Mutants suppressing in trans chorion gene amplification in Drosophila
(eggshell/female-sterile mutants)

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ABSTRACT Two recessive female-sterile mutants, K451 and K1214, disrupt chorion formation by causing underproduction of all major chorion proteins. We present evidence that this effect is due to underaccumulation of the chorion mRNAs and that, in turn, this is caused by a substantial reduction in the level of chorion gene amplification. The mutants are X-linked but located at two sites far from the chorion gene cluster at 7F1-2; their effect is even more pronounced on the third chromosome chorion gene cluster, and thus the wild type gene must act in trans. The time course of amplification in mutant and wild-type follicles is documented.

Formation of the chorion in Drosophila melanogaster is a precisely regulated developmental program that takes place in the late stages of oogenesis. During a period of only 5 hr, the various chorion layers are successively laid down by the highly specialized follicular epithelium that envelops the oocyte. Approximately 20 proteins have thus far been identified as chorion components, of which 6 are quite abundant (1, 2). The “major” genes encoding these abundant proteins are tightly clustered into two distinct chromosomal loci. The X chromosome locus at 7F1-2 includes two major chorion genes, s36-1 and s38-1, which are expressed in the early stages of choriogenesis, plus several additional minor genes (refs. 3–5; unpublished observations). The other four major chorion genes, s15-1, s16-1, s18-1, and s19-1, which are expressed late in choriogenesis albeit not in a strictly coordinate fashion, are clustered on the third chromosome at 66D12-15 (5–8).

Both of these loci are differentially replicated in the follicular cells (9). Tissue-specific amplification begins by stage 9, at least 5 hr prior to the onset of choriogenesis, and has the effect of increasing the copy number of the major genes at a time when rapid chorion protein synthesis is required. Gene amplification is apparently needed for production of the normal, massive amounts of chorion proteins, although the expression of individual genes is subject to further temporal and quantitative regulation (8). In the chromosome each chorion gene cluster resides in a 50- to 100-kilobase (kb) amplification domain; maximal amplification occurs in the center of the domain, where the chorion genes are located (5). In the mutant, ocelliless, a small inversion with a breakpoint within the X chromosome cluster disrupts the amplification of that cluster (10). Apparently as a result, the s36-1 and s38-1 proteins are underproduced, an abnormal chorion is formed, and the embryos fail to develop (3, 11). In summary, amplification is itself a developmentally regulated phenomenon, an important subroutine within the overall developmental program of choriogenesis.

We have begun a genetic analysis in an effort to dissect and identify the various steps in the program of choriogenesis. cis-acting mutants (like ocelliless) that map in the vicinity of the chorion genes can be expected to help identify regulatory DNA sequences, such as the origins of amplification, promoters, temporal specificity elements, etc. We are even more interested in trans-acting mutants mapping elsewhere in the genome, for they are likely to identify previously unsuspected functions and ultimately illuminate the logic of the developmental program as a whole. In a series of ethyl methanesulfonate mutagenesis experiments conducted in Madeleine Gans’ laboratory, 28–31 genes affecting egg morphology and female fertility were identified on the X chromosome (12, 13). Upon further analysis, mutants in 9 of these genes were found to have prominent ultrastructural defects in the chorion (unpublished data). Here we report that two of these mutants define functional elements that operate in trans and are necessary for normal chorion gene amplification.

MATERIALS AND METHODS

Drosophila Strains. v21/v21 is the reference strain used in these experiments (12). The K1214 and K451 female-sterile mutants were induced by ethyl methanesulfonate mutagenesis on v21-marked X chromosomes (13) and kept in stock over the balancer FM3 (14). The FM3/K1214 sn1/v21 m K451 strain was constructed by us. The strain Df(1)HA92/FM7 (15) was obtained from the stock center at the California Institute of Technology. For convenience, homozygous strains will normally be indicated with a single allele notation.

Genomic Clones and Subclones. The genomic λAB clone and subclones encompassing third chromosome chorion genes have been described (8). An s36-1 cDNA clone, pDmC1-1, was isolated by George Thireos (personal communication) from the same follicle cDNA library previously used to isolate the s15-1 CDNA clone, pDmC1-1 (6). A corresponding genomic clone, Δ7F-1, was isolated by Ken Jacobs from a partial EcoRI genomic library (same as for λAB) constructed from embryonic Oregon R Drosophila DNA and the vector Charon 4 (16). The 18.9-kb genomic insert was digested with EcoRI and subcloned into pBR322 by Ken Jacobs. Two of the subclones, pDm3-6 and pDm3-8, contain the s36-1 and s38-1 genes, respectively, and correspond to the previously described subclones p103.48 and p104.41 (5). Two genomic subclones were used as controls: pCg 441-34-1, which contains a 6-kb fragment that hybridizes in situ to 55D-E (J. Rebers, personal communication), and p94r, which has a 3.6-kb genomic insert isolated from the decapentaplegic (DPP-C) locus at 22F1-3 (M. Hoffmann, personal communication).

Nucleic Acid Preparations. Newly eclosed flies grown at 25°C were conditioned for 2–5 days by daily transfer to fresh medium prior to dissection in cold Ringer’s solution (17). Staged follicles were selected (18) and total nucleic acids

Abbreviation: kb, kilobase(s).
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FIG. 1. Accumulation of chorion mRNAs during development in control and mutant strains. Equal amounts of total nucleic acids were isolated from follicles of the indicated stages and the RNAs were analyzed by RNA transfer blotting, using a mixture of probes corresponding to genes s38-1, s18-1, and s15-1 (for brevity, in this and subsequent figures the suffix –1 is omitted). Note the approximate equality of rRNAs in all samples (B). Also note the normal developmental specificities but underaccumulation of the mRNAs in the mutants and the more severe effect on mRNAs encoded by the third chromosome gene cluster (s18 and s15) in A.

were extracted as described (8). Plasmid DNAs were purified by standard procedures (16). After restriction enzyme treatment, DNA fragments were recovered from low-melting agarose gels (19).

**Nucleic Acid Electrophoresis, Transfers, and Hybridizations.** For RNA blots, total nucleic acids were treated with glyoxal and dimethyl sulfoxide (20) and fractionated on a 1.5% agarose gel, followed by transfer to diazobenzyl oxyethyl-paper (21). For Southern analysis, total nucleic acids

FIG. 2. The conclusions of Fig. 1 are generalizable for all 6 major chorion genes. The same blot as in Fig. 1 was sequentially dehybridized and rehybridized with probes corresponding to one or two well-separated major chorion transcripts (cDNA clones for s15 and s38; genomic subclones for s16, s18, s19, and s38). Only the portions corresponding to individual transcripts are shown. As noted (8), the amount of transient s15 RNA expression at stages 8–10 is dependent on the physiological condition of the flies; here the amount in the wild type was too low to permit evaluation of whether transient expression also occurs in the mutants.

FIG. 3. Time course of chorion gene amplification and underamplification in the mutants. EcoRI-digested DNAs from follicles of the indicated stages from wild-type (v24) and homozygous mutant flies were blotted hybridized with a mixture of probes representing two control, unamplified loci (c; pCc441-34-1 and p94r) and the two chorion gene loci, on the third chromosome (2.4-kb HindIII genomic fragment containing genes s15 and s18) and on the X chromosome (pDm3-6 containing gene s38). (A) Comparisons of the band intensities within a lane clearly show underamplification of both chorion loci in the mutants. The intensities of control bands are proportional to the amount of total DNA in each sample. (B) Two exposures from a similar experiment reveal the different time course of amplification in the two chorion loci. The stage 14 sample was incompletely digested, resulting in overlap of some partials from amplified DNA with the top control band.
were digested with EcoRI, fractionated on a 1% agarose gel, and transferred to nitrocellulose (22) after acid depurination (23). DNA probes were labeled by nick-translation (24). RNA-DNA hybridizations were carried out at 42°C in 50% (vol/vol) formamide/0.6 M NaCl, whereas DNA-DNA hybridizations were done in aqueous 0.6 M NaCl solution at 65°C. Autoradiograms were quantified by using an Ortec model 4310 densitometer.

Materials. Restriction endonucleases and DNA polymerase I were from New England Biolabs; DNase I was from Boehringer Mannheim and [α-32P]dNTPs (800 Ci/mmol; 1 Ci = 37 GBq) were from Amersham. Low-temperature melting agarose was purchased from Bethesda Research Laboratories and diazobenzoylomethyl-paper and nitrocellulose filters from Schleicher & Schuell.

RESULTS

Mutant Phenotype. As described elsewhere (13), two of the ethyl methanesulfonate-induced, X-linked mutants, K451 and K1214, are recessive female steriles that map to 12A6-7-12D3 and 5D5-6-6C12, respectively—loci that are distant from either chorion gene cluster. Chorion structure is severely disrupted in both mutants. Electrophoresis of eggshell proteins shows that, in both mutants, the levels of all 6 major chorion proteins are substantially reduced, relative to the wild type (unpublished data).

Accumulation of Chorion mRNAs in the Developing Oocytes of K1214 and K451. Follicles corresponding to stages 1–7, 8 + 9, 10, 11 + 12, 13, and 14 of oogenesis (as described by King, ref. 18) were isolated from ovaries of the mutants K1214 and K451 as well as the parent strain v^24. Total nucleic acid was extracted and 10 μg (1 OD260 = ca. 45 μg) of each preparation was fractionated on a 1.5% agarose gel. Prior to transfer to diazobenzoylomethyl-paper the gel was stained with ethidium bromide to verify that approximately equal amounts of RNA were present in each lane (Fig. 1B). The filter was initially hybridized with a mixture of 32P-labeled probes, representing the s15-1, s18-1, and s38-1 chorion genes. As shown in Fig. 1A, all three types of chorion mRNAs are represented in the mutants in amounts substantially lower than in the control; the effect is more pronounced in K1214. Despite this quantitative reduction, the temporal pattern of mRNA accumulation is not affected. Thus, in the mutants as in the wild type, s38-1 mRNA is most abundant at stages 11–13, s18-1 mRNA is most abundant at stage 13, and s15-1 mRNA is most abundant at stage 14.

To determine whether this phenomenon holds true for all
of the major chorion genes, the same RNA blot was dehybridized and sequentially rehybridized with probes specific for each of the major chorion mRNAs. Fig. 2 confirms that, relative to wild type, all of the major chorion mRNAs are present at lower levels in the mutants K451 and K1214; furthermore, in both mutants all major chorion mRNAs exhibit a normal temporal pattern of accumulation, despite the quantitative reduction. According to densitometric estimates, the mRNA accumulation relative to wild type is significantly lower for the s15-1 through s19-1 components encoded in the third chromosome (6–12% of wild type for K451; 3–7% for K1214), as compared with the s36-1 and s38-1 components encoded in the X chromosome (23–31% for K451; 12% for K1214). For these six mRNA sequences, the K451/K1214 abundance ratio ranged from 1.3 to 2.9.

Amplification Levels of the Major Chorion Gene Clusters in K451 and K1214. The consistently lower mRNA levels for all major chorion components suggested the possibility that the mutants might be defective in amplification. To test this possibility we performed the following carefully controlled Southern hybridizations. Total nucleic acids were isolated from staged follicles of K1214, K451, and v^{54} flies. Equal amounts were subjected to EcoRI digestion and fractionated by electrophoresis on a 1% agarose gel. The DNA was then transferred to nitrocellulose and hybridized with a mixture of four different ^{32}P-labeled probes, representing the third chromosome chorion gene cluster (s15-1 and s18-1), the X-chromosome chorion gene cluster (s38-1), and two control, single-copy sequences that are not amplified during oogenesis (from the vicinity of an ec dys-steroid-inducible gene at 55D-E and from the decapentaplegic locus at 22F1-3). The hybridization intensities of bands derived from the two chorion loci were then evaluated densitometrically and compared to the internal controls of unamplified DNA fragments. DNA from follicular stages 1–7 was used as a baseline, establishing the expected signal ratio (chorion/control) prior to amplification. By themselves the signals of control bands indicated the relative amounts of DNA in each lane. Furthermore, because these control probes hybridized to genomic restriction fragments of significantly different sizes (13.5 and 3.6 kb), it was possible to monitor each lane for incomplete digestion or differential degradation by comparing the signals of the two control bands. For greater accuracy, the control bands were chosen so as to bracket the sizes of bands derived from the chorion clusters.

The results of this analysis unequivocally established that the level of amplification of the two major chorion gene clusters is severely reduced in both mutants, relative to the parental v^{54} strain (Fig. 3). The experiments permitted us to evaluate not only the final levels of amplification but also its developmental time course in the wild type and in the two mutants and for both chorion gene clusters. Results are plotted in Fig. 4.

According to our measurements, in the wild type the final levels of amplification are ∼20× and ∼80× for the X chromosome and third chromosome chorion gene clusters, respectively (Fig. 4A and B); both estimates are somewhat higher than previously published (5). The time course of amplification in the two clusters is quite distinct (Figs. 3B and 4). Amplification begins at stage 8 + 9 and increases slowly in both clusters until stage 10; the increase is slightly more rapid in the third chromosome and the third/X chromosome ratio reaches 2.5 at stage 10. As amplification accelerates, the disparity becomes much more prominent. Between stages 10 and 11 + 12, the gene copy level nearly doubles in the X but more than quintuples in the third chromosome. This disparity is maintained at stages 13 and 14, resulting in the final 4-fold difference in amplification levels between the two loci.

In both mutants, amplification seems to be delayed and only becomes detectable at stage 10 or later. As in the wild type, most of the amplification takes place at stages 11–13. The third chromosome again amplifies more extensively, but the difference between the third and X is not nearly as great as in the wild type (Fig. 4C). As a result, the effects of the mutations appear more severe for the third chromosome genes, relative to wild type: the final amplification level is approximately 4× (∼20% of the wild-type level) in the X chromosome locus and 5–7× (∼6–9% of the wild-type level) in the third chromosome locus. This difference correlates well with the more severe reduction in accumulation of mRNAs derived from the third chromosome (Figs. 1 and 2). Similarly, the effects of K1214 on amplification level seem to be greater than those of K451 (Fig. 4D), just like the respective effects on mRNA level. Indeed, in the mutants all of the reductions in mRNA level are in remarkably good agreement with the reductions in the corresponding gene dosage, con-

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Fig. 5. Extent of amplification in various strains. (Upper) Lanes 2–12, DNAs from stage 12 and 13 follicles, nearing the plateau of amplification (see Fig. 4), were collected from each of the strains indicated (Lower). Lanes 1 and 13. DNAs from Oregon R embryos were used. After EcoRI digestion, blot hybridization was performed by using the same probes as in Fig. 3. After densitometry, the third/X and C/X ratios from lanes 1 and 13, corresponding to unamplified DNA, were used to normalize all data. Results are presented in lower, where significantly underamplified samples are boxed.
considering both the accuracy of these measurements and the fact that the mRNAs accumulate while the gene dosage is changing, with different kinetics in wild type and mutants. Thus, the reduction of mRNA levels in the mutants appears to be due to the underamplification of the corresponding genes.

**Genetic Analysis of the Amplification Defect.** To further elucidate the nature of the mutants, we compared the levels of amplification in stages 12 + 13, for a variety of strains (Fig. 5 Upper and Lower). The mutations are totally recessive, since essentially normal levels of amplification are attained with a single wild-type copy of the K1214 and K451 genes (lanes 5, 7, and 8), even in the double heterozygote (lane 9). The double homozygote (lane 3) shows approximately half the level of amplification seen in either single homozygote (lanes 4 and 10), although this comparison is not controlled for the slightly different genetic background of the double mutant chromosome.

A deficiency exists that encompasses the K451 locus [Df(1)HA92, location 12A6-7-D3]. This deficiency, equivalent to a null allele, permits further characterization of the K451 mutant. As shown in Fig. 5 Upper and Lower, the levels of amplification are: +/+ , K451/+ , HA92/+ >> K451/K451 > K451/HA92. Thus, the K451 gene product is hypomorphic: it serves a function that is necessary for amplification, with an efficiency substantially lower than half that of the wild-type gene product but clearly higher than no gene product at all.

**DISCUSSION**

The underproduction of all major chorion proteins in the mutants K451 and K1214 was traced to underaccumulation of the corresponding mRNAs, and this, in turn, was traced to severe underamplification of the corresponding genes. Quantitative differences between the two chorion loci as well as between the two mutants helped establish that the mRNA deficiencies closely parallel the gene dosage deficiencies, within the accuracy of the measurements and the inherent limitations of comparisons involving changing concentrations of template and product. The temporal specificities of mRNA production were shown to be unaffected, with only the amounts of each mRNA reduced in approximate proportion to the reduction in gene dosage. Thus, it appears that the chorion defects of these mutants are completely explainable by the amplification defects.

K451 and K1214 are recessive alleles of two genes that are required for normal amplification. These genes apparently act in trans, through a diffusible factor, since they are either very far removed or on a different chromosome from the chorion structural gene loci. There is no indication that the genes are functionally equivalent: no defect is associated with the double heterozygote. At least K451 is hypomorphic.

We do not know whether the effects of these mutants on amplification are direct or indirect. We cannot exclude the possibility that they affect a nonspecific process, such as generalized chromatin structure, which, in turn, influences the probability of amplification. However, the normal temporal specificities of mRNA production and the approximately normal quantities of mRNA produced per gene copy clearly exclude trivial explanations such as cell death or grossly deficient metabolism. It is intriguing that the onset of amplification appears to be delayed in the mutants and that the normally much more amplified third chromosome locus is disproportionately affected, although it still shows an amplification level somewhat higher than that of the X chromosome locus.

The most interesting possibility would be that the mutants impinge directly on the amplification mechanism. Certainly the regulation of differential amplification can be expected to involve multiple diffusible factors, especially in view of the complexity of the replication machinery in prokaryotes. To understand how the genes defined by these mutants act, we have begun an effort to clone and functionally characterize them by molecular procedures and P-element-mediated transformation.

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