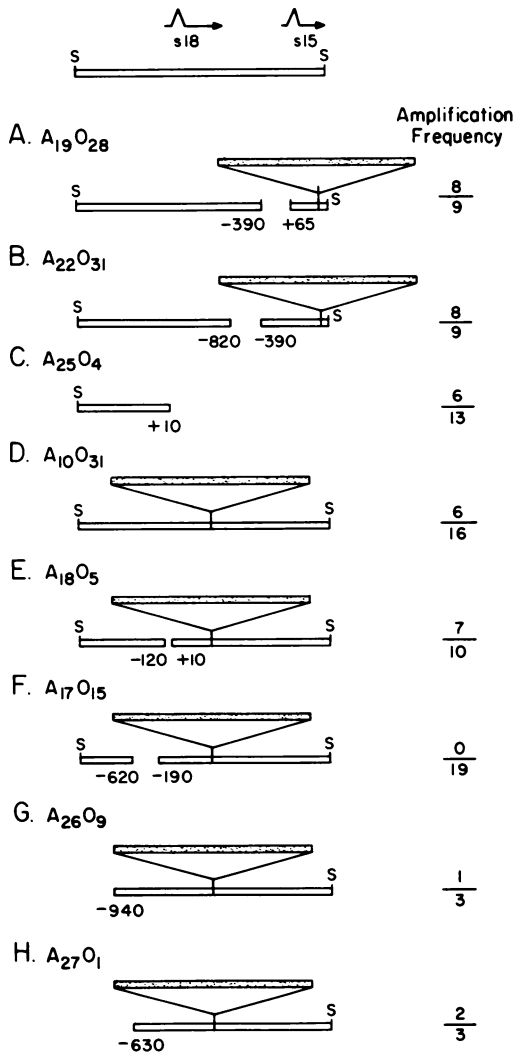

FIG. 5. Deletion analysis of *ACE3*. All of the deletions tested were constructed within in the R7.7 transposon. Only the internal 3.8-kb *SalI* fragment is shown, since the remainder of the transposons were identical. The location of the two chorion genes within the 3.8-kb *SalI* fragment is diagrammed at the top of the figure; all constructs are drawn to the same scale. The number of lines for which the inserted transposon underwent amplification over the total number of lines tested for each construct is given on the right. Details of the construction of each transposon are included in Materials and Methods. (A) $A_{19}O_{28}$ has *lacZ* DNA inserted into the 3′ untranslated region of the *s15* gene and a deletion between −390 and +65 (where +1 designates the start site of the *s15* transcript). (B) $A_{22}O_{31}$ is the same *s15-lacZ* fusion as $A_{19}O_{28}$ but with a deletion between −820 and −390 5′ to the *s15* gene. (C) In $A_{25}O_4$, the region from +10 within the *s18* transcript (here +1 designates the start site of *s18*) to the *SalI* site at the end of the *s15* gene has been deleted. (D) $A_{10}O_{31}$ has a fusion of *lacZ* to the *s18* gene in the intact 3.8-kb fragment. (E) $A_{18}O_5$ contains the *s18-lacZ* fusion transposon of $A_{10}O_{31}$ but with the DNA sequences between −120 and +10 in the *s18* gene deleted. (F) $A_{17}O_{15}$ has a deletion between −620 and −190 5′ to the *s18-lacZ* fusion gene. (G) In $A_{26}O_9$, the 5′ region of *s18* is deleted from 500 bp outside the *SalI* site to position −940. (H) $A_{27}O_1$ contains a deletion from the *SalI* site to position −630. Symbols, ▭, chorion DNA; ▬, *lacZ* DNA; S, *SalI*.

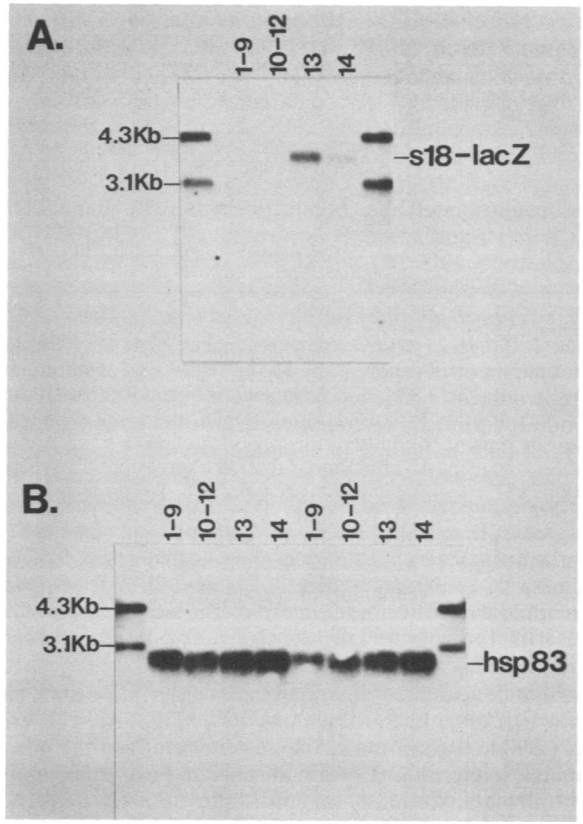

FIG. 6. Production of *s18-lacZ* RNA in $A_{10}O_{31}$ and $A_{17}O_{15}$ lines. RNA was purified from stage 1 to 9, 10 to 12, 13, and 14 egg chambers from line $A_{10}O_{31}$-1 (A) and lines $A_{17}O_{15}$-3 and $A_{17}O_{15}$-9 (B). Northern transfers were hybridized to ^{32}P-labeled *lacZ* DNA in panel A and to ^{32}P-labeled *lacZ* and *hsp83* probes in panel B. Single-stranded DNA standards were included as size markers. Panel A shows a transcript of 3.8 kb, the predicted size of the *s18-lacZ* fusion, which hybridized to the *lacZ* probe in RNA from stages 13 and 14. This is the normal developmental profile of *s18* mRNA. Subsequent hybridization of this blot to *hsp83* verified that equal amounts of RNA were loaded in all lanes. Panel B shows that although RNA is present in all lanes, as indicated by hybridization to *hsp83*, no *s18-lacZ* transcript was detected. Numbers at the top of lanes designate egg chamber stages from which RNA was purified.

transposon R7.7 made the further deliniation of *cis*-regulatory sequences within *ACE3* technically feasible. Our strategy was to construct a series of transposons identical to R7.7 except for the presence of small deletions within the internal 3.8-kb *SalI* fragment containing *ACE3*. The deletions were presumed to be too small to significantly affect the frequency of position effects. However, removal of an essential *cis*-acting element, for example, a replication origin or its developmental control sequences, should abolish the ability of the transposon to induce amplification in the follicle cells of transformants. Functionally redundant control signals would not be detected by this approach.

Transcription in *cis* of *s15* is not required for amplification. We first examined the role of *s15* and its upstream DNA sequences on amplification. In two constructs, the *s15* gene in R7.7 was tagged by insertion of *E. coli lacZ* DNA at the same site as in transposon S6.9, permitting us to study the effects of deletions on *s15* transcription, as well as on transposon amplification. The *s15-lacZ* fusion used in these experiments was shown previously to undergo a normal

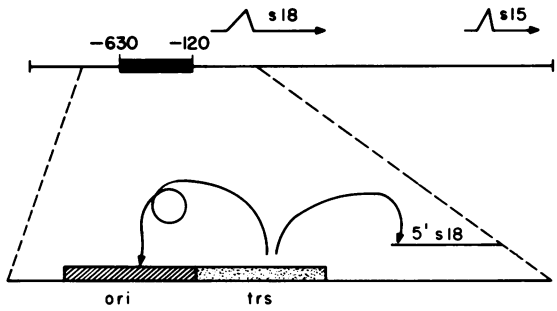

FIG. 7. Model of *cis*-regulatory sequences in *ACE3*. The upper portion shows the location of the 510-bp region essential for amplification within the 3.8-kb *Sal*I fragment. We propose that an origin of DNA replication (*ori*) used during amplification is present within the 510-bp region. In addition, this region contains a transcriptional regulatory sequence (*trs*) required for *s18* expression. The relative positions of the two components are unknown. We propose that the *trs* is also required in *cis* for initiation of replication at *ori*, inducing rounds of replication specifically in the follicle cells, possibly as a result of binding of *trans*-acting factors. Symbols: ▬, essential sequences for amplification; ▨, origin of replication (*ori*); ▩, transcriptional regulatory sequence (*trs*). The location and direction of transcripts are indicated by arrows.

pattern of developmentally regulated transcription at all sites of insertion (30). In transposon $A_{19}O_{28}$ (Fig. 5A), a deletion from −390 to +65 in the *s15-lacZ* gene removed the normal promoter sequences. (Coordinates are in base pairs relative to the *s15* transcription initiation site as +1.) Sequences upstream of *s15* (−820 to −390) were eliminated in transposon $A_{22}O_{31}$ (Fig. 5B). Finally, the entire *s15* coding and upstream region, as well as all but the first 10 nucleotides of the *s18* transcription unit, were removed in a third construct, transposon $A_{25}O_4$ (Fig. 5C). Single-insert transformants containing these transposons were constructed and tested for amplification as described above and for *s15* transcription by Northern analysis.

None of the three deletions prevented amplification (Fig. 5; Table 1) or altered its developmental specificity. The frequency of $A_{19}O_{28}$ and $A_{22}O_{31}$ amplification (eight of nine) was indistinguishable from that of R7.7. However, the levels of $A_{22}O_{31}$ amplification were significantly lower than for R7.7 transformants ($P = 0.05$), and so it is possible this deletion removed sequences with a quantitative effect on differential replication. Consistent with this possibility, lines containing $A_{25}O_4$ also amplified less frequently and at lower levels than the R7.7 control ($P < 0.01$). However, the large 2.4-kb deletion may have sensitized the transposon to position effects, as for $A_{28}O_{10}$. Nonetheless, these regions were clearly inessential for either the initiation or the developmental specificity of amplification.

To examine the effects of the deletions on *s15* transcription, RNA was isolated from staged egg chambers of the transformants, separated on a formaldehyde agarose gel, transferred to nitrocellulose, and hybridized to a *lacZ* probe (see Materials and Methods). No transcripts were detected from the fusion gene in lines $A_{19}O_{28}$-3 or $A_{19}O_{28}$-6, as expected, since the normal promoter and transcription start site were deleted in this construct. These experiments would have detected transcripts at levels 50-fold lower than those found in transformants containing the undeleted gene. Clearly, then, transcription of *s15* was not essential in *cis* for amplification. The transcription of the *s15-lacZ* fusion gene in transposon $A_{22}O_{31}$ was tested by using two lines (Table 1). The upstream deletion in this construct did not abolish transcription; both lines of $A_{22}O_{31}$ showed detectable levels of the *lacZ* homologous fusion transcript only in RNA from stage 14, the correct stage specificity (data not shown).

Mapping an essential sequence within *ACE3* upstream from *s18*. To search for regulatory sequences in the remaining 1,400 bp upstream of *s18* in the 3.8-kb region, we constructed five additional R7.7 derivatives (Fig. 5D to H). All these transposons contained a *lacZ* insertion within the *s18* transcription unit to allow effects on *s18* expression to be monitored. Transposon $A_{10}O_{31}$ served as a control to determine whether the *lacZ* insertion itself affected amplification and to determine whether the *s18-lacZ* fusion transcript was expressed with a normal developmental specificity. Sixteen single-insert lines containing $A_{10}O_{31}$ were studied. Northern blots of egg chamber RNA demonstrated that RNA derived from the *s18-lacZ* fusion gene accumulates only during stages 13 and 14 in all three lines which were tested (Fig. 6A and data not shown). This is the same pattern of expression as for the endogenous *s18* gene. Surprisingly, insertion of the *lacZ* gene within the *s18* structural gene partially inhibited amplification ($P < 0.01$). The $A_{10}O_{31}$ transposon amplified in only 6 of 16 lines, and amplification levels were lower than in the R7.7 control (Table 1). However, developmental specificity was unaffected.

Analysis of lines transformed with $A_{18}O_5$, a transposon deleted between −120 and +10 relative to the *s18* transcription start site, showed that transcription from the *s18* promoter was not required in *cis* for amplification. Transposon $A_{18}O_5$ amplified with normal specificity in 7 of 10 lines tested (Table 1). This frequency was not significantly different from that for the $A_{10}O_{31}$ control. However, RNA complementary to *lacZ* could not be detected in egg chamber RNA from five lines containing $A_{18}O_5$ (Table 1). Since lines undergoing amplification were tested, the sensitivity of these experiments would have permitted detection of transcripts at levels 50-fold lower than those found in transformants containing the undeleted fusion gene.

Transposon $A_{17}O_{15}$ lacked sequences between two *Bal*I sites at −620 and −190 5′ to *s18* (Fig. 5F). Initially, 10 single-insert lines were isolated which contained this construct. The transposon failed to amplify in any of the lines (Table 1), suggesting that the deletion significantly reduced or eliminated the ability of $A_{17}O_{15}$ to amplify ($P < 0.01$). To determine whether $A_{17}O_{15}$ might retain a weak ability to induce amplification at some chromosomal locations, we constructed and analyzed nine additional lines. All of these were also negative, supporting the view that the region between −620 and −190 upstream from *s18* contained sequences essential for amplification. The region deleted in $A_{17}O_{15}$ also was found to contain signals essential for *s18-lacZ* transcription. RNA was prepared from staged egg chambers isolated from seven of these lines (Table 1), and Northern blots were hybridized simultaneously with probes to *lacZ* and *hsp83*. No hybridization to transcripts from the *s18-lacZ* fusion gene was found, although the *hsp83* transcript was readily detected in all the stages tested (Fig. 6B and data not shown). Since these transformant lines did not amplify the transposon DNA, our ability to detect the *s18-lacZ* mRNA was reduced compared with that for lines containing amplified insertions. However, we concluded that in the presence of the $A_{17}O_{15}$ deletion, *s18* expression was reduced more than 10-fold per gene and may be eliminated altogether.

$A_{26}O_9$ and $A_{27}O_1$ contained deletions further upstream from *s18*. $A_{26}O_9$ lacked sequences from an *Xba*I site 500 bp outside the 3.8-kb fragment to an *Xba*I site at −940, while in

$A_{27}O_1$, the sequences up to a *Kpn*I site at −630 were removed (Fig. 5G and H). Neither of these deletions affected *s18* transcription or amplification (Table 1).

DISCUSSION

A 510-bp region upstream of *s18* is essential in *cis* for amplification. The experiments reported here identify a 510-bp region located between −630 and −120 bp upstream from the *s18* chorion gene that contains the only elements essential for amplification in transformants (Fig. 7). Amplification-competent transposons were constructed that lack any other portion of the 11.9-kb region encompassing the entire 66D gene cluster (Fig. 2). However, no transposon lacking the 510-bp *ACE3* region was observed to amplify at any site of insertion. Furthermore, none of the deletions analyzed altered the timing or tissue specificity of transposon replication from that characteristic of normal chorion gene amplification. Increases in transposon copy number in male or nonovarian female tissues or in egg chambers prior to stage 9 were never detected. Therefore, if sequences outside the 510-bp critical area developmentally regulate amplification, they must either be redundant or play only a quantitative role in differential replication.

Our experimental strategy was designed to minimize the influence of chromosomal position effects on amplification. Comparison of the replication induced by large transposons differing only in the location of small internal deletions reduced the effects of flanking chromosomal sequences unique to each insertion site. However, the parent transposon R7.7 was still subject to position effects, since 1 of 10 lines containing it failed to amplify. The extent of R7.7 amplification was site dependent and was always less than the normal 60-fold replication. These remaining position effects limited our ability to detect *cis*-acting sequences modulating amplification levels and served to emphasize the unusual sensitivity of amplification of chorion transposons to their chromosomal surroundings. However, significant reductions in the level of amplification induced by transposon $A_{22}O_{31}$ suggest that the region 390 to 820 upstream from *s15* may be important for high levels of amplification. Some weak sequence homologies between this region and the essential region were noted previously (15).

During the process of amplification, replication forks initiate repeatedly within the 66D chorion gene cluster. At least one replication origin used during amplification must lie within the 11.9-kb gene cluster, because termination of some replication forks initiated from this region produced characteristic gradients of decreasing amplification in DNA flanking the *Eco*RI sites at both sides (Fig. 2). We considered two general models relating our results to replication origins used during amplification. The simplest interpretation is that a single replication origin is used during amplification and must remain functional in all amplifying transposons. Such an origin, along with any elements essential to its tissue-specific and temporally controlled activation in maturing egg chambers, would therefore have to be present in the 510-bp essential region. An alternative possibility is that replication origin sequences are not essential for transposon amplification. Sequences within flanking chromosomal DNA might have been recruited as replication origins under the control of the *ACE3* element at each insertion site at which amplification occurred. Only direct measurements of replication start sites used during amplification will allow these models to be distinguished. Furthermore, even if a specific origin required for amplification resides upstream from *s18*, as we suspect, its ability to function during normal cell growth and in other tissues is unknown.

***s15* and *s18* transcription is not required for amplification.** The observation of nonpolysomal *s15* transcripts in stage 8 to 9 egg chambers near the time when amplification begins led Thireos et al. (27) to propose that early *s15* transcription might activate amplification. Our experiments demonstrated that neither *s15* nor *s18* transcription is necessary in *cis* for the amplification of chorion transposons. Deletion of the *s15* or *s18* 5′ start sites (Fig. 5) eliminated production of detectable transcripts within the remainder of the affected transcription unit but did not prevent transposon amplification. Transposon $A_{25}O_4$ lacked both genes but was amplification competent. Therefore, neither *s15* nor *s18* transcripts remain good candidates for a trancriptional activator of amplification.

These results are subject to several limitations. The *s19* gene (32) is located within the *Sal*I-*Eco*RI buffer sequence that is present in all the R7.7 derivative transposons tested, including $A_{25}O_4$. Therefore, all amplifying transposons studied in this and previous experiments contained at least one chorion gene. If transcription of *s15*, *s18*, or *s19* can each independently activate amplification, this requirement would not have been detected. However, because of the poor temporal correspondence between the transcription (stages 13 and 14) and amplification (stages 9 to 13) of these genes, such a requirement seems unlikely. A more significant possibility is that an activator or primer transcript within the gene cluster exists but has so far escaped detection. If so, our results imply that *cis*-regulatory sequences essential to transcript production are confined to the previously identified region upstream from *s18*.

Coincidence of sequences essential for *s18* transcription and the *ACE* within the same fragment. Our results localized sequences required for *s18* transcription within the same 510-bp region essential for amplification. Transcripts initiating at the *s18* promoter were reduced to an undetectable level (at least 10-fold) in transposon $A_{17}O_{15}$, lacking sequences between −190 and −630. *s18-lacZ* transcripts were observed in RNA from stage 13 to 14 egg chambers when sequences upstream from −630 were removed (transposons $A_{26}O_9$ and $A_{27}O_1$). Therefore, sequences within the 3.8-kb *Sal*I fragment essential for the normal developmental expression of *s18* lie no further than 630 nucleotides 5′ to the initiation site of *s18* mRNA. Location of elements regulating *s18* transcription 190 to 630 bp upstream from the start site is not unusual. Sequences essential for the regulation of a wide variety of *D. melanogaster* genes have now been mapped within corresponding 5′ upstream regions by using germ line transformation (see Cold Spring Harbor Symp. Quant. Biol., volume 50). The present resolution of these studies is, however, insufficient to determine whether the element regulating *s18* transcription and the element required for amplification within the 510-bp *ACE3* region are separable.

Model for developmental regulation of amplification. The linkage of a putative replication origin and an *s18* transcriptional control element within the 510-bp essential region suggested that a single regulator may influence both amplification and *s18* transcription. The requirement of a transcriptional control sequence in *cis* for replication origin function has been demonstrated previously in polyomavirus and is suggested for bovine papillomavirus (17) and Epstein-Barr virus (20). Polyomavirus contains a 244-bp enhancer region that is required for early viral transcription (6) and for viral DNA replication (28). The enhancer is required in *cis* for replication even when large T antigen is provided in

trans, and other viral and cellular enhancers can substitute for the polyomavirus enhancer in stimulating replication (7). Mutations in the enhancer region or substitution by other enhancers may alter the viral host range, possibly because the enhancer element regulates the cell type specificity of origin function (reviewed in reference 1).

The similarity of these observations of eucaryotic viral replication to the results described here suggests a model for the regulation of chorion gene amplification (Fig. 7). We propose that an enhancerlike *s18* transcriptional control element residing in the −120 to −630 region is also required to activate rounds of replication from a nearby origin during amplification. Activation of amplification results from a change in the disposition of *trans*-acting factors that are produced in follicle cells and that interact with the enhancer. Alternatively, the transcriptional control element may regulate amplification by modulating the production of a primer RNA or by influencing the position of the origin within the nucleus.

This model makes several predictions which can be tested. If the follicle cell specificity of amplification is a property of the *s18* transcriptional regulator, then substitution of strong regulators from other genes might lead to alterations in the tissue specificity of amplification. It might be possible to resolve the −120-to−630 region into two separate functional elements, an enhancerlike element required for both *s18* transcription and amplification and an origin sequence required only for replication (Fig. 7). If other *D. melanogaster* replication origins are subject to similar transcriptional regulatory sequences, rescue of an origin-specific deletion within *ACE3* might provide an assay for origin function. Finally, a similar association between amplification control sequences and transcriptional regulators is predicted to exist for the X chromosome chorion gene cluster, an association already supported by recent results (A. Spradling, D. de Cicco, B. Wakimoto, and L. Kalfayan, unpublished results).

If a common element mediates the transcription of *s18* and cluster amplification, position effects on amplification that disturb the function mediated by this sequence should have parallel effects on *s18* expression. Correlations between the amplification levels and *s18* expression levels per gene in lines containing insertions at different sites are currently being studied. *s15-lacZ* expression per gene in 11 lines containing the S6.9 transposon did not correlate strongly with transposon amplification (30).

Position effects on amplification. No simple model appears sufficient to explain the position effects on amplification that are caused by changes in transposon structure. Our experiments demonstrated that amplification-competent transposons such as R7.7 containing 3 to 4 kb of normal flanking sequences surrounding the amplification control element were significantly less sensitive to inhibition than were transposons such as S3.8 or $A_{28}O_{10}$ bearing only about 2 kb of flanking sequence on the 3′ side of the element. When further reductions in the amount of flanking DNA were made, amplification frequencies were further reduced. For example, transposon SB2.1 (5), retaining only about 1 kb between the transposon end and the control region, failed to amplify at six sites tested, despite the presence of all sequences now known to be essential. This strong position sensitivity has frustrated attempts to directly demonstrate the sufficiency of the essential region defined here for amplification. However, attempts are in progress to examine the amplification of tandem arrays of the 510-bp element.

The striking improvement in both the frequency and the level of R7.7 compared with S3.8 amplification suggests that position effects frequently act over a range of only a few kilobases. Consistent with this, no correlation between cytological location and amplification levels was observed in these or previous experiments. Further modest increases in the amount of flanking DNA are unlikely to completely eliminate position effects. Kafatos et al. (12) reported that amplification levels of a transposon similar to S3.8 but containing an additional 6 kb of normal flanking DNA 3′ to *s15* were still position sensitive. An amplification control element (*ACE1*) has also been mapped within the X chromosome gene cluster (30; Spradling, et al., unpublished results) near the *s38* gene. The chromosome rearrangement *In(1)ocelliless* juxtaposes abnormal sequences 6 kb from the 5′ end of *ACE1*, while the 3′ flanking DNA remains unchanged. Nonetheless, amplification is reduced two- to fourfold (24), suggestive of a long-range position effect, although the possibility of a mutation within the *ACE* on the oc chromosome has not been eliminated.

The frequency of position effects implies that many genomic regions are inhibitory to the *ACE*-mediated induction of local replication. At present, very little is known about the molecular nature of this inhibition. The studies reported here, however, identify one example of an inhibitory sequence. When inserted close to *ACE3*, the 3.1-kb *E. coli lacZ* gene caused a significant ($P < 0.01$) reduction in amplification as determined by comparing R7.7 and $A_{10}O_{31}$ insertions. This inhibition is probably specific for *lacZ* sequences, rather than being an effect of context changes within R7.7, because insertion of a *D. melanogaster Adh* fragment of similar size at the same site in the *s18* gene did not inhibit amplification (R. Kelley and A. Spradling, unpublished results). Inhibitory effects of procaryotic sequences on plasmid replication in mammalian cells have been described previously (16), and these effects are sometimes sensitive to distance (10).

The apparent complexity of position effects on amplification may indicate that the evolution of high amplification levels required extensive selection to modify sequences within and flanking the chorion gene clusters. If other genomic regulators of DNA replication are similar to amplification control elements, they may also be subject to interactions that could play a role in shaping the organization of eucaryotic chromosomes.

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