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THE USE OF GENETIC TRANSFORMATION IN THE STUDY OF OVARIAN-SPECIFIC GENE EXPRESSION

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Abstract

We are using genetic and molecular approaches to understand the mechanisms controlling the establishment of the cellular specificity of expression during oogenesis. Female-sterile mutations have been isolated and the molecular analysis is revealing interesting cell-cell interaction systems that work not only during oogenesis but also at other developmental stages. We will review in this paper our most recent studies on genes involved in ovarian development.

1. INTRODUCTION

Different cell types of germ-line and somatic origin are involved in oogenesis which in *D. melanogaster* has been divided into 14 morphological stages [1,2] and can easily be approached at genetic, cellular and molecular levels. To study the role of maternal genes involved in oogenesis and early embryogenesis the mutants to be selected have a large variety of phenotypes sharing a common characteristic: females are unable to generate offspring either because they are unable to lay eggs (female sterility) or because the embryos die (maternal effect). Notwithstanding the large collection of mutants isolated [3], it has been difficult to assign a functional role to the various genes. In addition, classical genetic approaches are unable to detect all the genes necessary for the production of a functional egg. It has been demonstrated that when a system of cell-cell communication has been well established, the genes involved will be used repeatedly during development [4]. Consequently, mutations in such genes will be zygotic lethal, and it will be impossible to assess the female sterility or the maternal effect produced by these genes. A very elegant method has been developed that allows the identification *in situ* of control elements of genes regulated during different developmental stages [5]. The method is called "P-element-mediated enhancer detection". The P transposon detects neighbouring genomic transcriptional regulatory sequences by means of a β-galactosidase reporter gene, which, in the majority of cases, responds to nearby transcriptional regulatory sequences in the *D. melanogaster* genome.

Other methodologies, such as *in situ* hybridization [6] or DAPI staining [7] of ovaries, isolated egg chambers and early embryos can provide significant insight into the role played by genes expressed at different stages of ovarian development.

Our interest in genes involved in oogenesis started with the study of mutations isolated by Sandler in region 32 of the standard salivary gland chromosome map [8]. The mutations are named *hold up (hup)*, *wavoid-like (wdl)*, *daughterless-abo-like (dal)*, and *abnormal oocyte (abo)* and are all recessive, hypomorphic and female semi-sterile. The semi-lethal phenotype shown by these mutations [8] and the demonstration of their zygotic action [9] seem to indicate that the corresponding genes are expressed not only during oogenesis but also in other tissues and developmental stages and that null alleles will be lethal for the flies.

Our present study on the control of gene expression during oogenesis avails of all the powerful recent methodologies (P-mediated mutagenesis, P local transposition and enhancer trapping) to isolate new mutations conferring female sterility to the flies in 32 and other regions of the second chromosome.

On the other hand, the molecular work carried out in the last years has led to the identification in region 32 of two genes with specific ovarian patterns of expression: the Vitelline Membrane Protein gene 32E [10,11] and the gene coding for a receptor form of Guanylate Cyclase [12]. We have started a detailed analysis of their structure and expression by P-mediated genetic transformation that in *Drosophila* is very well established [13].

We review in this paper the more recent data on the expression and regulation of these two genes, show the phenotypes of female sterile mutants recently isolated in our laboratory and illustrate the cellular specificity of expression of other genes identified in genetic screen by P-mediated enhancer detection or P-local transposition.

2. RESULTS AND DISCUSSION

Starting with a plasmid clone containing a segment from region 32D, we screened a library constructed in the EMBL4 lambda phage vector with embryonic DNA from *D. melanogaster Oregon R* stock and walked about 200 kb in this region, from band 32D to band 32E-F. We used the recombinant phages by the following approaches: 1) analysis of the transcription pattern of the DNA region isolated in our recombinant phages by screening of stage-specific cDNA libraries and 2) investigation of the molecular organization of region 32 to identify possible restriction enzyme site polymorphism correlated with the presence of one of the mutations under study.

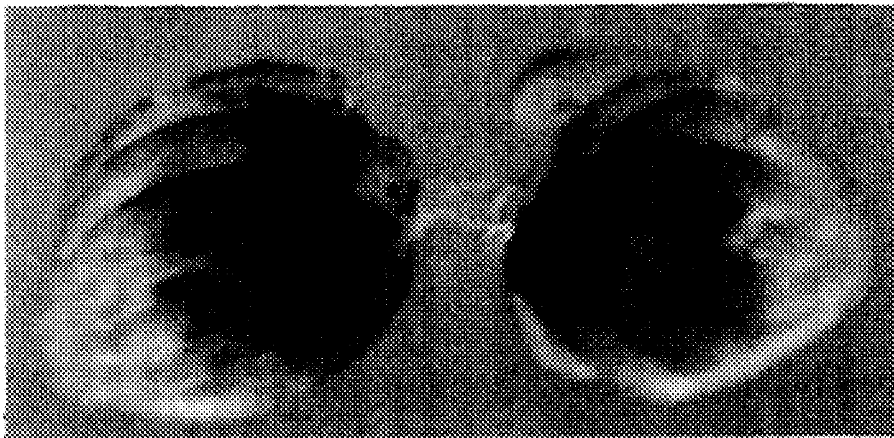
1.) We hybridized restriction fragments of the recombinant phages isolated during the chromosome walk in that region with labelled cDNA prepared from poly-A RNAs extracted from different developmental stages of *Drosophila* (embryos, larvae, pupae, adult females, and adult males). We identified in this way genes selectively expressed in particular developmental stages. We started our characterization with a fragment strongly hybridizing with a transcript present in females and not in 18 hr embryos, as happen with a maternal transcript and discovered the gene coding for a Vitelline Membrane Proteins (VMP). We called the gene VMP32E from his chromosomal location [10].

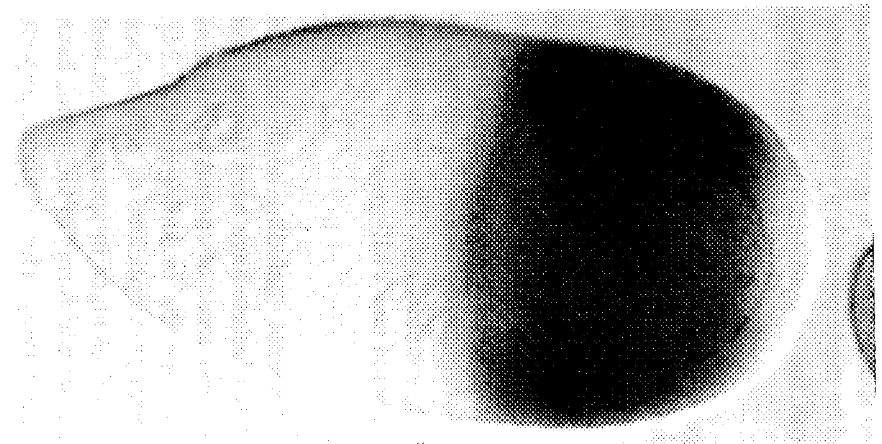
2.) By using as probes the DNA fragments from the phages isolated in the chromosomal walking, we performed restriction enzyme site polymorphism analyses in wild type and homozygous mutant flies. We identified, in stocks carrying the *abo* mutation, a copia-like *blood* transposon in region 32E. We demonstrated that the *abo* phenotypic expression is correlated with the presence of the *blood* transposon [14]. The membrane form of Guanylate Cyclase (GC) *Drosophila* homolog gene was cloned as the first gene downstream (4.2 Kb) from the *blood* transposon insertion site [12].

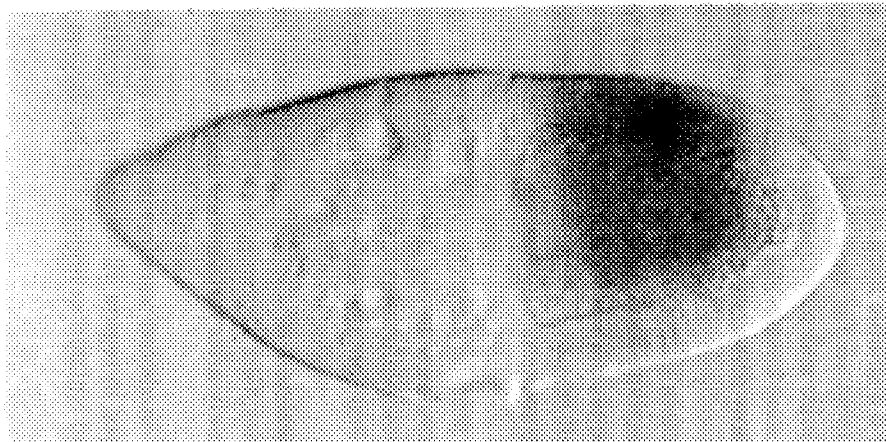
2.1. The 32E Vitelline Membrane Protein Gene

The VMP32E gene was characterized in detail. A peculiar feature of the VMP gene family was discovered: a conserved hydrophobic domain, constituted of about 30 amino acids, is present in all the genes so far isolated [10,15,16]. In the late stages of oogenesis, the follicle cells surrounding the growing oocyte are engaged in the elaboration of the egg-shells. The eggshell proteins are produced in a characteristic temporal and spatial pattern that reflects the transcriptional regulation of the respective genes. During stages 9-10 Vitelline Membrane Proteins are maximally synthesized, later, in stages 11-14, chorion proteins are produced. Synthesis of the proteinaceous eggshell in *D. melanogaster* is a system particularly well suited for cis-regulatory signals controlling gene expression in eukaryotes [17,18].

We are analyzing the VMP32 gene expression and regulation *in vivo* by P-mediated transformation. We have demonstrated the existence of a compartmentalization within the follicle cells. In transformation experiments performed with a VMP-β-gal gene fusion we demonstrated the existence of a cellular specificity of VMP32E gene expression [11]. Expression is localized initially in a very small ventral group of columnar follicle cells at stage 10A egg chambers and progressively extends to the dorsal region as a large stripe surrounding the oocyte in stage 10B. We do not observe expression in the polar terminal domains, which remain silent (Fig. 1). This is a peculiar feature of the VMP32E gene,







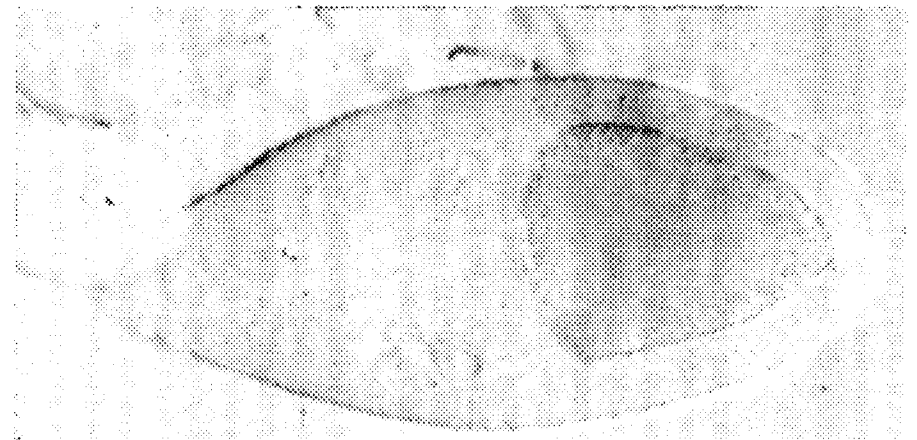


FIG. 1. ß-galactosidase activity in ovaries of females transformed with the VMP32E-ß-gal fused gene. The gene fusion carries the 5' upstream region plus the first 232 bp of the VMP32E coding region fused in frame with the b-gal gene. After P-mediated transformation, the ovaries of the transformed females were dissected by hand and examined by histological staining for b-galactosidase activity.

while the other genes so far tested, VM26A1 and VM26A2, are expressed all round the follicular cells, including the most posterior ones [15,19]. The peculiar temporal and spatial VMP32E gene expression may reflect a particular function of this VMP component and could be made possible by those genetic mechanisms co-ordinating the development of asymmetry in both the oocyte and the enveloping follicular epithelium [20].

We have dissected the promoter region of the VMP32E gene and we have found that DNA sequences extending from -465 to -39 bp upstream from the transcription initiation site are sufficient to direct the specific expression of the VMP32E gene. Deletion analysis in transformant lines revealed the presence of at least two cis-acting regulatory elements (-465/-249, -135/-39). Both these regulatory regions are necessary to determine the specificity of the VMP gene expression in ovaries [11]. We have recently demonstrated, in collaboration with F. Kafatos, that in the functionally defined -465/-249 fragment, where substantial similarities are found to the hormone response elements of chorion s-15 gene, the transcription factor CF1/Usp [21,22] which bind to the cis-regulatory sequences of the *s15* chorion gene also binds the VMP32E regulatory region (Gargiulo et al., unpublished). This provides the first indication that similar regulatory strategies may be shared by chorion and VMP genes.

2.2. The Guanylate Cyclase receptor gene

Cyclic GMP levels change in response to a great variety of agents, including hormones and neurotransmitters. Several soluble and membrane forms of Guanylate Cyclase have been studied and described in detail. However, only recently, by taking advantage of the existence of conserved domains of the proteins, the corresponding genes have been identified in several species, including man[23,24]. It has been discovered that Guanylate Cyclases in sea urchins are strongly implicated as cell-surface receptors on spermatozoa for chemotactic peptides causing behavioral changes in spermatozoa that include both kinetic and directional effects on motility. In mammals, several forms of GC genes have been described and they are expressed in aortic smooth muscle, brain, kidney, adrenal gland, adipose tissue, ileum, human placenta, pituitary gland, olfactory tissues, and retina.

Only recently also a soluble form of *Drosophila* GC gene has been isolated in 63A1-2 [25]. All the GC membrane proteins so far described have a single hydrophobic sequence that divides the molecule into extra cellular and cytoplasmic regions. The intracellular catalytic region contains two different domains: a tyrosine kinase-like and a cyclase domain (Reviewed by Garbers [23]). The various regions of the protein show different degrees of conservation among the species.

Although the protein kinase-like region contains 25 out of the 33 amino acids highly conserved in protein kinases, this activity has not been demonstrated for GCs. The molecular organization of the *Drosophila* receptor gene is complex: it is made up of many small exons, each one coding for less than 50 aa, separated by small introns. All the functional domains, including the small transmembrane and the catalytic regions, are separated by introns.

The *Drosophila* protein shows varying degrees of conservation with other Guanylate Cyclases and also with adenylate cyclases. In Fig. 2 is reported the putative translational product of the *Drosophila* receptor GC with the *Drosophila* soluble GC, as obtained by the pileup GCG Wisconsin program. The soluble form is smaller and contains only the cyclase catalytic domains underlined in the figure [25].

The level of expression of the GC gene is very low. The transcript is detected by *in situ* hybridization [6] in ovaries, first in germaria and later at stage 10 (Fig. 3). A signal is also present in very early embryos, immediately after fertilization and in larval imaginal discs ([12] and unpublished).

We are using P-mediated transformation to investigate if the GC gene can rescue any of the mutations already mapped in region 32, including *l(2)gd* that is a tumor suppressor gene affecting the imaginal disc growth and isolated in region 32E by Bryant and Schubiger [26].

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VTDGG LPLAI EDVNK NPNLL PGKKL AFKPV DIGHK MSAYR VKPLR AMTQM  50
REAGV TAFIG PDESC TTEAL LASAW NTPML SFKCS DPIVS NKSTF HTFAR 100
TLAPA SKVSK SVISL LNAFH WNKFS IVVSS KPIWG SDVAR AIQEL AEARN 150
FTISH FKYIS DYIPT TKTLS QIDKI IEETY ATTRI YVFIG EHIAM VDFVR 200
GLQNR RLLES GDYTV VSVDD EIYDS NRRVN IMERN YLDPY IRKEK SKSLD 250
KISFR SVIKI SMTYP QNPHI RVPIY GLHLY DSVMI YVRAI TEVLR LGGDI 300
YDGNL VMSHI FNRSY HSIQG FDVYI DSNGD AEGNY TVTTL QNDVG SGASI 350
GSLAK MSMQP VGFFA YDKNS VIPEF RYIKN DRPIQ WLNGR PPLAE PLCGF 400
HGELC PRKKL DWRYL VSGPL CALVV VVAIA LLIKH YRYEQ TLAGL LWKVD 450
MKDVT VINLG EYNNP TNKNI FQICR QSILV VGEPN KRSFT NIALF RGNIV 500
.................... .......... .......... ..........
MACPFFRRAD SLTRQPSVIA EPGGHWALED EELSDDALTL THLQMAIQLL

.......... .......... AMKKIHKKS VDI.TRSIRK ELKLMREVRH
TAPSNEDLNT AVTSLVAKYR QNWPNIHKLK LDPQTFKSCA NYDYLADI.Q

ENIINFIGAS TDHGSVII.. .FTTYCAR.. .......... ......GSLE
ELLLKMDEAS ASEILVLLGE ELITCCCTGI IERAFRCLGT DLQEFLGSLD

DVLANEDLHL DHMFISSLVS DILKGMIYLH DSEIISHGNL RSSNCL....
GVYDVLKLQE EDVTDTGFVC AGEGELIFTS ERPVIAWLLL GSLKALTRML

......IDSR WVCQISDFGL HELKLGQEEP .......... ..........
YKVDVNIKIE PVERCPAVSL PLLAGQGQLP DHADGSSTSV SKTIPETVQR

....NKSELE LK........ .RALCMAPEL .......... .LRDAYRPGR
SNSSNASDLQ MNSSSFCKMF PWHFIMNEQL ELVQLGRGFS KLYKPYMADF

GSQKGDVY.. .......... ......SFGI LLYEMIGRKG PWGDTAYSKE
GCQATTYFDF KRPKGLTMKF RDIVRRTYTP FLIGLNNPPG AVDFPAIGLE

EIIQFVKCPE MLQHGVFRPA LTHTHLDIPD YIRKCLCQCW DEDPEVRPDI
IKGQMVHCPE SNSLLFIGSP FLD...GLDG LTCNGL...F ISDIPLHDAT

RLVRMHLKEL QAGLKPNIFD NMLSIMEKYA YNLEGLVQEA NQFVVRREEE
RFVILVGEQA RAQ......D GLRRRMDK.. ..IKNSIEEA NSAVTKERKK

TDMLLYQMLP RPVAELLKRG DPVEAECFDC VTILFSDIVG FTELCTTSTP
NVSLLHLIFP AEIAEKLWLG SSIDAKTYPD VTILFSDIVG FTSICSRATP

FMVVEMLNDW YTCCDSIIWN YDVYKVVTIG DAYMVVSGLP LQNGSRHAGE
FMVISMLEGL YKDFDEFCDF FDVYKVETIG DAYCVASGL...HRASIYDAH

IASLALHLLE TVGNLKIRHK RTETVQLRIG VHSGPCAAGV VGQKMPRYCL
RCLDGLKMID ACSKHITHD. .GEQIKMRIG LHTGTVLAGV VGRKMPRYCL

FGDTVNTASR MESTGDSMRI HISEATYQLL QVIGSYVC.I ERGLTSIKGK
FGHSVTIANK FESGSEALKI NVSPTTKDWL TKHEGFEFEL GPRDPSFLPK

......GDMR TYWLTKRQQP ELTPGSD... .......... ..........
EFPNPGGTET CYFLESFRNP ALDSELPLVE HINVSMKTIS EGAMPRSPVA

....
PIPP
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FIG. 2. Amino acid sequence comparison between Drosophila *receptor and soluble form guanylate cyclases, as shown by alignment produced by the pileup program in the GCG software package. The proposed catalytic domain and the region which is likely to be involved in recognition of the base by GCs are underlined. The sequence of the soluble form is from Yoshikawa et al., 1993.*

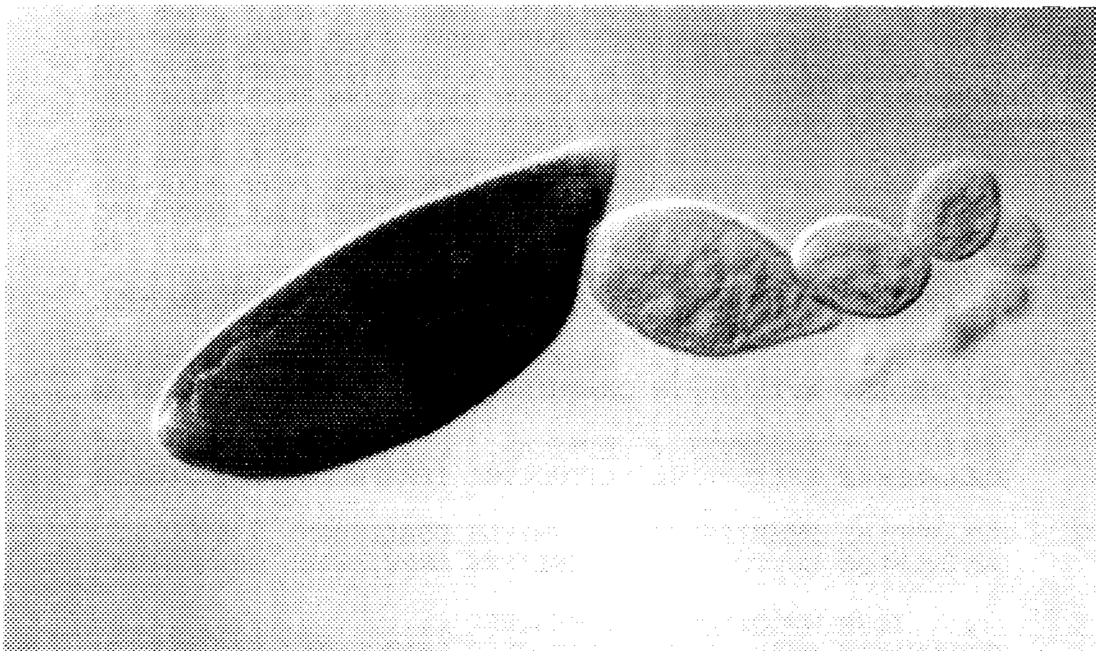

FIG. 3 Whole-mount in situ *hybridization on wild type egg chambers with digoxigenin-labelled GC cDNA probe, performed according to Tautz and Pfeifle (1989).*

For the moment, nothing can be said about the potential ligands of the *Drosophila* receptor protein except that they must be present in various tissues and developmental stages. Sea urchin egg peptides interact with the GC and cause behavioral changes in spermatozoa. Natriuretic peptides apparently elicit their physiological effects of natriuresis, diuresis, and vasorelaxation through the interaction with the receptor GCs and finally it has been shown that a heat-stable enterotoxin responsible for acute diarrhoea binds to an intestinal membrane form of GC [24]. The identification of possible ligands of the *Drosophila* receptor protein will be highly dependent on the identification of the tissues and cell populations expressing this gene and of mutant phenotypes due to the disruption of the GC gene function, approaches which are presently followed in our laboratory.

2.3. Other mutants and lines recently isolated

We performed various sets of experiments either by using insertional mutagenesis with the "mutator" *Pry11* or *PlacZ* and the "jump starter" *P(D2-3-ry')* combination [27] or by mobilizing Pry+ elements already inserted in region 32 [28].

With these approaches we have isolated: new female-sterile mutations on the second chromosome; enhancer trapping lines showing β-gal expression in various ovarian cell sub populations; two new *hup* alleles and homozygous lethal deletions in 32 region. In Fig. 4 are shown egg chambers stained with DAPI and isolated from females homozygous for the mutations teg, abo and pla. In these mutations the control of germ-line cell proliferation is affected.

In Fig. 4A, the *tegamino* (*teg*) mutation is shown. In this new mutant, obtained in a P element mediated enhancer detection set of experiments performed in our laboratory, sterility is complete. The oocyte position within the egg chamber is no longer fixed. Nurse cells are quite irregular in size, shape and number.

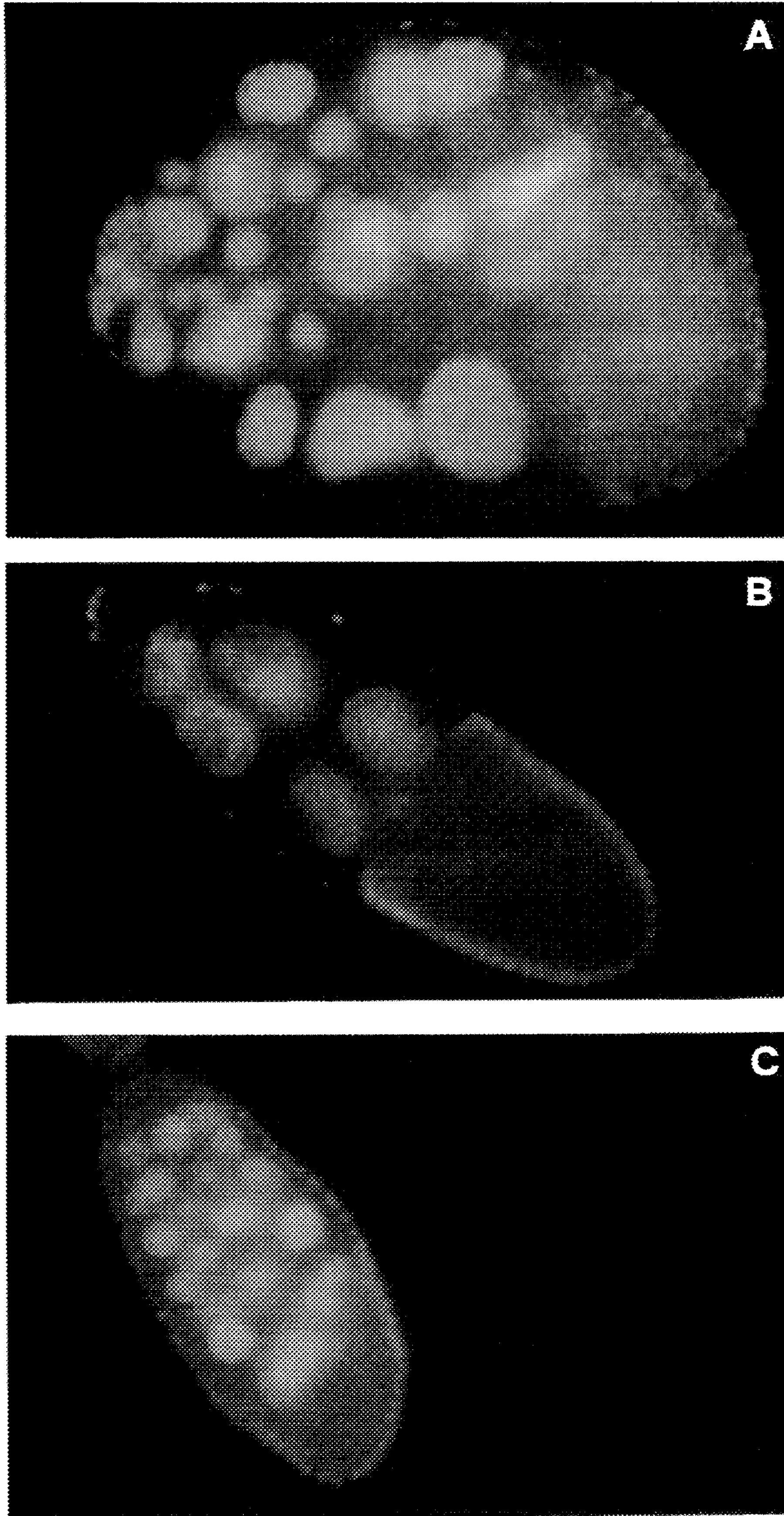

*FIG. 4. DAPI staining of egg chambers isolated from females homozygous for the following mutations: A= **teg**; B= **abo**; C=**pla**. For details see text.*

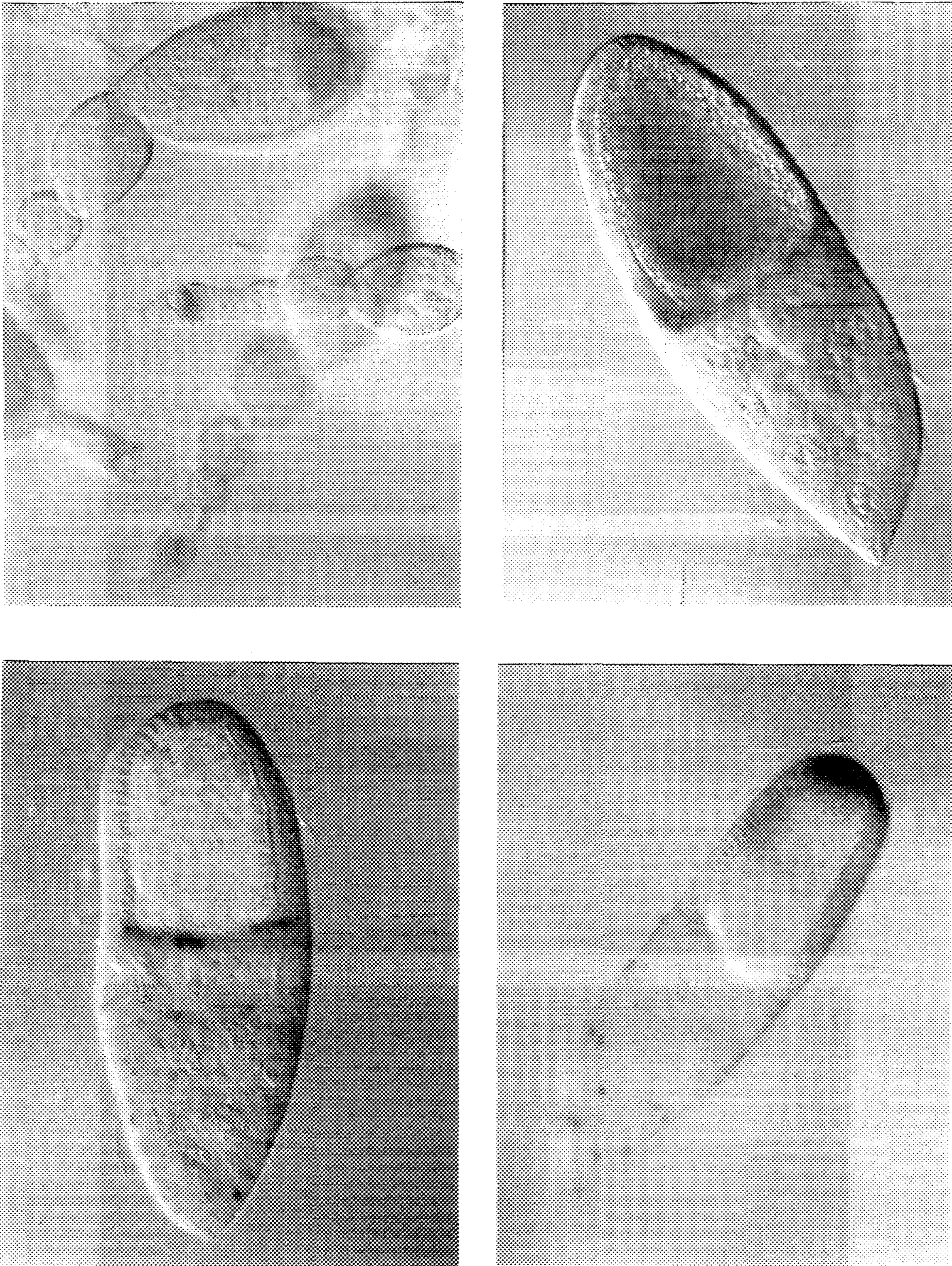

FIG. 5 β-galactosidase activity in egg chambers of different enhancer trap lines isolated in our laboratories. For details see text.

In ovaries of *abo* homozygous females (Fig. 4B) we observed a reduced number of nurse cells in about 30% of egg-chambers, suggesting that the *abo* mutation affects germ-line cell divisions. Females homozygous for the *palla* (*pla*) mutation (Fig. 4C) do lay eggs, indicating that in most of the egg chambers many aspects of oocyte development occur relatively normally. Follicle cell nuclei show an uneven distribution around the oocyte and the eggs are shorter and flatter and show reduced dorsal appendages. A stronger phenotype is shown by 10% of the ovarioles, were fusion of adjacent egg chambers or tumorous follicles are observed (Fig. 4C). For some of these mutations we have already isolated lethal, wild type and weaker or stronger alleles due to imprecise P excision. We have mapped the genes by *in situ* hybridization and have isolated genomic DNA fragments. The β-gal expression of enhancer trap lines recently isolated in our laboratory are reported in figure 5.

Experiments are in progress to better characterize some of these lines and to clone some of the detected genes. In addition, we are using the β-gal expression of the different lines as ovarian specific markers to gain more insight on the positional cues existing in oogenesis.

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