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Amplification-control element ACE-3 is important but not essential for autosomal chorion gene amplification

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ABSTRACT **We have further characterized the cis-acting elements that control the amplification of the third chromosomal cluster of chorion genes in *Drosophila melanogaster*; these include the amplification-control element ACE-3 and four amplification-enhancing regions (AER-a to -d). We have used two types of deletions in the chorion cluster: the first was *in vitro* generated deletions of the ACE-3 region that were subsequently introduced into the germ line, and the second was deletions induced *in vivo* within a transposon at a preexisting chromosomal location, thus avoiding the complication of position effects. Some of the lines bearing deletions of either type showed amplification, albeit at drastically reduced levels. These unexpected results indicate that, despite its importance, ACE-3 is not essential for low-level amplification and that cis-acting amplification elements are functionally redundant within the autosomal chorion replicon.**

In *Drosophila melanogaster* the major chorion genes form two chromosomal clusters: the early genes (*s36, s37,* and *s38*) at position 7F1 of the X chromosome, and the middle and late genes (*s15, s16, s18,* and *s19*) at 66D12–15 on the third chromosome. Chorion genes are expressed in the follicular epithelium that surrounds the developing oocyte, each during a characteristic period in the last 5 hr of oogenesis (stages 11–14). Prior to their expression, both gene clusters begin to replicate differentially, and by the end of choriogenesis, they reach amplification levels of 50- to 100-fold (autosomal cluster) or 20-fold (X-chromosome-linked cluster). Amplification extends over chromosomal domains of 50 to 100 kilobases (kb), with maximal copy levels in the chorion genes at the center (ref. 1; for further review, see ref. 2).

Several cis-acting elements that are important for amplification have been identified by transformation analysis. Spradling and coworkers (3, 4) identified short, apparently essential amplification-control elements (ACE) within the chorion clusters of the X (*ACE-1*) and third (*ACE-3*) chromosomes: chorion transposons with *in vitro* constructed deletions eliminating these 467- to 510-base pair (bp) regions did not amplify in any of the transformed lines tested. Although this approach has yielded important results, a limitation is that when amplification does occur, the copy number is highly variable between transformed lines because of chromosomal position effects. To avoid this problem, large numbers of lines per construct must be examined, and minor effects on amplification are impossible to discern. Recently, Delidakis and Kafatos (5) have taken a different, *in vivo* deletion approach that circumvents positional effects: by utilizing *P*-transposase to generate internal deletions in the resident transposon of a preexisting line, the effects of such deletions were studied at the same chromosomal position. This permitted identification of four amplification-enhancing regions (AERs) in the autosomal cluster, distinct from ACE-3 (see Fig. 1). Targeted *in vitro* deletions were also tested and suggested that AERs may have redundant functions: elimination of only one had no statistically significant effect on amplification, whereas simultaneous deletions in three AERs, with ACE-3 intact, greatly reduced amplification levels (5). At least one of the AERs, AER-d, may correspond to an origin of DNA replication, according to biochemical analysis of amplification intermediates by the method of Brewer and Fangman (7); within the detection limits of this approach, ACE-3 does not appear to function as a replication origin (5).

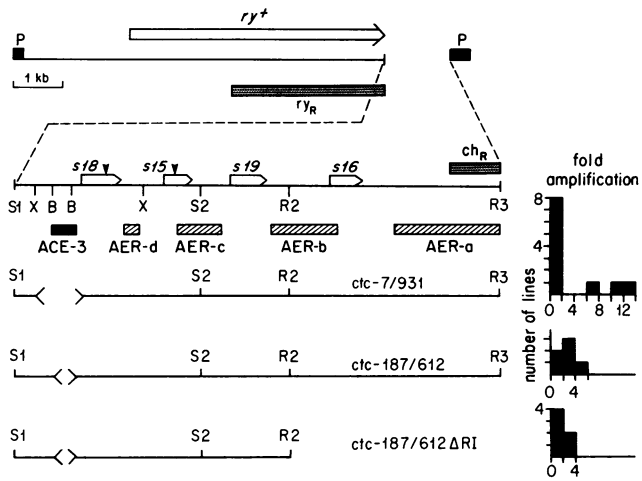

FIG. 1. Structure and amplification of the *in vitro* deleted chorion transposons. From top to bottom, the diagrams represent the Carnegie 20 vector (6), the 10-kb autosomal chorion cluster, and the three ctc constructs with ACE deletions (<>). Restriction sites are: S, *Sal* I; X, *Xba* I; B, *Bal* I; and R, *Eco*RI; the *Sal* I and *Eco*RI sites are numbered from left to right as previously done (5). The ry_R and ch_R probes used for the amplification blots of Fig. 3 are stippled. The chorion and rosy genes are shown as arrows; black arrowheads represent the moth DNA insert in genes *s18* and *s15*. The putative cis-acting regulatory elements for amplification, ACE-3 and AER-a through AER-d, are indicated. The histograms represent transposon amplification levels at ry_R, relative to the endogenous ry_c single-copy control, in all independent transformant lines tested.

In the present work, we have combined *in vitro* and *in vivo* deletion analysis to reexamine the role of ACE-3. While confirming that ACE-3 plays a central role in amplification, we present evidence that it is not altogether essential: elimination of ACE-3 alone does not abolish amplification, although it does decrease greatly the amplification level.

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Abbreviations: ACE, amplification-control element; AER, amplification-enhancing region; ch_R and ry_R, right end of chorion and rosy loci; ry_L, left end of *ry* locus; ch_c and ry_c, endogenous rosy and chorion bands; ch_t, transformant-specific chorion band; ry_t, transposon-specific *ry* band.

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MATERIALS AND METHODS

Plasmid Construction. The *Sal* I fragment containing both *s18* and *s15* (S1S2; nucleotides 1–3845, numbered from the left end of the locus as diagrammed in Fig. 1) was subcloned into a pUC9 derivative lacking the *Eco*RI site. Both genes in this subclone were marked with a 288-bp fragment of moth (*Antheraea polyphemus*) chorion DNA, inserted in-frame after nucleotides +564 or +418 of *s18* and *s15*, respectively. A deletion between +7 and −931 of *s18* was then generated by cutting at the *Cla* I site (+17) and ligating the downstream end to a synthetic oligonucleotide that contained the *Cla* I overhang and the sequences extending to +7. The product was fused to an upstream fragment at the *Xba* I site (−931), which had been repaired to a blunt end by incubation with the Klenow fragment of DNA polymerase. In a second construct, the DNA between −187 and −612 of *s18* was removed by digesting with *Bal* I, and an *Eco*RI linker was inserted at the deletion junction. The extent of each deletion was confirmed by sequencing (8). These modified *Sal* I fragments were then shuttled into a *P*-element vector, which contains the rest of the chorion cluster (fragment S2R3) cloned into Carnegie 20 (see Fig. 1); the constructs were designated ctc-7/931 and ctc-187/612, respectively. The third construct, ctc-187/612 ΔRI, was created by shuttling the same modified *Sal* I fragment as ctc-187/612 into a Carnegie 20 derivative lacking the right-end 4.3-kb *Eco*RI fragment of the chorion cluster (R2R3). All enzymes were used as recommended by the vendor and ref. 9.

Fly Transformation. *Drosophila* embryos were injected, and transformed lines were selected with a genetic scheme as described (10).

Generation of *in Vivo* Deletions. Internal deletions in the resident transposon of preexisting transformed lines were obtained as described (5). They were first selected by the loss of the rosy phenotype and were distinguished from retranspositions by confirming the integrity of known junction fragments that span the ends of the transposon and insertion-site DNA. To that end male genomic DNA was digested with *Eco*RI, *Sst* I, and *Xba* I, and the same filters were sequentially probed with ch_R (the right end of the chorion locus) and ry_L (the left end of the rosy ry^+ gene) DNA (see Fig. 4 for the extent of probes).

DNA Analysis. Transformed lines and *in vivo* deletions were characterized by Southern analysis of DNA prepared from male flies as described (5). ^{32}P-labeled probes were prepared either by nick-translation of gel-purified fragments (11) or by primer extension from templates of phage M13 subclones. Amplification of transposons was quantitated in DNA from a mixed population of stage 13/14 follicles (probably comparable to the stage 13 follicles used by Orr-Weaver and Spradling, personal communication) as described (10); male DNA was used as an unamplified control. All DNA samples were heterozygous for the transposon, except for S1R3-8 and its derivatives, which were homozygous. For distinguishing low amplification levels from nulls, amplification values were expressed in absolute terms as multiples of single-copy levels, according to the formula $[(ry_{Rt}/ry_{Re})f]/[(ry_{Rt}/ry_{Re})\delta]$, in which ry_{Re} is the right end of the endogenous ry^+ gene; ry_{Rt} is the right end of the transposon-specific *ry* gene; ch_{Re} and ch_{Rt} are the right ends of the endogenous and transformant-specific chorion band; and f is follicular DNA. Percent amplification levels, relative to endogenous, were also calculated, using the formula $[(ch_{Rt})f/(ch_{Rt})\delta]/[(ch_{Re})f/(ch_{Re})\delta]$. We consider these values a more accurate measure for quantitative comparisons among amplifying lines because in our experience the endogenous amplification levels can vary from 24- to 180-fold.

RESULTS

***In Vitro* Deletions of the Amplification Control Region.** Fig. 1 shows a restriction map of the autosomal chorion cluster, with key *Sal* I and *Eco*RI sites numbered from left to right; these sites (S1, S2, R2, and R3) are used to name fragments of the cluster. Two deletions removing ACE-3 sequences were introduced into the parent amplification-positive vector, which encompasses the entire 10-kb chorion cluster (S1R3; Fig. 1). These constructs lack either the region between +7 and −931 bp (ctc-7/931) or between −187 and −612 bp (ctc-187/612) upstream of the *s18* gene. A third construct, ctc-187/612ΔRI, is identical to ctc-187/612 except that it lacks R2R3, the right end 4.3-kb *Eco*RI fragment that encompasses the *s16* gene as well as the putative amplification-enhancing regions AER-a and most of AER-b. In all of the constructs, the *s18* and *s15* genes are marked by the insertion of a fragment of silk moth chorion DNA.

The constructs were introduced into *D. melanogaster* via *P*-element transformation, and multiple, independent, single-insert lines were isolated for each construct: 6 each for ctc-187/612 and ctc-187/612ΔRI, and 11 for ctc-7/931. Absence of the ACE-3 sequences between −187 and −612 was confirmed in all lines. Male genomic DNA was digested with *Xba* I, blotted, and probed with either a labeled ACE probe or the moth DNA with which *s18* and *s15* are marked (Fig. 2 and data not shown). The ACE probe hybridized only to the

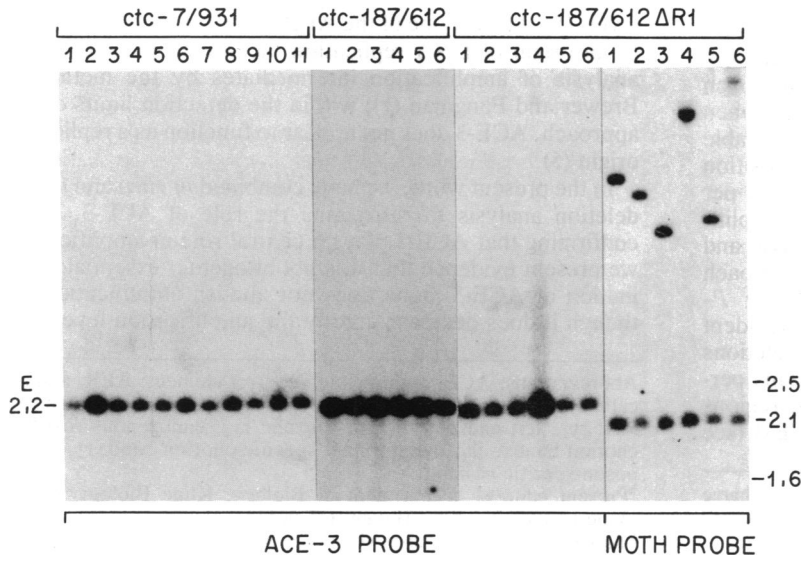

FIG. 2. Southern analysis of transformants bearing ctc deletion constructs. Male DNAs from each of the indicated lines were digested with *Xba* I and blot-hybridized with either an ACE-3 probe or a probe corresponding to the moth insert that marks genes *s18* and *s15*. The ACE-3 probe was the gel-purified *Bal* I fragment removed from ctc-187/612. The moth probe was a phage M13 subclone of the 288-bp fragment used to mark the chorion genes. The ACE-3 probe only detects the 2.2-kb fragment of the endogenous (E) locus. Other size markers indicate the absence of ACE sequences at 2.1 and 1.6 kb (the expected size of the *Xba* I transposon fragment that bears the moth DNA insert in *s18* and the −187 to −612 deletion or +7 to −931 deletion, respectively), and at 2.5 kb (the expected size of a fragment with the moth insert but no ACE deletion). The moth insert probe only detects transposon fragments: (*i*) the internal, constant, 2.1-kb fragment that encompasses *s18* plus the deletion and (*ii*) a junction fragment that encompasses *s15* and varies in each line, confirming that the transposon is inserted in different chromosomal sites.

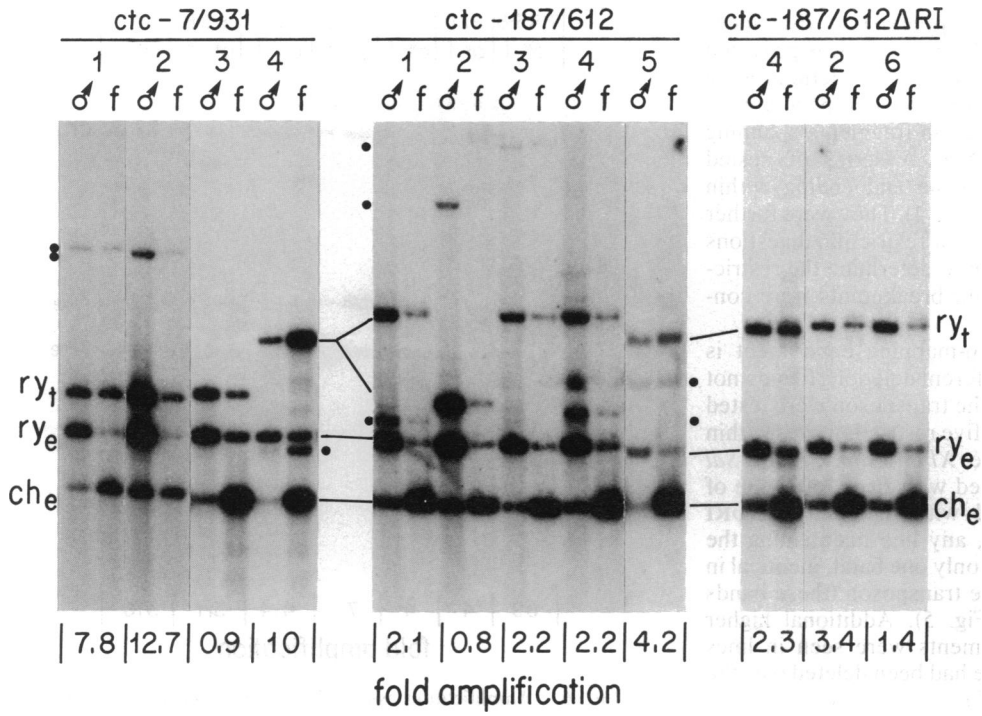

FIG. 3. Amplification analysis of transformant lines. Genomic DNA blots of males (lanes ♂) and stage 13/14 follicles (lanes f) were probed with a mixture of labeled ch_R and ry_R fragments (see Fig. 1). Samples from lines 1, 2, and 4 of ctc-7/931 and line 2 from ctc-187/612 were digested with *Sst* I, *Xho* I, and *Xba* I, and all others were digested with *Sst* I and *Xho* I. Endogenous rosy and chorion bands (ry_c and ch_c) and transposon-derived rosy bands (ry_t) are labeled. The fourth band in each lane is the variable transformant-specific chorion band, ch_t (dots); it is absent in ctc-187/612ΔRI, since that transposon lacks the right end of the chorion cluster (see Fig. 1). Compare the f/♂ intensity ratios for ry_t, ry_c, and ch_t in the same line: ry_t and ch_t ratios greater than the ry_c ratio indicate transposon amplification. The levels of ry_t amplification for each line are indicated at the bottom.

expected fragment of the endogenous cluster (2.2 kb) and showed no band derived from the transposon. As expected, the probe of inserted moth DNA hybridized to two fragments: an invariant fragment spanning *s18* and the ACE deletion (2.1 kb for ctc-187/612 or its ΔRI derivative, and 1.6 kb for ctc-7/931), and a line-specific junction fragment encompassing *s15*. No line showed a 2.5-kb band, which would correspond to a fragment with intact ACE-3 and *s18* DNA marked with the moth DNA insert. In addition, detailed restriction mapping showed that the transposons are not rearranged in any of the lines (data not shown).

Amplification levels were determined by comparing DNA from males and mixed stage 13/14 follicles. Fig. 3 shows the results for all amplifying and representative nonamplifying lines. The blots were probed with ch_R and ry_R (see Fig. 1). The endogenous rosy fragment, ry_e, served as a single-copy standard, while the endogenous chorion fragment, ch_e, served as an internal amplification control.

The results clearly established that deletion of the previously defined ACE-3 region does not completely abolish amplification. Based on duplicate measurements, we consider amplification values of up to 2.0-fold as within the margin of error. Four lines with amplification levels between 2.0- and 3.0-fold might be considered marginally positive, but a total of five ACE-minus lines clearly amplify above that level: three of these lines were derived from ctc-7/931 (7.8×, 10.0×, and 12.7× amplification), one from ctc-187/612 (4.2×), and one from ctc-187/612 ΔRI (3.4×). Eight marginally or definitively amplifying lines were analyzed twice with consistent results. Furthermore, results were qualitatively the same whether amplification was estimated from ry_t (all lines) or ch_t (ctc-7/931 and ctc-187/612 lines). Amplifying and control DNA fragments were of comparable size in several lines, reducing the possibility of artifacts (see *Discussion*). Differences among lines derived from the same construct were due to position effects (10). We conclude that, although the ACE region is required to reach the high amplification levels seen with the undeleted S1R3 construct (10), it is not essential for amplification.

***In Vivo* Deletions.** A second approach that avoids the problems of position effects provided independent evidence for the same conclusion. *In vivo* deletions were generated in an invariant chromosomal position within a resident, highly amplifying S1R3 transposon encompassing the entire 10-kb chorion cluster. The method relied on the findings of Daniels *et al.* (12) that *P*-transposase activity induced by a $P \times M$ dysgenic cross can result in internal deletions as well as

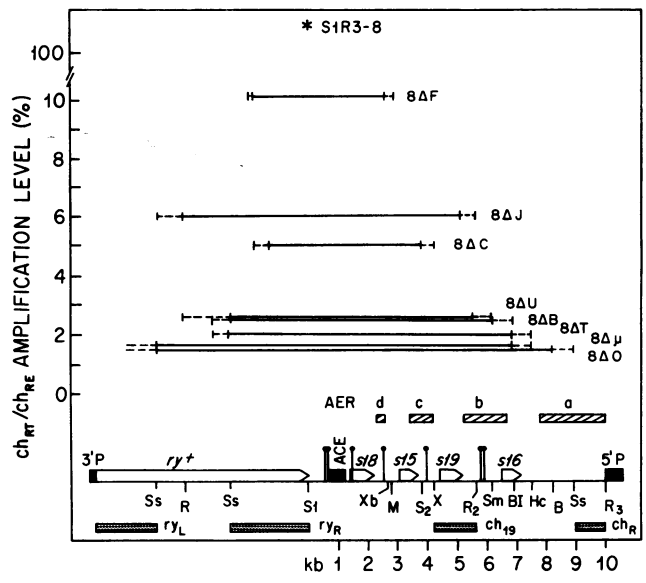

FIG. 4. Diagram of *in vivo* deletions and their amplification levels. The restriction map of the transposon present in the parental S1R3-8 line is indicated. Chorion and ry^+ genes are indicated by open arrows, the ACE and *P* element ends by filled boxes, the AER elements by hatched boxes, ARS-core-like repeats in chorion DNA (10) by ↑, and the probes by stippled boxes. All sites for restriction enzymes *Eco*RI (R) and *Sal* I (S) are marked. For the remaining enzymes, only the sites relevant to deletion end-point mapping are shown: B, *Bam*HI; BI, *Bgl* I; Hc, *Hin*cII; M, *Mlu* I; Sm, *Sma* I; Ss, *Sst* I; X, *Xho* I; Xb, *Xba*I. The different deletions are shown above the restriction map at an ordinate position indicating their amplification level (% of endogenous level) measured at ch_R. Amplification in the parental line (*) was 116% of endogenous. For the deletions, solid lines indicate known deleted regions and dashed lines at the ends delineate the intervals within which the deletion endpoints have been mapped.

excisions and retranspositions of preexisting *P*-element transposons. In our scheme the *P*-transposase was provided by the Jumpstarter (*Js*) chromosome (13). Deletions were initially selected by the loss of the rosy phenotype and were then checked for intactness of junction fragments spanning transposon and insertion site DNA. A series of nested deletions beginning with the ry^+ gene and ending within chorion DNA were thus generated (Fig. 4). They were further mapped internally by a combination of restriction digestions and probes from the chorion region to determine the restriction site intervals within which their breakpoints were contained.

An example of such a deletion-mapping experiment is shown in Fig. 5. Four lines with different deletions that do not impinge on the R2R3 right end of the transposon were tested here for the loss or maintenance of five restriction sites within 3 kb to the left of the R2 *Eco*RI site: *Xba* I, *Mlu* I, *Pvu* I, *Sal* I, and *Xho* I. When doubly digested with *Eco*RI and one of these enzymes and then probed with the *Xho* I–*Eco*RI chorion fragment (ch_{19} in Fig. 4), any line maintaining the pertinent restriction site produced only one band, identical in both the endogenous locus and the transposon (these bands are indicated as 1.4–3.0 kb in Fig. 5). Additional higher molecular weight restriction fragments were seen in lines where the particular restriction site had been deleted (see the map of Fig. 4 and legend of Fig. 5).

Fig. 6 shows the amplification blots for a series of six deletions induced on the same parental line, S1R3-8. Three of these deletions, S1R3-8ΔC, S1R3-8ΔF, and S1R3-8ΔJ do not reach the R2 site, while the rest extend past it, further to the right (Fig. 4). Genomic DNA from males and late follicles (stages 13/14) was digested with a combination of *Sst* I, *Xho* I, and *Xba* I and was probed with a mixture of ry_R and ch_R.

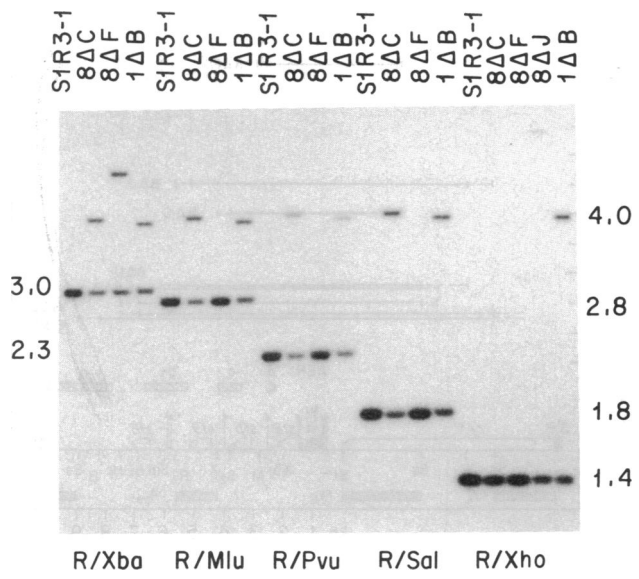

FIG. 5. Mapping of the *in vivo* deletion endpoints in chorion DNA. Double-restriction enzyme digestions were performed on aliquots of DNAs from lines bearing an undeleted transposon (S1R3-1), one of its deletion derivatives (S1R3-1ΔB, abbreviated 1ΔB), and three deletion derivatives of a second parental line (S1R3-8ΔC, -8ΔF, and -8ΔJ). The restriction enzymes used are abbreviated as in Fig. 4. The probe was ch_{19} (see Fig. 4). For each digest, the size of the band derived from the endogenous cluster is indicated in kb (1.4–3.0). All deletions fall short of the R2 site (see Fig. 4). If the deletion also falls short of the second restriction site, it generates a band identical in size to the endogenous one, whereas an additional band indicates that the pertinent restriction site has been eliminated. In conjunction with the presence or absence of an extra band in the various digests, the size of that band permits reasonably accurate mapping of both deletion endpoints (see Fig. 4).

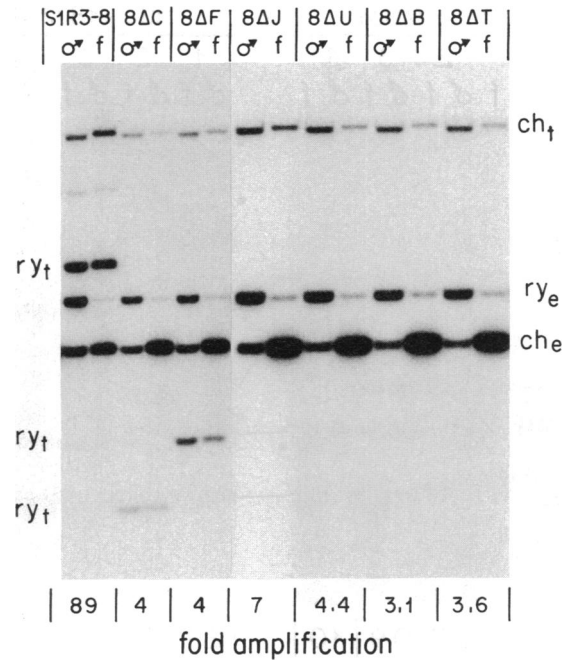

FIG. 6. Amplification assays for S1R3-8 and its *in vivo* deleted derivatives. Male (lanes ♂) and follicular (lanes f) DNA was digested with *Sst* I/*Xho* I/*Xba* I and probed with ch_R/ry_R. The ry_e and ch_e bands are identical for all lines; they serve as single-copy control and endogenous chorion (amplifying) control, respectively. Transformant-specific chorion band ch_t is a transposon-insertion site junction fragment; its invariance confirms that the deletion derivatives have not retransposed. Transposon-specific ry_t band is altered (lanes 8ΔC and 8ΔF) or absent (lanes 8ΔJ to 8ΔT) in the S1R3-8 deletion lines, depending on the extent of the deletion (see Fig. 4). The amplification level of each line is indicated at the bottom.

It can be seen in Fig. 6 that the ch_t bands of the deletion derivatives are the same size as in the parental line, confirming that the construct did not retranspose in the process of deleting. The blots also help to map the left breakpoint of the deletion within ry^+: part of the ry_R-homologous region is missing in S1R3-8ΔF and S1R3-8ΔC, and the entire region is deleted in the remaining lines. Most importantly, the blots document the occurrence of amplification despite the total elimination of ACE sequences.

The two most extensive of the eight deletions summarized in Fig. 4, S1R3-8Δμ and S1R3-8ΔO, show no more than marginal amplification. The remaining six deletions definitely amplify at low levels, as judged by duplicate determinations. The amplification levels were calculated both in absolute terms (Fig. 6) and as a percentage of endogenous amplification (Fig. 4). While the former calculation is optimal for detecting low-level amplification, the latter more accurately compares the levels between lines, as it controls for variations in the endogenous amplification levels; the absolute amplification levels of the transposons do not necessarily parallel the percentage levels (compare Figs. 4 and 6). The highest percentage amplifications are shown by deletions that, in addition to ACE, eliminate only one or two of the AER elements (AER-d in S1R3-8ΔF; AER-d plus AER-c, completely or in part, in S1R3-8ΔJ and S1R3-8ΔC).

DISCUSSION

The results reported here confirm that the ACE-3 sequences are very important for amplification, as initially reported by Orr-Weaver and Spradling (4): in a total of 37 transformant lines with ACE-3 deletions, not 1 amplified at more than low levels. Nevertheless, there is also an important discrepancy:

11 of our deletion lines amplify at more than marginal levels, whereas Orr-Weaver and Spradling (4) did not detect any amplification in 19 ACE-deletion lines. The discrepancy is important, because it bears on the nature of ACE.

Several differences between the two studies might account for the discrepancy. One is differences in the genetic background of the host fly strains. A second is the choice of single-copy standards: in most experiments we used male DNA as the unamplified control, whereas Orr-Weaver and Spradling (4) used DNA from stages 1–8. A third difference is the presence of 1.95 kb of chorion DNA upstream of the S1 site in the experiments of Orr-Weaver and Spradling (4). A fourth difference is the presence of moth DNA inserts in our constructs and *lacZ* sequences in the constructs of Orr-Weaver and Spradling. Finally, we used Carnegie 20 as a vector, whereas Orr-Weaver and Spradling used a precursor to that vector with the ry^+ gene oriented away from the chorion genes. The last two differences may be the most significant.

Whole-animal DNA can be subject to more degradation than DNA purified from isolated tissues, and thus high molecular weight fragments could be underrepresented in male DNA. Although no DNA degradation was detectable in our samples, we chose restriction enzymes that frequently led to comparable sizes of amplifying and control *ry* fragments (3.1 and 2.7 kb in ctc-7/931-1 and -2; Fig. 3). Similarly, ch_t amplification could be monitored in three cases with transformant-specific bands smaller than the ry_e single-copy control (ctc-7/931-4 in Fig. 3 and S1R3-8ΔC and S1R3-8ΔF in Fig. 6). Therefore, DNA degradation is not the explanation of the discrepancy.

A disadvantage of using stages 1–8 DNA as standard is that low-level amplification in late follicles may be masked because amplification begins at about stage 8. It is also pertinent to recall that during endoreduplications in *Drosophila*, certain chromosomal sequences, such as histone or satellite DNA, are underreplicated (14). This appears to occur in both nurse and follicular epithelial nuclei, which reach approximately 1000 C (C equals one haploid genome equivalent of DNA) and 14 C levels in stage 10A follicles (14). If the under-replicated loci differ in different tissues, there might be some advantage in averaging out the aneuploidy by using DNA from a mixture of cell types (e.g., males) as a standard, rather than DNA from follicles alone. Of course, that line of argument raises a contrary objection: the transposon in some of the positive lines derived from *in vitro* deletions may not be itself amplifying but simply happens to lie in chromosomal sites that are overrepresented due to local variation in ploidy. However, in some experiments, both we and Orr-Weaver and Spradling (personal communication) have used both male and stages 1–8 follicular DNA as unamplified controls, with consistent results.

For the reasons stated in *Materials and Methods*, we consider percent rather than absolute amplification levels more accurate for quantitative comparisons between low amplifying lines. Therefore, although we cannot eliminate the possibility that the chromosomal insertion site for S1R3-8 is inherently favorable for amplification, we consider that the results summarized in Fig. 4 document the importance of chorion DNA other than ACE: the least extensive deletions S1R3-8ΔF, S1R3-8ΔJ, and S1R3-8ΔC, amplify better than the rest of the deletions.

In conclusion, the above considerations support our contention that low-level amplification can occur in the absence of ACE-3 sequences, although high-level amplification requires that element. It is interesting that multiple elements affecting amplification lie in the ACE-3 region (Orr-Weaver and Spradling, personal communication). It appears that the third chromosome chorion replicon is functionally stabilized by multiple redundancies and that no region in it is absolutely required for amplification: neither the AER elements or replication origins (5) nor ACE itself. One possibility is that ACE and AER elements are functionally redundant with ACE being only the most active one of multiple amplification control regions within the chorion cluster.

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