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Spatial Activation and Repression of the *Drosophila* Vitelline Membrane Gene *VM32E* Are Switched by a Complex *cis*-Regulatory System

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ABSTRACT **The *VM32E* gene is differently expressed in the distinct cell domains composing the follicular epithelium. Our previous work on the *VM32E* gene defined the promoter regions required for the control of gene expression in the ventral and dorsal follicle domains. In this report, we present data from a finer dissection of each upstream regulatory region, allowing to draw the functional interactions among different regulatory elements. A 73-bp proximal region (−112/−39) contains regulatory element(s) to dictate the activation of the gene in the follicular epithelium. This region interacts with two other *cis*-regulatory elements and is absolutely required for their output. The first element (−206/−113), individually unable to raise reporter expression, elicits gene activity in the ventral domain when joined to the proximal fragment; a second element (−348/−254) joined to the same proximal fragment sustains the full dorsal and ventral activity. Moreover, the ectopic expression driven by some promoter fragments in border or posterior cells uncovers the existence of specific negative regulatory elements. So, the follicular domain specificity of *VM32E* gene expression is achieved through the combined activities of cell-type specific positive and negative elements. *Dev Dyn 2000;218:499–506.***

Key words: oogenesis; follicle cells; vitelline membrane gene; promoter

INTRODUCTION

In *Drosophila melanogaster*, the events of oogenesis take place in egg chambers, which consist of the oocyte and 15 nurse cells, surrounded by a monolayer of approximately 1,000 follicle cells. Based on the morphology of the maturing egg chamber, oogenesis has been divided in 14 stages (King, 1970). During oogenesis, the framework for early embryonic development is laid down. The follicle cells overlaying the oocyte and germline cells interact critically to establish the polarity of the egg chamber and the developing embryo (St. Johnston and Nüsslein-Volhard, 1992; Gonzalez-Reyes et al., 1995; Ray and Schüpbach, 1996). The EGFR signaling system is required for the induction of posterior follicle cell fate and plays a central role in the differentiation of dorsal follicle cells. Follicle cells that do not receive the EGFR signaling will assume the default ventral cell fate. During egg chamber development, the different dorsal, ventral, anterior, and posterior follicle cell populations acquire regional specific functions, relevant for both the construction of the different eggshell layers and embryonic polarity. The eggshell proteins are synthesized in an ordered spatial and temporal pattern during the terminal stages of oogenesis. The vitelline membrane components are produced during stages 9 and 10, while in stages 11 to 14 the synthesis of chorion proteins is predominant (King, 1970; Mahowald, 1972; Petri et al., 1976; Waring and Mahowald, 1979; Fargnoli and Waring, 1982; Spradling, 1993). The vitelline membrane is a rigid structure that can keep the shape of the egg after removal of the chorion layers. This membrane is composed of at least 10 proteins that are rich in proline and alanine (Petri et al., 1976; Fargnoli and Waring, 1982). Four *Drosophila* vitelline membrane protein genes have been cloned: *VM32E*, *VM34C*, *VM26A1*, and *VM26A2* (Higgins et al., 1984; Mindrinos et al., 1985; Burke et al., 1987; Gigliotti et al., 1989). The transcription pattern of these genes has been analyzed, the *VM26A1*, *VM26A2*, and *VM34C* genes are expressed from stage 8 to stage 10 of oogenesis (Mindrinos et al., 1985; Burke et al., 1987; Popodi et al., 1988; Jin and Petri, 1993); the *VM32E* gene is a late gene of this family, whose activity is restricted at stage 10 (Gigliotti et al., 1989) and follows a peculiar pattern. Transcript is detected first at stage 10A in a group of ventral columnar follicle cells, and progressively extends to the dorsal region as a large stripe surrounding the oocyte at stage 10B (Gargiulo et al., 1991; Gargiulo and Malva, 1994). We do not observe expression in the polar terminal domains, which remain silent (Gargiulo et al., 1991). Absence of posterior expression has also been shown for the *VM34C* gene (Bryant et al., 1999), while *VM26A1* and *VM26A2* are expressed in the whole follicular epithelium (Popodi et al., 1988; Jin and Petri, 1993). It appears that the patterning of follicular epi-

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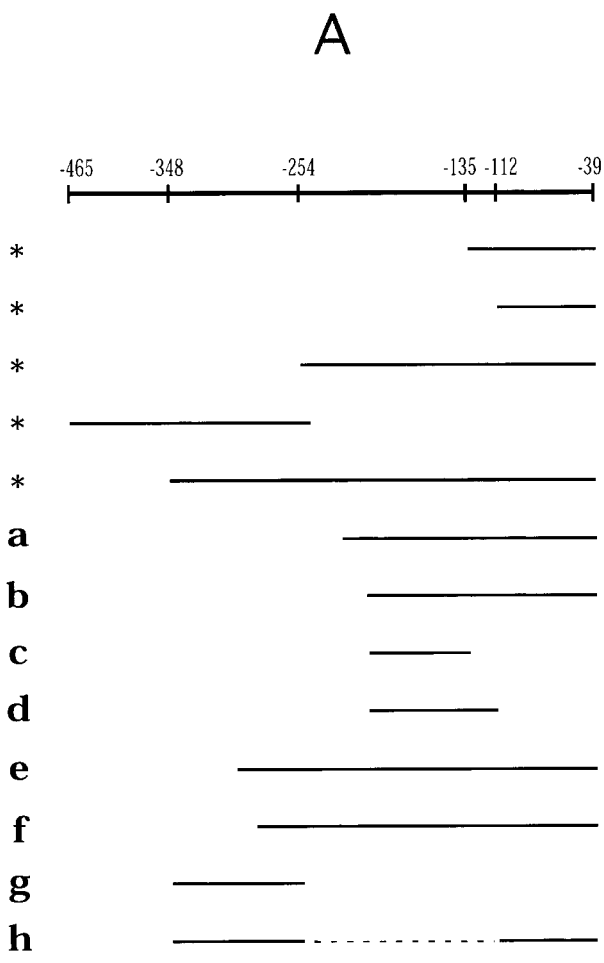

Fig. 1. Scheme of the *VM32E* deletion constructs (**A**) and table summary of their promoter activity (**B**). The asterisks indicate constructs presented in previous reports (Gargiulo et al .,1991; Cavaliere et al., 1997). a–h: Fragments from the *VM32E* upstream region were subjected to transgenic analysis; the dashed line indicates an internal deletion.

thelium imposes a specific temporal and spatial regulation of *VM32E* gene expression, which may reflect specific functions played by this protein in the vitelline membrane construction.

In an effort to elucidate the regulatory signals that dictate the developmental expression of the *VM32E* gene, we have performed an extensive analysis of its promoter. Our previous studies assigned the *cis*-acting regulatory DNA region, responsible for the correct expression of the gene, to the segment −348/−39 upstream the transcription initiation site. Expression in the dorsal domain depends on the two regions −348/−254 and −118/−39. The −253/−39 region contains *cis*-acting elements sufficient to drive expression of the reporter gene in the ventral follicle cells, as well as a negative element repressing gene activity in centripetal cells. Within this region, the −135/−39 fragment appears as the minimal element able to sustain a weak expression in the ventral cells (Cavaliere et al., 1997). In this report, we further dissect the −253/−39 and −348/−254 regulatory regions, giving new insight on their functions, and present a model of the interactions among the different regulatory elements in a scheme that also includes results from previous studies.

RESULTS

Expression of the *VM32E* gene, as assayed by whole mount in situ hybridization, is spatially regulated, with the ventral region that stains earliest, followed by the lateral and dorsal follicle cells (Gargiulo et al., 1991). As we previously reported (Cavaliere et al., 1997), in transgenic flies the same expression pattern was detected for a reporter *lacZ* gene using the minimal promoter region −348/−39 (see Fig. 2A and B). To finely dissect the −253/−39 and −348/−254 regulatory regions, we produced the 8 different deletions in the −348/−39 *VM32E* promoter fused to the *lacZ* reporter gene depicted in Figure 1 (Fig. 1 also reports some of

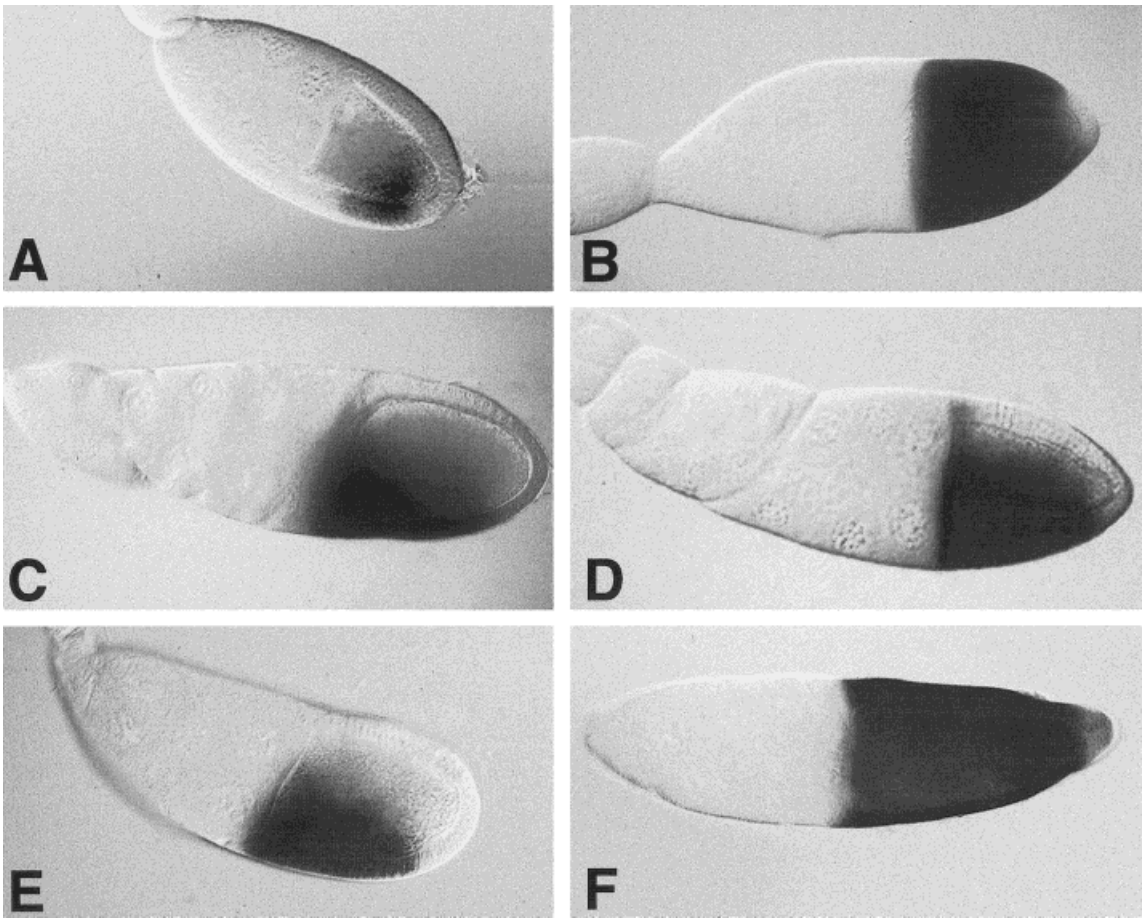

Fig. 2. *lacZ* expression pattern in stage 10 egg chambers of females transformed with the construct containing the reported *VM32E* upstream region. All the egg chambers are at stage 10B except for the stage 10A in A. **A,B:** −348/−39; **C:** −223/−39; **D:** −206/−39; **E,F:** −253/−39 in wild type and *gurken* background, respectively. The egg chambers are oriented with the anterior end on the left and with the dorsal side at the top.

the previously characterized constructs). The spatial expression pattern conferred in vivo by each deletion mutation was obtained after P mediated germ line transformation.

Dissection of the −253/−39 Region

In our previous work, we reported that the −253/−39 region directs expression of the reporter gene in the ventral follicle cells. A shorter DNA fragment, −135/−39, was still capable to drive ventral expression, albeit at a reduced level. Within the −135/−39 region, the −135/−113 element appeared necessary to drive ventral expression; in fact, the −112/−39 fragment alone cannot support normal expression in the main body follicle cells, instead it directs ectopic expression in the anterior centripetal cells (Cavaliere et al., 1997; see Fig. 3D). The ventral activity displayed by the −253/−39 is stronger than the one showed by the −135/−39 region. This indicates that additional ventral activating sequences have to be present in the −253/−136 region. In this report, the −253/−39 region was further dissected by analyzing 4 different deletions (a,b,c,d in Fig. 1). Constructs with the −223/−39 or −206/−39 fragments originated a pattern comparable to that of −253/−39, expressing the reporter gene in the ventral follicle cells (Fig. 2C–E, respectively). In the transformed lines carrying the −206/−39 construct, staining was also visible in the anterior dorsal follicle cells (Fig. 2D). A similar phenotype was observed in a few lines of the −253/−39 and −223/−39 constructs, expressing the reporter gene at a high rate (data not shown). The average levels of expression of the reporter gene shown by the −223/−39 and −206/−39 constructs were similar to that shown by the −253/−39 region, and consistently higher than the activity of −135/−39; this suggests that the −206/−136 fragment should contain regulatory sequences that enhance the activity of the −135/−113 element. We analyzed −206/−136 individual activity and did not find any expression of the reporter gene (data not shown). The same result was obtained for the −206/−113 fragment that also includes the −135/−113 ventral element (data not shown). Hence, it appears that the −112/−39 fragment, which per se drives gene expres-

sion ectopically in centripetal cells, gains a ventral function through regulatory elements located in the −206/−113 region.

Cell-Type Specificity of the −253/−39 Region Activity

The −253/−39 element is able to sense the ventralization of the follicular epithelium induced by the *gurken* mutation. We examined the expression of the −253/−39-*lacZ* construct in egg chambers of females homozygous for $gurken^{HK36}$ mutation and uniform expression of the reporter was observed throughout the follicle cells, except the posterior ones (Fig. 2F). This observation contrasts the expression caused by this same construct in egg chambers from females homozygous for $fs(1)K10^{1}$. In this dorsalizing genetic background, the promoter activity of the −253/−39 fragment, when allowed, was detected only in residual patches of the pattern observed in wild type egg chambers; in addition, in several egg chambers reporter gene expression was completely abolished by $fs(1)K10^{1}$ (data not shown). These data clearly strengthen the inference that the −253/−39 region is competent to drive expression of the reporter gene in follicle cells with a ventral identity. To further analyze the spatial constraints to the activity of this fragment, we mobilized a P-derived construct containing the −253/−39-*lacZ* gene fusion in flies carrying it on the X chromosome, in order to check for some position effects on the reporter gene expression. We have analyzed 100 independent lines where the P-derived element had moved to the second or third chromosome. These lines varied from the starting one only as to the amount of staining produced. A few lines were characterized by high activity that also allowed strong expression of the reporter gene in the lateral follicle cells and in a small anterior dorsal group (data not shown). No expression was ever detected in cells on the dorsal side of the main follicle body. In our experiments, we did not note any further significant ectopic expression suggestive of enhancer trapping (O'Kane and Gehring, 1987). As a whole, these data may suggest that the −253/−39 region, besides the positive elements that support expression by the ventral follicle cells, contains specific control elements that do not allow expression of the reporter gene by dorsal follicle cells, unless in the presence of the specific upstream sequences.

Dissection of the −348/−254 Region

In our previous experiments, we have found that expression in the dorsal domain requires the two regions −348/−254 and −118/−39 (Cavaliere et al., 1997). In this section, we further dissect the former one by analyzing the two shortened elements produced by deletions e and f (Fig. 1A). Egg chambers of females transformed with the construct −301/−39 showed a strong reduction of the dorsal expression domain of the reporter gene (Fig. 3A). Deletion of 20 additional nucleotides from position −301 to position −282 abolishes expression in all the dorsal follicle cells (Fig. 3B). Therefore, the distal fragments −348/−302 and −301/−282 have to be essential parts of a dorsal controlling element(s) contributed by the −348/−254 region. The activity of the −348/−254 fragment appears dependent on the presence of the downstream regulatory elements; in fact, as previously reported, the −465/−249 fragment was unable to express the reporter gene, suggesting also that the included −348/−254 element should be inactive (Gargiulo et al., 1991). Since the −465/−249 fragment contains additional sequences upstream of the −348 end, to better define the function of the −348/−254 element we checked its individual activity. As shown in Figure 3C, staining was observed in the border cells that normally do not synthesize the vitelline membrane proteins (Gargiulo et al., 1991; Jin and Petri, 1993) but no activity was detected in the cell domains expressing *VM32E*. This result, scored in ten independent transformant lines excluding any positional artifact, clearly supports our expectation on this element. Moreover, the ectopic promoter activity in the border cells hints to more intriguing regulatory interactions among this element and its flanking regions.

Besides for dorsal expression, the −348/−254 region also appears necessary for high level of ventral expression. In fact, the ventral expression driven by the −253/−39 region is lower than the one shown by the −348/−39 promoter. We constructed a promoter fragment in which we deleted the −253/−113 region containing the ventral regulatory elements and joined the −348/−254 to the −112/−39 region (construct h in Fig. 1). It is worth noting that, individually tested, these regions express the reporter gene ectopically: the −348/−254 is active in the border cells and the −112/−39 in the centripetal cells (Fig. 3C and D, respectively). The in vivo activity of the −348/−254Δ−112/−39 fragment was analyzed in 8 independent transformant lines with consistent results. The expression resembled the wild type pattern; it started ventrally at stage 10A (Fig. 3E) and at stage 10B the ventral, lateral, and dorsal follicle cells were highly stained (Fig. 3F). The activity was higher than the one shown by the −348/−39 promoter and appeared to also involve the anterior and posterior follicle cells at the exclusion of the terminal posterior ones. We further analyzed the activity of this element by in situ hybridization using a fragment of the *lacZ* gene as probe. As shown in Figure 3G, *lacZ* transcription extends to the centripetal and posterior follicle cells but remains absent in the terminal ones. This activity contrasts with that of the wild type promoter (Fig. 3H), indicating that the deleted −253/−113 fragment, that per se contributes ventral positive elements, also contains *cis*-acting elements repressing gene expression in these follicle cell domains. From these data, we can conclude that the putative dorsal element −348/−254 actually also plays a relevant function for the ventral expression of *VM32E* gene. In particular, its function appears to depend on the presence of the same −112/−39 region required for the ventral activity of

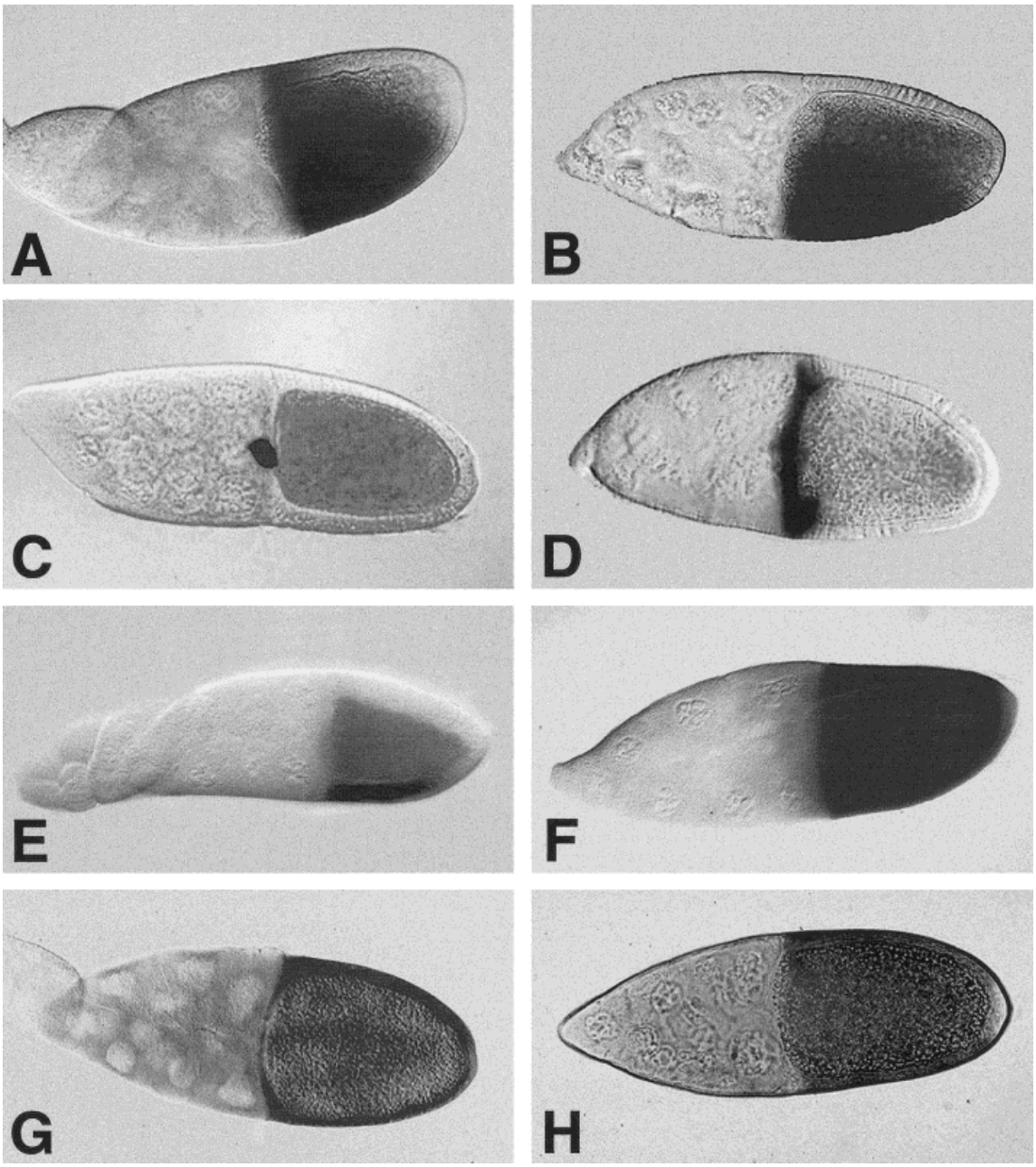

Fig. 3. **A–F:** *lacZ* expression pattern of egg chambers from females transformed with the construct containing the reported *VM32E* upstream region, analyzed as β-galactosidase activity. **G,H:** Whole-mount in situ hybridization. All the egg chambers are at stage 10B except for the stage 10A in E. A: −301/−39; B: −281/−39; C: −348/−254; D: −112/−39; E–G: −348/−254Δ−112/−39; H: wild type egg chambers. G: Expression, probed with a digoxigenin-labelled *lacZ* DNA fragment, extends posteriorly, and anteriorly involves centripetal cells. H: Hybridization, probed with the 3' end of the *VM32E* cDNA, is observed only in the columnar main body follicle cells while the posterior and the centripetal follicle cells remain totally unstained.

the −206/−113 fragment. Therefore, the −112/−39 fragment contains essential regulatory element(s) to dictate the *VM32E* gene activation in the follicular epithelium.

DISCUSSION

The complex architecture of the eggshell implies the underlying differentiation of the follicular epithelium in distinct spatial cell domains, each endowed with specific functions. This fact, which fairly reflects the complex regulatory strategy followed by the chorion genes for their expression (Mariani et al., 1988, 1996; Tolias and Kafatos, 1990; Tolias et al., 1993), is also evident when considering the *VM32E* gene: its peculiar expression pattern results from the interaction of dis-

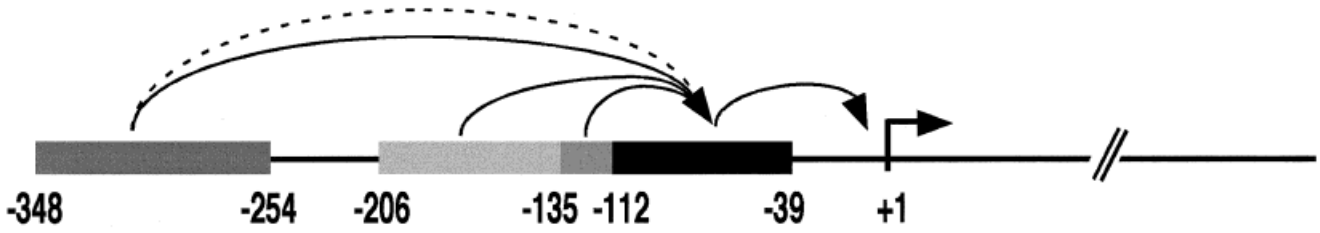

Fig. 4. Schematic representation of the interactions among the *cis*-regulatory positive elements (boxes) of the *VM32E* promoter. The −348/−254, −206/−136, and the −135/−113 elements interact with the −112/−39 fragment to control the *VM32E* expression in the ventral (continuous arrows) and dorsal (dashed arrow) domains of the follicular epithelium.

tinct *cis*-acting regulatory elements, each of which controls expression in specific follicle cell subpopulations (Fig. 4). *VM32E* gene expression in the follicular epithelium depends on the key activity of control element(s) harbored in the −112/−39 proximal fragment. Furthermore, in the −348/−39 minimal promoter, the region −253/−113 may act to modulate and define the expression mediated by the −348/−254 region. The latter, in fact, fused to the proximal control fragment, confers an overall high rate of activity also displayed in the polar follicle domains excluding the terminal posterior cells. The finding that the −348/−254 fragment confers expression to the reporter gene in the border cells indicates that, in these cells, native *VM32E* gene is switched off by a negative control element, which could be located within the −112/−39 fragment itself. In fact, reporter expression in this anterior domain disappears when –348/−254 and –112/−39 fragments are fused. Border-cell specific, *cis*-acting repressor elements have also been found in the promoter of *VM26A1* (Jin and Petri, 1993) and in the ovarian enhancer element 1 (oe1) of *yp1* and *yp2* genes (Logan and Wensink, 1990). Another negative element related to centripetal cells had been mapped within the −254/−118 region (Cavaliere et al., 1997). Since reporter expression driven by −348/−254Δ−112/−39 construct extends both anteriorly, to the centripetal cells, and posteriorly, the −254/−113 region could contain a further negative element, specific for the posterior domain, added to the previously described centripetal element. Polar follicle cells constitute a separate lineage from the rest of follicle cells (Margolis and Spradling, 1995). During egg chamber development the fate of polar follicle cells is restricted to a choice between anterior or posterior. It has been shown that Notch pathway is required for early follicle cell differentiation at both termini, but is then repressed at the posterior for proper determination of the posterior follicle cells by the EGF receptor pathway (Larkin et al., 1999). We never obtained expression in the terminal domain of the posterior follicle cells from the various constructs analyzed in this and previous reports (Gargiulo et al., 1991; Cavaliere et al., 1997). The putative *cis*-acting repressor signal could be tightly associated to some positive activating element or, as well, these cells could lack some proper *trans*-activating factor present in the rest of the follicular epithelium.

The −253/−39 fragment promotes reporter expression specific for the ventral follicle cell type, as shown by the pattern seen on ventralized egg chambers of *gurken* females (see Fig. 2F) and underscored through the analysis of the lines obtained after its mobilization. This suggests that dorsal expression of the gene is under a negative *cis*- acting control contained in this fragment. Silencing elements involved in tissue-specific regulation of the *s15* and *s36* chorion genes have also been reported by Bienz-Tadmor et al. (1992). In the wild type promoter, the effect of this negative element should be relieved by the activity of the −348/−254 positive region driving expression also in the dorsal follicle cells. The process is stage dependent and the dorsal activity arises only at stage 10B (see Fig. 3E and F). This developmental control is maintained in the construct −348/−254Δ−112/−39, indicating that the negative dorsal element should be located in the proximal promoter fragment.

Ventral cell type specificity was also shown by constructs carrying the −223/−39 and −206/−39 regulatory regions. The only exception to this specificity is a low activation of the reporter gene in the anterior dorsal follicle cells, occurring in egg chambers that highly expressed the reporter gene in the ventral domain. We have also analyzed those lines of these constructs that express the reporter gene at a lowered rate due to position effect, and found that the expression was not uniformly reduced in the ventral follicle. Three independent lines with the −223/−39 fragment supporting different degrees of reduced expression of the reporter gene are illustrated in Figure 5A–C. Each of them illustrates the differential activity of ventral cells. These observations compare those collected among the lines obtained after mobilization of the −253/−39 construct. The data indicate that some local differentiation relevant to the process of oogenesis could be operating among the cells of the ventral domain of the follicular epithelium. Our results are consistent with the modular functional character proposed by Kirchhamer et al., (1996) for *cis*-regulatory systems. Therefore, the highly precise developmental expression pattern of the *VM32E* gene appears as based on an appropriate bal-

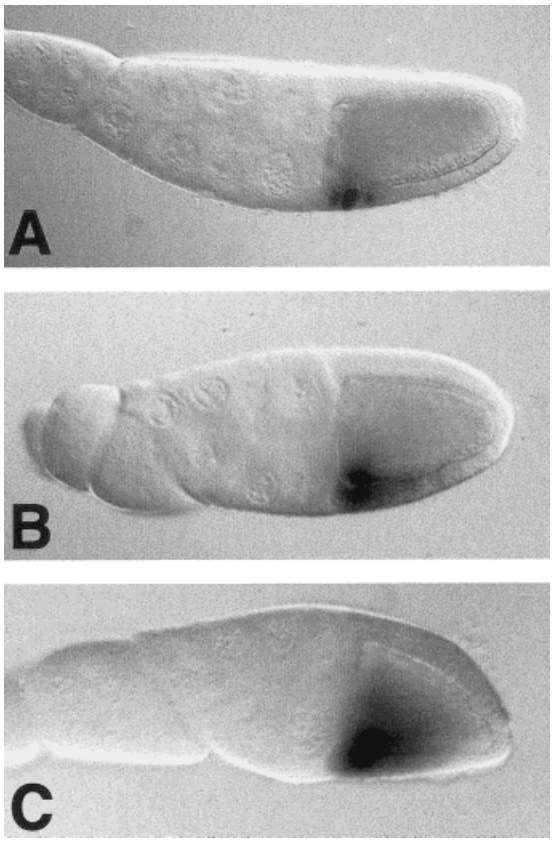

Fig. 5. Analysis of β-galactosidase ventral expression pattern in stage 10 egg chambers from three independent lines (**A–C**) of flies transformed with the construct containing the −223/−39 fragment.

ance of multiple positive and negative regulatory elements specifically monitored by the different follicle cell types.

EXPERIMENTAL PROCEDURES

Fly Strains

Stocks were raised at 21°C under standard culturing conditions. The grk^{HK36} and $fs(1)K10^{1}$ stocks were obtained from T. Schüpbach. The *CyO/Sp;* ry^{506}*, Sb, P[Δ2-3-*ry^{+}*](99B)/TM6, Ubx* stock was obtained from W. R. Engels.

Molecular Biology General Procedures

Standard techniques such as the isolation of plasmid DNA, restriction endonuclease digestions, DNA electrophoresis, plasmids construction, and DNA sequencing were carried out essentially as described in Sambrook et al. (1989).

Promoter Constructs

All the different promoter regions analyzed were obtained by PCR amplification from genomic DNA, using designed primers and Pwo DNA polymerase in an Omnigene Thermocycler. PCR products were gel-purified and treated 30 min at 37°C with 20 U of Polynucleotide Kinase. Following phenol/chloroform extraction and ethanol precipitation, the amplification products were treated 5 min at 37°C with 20 U of T4 DNA Polymerase. After a final round of purification and precipitation, they were cloned in the filled Not I site of the HZ50PL vector (Hiromi and Gehring, 1987), just upstream of the *hsp70* basal promoter fused to the *Escherichia coli lacZ* gene. Analyzing the restriction digests assessed the correct orientation of each construct and the selected clones were analyzed by DNA sequencing.

P-mediated Transformation and Analysis of Transformants

P-element-mediated transformation was carried out essentially as described (Spradling and Rubin, 1982; Rubin and Spradling, 1982). Embryos from a *cn;* ry^{506} strain were injected with the different DNA constructs and helper plasmid p-25.7wc (Karess and Rubin, 1984) at concentrations of 400 and 75 μg/ml, respectively. To obtain isogenic lines, the transformants were mated to flies carrying multiply balanced chromosomes. At least four transformant strains for each of the constructs were analyzed.

The X-Gal assay for β-galactosidase activity was performed as described by Margolis and Spradling (1995). After staining the ovaries were dissected and mounted in 50% glycerol in PBS, and viewed with Nomarski optics on a Zeiss microscope.

Mobilization of the −253/−39-*lacZ* Construct

We mobilized the −253/−39-*lacZ* gene construct by crossing the stock carrying the *P[−253/−39-lacZ,* ry^{+}] element on the X chromosome (Cavaliere et al., 1997), with *CyO/Sp;* ry^{506}*, Sb, P[Δ2-3* ry^{+}*](99B)/TM6, Ubx* (Robertson et al., 1988) and selecting new locations of the *P[−253/−39-lacZ,* ry^{+}] among ry^{+} male progeny. Appropriate crosses were performed to obtain homozygotes for the new insertions.

In Situ Hybridization

Whole-mount in situ hybridization with digoxigenin-labelled probes was performed as described by Tautz and Pfeifle (1989). The probes used were the coding region of the *lacZ* gene and the 3' end of the *VM32E* cDNA (Gigliotti et al., 1989). After staining the ovaries were dissected, mounted in Fluormount, and viewed with Nomarski optics on a Zeiss microscope.

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