Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 3341–3345, May 1986
Developmental Biology

trans-acting amplification mutants and other eggshell mutants of the third chromosome in *Drosophila melanogaster*

(chorion/DNA replication/oogenesis/gene amplification)

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Contributed by Fotis C. Kafatos, December 31, 1985

ABSTRACT **We report on the characterization of five third chromosome mutations with strong effects on the formation of the eggshell or chorion. Three mutations, defining two loci, result in substantially reduced follicle cell-specific amplification of the major chorion structural genes and, hence, in underproduction of the corresponding mRNAs and proteins. The other two mutations, though displaying structural chorion abnormalities, appear to have no significant effect on amplification and to express normally the major chorion structural genes. The possible nature of these mutations is discussed.**

The deposition of the chorion (eggshell) of *Drosophila melanogaster* is a model system for analyzing the control of development at multiple levels, from gene replication and expression to the coordinated morphogenesis of cell populations and their secretory products. During the later part of oogenesis (stages 9–14), ≈1200 follicle cells secrete the complex egg envelopes around each oocyte (1, 2). The envelope known as endochorion is particularly elaborate. It appears to consist of six abundant proteins as well as ≈15 minor components that have not been studied extensively (2–4). The abundant proteins are encoded by two unlinked gene clusters (5, 6): the one on the X chromosome (7F1-2) encompasses at least two genes, *s36-1* and *s38-1*, which are expressed early in choriogenesis, whereas the third chromosome cluster (66D12-15) includes four genes, *s15-1, s16-1, s18-1*, and *s19-1*, which are expressed later, at various overlapping periods. None of these genes is expressed in other tissues at detectable levels. Thus, choriogenesis entails tissue-specific and temporally specific gene expression.

Choriogenesis is also controlled in part at the level of DNA replication. Spradling and Mahowald showed that the two chorion gene clusters undergo specific amplification in the follicle cells, beginning shortly before the onset of choriogenesis (7). Amplification appears to be necessary for large-scale production of the chorion proteins during the brief period in which they are synthesized (8, 9). Current models envisage the existence of a special origin of replication within each cluster, which allows multiple rounds of initiation to occur (10, 11). Analysis of the *oc* mutation (8) and experiments using P element-mediated transformation have revealed that a portion of each chorion locus is capable of autonomous amplification and that amplification is modulated by nearby sequences (12, 13).

Little is known about how choriogenesis is regulated at higher levels. Since it can proceed in cultured individual follicles (14), much of the necessary information must be internal to the follicle. The regionally specific architecture of the chorion suggests that multiple cell populations must be coordinated for production of a normal shell, and the localized morphogenesis suggests an important involvement of the secretory cell surface (2). The existence of germ-line specific mutations that affect chorion structure (15) testifies to the importance of interactions between the follicle cells and the oocyte–nurse cell syncytium.

The power of *Drosophila* genetics obviously offers an important approach to a mechanistic analysis of choriogenesis. Several sex-linked mutations with major effects on the chorion have been identified (16, 17), and two of these have been shown to interfere with amplification in *trans* (9). Here we present our first results on a similar genetic analysis of the third chromosome.

MATERIALS AND METHODS

Materials. Phluoroglucinol and Robb's tissue culture medium were the generous gift of W. Petri. [^{3}H]Proline (108.8 Ci/mmol; 1 Ci = 37 GBq) and [α-^{32}P]nucleotide triphosphates (800 Ci/mmol) were from New England Nuclear and Amersham, respectively. Biodyne nylon transfer membranes were from Pall Ultrafine Filtration (Glen Cove, NY).

***Drosophila* Strains and Culturing.** Except for mutation *SD758*, mutant strains were isolated by C. Nüsslein-Volhard and co-workers. Mutations *108-17* and *350-7* were induced on a chromosome bearing *ru th st ri roe* p^d e^s *ca* (called rutipa); *272-9* and *293-19* were induced on a chromosome bearing *ru st e ca* (called rusteca) (for a description of the marker genes used in this report, see ref. 18). *SD758* was isolated by D. Lindsley and was induced on a chromosome bearing *st*. All mutations were induced with ethyl methanesulfonate by standard procedures and were maintained over the *TM3 Sb Ser* balancer chromosome. We will use the mutant strain numbers as temporary designations for the respective genes. Flies were grown on standard medium in humidified chambers at either 18°C or 25°C. Prior to dissection, adults were collected within 48 hr of eclosion and conditioned on yeast-supplemented medium for 1–4 days, with fresh medium supplied every 2 days.

Microscopy. For electron microscopic analysis, flies were lightly etherized and dissected in *Drosophila* Ringer's solution. Follicles were fixed in 2% glutaraldehyde/2% paraformaldehyde for 90 min at 4°C, washed in buffer containing 4% sucrose, postfixed in 2% aqueous OsO_4 for 60 min at 4°C, dehydrated in ethanol, and embedded in a modified Mollenhauer's resin (25 g of Epon-812/20 g of Araldite-506/60 g of dodecenylsuccinic anhydride/3 g of DMP-30). The fixation buffer was sodium cacodylate at pH 7.4. Thin sections were cut with glass knives using an LKB Ultratome-V, collected on 300-mesh copper grids, and stained with uranyl acetate and lead citrate. Images were obtained on Kodak electron microscope film 4489 (6.5 × 9 cm plates) using a JEOL 100C electron microscope equipped with side-entry goniometer stage and operated at 80 kV.

For whole-mount light microscopy, late stage 14 follicles were photographed on Kodak Panatomic-X 35-mm film

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under dark-field conditions using a Leitz Dialux 20EB light microscope.

Protein Labeling and Electrophoresis. Individual follicles were dissected in cold *Drosophila* Ringer's and labeled with [^{3}H]proline for 7.5 hr in culture, according to published procedures (19), in the presence of phluoroglucinol (1 mM) for preventing cross-linkage of the chorion (20). Samples were solubilized and electrophoresed through 10–15% $NaDodSO_4$/polyacrylamide gradient gels (19), and proteins were visualized by autofluorography using EN^{3}HANCE (New England Nuclear).

Nucleic Acid Preparations. For RNA analysis, total nucleic acids from staged follicles were isolated as described (6), with the exception that the homogenization buffer consisted of 7 M urea/2% (wt/vol) $NaDodSO_4$/10 mM Tris·HCl, pH 8.0/1 mM EDTA/0.35 M NaCl. Genomic DNA was prepared by homogenization in 50 mM Tris·HCl, pH 8.0/5% (wt/vol) sucrose/10 mM EDTA/50 mM NaCl. After homogenization, proteinase K and $NaDodSO_4$ were added to a final concentration of 0.2 mg/ml and 0.5% (wt/vol), respectively, and samples were incubated for 30 min at 60°C, followed by phenol/Sevag extraction and ethanol precipitation. Plasmid DNAs were prepared according to published procedures (21).

Nucleic Acid Analysis. The protocols for DNA blots and the probes used were as described (9). For RNA blots, total nucleic acids were glyoxylated (22), fractionated by electrophoresis through a 1.5% agarose gel, and transferred in 0.025 M NaH_2PO/Na_2HPO_4, pH 7.0, to a nylon membrane. In both cases, nucleic acids were affixed to the membrane by UV irradiation, and hybridizations using nick-translated (23) probes were performed according to the method of Church and Gilbert (24). For purposes of quantitation, autoradiograms were scanned with an Ortec 4310 densitometer.

RESULTS

Mutant Phenotypes. We examined 20 ethyl methanesulfonate-induced, third chromosome mutant stocks with macroscopically visible abnormalities in egg morphology (P.B.S. and V.K.G., unpublished data) and found that 5 had substantial recessive effects on chorion morphology. Four of these (*108-17, 272-9, 293-19*, and *350-7*) were derived from a large screen of Nüsslein-Volhard and co-workers for maternal effect mutants, whereas *SD758* was obtained by D. Lindsley. All 5 are recessive female steriles (except for *293-19*, which has a low level of fertility); *350-7* is also male sterile. They fully complement one another both for female sterility and for gross egg morphology, except for *272-9* and *SD758*, which show allelism for both phenotypic traits. Preliminary recombinational analysis, scoring female fertility and egg morphology, assigned *272-9/SD758* to the interval between *st* (3-44.0) and *cu* (3-50), *293-19* to the interval between *st* and *ss* (3-58.5), and *108-17* to the interval between *ss* and *k* (3-64); although the female sterility trait was not specifically mapped, it appeared to cosegregate with abnormal egg morphology. Mutation *350-7* has only been assigned to the 3R arm; it is not known whether its male sterility and the other two phenotypes are recombinationally separable. It should be noted that all four loci map distantly from the cluster of known chorion structural genes at chromosomal locus 66D12-15 of the 3L arm; that locus maps between *se* (3-26.0) and *h* (3-26.5) (refs. 25 and 26). The *SD758* mutation is temperature-sensitive: rearing at 25°C results in female sterility and abnormal egg morphology, whereas at 18°C both defects are largely, although not completely, alleviated (data not shown).

Fig. 1 compares the overall appearance of mature (stage 14) follicles as well as the chorion ultrastructure in these mutants and the wild type. In the whole mounts, the chorion defect is most obvious in the respiratory appendages, which are extremely flimsy or missing in *272-9, SD758*, and *293-19* and unusually thin and/or short in *108-17* and *350-7*. In addition, abnormalities in the main body of the chorion are evident as surface wrinkles around the oocyte. The ultrastructural defects range widely in severity. In the wild type (1, 2) the following layers are found between the vitelline membrane and the exochorion: a thin, continuous innermost chorionic layer (ICL) and a quadripartite endochorion, consisting of a thin perforated inner layer or floor (inner endochorion), solid

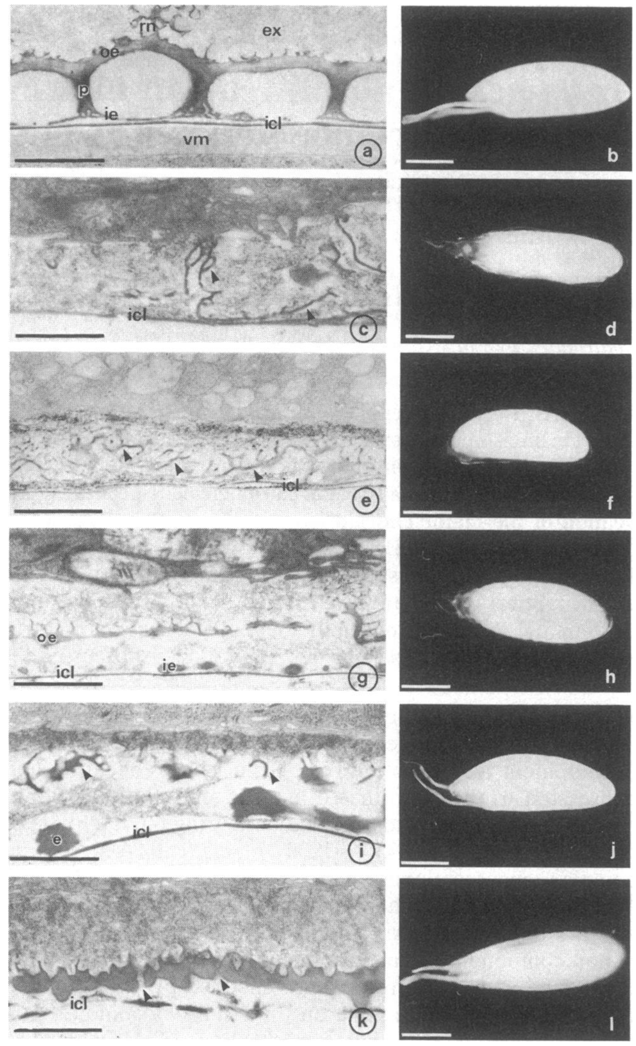

FIG. 1. Transmission electron micrographs (*Left*) and whole-mount dark-field images (*Right*) of stage 14 follicles from homozygous wild-type or mutant flies at 25°C. (Bars, 1 μm and 200 μm, respectively.) (*a* and *b*) Wild-type follicle. Note the vitelline membrane (vm), the innermost chorionic layer (icl), the exochorion (ex), and the endochorion complex. The latter consists of the floor or inner endochorion (ie), the pillars (p), the roof or outer endochorion (oe), and the roof network (rn). In the whole mount, the respiratory appendages are on the left. (*c* and *d*, and *e* and *f*) *272-9* and *SD758*, respectively. In the whole mounts, note the rudimentary appendages and the surface wrinkles on the main body. In the electron micrographs, note the disrupted roof network (arrowheads) and the icl. (*g* and *h*) *293-19*. Note the rudimentary appendages and the presence of small amounts of inner and outer dense endochorionic material (ie, oe) between the icl and the roof network. (*i* and *j*) *350-7*. Note the presence of abundant but disorganized clumps of dense endochorionic material (e) between the icl and the disrupted roof network (arrowhead). The appendages are thinner than normal. (*k* and *l*) *108-17*. Note that the roof is thick but irregular, with many holes (arrowheads), the pillars are short, and the icl is disrupted. The two appendages show variable abnormalities.

vertical pillars, a thick domed roof (outer endochorion), and an outermost roof network that interdigitates with the exochorion. In *272-9* and *SD758* essentially only the ICL and a disrupted roof network remain: the characteristic dense endochorionic material of floor, pillar, and roof is almost totally missing. The phenotype of *293-19* is similar but less severe: the roof network is more regular, and small patches of dense endochorionic material are seen in the expected locations of inner and outer endochorion. Less severe still is the phenotype of *350-7*, which shows, in addition to an extensive although disrupted roof network, dense endochorionic material that forms large clumps but no recognizable floor, pillars, or roof. Finally, the phenotype of *108-17* is more subtly abnormal: the roof network is normal and the roof is of normal thickness although somewhat irregular in shape and traversed by numerous small apertures; pillars are not numerous and are short when present; the floor may be missing and the ICL is disrupted.

In summary, the ultrastructural phenotypes belong to two classes. In the first, the dense endochorionic material is severely underrepresented (*293-19* and especially *272-9/SD-758*). In the second, the dense endochorionic material is approximately normal in abundance but is structured abnormally, either in disorganized clumps (*350-7*) or in an irregular roof with underdeveloped pillars and floor (*108-17*).

Effects on Major Chorion Proteins. The biochemical phenotypes were examined by biosynthetically labeling the chorionic proteins in culture (14) and assaying them by $NaDodSO_4$/polyacrylamide gel electrophoresis followed by autofluorography. Only the six major chorion components were well resolved by the one-dimensional electrophoretic procedure used.

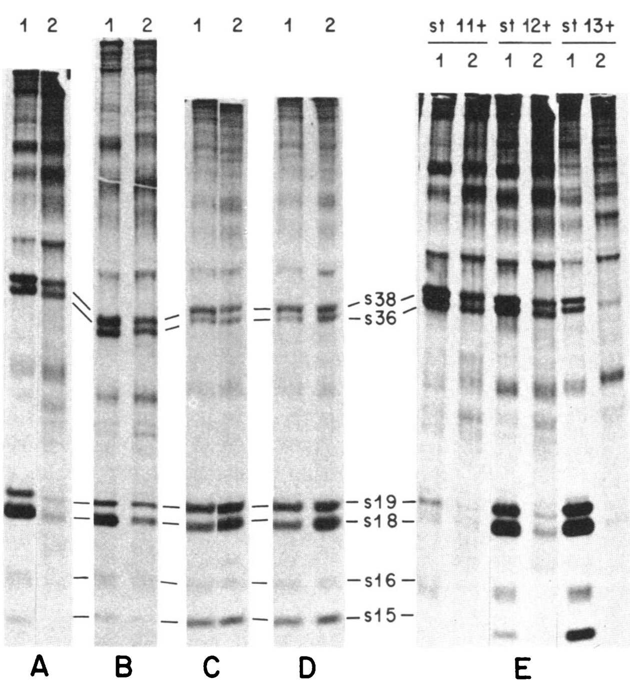

FIG. 2. Labeling of the major chorion proteins in culture. Follicles were labeled with [^{3}H]proline for 7.5 hr. (*A–D*) Labeling beginning at stage 12 for parental wild-type (lane 1) and homozygous mutant (lane 2) follicles. Parental strains were rusteca (*A* and *B*) or rutipa (*C* and *D*). Note the underaccumulation of all six major chorion proteins in *272-9* (*A*, lane 2) and *293-19* (*B*, lane 2); *350-7* (*C*) and *108-17* (*D*) do not differ significantly from the parental strain. (*E*) Labeling beginning at the indicated stages for *108-17* (lane 1) and *272-9* (lane 2) follicles. Compared to the wild type (not shown), both strains display an essentially normal developmental profile, although the level of synthesis is strongly reduced in *272-9*.

As seen in Fig. 2 *A–D*, the protein phenotypes were consistent with the ultrastructure. Mutants *272-9* and *293-19* showed substantially reduced amounts of all six major chorion protein bands; this deficiency was most severe in *272-9* and especially for the lower molecular weight components, s15 to s19. By contrast, *350-7* and *108-17* showed essentially normal levels of the major chorion proteins.

Fig. 2*E* analyzes the developmental regulation of specific chorion protein synthesis in mutants *108-17* and *272-9*. Follicles were staged and labeled for 7.5 hr in culture, beginning at stage 11, 12, or 13. (It should be recalled that stages 11–14 last a total of 5–6 hr *in vivo*; refs. 3 and 27.) The temporal as well as quantitative specificity of major chorion protein synthesis in *108-17* appeared normal (cf. refs. 3 and 4): the labeling profiles were essentially indistinguishable from those of wild-type follicles analyzed in parallel (data not shown), with s36 and s38 synthesized mostly at early stages, the four low molecular weight proteins synthesized mostly at late stages, and, in particular, s15 being the most late component. In *272-9*, although the amounts of proteins (especially those of lower molecular weight) were severely reduced, the temporal specificities appeared normal.

Effects on Chorion Gene Transcripts. Fig. 3 shows the developmental pattern of accumulation and disappearance of transcripts from three major chorion genes, *s38-1* (located on the X chromosome) and *s15-1* and *s18-1* (located on chromosome 3). The abundance of the α_1-tubulin transcripts is also shown as an internal control for variations in the amount of RNA loaded for each sample.

It is evident that the transcript levels are in agreement with expectations from the morphological and protein-level phenotypes. Only approximate quantification was possible because of the diffuseness of the RNA bands and the difficulty of exactly staging the follicles in the mutants. However, *108-17* and *350-7*, the two mutations that showed abundant

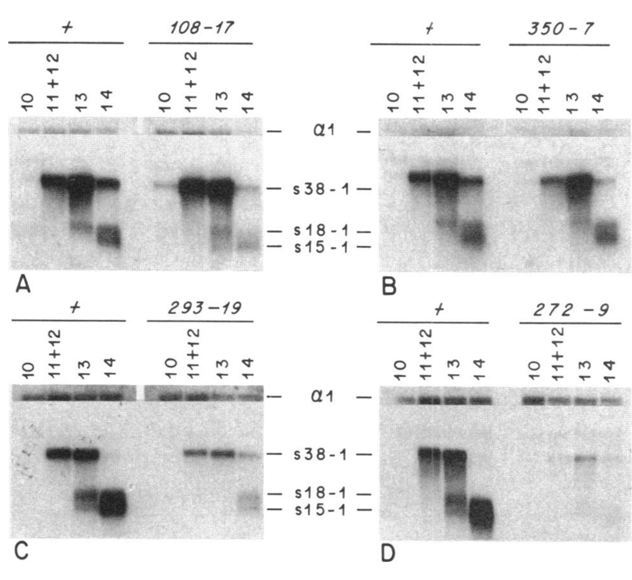

FIG. 3. Accumulation of chorion gene transcripts during choriogenesis. RNA blots of total nucleic acids from follicles of the indicated stages were hybridized with a mixture of probes specific for the early-expressed *s38-1* gene and the late-expressed *s15-1* and *s18-1* genes (see Fig. 4 and ref. 9 for description of probes). An α_1-tubulin genomic subclone (pDmTal, ref. 28) served as an internal control to correct for differences in amount of RNA loaded. The faint band running just above the *s38-1* transcript shows the actual position of tubulin mRNA; a darker exposure is shown at the top of each panel. In each panel, the indicated homozygous mutant is compared with the wild type (rutipa for *A* and *B* and rusteca for *C* and *D*). Note that the developmental specificities are normal but that the amounts of transcripts are reduced in *C* and *D*.

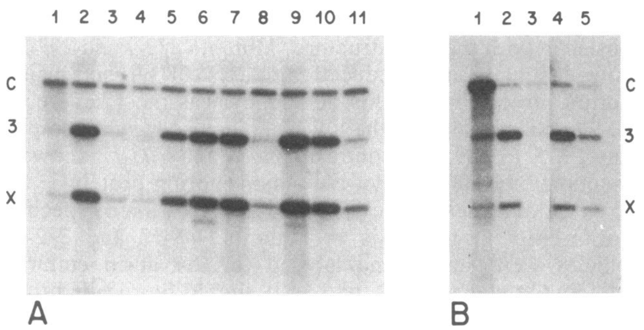

FIG. 4. Amplification of chorion gene clusters. Flies were cultured and conditioned at either 25°C or 18°C as indicated. Genomic DNAs from males or late-stage follicles were digested with *Eco*RI, transferred to a nylon membrane, and hybridized with a mixture of three probes: probe C, genomic subclone pCg441-34-1, which hybridizes *in situ* to 55D-E and serves as an unamplified control; probe 3, genomic subclone p2.4, which includes the *s18-1* and *s15-1* genes of the third chromosome chorion cluster; probe X, genomic subclone pDm3-6, which contains the *s38-1* gene of the X chromosome chorion cluster. (See ref. 9 for further description of probes.) (*A*) Analysis of *272-9/SD758* and *293-19*. Lane 1, rusteca/rusteca males (25°C); lane 2, rusteca/rusteca follicles (25°C); lane 3, *272-9/272-9* follicles (25°C); lane 4, *SD758/SD758* follicles (25°C); lane 5, *SD758/SD758* follicles (18°C); lane 6, *272-9/TM3* follicles (25°C); lane 7, *SD758/TM3* follicles (25°C); lane 8, *272-9/SD758* follicles (25°C); lane 9, *SD758/TM3* follicles (18°C); lane 10, *272-9/SD758* follicles (18°C); lane 11, *293-19/293-19* follicles (25°C). (*B*) Analysis of *108-17* and *350-7* (all at 25°C). Lane 1, rusteca/rusteca males; lane 2, rutipa/rutipa follicles; lane 3, *272-9/272-9* follicles (included for comparison); lane 4, *108-17/108-17* follicles; lane 5, *350-7/350-7* follicles. See Table 1 for quantitation and the text for discussion of results.

dense endochorionic material and essentially normal labeling of the major chorion proteins, also showed normal (or slightly higher than normal) levels of transcripts. By contrast, *293-19* and especially *272-9* showed substantially reduced RNA levels, and the effect was greater for the lower molecular weight components (approximately 8–20% of wild-type levels for *s38-1* and 6–9% for *s15-1* plus *s18-1* in *272-9*; 30–60% and 7–20%, respectively, for *293-19*).

Mutant Effects on Amplification. The pleiotropic effects of *293-19* and *272-9* on the apparent rates of synthesis of the major chorion proteins and on the levels of accumulation of the corresponding transcripts were reminiscent of the effects observed with two sex-linked, *trans*-acting amplification mutants (9). We analyzed the levels of amplified DNA in mutant follicles and concluded that *272-9/SD758* and *273-19* indeed are amplification-defective.

A typical experiment is shown in Fig. 4, and the levels of amplified DNA are quantified in Table 1. For these experiments, genomic blots of DNA from males and from late-stage follicles (equal numbers of stages 12–14) were hybridized with a mixed probe corresponding to the X and third chromosome chorion clusters and to a control, unamplified DNA locus. The control band permitted normalization for differences in the amount of DNA in each sample. The normalized intensities of the chorion-specific bands in follicular DNA, divided by the corresponding intensities in male DNA, indicated the levels of amplification. Finally, the mutant values were expressed as percent of wild type, by dividing by the similarly determined amplification values for follicles of the parental line.

As seen in the upper part of Table 1, the level of amplified DNA was severely reduced in homozygous *272-9* follicles, more so for the third chromosome locus (4–7% of normal) than for the X chromosome locus (11–16%). Results were similar with *SD758* homozygotes and the *272-9/SD758 trans* heterozygote, verifying the allelism of the two mutations. Clear, but less extreme, suppression of amplification was also evident in *293-19* follicles (11–17% of normal for the third chromosome and 25–36% for the X).

The lower part of Table 1 shows amplified DNA levels in homozygotes and heterozygotes at two different temperatures. The high levels for *TM3* heterozygotes at 25°C demonstrate that *272-9* and *SD758* are recessives, as expected. Indeed, these levels are slightly higher than in the parental line; we have observed such minor differences in amplification levels between wild-type lines (unpublished observations). The levels for *SD758/TM3* heterozygotes are somewhat lower at 18°C than at 25°C: apparently, the lower temperature interferes slightly with amplification. Nevertheless, the levels for *SD758* homozygotes are substantially *higher* at 18°C than at 25°C, demonstrating the temperature sensitivity of that mutation. At 18°C neither *SD758/SD758* nor *SD758/272-9* show levels as high as those of the *SD758/TM3* heterozygote: apparently, the restoration of *SD758* function at 18°C is extensive but not complete.

The amplified DNA levels in *108-17* are very close to those of the parental line. A slight decrease in amplification is seen in *350-7*, but this effect is much more minor than in *272-9*, *SD758*, or *293-19* and within the range of differences observed among wild-type strains or at different temperatures (see above).

DISCUSSION

These and previous results (16, 17) demonstrate that it is possible to identify genetic defects of the eggshell by screening a collection of mutants with visibly abnormal egg morphology and/or female sterility (5 of 20 stocks in the present study). In this manner we have identified four loci on the third chromosome that affect choriogenesis and have assigned them to two morphological and biochemical classes.

Table 1. Levels of amplified DNA in late follicles

Chorion in chromosome	% of amplified DNA levels					
	Homozygous mutants at 25°C					
	*272-9**/*272-9*	*SD758**/*SD758*	*272-9**/*SD758*	*293-19**/*293-19*	*350-7*†/*350-7*	*108-17*†/*108-17*
X	11–16	15	8–15	25–36	71–72	82–85
3	4–7	8–12	7–8	11–17	57–60	93–100
	Homozygotes and heterozygotes at 25°C and 18°C					
	272-9/*TM3* (25°C)†	*SD758*/*TM3* (25°C)†	*SD758*/*TM3* (18°C)†	*SD758*/*272-9* (18°C)†	*SD758*/*SD758* (18°C)†	*SD758*/*SD758* (25°C)*
X	100–112	108–124	77–88	41–51	47–54	15
3	107–140	133–173	87	53–59	60–67	8–12

*Relative to rusteca follicles at 25°C.
†Relative to rutipa follicles at 25°C.

It should be noted that all five mutants map far from the major chorion gene cluster at 66D12-15 and on a different chromosome than the chorion locus at 7F1-2. The visible chorion defects and the female sterility appear to map together, within the limits of our recombinational analysis; furthermore, these two phenotypes as well as the biochemical defects are temperature-sensitive in *SD758* and show allelism between *SD758* and *272-9*.

Three mutants (two loci) show clear effects on chorion gene amplification. According to the mapping results, these effects must be mediated by *trans*-acting factors. Within the limits of our analysis, the amplification defects fully account for the underrepresentation of major chorion mRNAs and for the reduced synthesis of the corresponding proteins; presumably they also account for the morphological chorion defects and the female sterility (this presumption is particularly strong for the *272-9/SD758* locus). The effects are most severe in *272-9/SD758* and most noticeable for the third chromosome locus, which is normally amplified to a higher level than the X chromosome locus (9, 10). At least three *trans*-acting amplification mutants are already known from the X chromosome (ref. 9 and unpublished observations). By contrast, only a single *cis*-acting amplification mutant is known, the ocelliless (*oc*) inversion that breaks within the 7F1-2 chorion locus (8, 29). However, strong *cis* effects of chromosomal position on amplification have been documented in transformed flies (12, 13).

The apparent genetic complexity of *trans* effects on amplification raises the question of whether the factors involved are tissue-specific (i.e., involved only in follicle cell-specific amplification) or nonspecific (e.g., part of the general replication apparatus). Partial evidence toward discriminating between these alternatives should be provided by analysis of additional alleles. Genes coding for general replication factors would presumably be lethal if completely inactivated, whereas some of their hypomorphic alleles might yield activity sufficient for survival but insufficient for the demands of rapid DNA synthesis during amplification. Indeed, we now consider the hypomorphic, X-linked amplification mutant *K451* as nonspecific since it is allelic to *mus101* (B. Baker, personal communication), a temperature-sensitive lethal with effects on mutagen sensitivity and on condensation of heterochromatin (30–32). Inability to be mutated to a lethal phenotype may be a necessary criterion for highly specific genes involved in regulating amplification. In this connection, it is suggestive (although, of course, not definitive) that the two available mutant alleles of the *272-9/SD758* locus have normal viability and show no obvious phenotypes that cannot be accounted for by the drastic effects on amplification.

The second class of mutations, those that disrupt the structure of the chorion without significantly affecting the amplification or expression of major chorion genes, is potentially both diverse and interesting. At least one X-linked locus [*fs(1)384*] that falls into this class has been identified previously (16, 17). Such mutations may correspond to the as yet-unidentified structural genes for the ≈15 minor proteins evident on two-dimensional electropherograms of purified chorion (2, 4): only one minor chorion component (the s70 protein) has been mapped to date to the tip of the X chromosome (33). Alternatively, mutations of this second class may affect minor chorion proteins indirectly (e.g., via regulation of their synthesis or modification), may affect morphogenetically important components that are not incorporated in the final chorion, or may impinge on choriogenesis through mechanisms of cell interaction, differentiation of cell subpopulations, or specialization of the secretory surface. Further genetic and biochemical analysis is clearly in order for these and other eggshell mutants. This analysis may also uncover mutations of interesting, but as yet, unobserved types: those that might affect the amplification or expression of some, but not all, major chorion genes.

We are grateful to C. Nüsslein-Volhard for hospitality to P.B.S. during her mutant screen and to her and D. Lindsley for making the mutants available. We thank U. Potter-Damoulakis, A. Koniaris, and B. Klumpar for technical assistance and photography and E. Fenerjian for secretarial assistance. The work was supported by a National Institutes of Health grant to F.C.K. and a grant from the Greek Ministry of Research and Technology to V.K.G.

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