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Genetics

Amplification of genes for chorion proteins during oogenesis in *Drosophila melanogaster*

(*ocelliless* mutation/polyploidization/DNA puffs/ovarian follicle cells)

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ABSTRACT **The endochorion and exochorion of *Drosophila* eggs are synthesized by the ovarian follicle cells during a brief period of about 5 hr. In this terminal phase of egg chamber development, the structural genes for several abundant chorion proteins are expressed at high levels according to a temporally regulated program. The female-sterile mutation *ocelliless* maps at the site of the genes for two of these proteins, the 36,000- and 38,000-dalton chorion proteins (c36 and c38), which are closely linked. The mutation results in a *cis*-acting reduction in the amounts of c36 and c38 that accumulate in late-stage egg chambers. We have investigated the mechanism that underlies this decreased production by using cDNA clones complementary to these gene sequences. Unexpectedly, it was found that, in normal females, the genes for c36, c38, and at least one other chorion protein are specifically amplified more than 10-fold in the DNA of late-stage egg chambers. The extra replication involves at least some adjacent chromosomal sequences and begins prior to the onset of mRNA and protein synthesis. The additional DNA remains stable after gene expression has ceased. The behavior of these genes is thus reminiscent of the properties of the DNA puffs that have been described in several groups of Diptera. The extent of amplification of c36 and c38, but not of the 18,000-dalton chorion protein c18 (which is unlinked), was decreased in the egg chambers of flies homozygous for *ocelliless*, suggesting that altered gene dosage may be responsible for the decreased synthesis of chorion proteins in the mutant.**

The idea that cell differentiation occurs through the selective activation and repression of genes which are themselves equivalent in the different cells of an organism is widely accepted. However, numerous exceptions to the "rule" of DNA constancy are also known (1–10). In the oocytes of many species, the genes for rRNA are specifically amplified, presumably to allow production of large numbers or ribosomes which are incorporated into the egg (8, 9). Amplification of genes coding for abundant proteins, however, has not been observed. No increase in the amount of DNA coding for silk fibroin occurs in the posterior silk gland (11, 12). The gene for ovalbumin is present in the same concentration in the tubular gland cells of the avian oviduct as in other tissues (13), and the genes for globins in the erythropoietic tissues of mammals and birds are not amplified (14, 15). Furthermore, calculations (16) and direct measurements (17, 18) suggest that rates of transcription and translation are sufficient to allow a single gene to produce the observed amount of product during the course of differentiation of these cells. In all these cases, however, the cells are able to obtain sufficient quantities of mRNA by accumulating stable mRNA molecules over a period of several days. In other cases, such as during the synthesis of the insect eggshell by the ovarian follicle cells (19–26), a series of proteins are produced sequentially and at high levels within individual cells. *Drosophila melanogaster* is a dipteran insect that produces eggs and eggshells at a remarkably rapid rate. In this case the length of time a follicle cell produces a specific mRNA is about 1–2 hr (22). In such cases the required synthetic rates may be higher than in cells accumulating stable RNA.

Prior to chorion gene expression, the follicle cells undergo several rounds of DNA replication in the absence of cell division, reaching a DNA content of 16 times the DNA content of the haploid germ-line genome (16c) by stage 8 (10). Further increases take place, but exact doubling of the DNA content is no longer observed, suggesting the possibility of unequal replication of some sequences. At the time of onset of chorion gene expression late in stage 10, the follicle cell nuclei contain about 45c of DNA, a value which is not observed to increase further (10).

A second consideration also suggested that alterations at the DNA level might occur in *Drosophila* ovarian follicle cells. We have reported (24) that the female-sterile mutant *ocelliless* results in a *cis*-acting reduction in the amount of two chorion proteins, of 36,000 and 38,000 daltons (c36 and c38), which are present in late-stage egg chambers and purified chorions. The structural genes for both proteins have been mapped at the site of the mutation near 7E11 on the X chromosome (24, 26), where they are present in a single copy per haploid genome in embryonic DNA (26). Preliminary studies of the DNA from *ocelliless* females, however, suggested that it contains considerably less DNA complementary to these genes than does wild-type female DNA. We now report that specific amplification of the genes for both c36 and c38 as well as some flanking DNA sequences does occur in late-stage egg chambers. The extent of this increase is less in *ocelliless* females. However, a high degree of amplification of at least one other chorion protein gene which is unlinked to c36 and c38 (26) occurs in both normal and mutant females.

MATERIALS AND METHODS

Purification of DNA. Purification of plasmid DNA was carried out under P2-HV1 levels of containment as specified by National Institutes of Health Guidelines for Recombinant DNA Research.

DNA from flies was prepared by grinding 200–1000 flies in 1–5 ml of 50 mM Tris·HCl, pH 8/100 mM EDTA/0.35 M sucrose in a small glass homogenizer with a ground-glass pestle. After centrifugation at 16,000 × *g* for 10 min, the supernatant was removed and the pellet was resuspended in 1–2 ml of extraction buffer (50 mM Tris·HCl, pH 8/10 mM EDTA/0.5% $NaDodSO_4$). After digestion at 37 °C for several hours with 500 μg of proteinase K (E. Merck) per ml, DNA was extracted twice with phenol. DNA from ovaries or egg chambers was purified by disrupting the tissue in 1–2 ml of extraction buffer in a

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Abbreviations: kb, kilobase(s); c, the DNA content of the haploid germ-line genome of an organism; c38, c36, and c18, 38,000-, 36,000-, and 18,000-dalton chorion proteins, respectively.

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Dounce homogenizer and proceeding as for whole-fly DNA. DNA concentrations were determined by the diphenylamine method (27).

Gel Transfer Hybridization. *Drosophila* DNA inserts purified by preparative electrophoresis of *Eco*RI- digested plasmids were labeled by nick translation in the presence of [^{32}P]dATP to a specific activity of 4×10^7 cpm/μg. DNAs were digested with *Eco*RI in 100 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/50 mM NaCl at 37°C. Completeness of digestion was tested by incubating a small aliquot of the reaction mixture separately in the presence of 0.4 μg of λ DNA. Digested DNAs were separated, stained with ethidium bromide, and transferred to nitrocellulose according to Southern (28). Prehybridization, hybridization for 36–48 hr, washing, and autoradiography were as described (26).

Preparation of *Drosophila* cDNA clones. Dmc5F9 and Dmc5G2 are plasmids containing about 600 nucleotides of *Drosophila* DNA inserted in pBR322. They were selected from a library of cDNA clones prepared from the poly(A)-containing RNA of stages 11–14 egg chambers (26). Dme8D10 was selected from the same library as a colony showing hybridization to poly(A)-containing RNA from early embryos but which did not react with RNA from stages 11–14 follicle cells.

RESULTS

Hybridization to Gel Blots for Assay of Chorion Gene Number. Probes can be hybridized to gel blots (28) under conditions that yield a signal proportional to the concentration of complementary sequences in the digested DNA (29). To test for changes in chorion gene number, DNAs of interest were digested with *Eco*RI and gel blots were hybridized with a mixture of ^{32}P-labeled cloned cDNAs complementary to two specific genes. One of these, Dme8D10, served as an internal control and the other was complementary to the chorion gene of interest. By comparing the absolute and relative intensities of specific restriction fragments labeled by each probe, differences in the concentrations of these genes in the DNAs could be determined.

Fig. 1 describes some properties of Dme8D10 that are relevant to its use as a control in these experiments. Ovarian RNA selected by hybridization to plasmid DNA was translated *in vitro* into a 17,000-dalton protein (Fig. 1*A*). *In situ* hybridization of RNA transcribed from Dme8D10 resulted in strong labeling at 11F and a weakly labeled site at 47A in the salivary gland chromosomes (not shown). Hybridization of the 450-nucleotide *Drosophila* cDNA insert from the plasmid to several different restriction digests of genomic DNA indicated the presence of three or four bands containing complementary sequences. In the case of *Eco*RI digests, bands 2.6, 2.3, and 1.0 kilobases (kb) long were observed (Fig. 1*B*). Thus, Dme8D10 is complementary to a small family of genes coding for a 17,000-dalton protein, which are present in euchromatic regions of the genome.

Wild-Type Females Contain Increased Amounts of Chorion DNA Which Are Present Only in the Ovary. Fig. 2 shows the hybridization of Dme8D10 and Dmc5F9 sequences to *Eco*RI digests of genomic DNA from various sources. Dmc5F9 is complementary to a single 4.0-kb *Eco*RI fragment that contains coding sequences for c36. The intensity of the 4.0-kb fragment relative to the 2.6-kb fragment labeled by Dme8D10, as well as its intensity per microgram of DNA, depends on the source from which the DNA is obtained. Similar amounts of hybridization were observed with DNA from embryonic nuclei (lane 1), wild-type males (lane 2), or *ocelliless* males (lane 3). However, DNA from wild-type females produced significantly greater labeling of the chorion gene-containing fragment. This increased labeling of the 4.0-kb fragment was not seen with

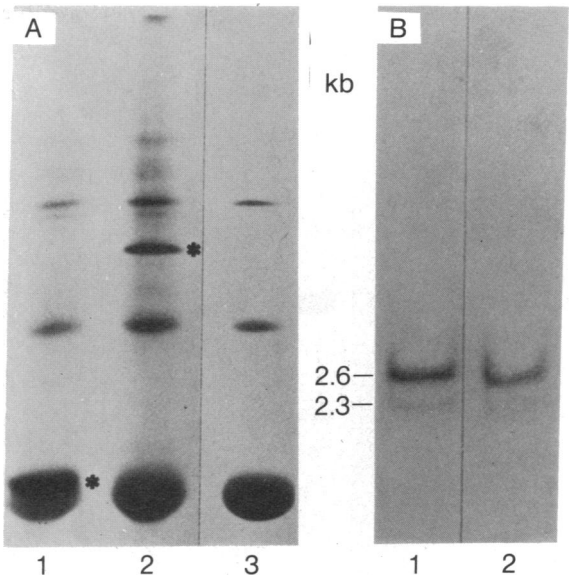

FIG. 1. Characterization of Dme8D10. (*A*) Plasmid DNA (10 μg) from Dme8D10 lane 1 and Dmc5F9 lane 2 was bound to nitrocellulose and hybridized to RNA from 50 ovaries as described (30). Hybrid-selected RNA was translated in a reticulocyte cell-free system in the presence of [^{35}S]methionine, and the products were separated on a 10–15% gradient acrylamide gel. The asterisks indicate the position of migration of specific polypeptides of 17,000 daltons (lane 1) and 37,000 daltons (lane 2) that were not observed in the absence of added RNA (lane 3). (*B*) DNA (1 μg) from wild-type (lane 1) or *ocelliless* (lane 2) female adult flies was digested with *Eco*RI, separated on a 0.8% agarose gel, and transferred to nitrocellulose. Hybridization was with ^{32}P-labeled *Drosophila* DNA purified from Dme8D10 (1.5×10^5 cpm/ml). Bands of DNA of 2.6 and 2.3 kilobases (kb) are shown. An additional fragment of 1.0 kb is also complementary to Dme8D10 but was not retained on this gel.

DNA from *ocelliless* females, however (lane 5). Hybridization of the probes to DNA purified from the ovaries (lane 6) and the nonovarian tissues (lane 7) of wild-type flies provided further information. The concentration of sequences complementary to Dmc5F9 was increased only in the ovary and could not be detected in other female tissues. The 2.6-kb band labeled by Dme8D10 showed only the 2-fold increase in labeling of female DNA compared to male DNA which is expected for an X-linked gene, suggesting that it may derive from 11F. The 2.3-kb fragment was equally labeled in male and female DNAs and hence may be located at 47A.

DNA Amplification During Oogenesis. The observation that DNA from the tissue expressing the c36 gene shows an increased hybridization with a probe specific for this sequence suggests that a differential replication of this gene takes place in the ovary. To relate the increased synthesis of chorion DNA to the time of its expression, DNA from groups of staged ovarian egg chambers were hybridized to the same probes used previously. A large increase in chorion gene labeling occurred, beginning at stages 9–10 (Fig. 3*B*). Egg chambers at stages 11–13, which synthesize c36 protein (21, 23) and its mRNA (22), contained the largest concentration of this gene. Densitometer scans of the autoradiograms (Table 1) showed that the 4.0-kb band was increased, relative to the 2.6-kb band, by about 12-fold compared to egg chambers at stages 1–8. The relative labeling of the 4.0-kb and 2.6-kb bands was unchanged in DNA from stage 14 egg chambers, although the absolute intensity of both bands was decreased per egg chamber due to degeneration of follicle cells late in stage 14. c36 synthesis ceases prior to stage 14.

The increased labeling of the c36 gene in DNA from late-stage egg chambers occurred in spite of the fact that the total

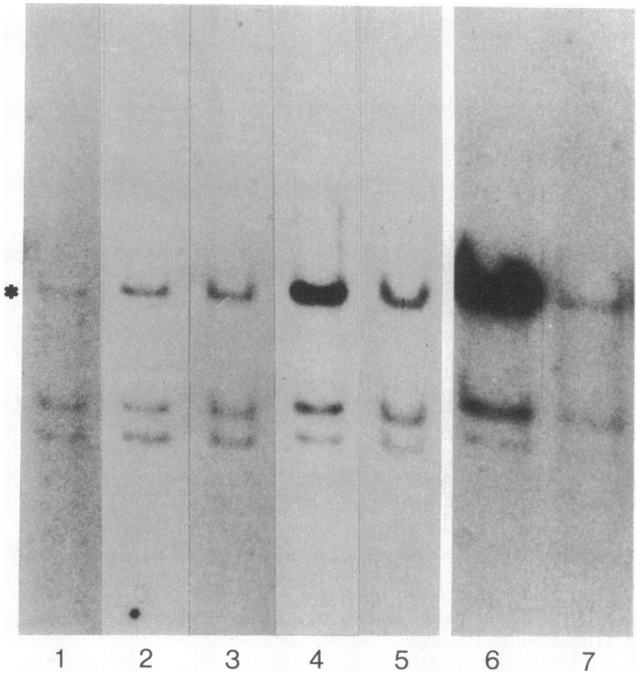

FIG. 2. Relative amounts of DNA complementary to Dme8D10 and Dmc5F9. DNA (0.8–2.0 μg) from embryonic nuclei purified as described (31) (lane 1), wild-type males (lane 2), *ocelliless* males (lane 3), wild-type females (lane 4), and *ocelliless* females (lane 5) was digested and transferred to nitrocellulose. *Drosophila* DNA from Dme8D10 (1.7×10^5 cpm/ml) and Dmc5F9 (1.9×10^5 cpm/ml) was hybridized to a single filter containing the DNAs. In a separate experiment, DNAs purified from the ovaries (lane 6) and the nonovarian tissues (lane 7) of wild-type adult females were analyzed with Dme8D10 (1.5×10^5 cpm/ml) and Dmc5F9 (3.4×10^5 cpm/ml). Incubation of the filters with the probes was for 48 hr to ensure that saturating amounts of hybridization were obtained. The 4.0-kb band (asterisk) is complementary to the Dmc5F9 probe; the lower two bands (2.6 and 2.3 kb) are complementary to Dme8D10.

amount of DNA per egg chamber decreases during these stages due to the breakdown of nurse cells after stage 10 and of follicle cells late in stage 14 (20). This decrease can be seen in Fig. 3A, which shows the pattern of staining of the gel with ethidium bromide; it also can be seen in the decreased hybridization of Dme8D10 per egg chamber after stage 10.

Ocelliless females produce decreased amounts of c36 and contain a lower amount of the c36 gene in the DNA of whole

Table 1. Relative amount of DNA complementary to c36 in DNA from various sources

Source of DNA	Ratio of fragments, 4.0 kb/2.6 kb
Wild-type males	1.4
Ocelliless males	1.3
Wild-type females	5.1
Ocelliless females	1.5
Wild-type ovaries	7.5
Wild-type carcass (ovaries removed)	1.0
Wild-type egg chambers, stages 1–8	1.0
Wild-type egg chambers, stage 10	6.2
Wild-type egg chambers, stages 11–13	12
Wild-type egg chambers, stage 14	12
Ocelliless egg chambers, stages 1–9	1.3
Ocelliless egg chambers, stage 10	4.9
Ocelliless egg chambers, stages 11–13	4.2
Ocelliless egg chambers, stage 14	4.1

Data are from the experiments of Figs. 2 and 3 and from replicate experiments. Autoradiograms were scanned and the peaks integrated electronically with a densitometer (Helena Laboratories Beaumont, TX). Several exposures of each filter, in the presence and absence of an intensifying screen, were measured. The data presented represent the intensity ratio of the 4.0-kb and 2.6-kb bands as measured on films exposed so that they were within the linear range of response of the film as determined by the analysis of known amounts of DNA present on each filter. In the case of DNAs with very different intensities of the two bands, the ratios presented may underestimate the true values because the 2.6-kb fragment could not be detected on exposures in which the 4.0-kb band was within the linear range. The measured values in the case of ovary and carcass DNA have been decreased by a factor of 1.6 to correct for the specific activity of a different preparation of Dme8D10 used in this experiment.

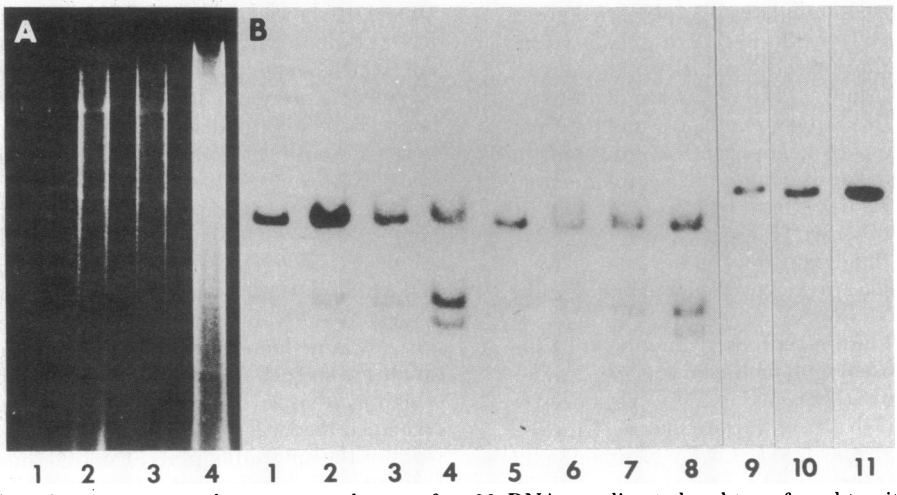

FIG. 3. Amplification of sequences complementary to the gene for c36. DNA was digested and transferred to nitrocellulose from various numbers of the following wild-type egg chambers: 65 in stage 14 (lane 1), 93 in stages 11–13 (lane 2), 65 in stage 10 (lane 3), and an undetermined number in stages 1–8 (lane 4). DNA was also transferred from the following homozygous *ocelliless* egg chambers: 85 in stage 14 (lane 5), 57 in stages 11-13 (lane 6), 68 in stage 10 (lane 7), and an undetermined number in stages 1–9 (lane 8). Known amounts of Dmc5F9 plasmid DNA that had been linearized by digestion with *Hin*dIII were also transferred from the same gel: lane 9, 3.7 pg; lane 10, 12 pg; lane 11, 36 pg. The pattern of ethidium bromide staining of lanes 1–4 of the gel prior to transfer is shown in *A*. DNA from the *ocelliless* egg chambers gave an identical pattern of staining (not shown). Despite the fact that the numbers of egg chambers in each lane were not exactly equal, it is clear that lane 4 contained the most DNA and lane 1 contained less than lanes 2 and 3 due to the degeneration of follicle cells late in stage 14. The discrete bands in *A* are *Eco*RI fragments of mitochondrial DNA (11 and 5.0 kb). After transfer, the filter was hybridized to the same probes as in Fig. 2, lanes 1–5. *B* shows an autoradiogram of the filter after an exposure of 48 hr. Bands of 4.0, 2.6, and 2.3 kb are present.

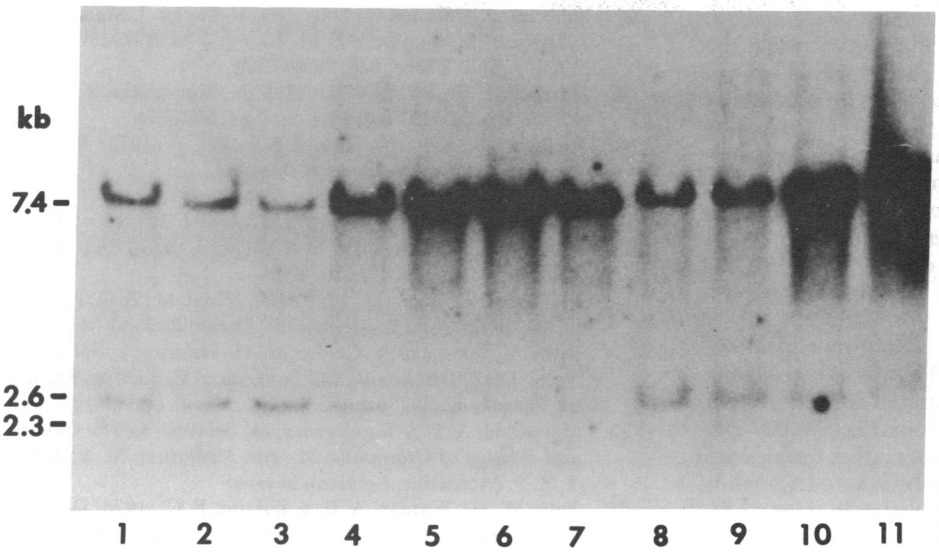

FIG. 4. Amplification of the c18 gene. DNA was digested and transferred to nitrocellulose from 45 wild-type egg chambers of the following stages: stages 1–8 (lane 3), stage 10 (lane 4), stages 11–12 (lane 5), stage 13 (lane 6), and stage 14 (lane 7). The same was done from 90 *ocelliless* egg chambers: stages 1–9 (lane 8), stage 10 (lane 9), stages 11–13 (lane 10), and stage 14 (lane 11). Also transferred was DNA (3 μg) from *ocelliless* (lane 1) and wild-type (lane 2) males. Hybridization was with Dme8D10 (1.5×10^5 cpm/ml) and Dmc5G2 (2×10^5 cpm/ml). The 7.4-kb band contains sequences complementary to Dmc5G2 (not shown). The autoradiogram was overexposed (100 hr) to illustrate the 2.6-kb and 2.3-kb bands complementary to Dme8D10. On shorter exposures, only a discrete band at 7.4-kb was apparent in the case of all the DNAs tested.

females compared to wild type. Fig. 3 demonstrates that c36 gene amplification is not as extensive in the mutant egg chambers. Relative labeling of the 4.0-kb band increased by a factor of only ≈4 compared to the level seen in stages 1–9 DNA from this strain (Table 1).

Amplification of a Chorion Gene Unlinked to *Ocelliless* Is Not Decreased. The lesion in *ocelliless* maps to the site of the c36 and c38 structural genes. Experiments similar to those described above but using a probe specific for c38 gene sequences demonstrated that the replication properties of this gene in wild-type and *ocelliless* DNA from females were indistinguishable from those of c36 (not shown). To see if the mutation affected the behavior of an unlinked chorion gene, the experiment of Fig. 3 was repeated using a probe specific for the c18 gene, which is located at 66D15 (26). As in the case of c36 and c38, a large amplification of the 7.4-kb DNA fragment containing the c18 gene occurred in egg chambers during and after stage 10 (Fig. 4). Unlike the genes closely linked to *ocelliless*, however, no reduction in the amount of DNA complementary to the c18 gene was seen in DNA from *ocelliless* egg chambers. The increased labeling of c18 sequences observed was even greater than in the case of c36 and c38. Because of the extreme differences in the intensities of the 7.4-kb and 2.6-kb bands in stages 11–14 egg chambers, the extent of the increase could not be measured accurately but was at least 30-fold. It could not be determined if the degree of amplification of the c18 gene in *ocelliless* egg chambers was equal to or greater than that seen in wild-type flies.

DISCUSSION

The results presented in this paper demonstrate that the DNA of late-stage *Drosophila* egg chambers contains a greatly increased concentration of several chorion genes. That this increase is truly an example of differential replication is supported by several arguments. At all stages of development, the follicle cells contain more than 50% of the egg chamber nuclear DNA. Direct measurements of the DNA content of follicle cell nuclei by Feulgen microspectrophotometry show that, at most, a 3-fold increase in DNA content occurs after stage 8 (10). The 12-fold increase in hybridizable chorion gene sequences present in stages 11–13 compared to stages 1–8 egg chambers can therefore only be the result of replication of these genes in the absence of replication of the bulk of the DNA.

Because hybridization was observed with DNA fragments of a specific size, the possibility that the increased labeling was due to an association not involving DNA·DNA base pairing is precluded. When different restriction enzymes were used in similar experiments (not shown), the increased labeling was always associated with fragments of the size observed after hybridization of the chorion gene probe to embryonic DNA (four enzymes and four chorion genes tested). A specific example of the behavior of a gene not expressed in the follicle cells is provided by Dme8D10. Hybridization to bands specific for this gene appeared to be in direct proportion to the total DNA, although small changes would not have been detected in these experiments.

Differential replication of specific nuclear DNA sequences can involve extrachromosomal molecules (32) or may take place on the chromosome itself (3, 33, 34). In the case of the DNA puffs that occur in the salivary gland of *Rhynchosciara* (3), a specific poly(A)-containing RNA whose production is correlated with the presence of a major DNA puff has been shown by *in situ* hybridization to contain sequences that are complementary to the puffed region (35). Evidence suggests that this puff may contain the gene for a major protein component of the cocoon produced by the salivary glands of the larvae (36). The fact that chorion gene number begins to increase prior to the onset of detectable mRNA production suggests that this process may be analogous to DNA puff formation because increased DNA staining is sometimes observed prior to the onset of transcription (RNA puff formation).

A significant difference between gene expression in the *Drosophila* follicular epithelium and in differentiated cells where amplification does not occur (11–16) is the time scale of the events. mRNAs for c38, c36, and c18 are detectable for a period of no more than about 2 hr (22). In contrast, the mRNAs for fibroin, ovalbumin, and globin are highly stable and accumulate over a period of several days. The rate of mRNA production per germ-line chorion gene may therefore be higher than in these cases, requiring an increase in gene dosage. Taking the transcription rate of the silk fibroin gene as a maximum (17, 18), it follows that the total amount of c36 mRNA that could be produced by a single-copy gene in the 1000 follicle cells (45c) of an egg chamber in 2 hr is: 10 mRNAs per gene per min $\times 4.5 \times 10^4$ genes per chamber $\times 1.2 \times 10^2$ min $= 5.4 \times 10^7$ mRNAs. Because each c36 mRNA is about 1.2 kb (26), this represents 34 pg. We estimate on the basis of poly([^{3}H]U) hybridization that the follicle cells of a stage 12 egg chamber contain 0.4 ng of poly(A)-containing RNA, 50% of which codes for c36 (22). Although this is consistent with the idea that amplification is required, more detailed measurements of the actual kinetic variables are needed.

A disruption in the amplification of the genes for c36 and c38 occurs in females homozygous for *ocelliless*. As a result, the number of copies of these genes is reduced in stages 11–13 egg chambers where they are normally expressed. This provides an explanation for the simultaneous reduction in the production of two proteins by the mutation. No changes in the size of restriction fragments containing chorion genes were observed during amplification in wild-type or *ocelliless* egg chambers. Because the c36 and c38 structural genes are only separated by about 2 kb in the genome (unpublished data), this suggests that the mutation reduces the replication of a contiguous region of DNA that contains both genes.

Is differential replication of protein-coding genes confined to the polyploid tissues of certain flies? If amplification of genes involved in the rapid production of a protein does occur in diverse classes of organisms, it might be found in cells that produce a succession of abundant polypeptides rather than a single superabundant one. Few such cases have been tested. Certainly, the occurrence of specific gene amplification in mammalian cells in response to selective agents (33, 37) suggests that this type of control mechanism may be of more general significance.

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