The role of ACE3 in *Drosophila* chorion gene amplification

Terry L.Orr-Weaver, Cynthia G.Johnston and Allan C.Spradling¹

The Whitehead Institute for Biomedical Research and Department of Biology, MIT, Nine Cambridge Center, Cambridge, MA 02142 and ¹Howard Hughes Medical Institute Research Laboratories, The Carnegie Institution of Washington, Department of Embryology, 115 W. University Parkway, Baltimore, MD 21210, USA

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ACE3, an amplification control element for the third chromosome chorion cluster of *Drosophila melanogaster*, was identified previously as a *cis*-regulatory element for amplification of transposons containing the three chorion genes *s18*, *s15* and *s19*. The deletion defining ACE3, located from –620 to –190 bp upstream of *s18*, disrupted both amplification and *s18* transcription, suggesting that ACE3 might contain a transcription enhancer that regulated replication, as had been observed in a number of eukaryotic viruses. We show here that transcription control can be separated from replication control in delineating ACE3 to a 320 bp region. Addition of heterologous enhancers fails to activate amplification in tissues other than the follicle cells. Therefore ACE3 does not appear to be analogous to a transcription enhancer. However, further deletions within the ACE3 region revealed that it contains multiple functional domains. In addition, ACE3 functions independently of orientation with respect to other chorion sequences, and can be moved 1.5 kb away from other chorion sequences without eliminating amplification.

Key words: amplification/chorion gene/DNA replication/*Drosophila*

Introduction

During *Drosophila* oogenesis, two eggshell gene clusters replicate selectively (reviewed in Kafatos *et al.*, 1985; Spradling and Orr-Weaver, 1987). Amplification occurs only in the ovarian follicle cells, which subsequently produce large amounts of chorion proteins from the reduplicated gene templates and secrete them around the oocyte. Replication forks repeatedly initiate in the amplifying regions, elongate and remain associated with the follicle cell chromosomes in the form of an 'onion-skin' structure. Specific genomic regions from both the third chromosome and X chromosome gene clusters induce developmentally regulated amplification following germ-line transformation, allowing *cis*-regulatory sequences to be mapped (de Cicco and Spradling, 1984; Orr-Weaver and Spradling, 1986; Delidakis and Kafatos, 1987, 1988). Amplification-defective mutations disrupt eggshell morphology and female fertility; this permits genes producing products needed for differential gene replication to be identified (Orr *et al.*, 1984; Kelley and Spradling,

1986; Snyder *et al.*, 1986). Thus, chorion gene amplification provides a rare opportunity to study developmentally regulated chromosome replication.

The amplification of P element transposons containing sequences derived from the third chromosome region is influenced by several factors. In this cluster, four small tandemly arranged genes encode the *s18*, *s15*, *s19* and *s16* chorion proteins, respectively. First, all chorion transposons are subject to chromosomal position effects that cause the level of amplification (but not its developmental specificity) to vary between strains where the transposon has inserted at different chromosomal sites. In addition, several *cis*-regulatory elements have been mapped within the cluster. Orr-Weaver and Spradling localized an element termed ACE3 (Amplification Control Element from the third chromosome cluster) between 120 and 630 bp upstream from the *s18* gene; deletion of ACE3 abolished the ability of a specific transposon containing the *s18*, *s15* and *s19* genes to amplify (Orr-Weaver and Spradling, 1986). Several additional elements with quantitative effects on amplification called AERs (Amplification-Enhancing Regions) have also been mapped at sites throughout the gene cluster using *in vitro* mutagenesis and transformation (Orr-Weaver and Spradling, 1986; Delidakis and Kafatos, 1987, 1988) or by analyzing P transposase-catalyzed derivatives of a transposon integrated at a single site (Delidakis and Kafatos, 1989). The nature of these elements, the rules governing their interaction and their relationship to position effects remain unclear.

Two general types of regulatory sequences have been recognized in a variety of prokaryotic and eukaryotic viral replicons. Minimal origin sequences include sites at which strand separation is effected and where nucleotide incorporation actually begins (see reviews by McMacken *et al.*, 1987; Kelly *et al.*, 1988). However some replicons also are influenced by a second class of regulatory sequences, located from a few base pairs to several kilobase pairs away from the origin. Properties of these replication elements are reminiscent of transcription enhancer sequences, and the requirement for these elements can sometimes be satisfied by heterologous transcription enhancers (DePamphilis, 1988).

Two observations indicated that ACE3 might represent a member of the enhancer-like class of replication regulatory elements. Although it lies several hundred base pairs upstream from the *s18* transcription start site, the original deletion defining ACE3 disrupted *s18* transcription, leading Orr-Weaver and Spradling (1986) to propose that ACE3 might contain an *s18* transcription enhancer also required for replication initiation. More recently, direct mapping of replicative intermediates in third chromosome amplification by two-dimensional gel electrophoresis revealed that most initiations occur downstream of ACE3 (Delidakis and Kafatos, 1989; Spradling and Heck, 1989). Thus ACE3 is physically separate from at least one replication origin used during amplification. Here we report that the major activity

of ACE3 is separable from sequences regulating *s18* transcription, but that ACE3 does display some properties of the enhancer-like class of regulatory elements.

Results

The transcription and amplification regulatory elements are separable

To investigate the relationship of ACE3 to *s18* transcription, we utilized transposons containing the 7.7 kb *EcoRI* fragment from the third chromosome chorion gene cluster (Figure 1). Previously, we observed that *Escherichia coli* *lacZ* DNA sequences inserted into the *s18* gene to provide a marker for transcription partially inhibited amplification. We therefore substituted a smaller, *Drosophila* DNA segment in place of *lacZ*, selecting a 500 bp *SacI* fragment from just outside the 3' end of the *rosy* gene (Lee *et al.*, 1987). *A₄₈O₂₈*, a transposon with the *rosy* locus tag in *s18* but with no deletions in the chorion locus, was transformed into flies to test whether the insertion inhibited amplification. Transformed lines with heterozygous single copy insertions of the transposon were assayed for amplification by preparing DNA from dissected stage 1–8 and 13 egg chambers and performing quantitative Southern blots. The level of amplification of the transposon was quantitated relative to internal control DNA fragments as previously described (Orr-Weaver and Spradling, 1986). The nine insertion sites of *A₄₈O₂₈* show a range of amplification levels from 1.1- to 12-fold with a mean of 5.1 and a median of 4.8 (Table I). The mean amplification level for the 10 lines tested of the same transposon lacking the *rosy* locus tag was 15.7, and the mean level with the *lacZ* insert was 2.8-fold (Orr-Weaver and Spradling, 1986). Therefore there is an inhibitory effect of the *rosy* tag, but it is significantly less

Fig. 1. Structure of the third chromosome chorion locus and the transposon vector. The location of the major chorion transcripts of the third chromosome chorion cluster and ACE3 are shown (top line). Our standard transposon (lower line) contains the indicated 7.7 kb *EcoRI* fragment and a 7.2 kb *HindIII* fragment containing the *rosy* gene as a selectable marker. The chorion fragment in the transposon encodes three chorion genes, *s18*, *s15* and *s19*; inserted into the *s18* gene on this transposon is a tag of 500 bp of DNA from the region adjacent to the 3' end of the *rosy* gene. This tag is outside the *HindIII* *rosy* fragment present on the transposon. The direction of transcription is indicated by arrows; the positions of introns are shown in the top line only. Top line, hatched box, ACE3; solid line, chorion DNA; *R₁*, *EcoRI*. Bottom line, hatched box, ACE3; open box, chorion DNA; dotted box, *rosy* DNA; solid box, P-element DNA.

than the *lacZ* effect. (The binomial distribution test (see Materials and methods) shows that the *A₄₈O₂₈* amplification levels are significantly higher than those of the *lacZ* transposon, *P* = 0.008).

If the tagged *s18* gene in *A₄₈O₂₈* was regulated normally, it should be expressed in stage 13 egg chambers, but not in earlier stages. Northern blots of RNA isolated from dissected egg chambers from five transformed lines were hybridized to an SP6 transcript homologous to the *rosy* locus tag in the *s18* gene. A transcript the size of the modified *s18* gene was detected in stage 13 but not in stage 10 egg chambers (Figure 2 and Table I). Stage 1–9 egg chambers also were shown not to contain the transcript (data not shown). Since the *rosy* tag allowed ready detection of the *s18* transcript and the amplification levels were adequate for our analysis, *A₄₈O₂₈* served as a standard transposon for further studies of the ACE3 region.

Table I. Deletion of ACE3

Transformant line	Amplification	<i>s18</i> RNA/gene ^a
<i>A₄₈O₂₈</i>		
4	1.1	1.5
6	1.1	ND ^b
1	1.9	1.2
9	2.4	1.0
3	4.8	ND
5	5.0	1.2
8	8.6	3.9
7	9.2	ND
2	12	ND
	$\bar{X}^c = 5.1$	$\bar{X} = 1.8$
<i>A₅₄O₁₈</i>		
9	0.71	ND
1	0.80	ND
12	0.81	13
2	0.91	2.2
5	1.0	ND
8	1.0	ND
4	1.1	4.0
6	1.2	1.7
7	1.2	2.1
10	1.2	ND
13	1.2	ND
3	1.3	1.4
11	1.3	ND
	$\bar{X} = 0.96$	$\bar{X} = 4.1$
<i>A₃₄O₁₈</i>		
1	0.59	ND
10	0.69	ND
8	0.71	ND
2	0.88	ND
6	1.5	ND
3	1.6	ND
7	1.9	ND
9	2.2	ND
5	4.9	ND
4	5.9	ND
	$\bar{X} = 2.1$	

^a*s18* transcript levels were measured relative to *hsp83* levels. Transcription levels are corrected for amplification of the transposon and are given on a per gene basis. The levels listed are relative to the *A₄₈O₂₈*-9 line which was run as a standard on every Northern blot.

^bND, not determined.

^cThe mean value for amplification and *s18* RNA/gene are given.

To test whether the transcription and amplification control elements could be separated, two derivatives of $A_{48}O_{28}$ were constructed (Figure 2). One transposon, $A_{54}O_{18}$, lacked the 5' portion (–630 to –310) of the original ACE3 region. Transposon $A_{34}O_{18}$ was deleted from sequences from –310 to +65. For each transposon, transformed lines were established carrying heterozygous single copy inserts that were then assayed for amplification. Thirteen $A_{54}O_{18}$ lines amplified between 0.71 and 1.3, with a mean of 0.96 and a median of 1.1 (Table I). These levels are not significantly different from 1.0. Thus the –630 to –310 region contains some or all of the previously mapped element(s) essential for transposon amplification.

Despite the failure of $A_{54}O_{18}$ to amplify, Northern blots revealed that the *s18* gene was transcribed in the correct tissue with normal developmental specificity. The level of *s18* transcripts in stage 13 egg chambers was quantitated relative to *s18* expression in lines containing the undeleted $A_{48}O_{28}$ transposon. The relative amount of *s18* transcript per gene in the $A_{48}O_{28}$ lines varied from 1.0 to 3.9, with an average of 1.8 (*s18* mRNA in line $A_{48}O_{28}$ -9 was arbitrarily set equal 1.0). *s18* mRNA levels from the $A_{54}O_{18}$ lines ranged from 1.4 to 13, with an average of 4.1 (Figure 2, Table I). The higher mean value for $A_{54}O_{18}$ transcription resulted from the single line with a value of 13, presumably the result of a position effect. We concluded that DNA sequences from –630 to –310, although essential for amplification, are not required for *s18* transcription in the context of transposon $A_{48}O_{28}$.

Ten lines transformed with $A_{34}O_{18}$ (deleted between

Fig. 2. Separation of replication and amplification control elements in ACE3. The transposon $A_{48}O_{28}$ has the structure shown in Figure 1, and ACE3 is intact. $A_{54}O_{18}$ is the same transposon except that the sequences from –630 to –310 5' to the *s18* gene have been deleted. In $A_{34}O_{18}$ the region between –310 and +65 was deleted. A Northern blot of RNA isolated from stage 10 and stage 13 egg chambers from three lines of $A_{48}O_{28}$ (1,5 and 9) and six lines of $A_{54}O_{18}$ (2,3,4,6,7 and 12) was probed with an SP6 transcript homologous to the tag in the *s18* gene on the transposon. The blot was subsequently probed with a nick-translated probe for the *hsp83* gene (lower blot). The positions of the two transcripts are indicated by arrows. The *s18* transcript appears only in RNA from stage 13 egg chambers, and the level of transcript is unaffected by the –630 to –310 deletion.

–310 and +65) ranged between 0.59- and 5.9-fold amplification, with a mean value of 2.1 and a median value of 1.55. Both the binomial distribution test and Wilcoxon's rank sum test revealed that $A_{34}O_{18}$ amplification differs from that of $A_{48}O_{28}$ at the 5% significance level. Therefore the deletion from –310 to +65 exerts a quantitative effect on transposon amplification.

These experiments revealed that at least one element essential for transposon amplification is located upstream from *s18* between –620 and –310. Deletion of other regions, including –310 to +65, like the regions downstream from *s18* described previously (Orr-Weaver and Spradling, 1986; Delidakis and Kafatos, 1987, 1989), reduced but did not eliminate amplification. The –630 to –310 deletion did not influence *s18* transcription, in contrast to the larger original deletion extending to position –190. Therefore the ACE3 element does not simply correspond to a tissue-specific *s18* transcription enhancer. Attempts to detect directly enhancer-like activity within the –630 to –190 region were unsuccessful. Transformants in which the region was located upstream from a P-element promoter fused to the *lacZ* gene did not specifically express *lacZ* activity in amplification-stage egg chambers (T.Orr-Weaver and C.Johnston, unpublished data).

Fig. 3. *Hsp70* and *Sgs-4* enhancer constructs. The position and orientation of enhancer insertions relative to the *s18* gene are shown. These insertions were constructed in the transposon vector in Figure 1, but only the relevant region is shown on this figure. $A_{57}O_{11}$ has a dimer of the *hsp70* enhancer (a 250 bp fragment) inserted at –310 from the 5' end of the *s18* gene. $A_{74}O_5$ contains the same fragment at –630 but in the opposite orientation. A dimer of the *Sgs-4* enhancer (1.5 kb) is present at –310 in the transposon $A_{55}O_{16}$. A single copy of this enhancer (750 bp) is inserted at –630 in one orientation in $A_{60}O_1$ and in the opposite orientation in $A_{60}O_5$. The arrows within the enhancer boxes indicate relative orientation, with the direction of the arrow pointing towards the sequences of the enhancer nearest the 5' end of the *hsp70* or *Sgs-4* gene. The location and direction of transcription of the *s18* gene is shown by the arrow with the indicated intron. Hatched box, *hsp70* enhancer; stippled box, *Sgs-4* enhancer; open box, chorion DNA.

Heterologous transcription enhancers do not alter the tissue specificity of chorion transposon amplification

Polyoma virus mutations altering the tissue specificity of replication reside in the enhancer, and substitution of heterologous enhancers may change the tissue specificity of polyoma replication (reviewed by DePamphilis, 1988). We undertook several experiments to determine if the tissue specificity of chorion gene amplification could be altered by the well characterized *Drosophila Sgs-4* (Shermoen *et al.*, 1987) and *hsp70* (Bienz and Pelham, 1986) enhancers. These enhancers were selected because they are active in tissues that were considered the best candidates to contain amplification *trans*-regulatory factors. Like ovarian follicle cells, the third instar larval salivary gland cells in which *Sgs-4* is active sequentially express a series of gene products from endoreduplicated templates. In dipterans of the genus *Sciara* and *Rhynchosciara*, although not *Drosophila*, the genes for some of the salivary gland-specific secretory proteins are specifically amplified (Millar *et al.*, 1985). The *hsp70* enhancer allowed us to test for amplification in the same tissue in which it normally occurs but at an earlier development stage, by activating the enhancer in stage 1–8 egg chambers (Parker-Thornberg and Bonner, 1987).

The *hsp70* enhancer resided on a 250 bp fragment containing two copies in direct orientation of the region from –37 to –108 5' to the *hsp70* gene. This dimer has been shown to be efficient in activating genes in response to heat shock (Bienz and Pelham, 1986). This DNA fragment was

inserted on either side of the ACE3 region, at –310 ($A_{57}O_{11}$) or at –630 ($A_{74}O_5$). Except for the inserted enhancer, these transposons were identical in structure to $A_{48}O_{28}$ (Figure 3).

Four transformed lines of $A_{57}O_{11}$ were studied to determine if amplification and *s18* transcription were affected by heat shock. During a 36 h period, flies were either incubated continuously at 29°C or were periodically heat shocked in a 37°C water bath for 1 h every 12 h. Growth at 29°C did not alter the normal pattern of *s18* expression (data not shown); however, the *hsp70* enhancer strongly induced *s18* transcription in all three tissues tested following heat shocks at 37°C (Figure 4A). A transcript homologous to the *ry* tag and the same size as the stage 13 egg chamber control is present in males, malpighian tubules and in lower amounts in stage 1–8 egg chambers (Figure 4A, lines 1 and 5; lines 2 and 3 data not shown). DNA was isolated from whole males, adult malpighian tubules, larval salivary glands, stage 1–8 egg chambers and stage 13 egg chambers of $A_{57}O_{11}$ flies with heat shock enhanced *s18* transcription. Quantitative Southern blots revealed that under these conditions the transposons did not amplify in males, malpighian tubules, salivary glands or stage 1–8 egg chambers (Figure 4A, Table II). Two lines transformed with $A_{74}O_5$ (Figure 3) were also tested for amplification in the tissues listed above following heat shock at 37°C. Amplification was not induced in these tissues in either of these lines (Table II). Thus even though the *hsp70* insert enhanced *s18*

Fig. 4. Analysis of the transcription and amplification of *hsp70* and *Sgs-4* enhancer constructs. (A) On the left is a Northern blot of two transformant lines of $A_{57}O_{11}$, 1 and 5. RNA was isolated from whole males that had been heat shocked at 37°C and from malpighian tubules (MT), stage 1–8 egg chambers (1–8), and stage 13 egg chambers dissected from heat-shocked females. The Northern blot was probed with an SP6 transcript homologous to the tag in the *s18* gene on the transposon. In the presence of the *hsp70* enhancer, *s18* expression is induced by heat shock in the males, malpighian tubules and stage 1–8 egg chambers (arrow). (Note: in this figure the Northern blot shown is a composite in which lanes from the same exposure of the same autoradiogram are brought together.) Quantitative Southern blots were performed (right side) to assay for amplification in these tissues following heat shock. DNA was purified from heat-shocked males, from salivary glands dissected from heat-shocked third instar larvae (SG) and from malpighian tubules (MT), stage 1–8, and stage 13 egg chambers dissected from heat-shocked females. The Southern blot was probed with the *rosy* gene. A deletion of the endogenous *rosy* locus allows the endogenous locus to be distinguished from the transposon; these host fragments (H1 and H2) provide an internal standard for the amount of DNA present in each lane. By comparing the ratio of the transposon bands (T1 and T2) to the host bands from the heat-shocked tissues to the same ratio from unheat shocked transformant males (STD), the level of amplification following heat shock can be calculated. Heat shock did not induce amplification in any of the four tissues tested. (B) DNA was isolated from salivary glands from third instar larvae (SG,L) and from 8 h prepupae (SG,P) from lines $A_{60}O_1$ -3 and $A_{60}O_1$ -4 to determine if the *Sgs-4* enhancer activates amplification of the transposon in these tissues. The Southern was probed with the *rosy* gene, and amplification levels in the larval and pupal salivary glands were calculated as described above relative to the male standard. DNA samples from stage 1–8 and 13 egg chambers are also shown.

transcription, it did not induce amplification in novel tissues.

The ability of the *Sgs-4* enhancer to induce transcription and amplification was also studied. DNA sequences located between -840 and -105 upstream from the *Sgs-4* gene activate *ADH* transcription in third instar larval salivary glands (Shermoen *et al.*, 1987). This 735 bp enhancer fragment was inserted adjacent to ACE3 at either position -310 or -630 in our standard transposon vector (Figure 3). Three constructs were injected into embryos to generate transformed lines. A₅₅O₁₆ contains two copies of the *Sgs-4* enhancer in direct orientation at -310. Transposons A₆₀O₁ and A₆₀O₅ differ in the orientation of the single enhancer inserted at position -630.

The *Sgs-4* enhancer did not induce transcription of the *s18* gene resident on these transposons in the salivary gland. The 1.1 kb mRNA from the *s18* gene with the *ry* tag was not detectable on Northern blots of third instar salivary gland RNA from four lines of A₆₀O₁, two lines of A₆₀O₅, or one line of A₅₅O₁₆ (data not shown). However, we observed a

Table II. Transposon lines of enhancer constructs

Transformant line	Amplification level egg chambers ^a	Amplification level heat-shocked malpighian tubules ^b
A₅₇O₁₁		
4	1.5	0.57
5	2.8	0.92
1	3.1	0.58
3	7.1	ND
2	7.3	ND
	$\bar{X} = 4.4$	$\bar{X} = 0.69$
A₇₄O₅		
1	2.6	0.81
2	2.9	1.1
	$\bar{X} = 2.8$	$\bar{X} = 0.96$
Transformant line	Amplification level egg chambers ^a	Amplification level salivary gland ^c
A₆₀O₁		
3	0.91	0.91
1	1.8	ND
4	3.9	1.6
2	9.6	0.58
	$\bar{X} = 4.0$	$\bar{X} = 1.0$
A₆₀O₅		
3	1.3	ND
6	1.9	ND
1	3.2	0.68
4	3.4	0.93
5	7.2	ND
2	11	1.2
	$\bar{X} = 4.7$	$\bar{X} = 0.95$
A₅₅O₁₆		
2	0.68	ND
1	0.97	ND
4	1.2	1.9
3	5.4	ND
	$\bar{X} = 2.1$	

^aAmplification level in stage 13 egg chambers from adult ovary.

^bAmplification level of the transposon in malpighian tubules from heat-shocked adult flies.

^cAmplification level in salivary gland from third instar larvae.

transcript of 1.4 kb on these Northern blots. Since this transcript is present in salivary gland RNA from the *ry*⁵⁰⁶ stock, it must arise from transcription in the salivary gland of the endogenous region homologous to the *ry* tag. This transcript was not observed in any other tissues tested. To determine if transposon replication was affected by the enhancer, DNA was isolated from third instar salivary glands and quantified on Southern blots. In five of the seven lines tested the transposon amplified < 1.5-fold in the salivary gland (Figure 4B and Table II). Line A₆₀O₁-4 contained 1.6 times and line A₅₅O₁₆-4 contained 1.9 times the expected amount of transposon DNA in the salivary gland. While it is possible that the transposon in these two lines overreplicated due to the influence of the *Sgs-4* enhancer, we believe their increased copy number was more likely a consequence of small site-specific differences in salivary gland chromosome polyteny. One out of four A₄₈O₂₈ control lines showed similar increases in salivary gland copy number. To determine if amplification increased at later developmental times, we isolated DNA from the salivary glands of 8 h prepupae, and from 12 h prepupae just prior to histolysis. The transposon copy number did not increase during pupation in any of the lines tested, including A₆₀O₁-4 and A₅₅O₁₆-4 (Figure 4B, and data not shown).

ACE3 functions independently of orientation

To investigate whether ACE3 required a specific orientation relative to the rest of the chorion gene cluster, the region from -630 to -310 was inverted in our standard transposon, yielding transposon A₇₁O₁₀ (Figure 5). Single copy transformed lines were analyzed for *s18* gene transcription and for amplification. The seven lines tested ranged in amplification level between 1.1- and 13-fold with a mean of 4.8 and a median of 3.1 (Table III). Thus inversion of ACE3 had no effect on amplification. There was also no discernible effect on *s18* transcription; the seven lines analyzed had an average of 1.7 units of *s18* RNA per gene, compared to 1.8 for the control (Table III). This is consistent with the results from the transposon in which ACE3 was deleted and no effect of *s18* transcription was observed. Therefore if ACE3 is interacting with other amplification elements, such as a replication origin, it does so in an orientation-independent manner.

Function of subregions within ACE3

ACE3 includes several regions that are strongly conserved between the chorion gene clusters of *Drosophila melanogaster*, *Drosophila virilis* and *Drosophila grimshawi* (C.Swimmer, J.Martinez-Cruzado, M.Fenerjian and F.Kafatos, personal communication; Martinez-Cruzado *et al.*, 1988). One of the most highly conserved regions in the entire cluster lies between nucleotides -482 and -394,

Fig. 5. Inversion of ACE3. In transposon A₇₁O₁₀ the region between -630 and -310 is inverted. This inversion is present in the standard transposon vector shown in Figure 1.

where 78 out of 88 bp are identical in the three species. Between -367 and -329, 37 out of 47 nucleotides are homologous. We constructed four additional deletion-bearing transposons to investigate the function of these conserved elements and further to delineate ACE3. The deletion from -630 to -520 ($A_{59}O_{34}$) lacked sequences from the distal ACE3 boundary to near the first conserved region. Most of the highly conserved region was deleted in transposon $A_{63}O_5$ (-480 to -420). A less conserved region (-410 to -370) was absent in transposon $A_{62}O_{14}$. Finally, the proximal conserved block was removed in transposon $A_{61}O_7$, in which nucleotides between -368 and -310 were deleted

Table III. Inversion of ACE3

Transformant line	Amplification	s/8 RNA/gene ^a
$A_{71}O_{10}$		
1	1.1	2.3
5	1.5	0.53
7	1.8	0.34
3	3.1	1.5
6	3.6	0.44
4	8.6	2.2
2	13	4.8
	$\bar{X} = 4.8$	$\bar{X} = 1.7$

^aAnalyzed as described in Table I.

Fig. 6. Fine deletion analysis of ACE3. The amplification levels of each transposon line are graphically depicted. The y-axis is the level of amplification; each deletion transposon is indicated on the x-axis. Each transposon insertion is represented by a dot. The bottom line shows the endpoints of the deletions tested in our standard transposon vector. Transposon $A_{59}O_{34}$ has a deletion from -630 to -520, transposon $A_{63}O_5$ from -480 to -420, transposon $A_{62}O_{14}$ from -420 to -370, and transposon $A_{61}O_7$ from -368 to -310.

(Figure 6). Transformed lines with single-copy inserts were generated for each of the transposons containing deletions in ACE3. Heterozygous lines were assayed for amplification by quantitative Southern blots. The amplification levels of these lines are given in Table IV and plotted in Figure 6.

Table IV. Fine structure analysis of ACE3

Transformant line	Amplification	s/8 RNA/gene ^a
$A_{59}O_{34}$		
6	1.1	3.2
5	1.2	ND
10	1.3	ND
3	1.5	ND
7	1.7	ND
9	2.0	ND
4	4.1	7.2
2	4.9	ND
1	6.4	1.2
8	6.4	1.9
	$\bar{X} = 3.1$	$\bar{X} = 3.4$
$A_{63}O_5$		
9	0.99	1.6
5	1.5	0.92
6	2.5	1.2
3	2.6	ND
8	3.5	3.0
4	6.8	0.92
1	8.6	ND
2	13	ND
7	14	ND
	$\bar{X} = 5.9$	$\bar{X} = 1.5$
$A_{62}O_{14}$		
3	1.1	ND
6	1.1	ND
7	1.2	ND
4	1.3	ND
1	1.4	ND
8	1.5	ND
10	2.0	ND
9	4.3	ND
2	5.0	ND
5	12	ND
	$\bar{X} = 3.1$	
$A_{61}O_7$		
9	0.75	ND
17	0.99	ND
4	1.0	ND
7	1.0	ND
1	1.1	ND
11	1.1	ND
12	1.1	ND
14	1.2	ND
5	1.3	ND
15	1.3	ND
2	1.4	ND
8	1.4	ND
18	1.4	ND
10	1.6	ND
3	2.2	ND
16	2.4	ND
6	2.8	ND
13	6.0	ND
	$\bar{X} = 1.7$	

^aSee Table I for explanations.

These studies revealed that the strong effect of the -630 to -310 deletion resulted from the combined action of two or more separate sub-elements. The most proximal deletion, -368 to -310 ($A_{61}O_7$), significantly reduced transposon amplification. The 18 lines containing $A_{61}O_7$ amplified between 0.75- and 6.0-fold, with a mean of only 1.7 and a median of 1.3. Wilcoxon's rank sum test ($0.01 < P < 0.05$) and the binomial distribution test ($P = 0.007$) showed that $A_{61}O_7$ amplification differed significantly from the control transposon $A_{48}O_{28}$. Two other deletions, -410 to -370 ($A_{62}O_{14}$) and -630 to -520 ($A_{59}O_{34}$) appeared to cause some reduction in amplification; however, these differences were not statistically significant. Surprisingly, removal of the strongly conserved distal region ($A_{63}O_5$) had no discernible effect on amplification. The level of *s18* transcription per gene in stage 13 egg chambers was determined for transposons $A_{59}O_{34}$ and $A_{63}O_5$ as before, using the specific *ry* probe and correcting for transposon copy number. Neither of the deletions significantly reduced *s18* expression (Table IV).

Discussion

ACE3 is not simply a transcription enhancer

In eukaryotic viruses, replication origins are the sites at which DNA synthesis initiates, while auxiliary components often contain enhancers and are involved in regulating transcription as well as replication (reviewed in DePamphilis, 1988). The location of the ACE3 and AER regions near the four third chromosome chorion genes suggested that some of them might control both transcription and gene amplification. Transformation experiments have demonstrated that several chorion genes, including *s15* and *s38*, contain *cis*-regulators of transcription within 200–300 bp upstream from their transcription start sites (Kalfayan *et al.*, 1985; Romano *et al.*, 1988; Mariani *et al.*, 1988). Although *cis*-regulators of *s18* transcription have not previously been localized in detail, the observation that a deletion between -620 and -190 eliminated both amplification and *s18* transcription suggested that ACE3 might be an example of an element that regulated both amplification and *s18* transcription (Orr-Weaver and Spradling, 1986).

The experiments reported here detected no connection between the control of amplification and transcription. The amplification regulatory region in ACE3 could be separated from sequences controlling *s18* transcription. Furthermore, heterologous transcription enhancers did not influence amplification. In polyoma, substitution of heterologous enhancers can sometimes alter the cell types permissive for cell growth (de Villiers *et al.*, 1984). In our experiments, two *Drosophila* cellular enhancers were unable to induce amplification at earlier times or in tissues other than follicle cells. The *hsp70* enhancer activated *s18* transcription in heterologous tissues when activated by heat shock. However, no sequence-specific increase in transposon copy number occurred. We were unable to detect transcripts from the *s18* gene on the transposon in the salivary glands of transformants of the *Sgs-4* enhancer constructs. This enhancer has been shown to activate *ADH* in the salivary gland, and to be distance independent (Shermoen *et al.*, 1987; Jongens *et al.*, 1988). The *s18* promoter may be insensitive to the action of the *Sgs-4* enhancer or induced transcripts may be unstable.

There are several reasons why an effect of cellular

enhancers on amplification might not have been detected. Lack of a required *trans*-acting factor in a target tissue might have blocked amplification even though ACE3 function was induced by the heterologous enhancer. Additional rounds of replication might have generated lethal chromosome breaks following mitosis, selecting against insertions where the enhancers could function. However, the tissues studied—salivary glands, malpighian tubules and early egg chambers—contain polytene cells which do not undergo mitosis, and already contain differentially replicated sequences. Finally, the location of the enhancer relative to other *cis*-regulatory elements might be critically important for the induction of amplification. Although we can never exclude this possibility, we tested each enhancer at two different sites, in both orientations, and as monomers or dimers, all with negative results.

Properties of ACE3

Our experiments revealed several new characteristics of the ACE3 element. The sequences between -630 and -310 stimulate transposon amplification equally well in either orientation. Furthermore, the ACE3 element could be shifted several kilobase pairs relative to other chorion sequences, including the downstream replication initiation sites, without eliminating amplification. This conclusion follows from the ability of the transposons we studied with *Sgs-4* or *hsp70* insertions at either -630 or -310 to amplify. Finally, as in the case of many transcription and replication regulators, the amplification-promoting activity of the ACE3 region appears to result from at least two non-essential sub-elements. The sequence in this region does not contain large direct or inverted repeats.

Removing the promoter proximal 58 bp of ACE3 (-368 to -310) significantly lowered the ability of the transposon to amplify ($A_{61}O_7$). Sequences between -367 and -339 are $>80\%$ conserved between the three species *D.melanogaster*, *D.virilis* and *D.grimshawi* (C.Swimmer, J.Martinez-Cruzado, M.Fenerjian and F.Kafatos, personal communication), which are distant enough so that most intergenic regions cannot even be aligned. This region is therefore likely to play an important role in amplification. Another highly conserved subregion was dispensable in our assays. The sequences deleted in transposon $A_{63}O_5$ (-480 to -420) are 95% identical between the three species. None the less, transposon $A_{63}O_5$ amplified as well as the parent transposon. The sequence conservation argues that this region is functionally important in some aspect of chorion gene function not assayed in our transformation experiments. For example, the conserved region may mediate an interaction with the AER elements that is important for achieving the highest levels of amplification. Our test transposon lacked AER-b and AER-a and did not amplify to normal levels. Alternatively, the high A-T content of this region suggests that it may facilitate DNA unwinding (Umek and Kowalski, 1988). Since a second highly A-T rich region is located between -610 and -550 (Levine and Spradling, 1985), just upstream from the deletion, it may have been able to complement the function removed in $A_{63}O_5$. Our experiments suggest that a control element is present between -410 and -370 as well. Deletion of this region reduced amplification to levels that were significantly different from the control transposon by the binomial distribution test, although not by the more stringent Wilcoxon's rank sum test.

Mechanism of ACE3 function

We recently have shown that sequences between -620 and -190 are sufficient to induce low levels of amplification with proper developmental specificity (T.Orr-Weaver, C.Johnston and J.Carminati, unpublished data). This result confirms the conclusions of deletion experiments that the ACE3 element is an important regulator of third chromosome chorion gene amplification. It remains unclear how ACE3 interacts with the AER elements to reduplicate the endogenous gene cluster (or large transposons) to high levels. In our experiments ACE3 was distinguished from the AERs in that when deleted in our transposons, no amplification was observed. Recently, however, C.Swimmer, C.Delidakis and F. Kafatos (submitted) have demonstrated that some transposons derived from the third chromosome cluster can amplify to low levels in the absence of ACE3. Thus ACE3 may be the strongest of a series of elements from the third chromosome region capable of inducing amplification. There are two major models that can explain the role and properties of ACE3.

First, ACE3 may itself contain an amplification origin and may be capable of functioning autonomously. The orientation and distance independence of ACE3 would simply reflect its ability to function independently, at least to a low level. In this view, ACE3 might resemble a yeast chromosomal ARS element. Replication fork initiations in the vicinity of ACE3 have been mapped using two-dimensional gels (Spradling and Heck, 1989), but the number of such initiations relative to initiations in other regions was difficult to quantify. The majority of replicative intermediates appear to initiate at least 1 kb downstream (Delidakis and Kafatos, 1989; Spradling and Heck, 1989). Like ACE3, the function of yeast ARS elements is reduced but not abolished by certain deletions outside of the essential 'core' region (Srienc *et al.*, 1985; Diffley and Stillman, 1988; Palzkill and Newlon, 1988). However, several observations argue against this interpretation. Our experiments failed to find any core of essential sequences within ACE3. Transposons containing only ACE3 do not amplify consistently or to high levels (T.Orr-Weaver, C.Johnston and J.Carminati, unpublished data). Moreover, it is not possible to explain the amplification levels observed in previous transformation experiments simply by postulating that ACE3 and the four AERs are all autonomous elements that stimulate amplification in an additive manner.

The results of our experiments and of previous studies on chorion gene amplification suggest a second model in which long-range interactions between ACE3 and the other *cis*-regulatory elements influence the level of amplification that is achieved. The presence of ACE3 may trigger increased initiation at one or more downstream sites. Experiments reported here suggest that some of these interactions are orientation and distance independent, like the stimulation of replication by the enhancer-like class of replication regulators. However, not all interactions between ACE3 and other *cis*-regulators may be distance independent. Inserting either the 3.1 kb *lacZ* sequence (Orr-Weaver and Spradling, 1986) or the 500 bp *rosy* tag ($A_{48}O_{28}$) at the *SacI* site in *sI8* strongly reduced the average amplification level of the resulting transposon compared to controls without the insertion. These insertions increase the distance between ACE3 and the AER elements. This model also provides a simple explanation for the strong position effects observed

on amplifying transposons inserted at different chromosomal sites. Sequences adjacent to the transposon insertion may be acted on by ACE3 to increase levels of amplification. A major goal of future research will be to elucidate the nature of the long-range interactions that appear to play an important role in controlling chorion gene amplification.

Materials and methods

Materials

All enzymes were purchased from Boehringer Mannheim, except T4 DNA polymerase which was from P and L Biochemicals. ^{32}P -labeled nucleotides were from Amersham, and Nytran was from Schleicher and Schuell.

Plasmid construction

The insertion of a homology tag into the *sI8* gene for transcription analysis was done first in a plasmid with a 3.8 kb *SaI* chorion fragment containing the *sI8* and *sI5* genes. A 500 bp *SaI* fragment that is ~300 bp from the 3' end of the *rosy* gene (Lee *et al.*, 1987) was ligated into the unique *SaI* site at position +571 in the *sI8* gene ($A_{31}O_4$). The chorion *SaI* fragment with the tag in *sI8* was ligated into the transposon plasmid pR7.7 (Orr-Weaver and Spradling, 1986), replacing the normal *SaI* fragment. $pA_{48}O_{28}$ contains this *SaI* fragment in the same orientation as in the third chromosome chorion cluster.

The deletion transposon $A_{54}O_{18}$ was constructed from $pA_{31}O_4$, in which the only *Bam*HI site is at -310 relative to the 5' end of the *sI8* gene, and there is a unique *Kpn*I site at -630. The plasmid was digested with *Kpn*I and *Bam*HI, blunt-ended and *Kpn*I linkers were added to generate a single *Kpn*I site at the deletion. The *SaI* fragment containing this deletion was then replaced for the undeleted *SaI* fragment in pR7.7.

A deletion from -310 to +65 was generated by cleaving $pA_{31}O_4$ to completion with *Bam*HI, and then doing a partial digest with *Hind*III. The fragment cut at the correct *Hind*III site was isolated from a gel, end filled with T4 DNA polymerase and blunt-end ligated. This deleted *SaI* fragment was then replaced into pR7.7 to generate $pA_{34}O_{18}$.

The heat shock enhancer was from plasmid H430B, which contains a dimer of the *hsp70* enhancer (-107 to -37) in the polylinker of pUC9 (Bienz and Pelham, 1986). *Kpn*I or *Bam*HI linkers were added to an ~250 bp *Bam*HI-*Eco*RI fragment carrying the enhancer. The *Hsp70* fragment with *Kpn*I linkers was first cloned into the *Kpn*I site of the polylinker of car3 (Rubin and Spradling, 1983), and then into the unique *Kpn*I site of $pA_{48}O_{28}$ (at -630) to produce $pA_{74}O_5$. The *hsp70* fragment with *Bam*HI linkers was ligated into the *Bam*HI site of $pA_{31}O_4$; the *SaI* fragment was transferred into pR7.7, resulting in $pA_{57}O_{11}$. The *hsp70* fragment with *Bam*HI linkers was sequenced (Maxam and Gilbert, 1980) and shown to be the dimer of the *hsp70* enhancer. The orientation of the enhancer dimer in each plasmid is shown in Figure 3.

The *Sgs-4* enhancer was isolated on a *SaI*-*DraI* fragment from -840 to -106 5' to the *Sgs-4* gene obtained from the plasmid pCSO.85, provided by S.Beckendorf. *Kpn*I linkers were added to this fragment and it was cloned first into car3. This *Kpn*I-*Sgs-4* fragment was then cloned into the *Kpn*I site of $pA_{48}O_{28}$. A dimer of the *Sgs-4* enhancer was inserted in $pA_{55}O_{16}$ (Figure 3). *Bam*HI linkers were added to the *Kpn*I-*Sgs-4* fragment, and the resulting *Bam*HI fragment was cloned into $pA_{31}O_4$. The *SaI* fragment from this was replaced into pR7.7 to produce $pA_{60}O_1$ or $pA_{60}O_5$ (Figure 3).

To generate the fine structure deletions of ACE3 the chorion fragment from -630 and -310 was cloned into M13mp18 (Messing and Vieira, 1982) after the addition of *Kpn*I linkers. The deletion from -630 to -510 was made between the *Hind*III site at -510 and another *Hind*III site in the polylinker adjacent to the -630 end of ACE3, and *Kpn*I linkers were added. The resulting *Kpn*I fragment contains a deletion from -630 to -510 in ACE3, and it was ligated into the *Kpn*I site of $pA_{54}O_{18}$, generating $pA_{59}O_{34}$.

The three other deletions in ACE3 were produced by oligonucleotide-directed mutagenesis using the following strategy (Newman *et al.*, 1983). An oligonucleotide with ~15 bp of homology on either side of the desired deletion was hybridized to the single-stranded M13 ACE3 clone. The annealed oligonucleotide served as a primer for extension by the Klenow fragment of DNA polymerase I. This was then ligated and transformed into JM101. Plaques containing the deletion were tentatively identified by hybridization to the kinased oligonucleotide employed in the mutagenesis. Phage with the deletion hybridized to the probe at higher stringencies (determined empirically for each probe) than the undeleted phage. The

presence of the deletion was confirmed by sequencing the phage by the ddNTP protocol (Sanger *et al.*, 1980). The deletion from -478 to -420 has a new *HpaI* site at this breakpoint, the deletion from -410 to -370 results in a new *EcoRI* site, and the *BamHI* site at -310 is retained in the deletion from -368 to -310. The *KpnI* fragment containing each of these deletions was inserted into the *KpnI* site of pA₅₄O₁₈.

P-element transformation

Each of the transposons was injected into γ^{506} embryos and the resulting flies crossed to γ^{506} flies to identify transformants as described (Orr-Weaver and Spradling, 1986). Transformant lines containing a single copy of the transposon were identified by Southern blots of DNA from transformed flies (Orr-Weaver and Spradling, 1986). In addition, all the lines were restriction mapped by Southern blotting to ensure that the transposon was unrearranged and that the particular deletion was correct. Lines homozygous for transposon inserts were produced by first crossing the lines to balancer stocks with γ^{506} , and then selecting homozygotes of the insertion chromosome. In ~30% of the transposon lines the homozygous inserts were lethal or sterile.

The injections and matings of the heat shock enhancer transposons were done at 18°C to eliminate the possibility of amplification of the transposon during the development of the transformed flies. Our concern was that amplification of chromosomal sequences adjacent to the transposon in many tissues might lead to lethality.

Amplification assays and statistical analysis

Amplification of the transposon in egg chambers was assayed as described previously by comparing the ratio of the intensity of a transposon fragment to a host fragment from stage 13 egg chambers to that ratio from stage 1–8 egg chambers (Orr-Weaver and Spradling, 1986). The intensity of the hybridized bands was quantitated using an LKB laser densitometer with a recording integrator. For the deletion constructs, transformed flies were crossed to γ^{506} flies, and heterozygotes for the transposon insertion were assayed for amplification.

In the heat shock experiments flies were heat shocked by immersion of the vials in a 37°C water bath for 1 h, and then every 12 h for 1 h for a 36 h period. Egg chambers and malpighian tubules were dissected from the flies after the fourth heat shock. Salivary glands were dissected from third instar larvae from the heat-shocked vials. The DNA was isolated from the malpighian tubules from 50 adult flies and from 50 salivary glands by a modification of the protocol of Endow and Glover (1979). The nuclei were isolated as described; they were lysed by incubation in 50 mM Tris (8.0), 100 mM EDTA (8.0), 0.5% sarkosyl and 20 µg/ml proteinase K at 37°C for 15 min then at 65°C for 30 min. The DNA was extracted with a phenol/chloroform mixture, chloroform alone and ethanol precipitated. The extractions and precipitation were repeated, then the DNA was digested as described (Orr-Weaver and Spradling, 1986). Amplification levels were quantified relative to DNA from transformant males that were not heat shocked.

The amplification assays of salivary gland DNA from lines A₅₅O₁₆, A₆₀O₁ and A₆₀O₅ were done on stocks that were homozygous for the transposon insert. Salivary glands were dissected from 50 third instar larvae, from 50 8 h old prepupae, and from 50 12 h old prepupae. The age of the prepupae was defined by removing third instar wanderers to a new vial and dissecting the pupae 8 or 12 h later. DNA was prepared from these salivary glands as described above.

For each deletion transformant construct the level of amplification was determined for at least nine insertion sites. To provide an indication of the overall level of amplification for each transposon the mean value for amplification at all insertion sites is given in the tables. The median level of amplification gives an estimate of the range of amplification levels, and is cited in Results. However, these values are not useful for statistical comparisons because the amplification levels for each transposon are not in a normal distribution. Two tests were used to determine whether amplification levels differed between transposon constructs: the binomial distribution test and Wilcoxon's rank sum test (Lindren *et al.*, 1978). The first test measures the probability by random chance of observing the number of insertion sites for one transposon that amplify at levels below the median of the amplification levels observed for another transposon. The second test is a very stringent test for differences between transposons. It orders the amplification levels for each insertion site of one transposon and tests whether that rank order differs from the rank order of another transposon.

Northern analysis of *s18* transcripts

RNA was isolated from adult males and from egg chambers, and Northern blots were prepared as described previously (Orr-Weaver and Spradling, 1986). RNA was isolated from 15 pairs of salivary glands and from

malpighian tubules of 20–30 flies by the protocol of McNabb and Beckendorf (1986). The blots were hybridized to an SP6 transcript homologous to the tag in the *s18* gene (Melton *et al.*, 1984). The amount of RNA present in each sample was determined by rehybridizing the blots to nick-translated *hsp83* for egg chamber samples (Zimmerman *et al.*, 1983) or *actin 5C* for salivary gland samples (Fryberg *et al.*, 1983). The levels of transcript were quantitated by densitometric scanning of film exposures. For each transformant line the ratio of *s18* signal to the control message was determined. Different Northern blots were standardized by running RNA from stage 13 egg chambers of line A₄₈O₂₈₋₉ on each gel. The *s18/hsp83* ratio from this line was set at 1, and the ratios for other lines given relative to this after correcting for amplification level. Blots were hybridized in 50% formamide at 46°C for the SP6 probe and at 42°C for the nick-translated probes.

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