Intercellular communication between germ line and somatic line is utilized to control the transcription of *ZAM*, an endogenous retrovirus from *Drosophila melanogaster*

Carine Meignin, Bernard Dastugue and Chantal Vaury*

Unité INSERM 384, BP38, 63001 Clermont-Ferrand, France

ABSTRACT

ZAM is an long terminal repeat (LTR) retrotransposon from *Drosophila melanogaster* that bears striking resemblance to the vertebrate retroviruses, in their structure and replication cycle. This element transposes via an RNA intermediate and its reverse transcription, and ultimately inserts copies within the germ line. In this paper, we show that intercellular communication established between the germ line cells and the somatic follicle cells is used to initiate the replication cycle of *ZAM*. *ZAM* has been shown to be transcribed in the follicle cells located at the posterior pole of the oocyte. Here, we determine the *cis*-regulatory elements necessary for its somatic expression, and show that they respond to the EGF-receptor signaling pathway and its activation by the ligand Gurken emitted by the germ line. We further show that the ETS-transcription factor *Pointed2* acting downstream of this pathway acts as a *trans*-regulatory factor and targets a specific *cis*-regulatory binding site located within the *ZAM* LTR. Our data give an insight into the molecular mechanism for how intercellular communications between germ cells and somatic cells may be used by endogenous retroviruses to control their replication, and thereby specify their intrinsic and highly restricted expression in the reproductive apparatus.

INTRODUCTION

LTR retrotransposons share structural and functional homologies with the proviral form of vertebrate retroviruses and form a large and widespread family of mobile genetic elements present in all eukaryotic genomes studied so far. When active, these elements propagate by reverse transcription of RNA intermediates. The synthesized cDNA copies insert themselves within their host genome, which results in the inactivation or activation of genes located in the vicinity. These transpositions can then be a major source of mutations and as such contribute to the induction and/or enhancement of pathological processes. Nevertheless, retrotransposons are ancient constituents of eukaryotic genomes, indicating that their transposition is tightly controlled so as not to be too deleterious, and that they have now reached a status and a life cycle compatible with their own host's life cycle. In that respect, they constitute an adequate system to study interactions that should exist between these potentially mutagenic, parasite-like elements and their host.

Although their control at different steps of their replication cycle can limit transposition, transcriptional initiation is the first rate-limiting step in this process. Thereby, LTR retrotransposons display a strict pattern of expression, confining their transcription to some very specific tissues (1–5). Such specificities involve interactions between host-encoded, *trans*-regulatory proteins and complementary *cis*-regulatory protein binding sites located within the retrotransposon (6).

For the past few years, our laboratory has been engaged in studying the control of an LTR retrotransposon, *ZAM* from *Drosophila melanogaster*, that is similar in structure and replication cycle to mammalian retroviruses (1,7). This element is generally present with a very low copy number (not exceeding two copies) in the lines studied. However, one line called Rev exists in which this control has been disturbed, resulting in a high expression of *ZAM* and an increased mobilization rate. In the process of analyzing this control, we have found that *ZAM* expression is subject to two types of controls: one restricts its expression to specific somatic cells in the ovaries; the other silences this expression in most of the lines tested but not in 'Rev'. We previously reported that this latter control, which depends on the genetic background of the line, is controlled by an heterochromatic locus located in 20A2-3 on the X chromosome (8). However, we had not investigated the molecular basis of its control that restricts expression to a specific subset of somatic cells located at the posterior pole of the oocyte and that was only observed in the Rev line when silencing is abolished. In this study, we carried out an in-depth analysis of the intrinsic regulatory properties of *ZAM* and the host-encoded factor that defines this strict tissue specificity.

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*To whom correspondence should be addressed. Tel: +33 4 73 17 81 71; Fax: +33 4 73 27 61 32; Email: Chantal.VAURY@inserm.u-clermont1.fr
Present address:
Carine Meignin, Ilan Davis' Lab, Institute of Cell and Molecular Biology, King's buildings, Edinburgh EH9 3JR, UK

The expression pattern of LTR retrotransposons is partly controlled by sequences located in *cis*, on the retrotransposon proper and generally on the LTR. The LTRs are composed of a U3 region, a central R and a U5 region. The transcription initiation site defines the boundary between U3 and R regions. The polyadenylation site corresponds to the boundary between R and U5 regions. We have found that the first 190 bp of the U3 region of *ZAM* can drive expression of reporter genes in a pattern that reproduces the ovary expression pattern of endogenous *ZAM* elements. This establishes this 190 bp as a short and defined enhancer. We used this accessible system to identify factors involved in the transcriptional specificity. Since *ZAM* is transcribed in the follicle cells located at the posterior pole of the oocyte, we focused our attention on *Drosophila*-encoded factors known to be necessary for the differentiation of these cells.

During *Drosophila* oogenesis, cell–cell signaling occurs between the germ-line-derived oocyte and the somatically-derived follicle cells that surround it. The follicle cell layer becomes polarized along the anterior–posterior axis when the oocyte induces the follicle cells at one end of the egg chamber to adopt a posterior rather than an anterior fate (9). This induction requires Gurken in the germ line and the *Drosophila* homologue of the epidermal growth factor receptor, EGFR, in the soma (10,11). Gurken is thought to bind directly to the EGFR to activate a typical receptor tyrosine kinase signal transduction cascade that specifies posterior fate (12,13). Some of the effectors and target genes of EGF receptor pathway have been identified (14–16). One of them is an ETS transcription factor encoded by the *pointed* gene that is expressed in response to activation of the EGF receptor pathway, and brings about its negative regulation (17).

The present study has revealed a connection between EGF receptor signaling and regulation of *ZAM* expression. Indeed, we have shown that the EGF receptor pathway is necessary for the expression of *ZAM* in the posterior follicle cells. The transcriptional factor involved has been identified as the Pointed2 protein, an ETS-binding protein that is able to bind an ETS-binding site located at position 79 on the *ZAM* sequence.

MATERIALS AND METHODS

Fly stocks

The fly strains used were w^{1118} an S-line in which *ZAM* is silenced, and Rev, a U-line in which *ZAM* expression is active (both from the collection of INSERM U384); [hs-FLP 12, FRT 42D top^{CO}, FRT 42D hs-*myc*] (18); $pnt^{\Delta 88}$ (19); *UASpntP1*, *UASpntP2* (20); GAL 4 line 55B (15); [w^{1118} 70FLP; cu kar² Sb/TM6, Ubx e^S] (kindly provided by Kent Golic); [w^{1118}; P{w + mC = UAS-GFP.nls}14], [w^{1118}; P{ry + t7.2 = neoFRT} 82B P{w + t* ry + t* = white-un1}90^E], [w^*; P{ry + t7.2 = neoFRT}82B P{w + mC = Ubi-GFP}83] (Bloomington Stock Center). To obtain the $pnt^{\Delta 88}$ allele together with an FRT site on the third chromosome, meiotic recombination assays were generated in a set of crosses involving two fly stocks: the $pnt^{\Delta 88}$ stock from the name of its mutated allele and [w^*; P{ry + t7.2 = neoFRT}82B P{w + t* ry + t* = white-un1}90^E]. The pZ499, pZ310 and pZ190 transgenic lines were obtained by injection of pZ499, pZ310 and pZ190 transformation vectors into w^{1118} flies. These transgenic lines were established with the X chromosome of Rev unstable line and autosomes of w^{1118} line. Flies used for analysis of expression were raised and kept at 25°C.

Generation of clones

We used FRT/FLP techniques to generate clones of follicle cells homozygous for a null mutation in the EGFR gene, top^{CO} (18,21,22), or a null allele of *pointed*, $pnt^{\Delta 88}$. Females with genotypes [hs-FLP 12; FRT 42D top^{CO}/FRT 42D hs-*myc*; *ZAM*-LTR/+] and [hs-FLP 70; *ZAM*-LTR/+; FRT 82B $pnt^{\Delta 88}$/FRT 82B Ubi-GFP], respectively, were fed yeast for 24 h, heat shocked for 1 h in a 37°C water bath and placed back in yeasted vials at 25°C for 60 h. Females [hs-FLP 12; FRT 42D top^{CO}/FRT 42D hs-*myc*; *ZAM*-LTR/+] were then heat shocked again for 1 h at 37°C to induce expression of the *myc* tag. Females were dissected and fixed 1.5–2 h later.

DNA constructs

The pZ499 also called *ZAM*-LacZ has been described by Desset *et al.* (8). Sub-fragments of *ZAM*-LTR were amplified by PCR using primers Cliqt35 (5′-AGTTACCGACCCAT-CG-3′) and ZAM190CiBam (5′-CGGATCCGTATGCGTTG-TTCTGTCTGAG-3′) for pZ190 construct, and Cliqt35 and ZAM 8271Ci (5′-CGAATTCGTCTTAAATGGGCTAA-CAG-3′) for pZ310 construct. The *ZAM*-LTR fragments extending, respectively, from nucleotides 1 to 190 and 1 to 310 were inserted into the EcoRI site of the pLacZ vector carrying the *Escherichia coli LacZ* gene and the minimum Hsp70 promotor. These constructs called pZ190 and pZ310 were injected into w^{1118} flies, and a minimum of two independent transgenics lines were established. Then, by crosses with the Rev line, these transgenes were put in a genetic background allowing *ZAM* expression.

Histochemical staining for β-galactosidase

Ovaries were dissected in 1× phosphate-buffered saline (PBS), fixed in 0.5% glutaraldehyde in PBS for 4 min at room temperature and rinsed twice in 1× PBS and in Fe/NaP buffer (0.003 M Na_2HPO_4, 0.072 M NaH_2NPO_4, 0.003 M $K_3Fe(CN)_6$, $K_4Fe(CN)_6$, 0.15 M NaCl, 0.001 M $MgCl_2$). Staining was performed in Fe/NaP buffer with X-Gal (0.2 mg/ml) final concentration at 37°C. All samples were stained simultaneously and for the same length of time (2 h). Stained tissues were washed 4 times in 1× PBS and examined under an Axiophot microscope (Zeiss) using Nomarski optics.

Immunohistochemical procedures and microscopy

Ovaries were dissected in PBS and fixed in 4% formaldehyde for 15 min. After washing in PBT, tissues were permeabilized and blocked for 4 h in PBT at room temperature. The anti-LacZ primary polyclonal antibody (Sigma) was diluted to 1/2000. The Cy3 conjugated anti-rabbit secondary antibody (Molecular Probes) was diluted to 1/300. The anti α-tubulin (Sigma) was diluted to 1/2000. The Cy5 conjugated anti-mouse secondary antibody (Molecular Probes) was diluted to 1/300. Light and fluorescence microscopy were performed on Axiophot (Zeiss) and confocal microscopy was performed on an Olympus confocal microscope.

Preparation of Pointed fusion protein

The GST-PntC construct was kindly provided by R. Carthew (23). The GST–PntC fusion protein contains the DNA binding domain of Pointed fused to GST. Expression of the GST–PntC fusion protein was induced in the *E.coli* BL21 by addition of 0.1 mM IPTG during the log-phase bacterial culture for 4 h at 30°C. Bacteria were collected by centrifugation and the pellet was resuspended in a lysis buffer containing an anti-proteinase cocktail. Cells were broken by brief sonication and cleared from insoluble material by centrifugation. Gag fusion protein was collected from the supernatant with 500 µl of glutathione agarose (GSH) beads (Sigma) for 1 h at room temperature. Fusion protein was eluted from the beads by gentle shaking in 50 mM Tris–HCl, pH 9, 5 mM reduced glutathione for 10 min at 4°C. Fusion protein yield was estimated by Bradford spectrophotometric analysis and the quality of each fraction was tested by SDS-PAGE.

Mobility shift assays

The 190 bp fragment of *ZAM* was excised from plasmid pZ190 by an EcoRI digestion, and purified. Oligonucleotides used are as follows: ZAMets 5′-CGAACCGGGAAGCTT-3′ (forward) and 5′-AAGCTTCCCGGTTCG-3′ (reverse). The sequences of the Mut oligonucleotides were 5′-CGAACCGCCTTGCTT-3′ (forward) and 5′-AAGCAAGGCGGTTCG-3′ (reverse) (underlined nucleotides are mutated). Double-stranded fragment probes were prepared with the Invitrogen T4 polynucleotide kinase Kit and [γ-^{32}P]dATP at 3000 Ci/mmol. The DNA binding reaction (20 µl) is carried out by incubating 44, 88 and 264 ng purified GST fusion protein or GST alone with 20 000 c.p.m. of labelled dsDNA fragment probe in 10 mM HEPES (pH 7.9), 1 mM DTT, 100 mM KCl, 10% (v/v) glycerol, 1 µg poly(dI–dC), 10 µg BSA for 20 min at 25°C. DNA–protein complexes are resolved on 4% non-denaturing polyacrylamide gels (19:1) containing 0.5× TBE. In competition experiments, a 25- or 100-fold excess of unlabeled oligonucleotides was added in binding reactions.

RESULTS

Reporter constructs containing the *ZAM* U3 region reproduce the follicle cell-specific expression of *ZAM*

The expression pattern of LTR retrotransposons is generally governed by sequences contained within the retrotransposon itself. We previously reported that indeed, the tissue specificity and a strain control are determined by the *ZAM* LTR (8). In order to focus on the tissue specificity of its transcription and better localize sequences involved, we constructed plasmids containing *ZAM* sequences and a *LacZ* reporter gene which could mimic *ZAM* expression *in vivo*. To this end, a PCR-amplified *ZAM* fragments containing the U3 region (1–310) was fused to the *E.coli LacZ* gene in a P-element transformation vector. Since the 310 bp do not contain the *ZAM* promoter (7), they have been cloned in a transgenic vector upstream of a minimum hsp70 promoter fused to *LacZ*. This construct has been called pZ310. It has been injected into w^{1118} flies, and five independent transgenic lines were established. Using a histochemical assay for β-galactosidase activity, we have studied the expression of the *LacZ* reporter gene controlled by
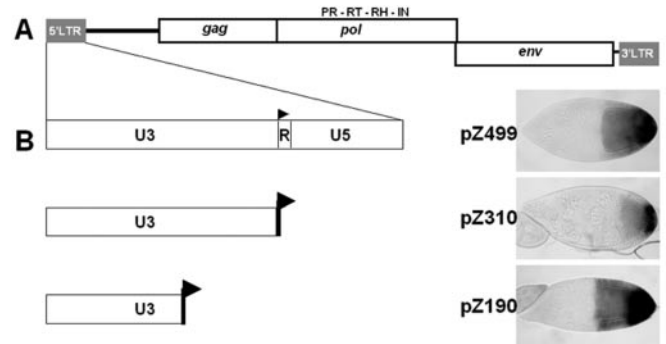

Figure 1. *ZAM* expression in the ovaries of adult females. (**A**) Molecular structure of *ZAM*. (**B**) On the left, *ZAM* fragments placed upstream of LacZ. Transgenes contain the full length 499 bp LTR (pZ499), the U3 region of 310 bp (pZ310), or the 5′ part of U3 up to nucleotide 190 (pZ190). For each construct, an example of histochemical detection of β-galactosidase activity in the follicle cells located at the posterior pole of stages 9 and 10 follicles is presented on the right.

ZAM U3 sequences. In two independent transgenic lines, β-galactosidase activity is detected in the somatic follicle cells located at the posterior pole of follicles all along the ovarioles (Figure 1). This transcription of *LacZ* exactly reproduces the expression pattern of endogenous *ZAM* elements (1) and of the full length LTR of ZAM when fused to *LacZ* [(Figure 1A and (8)]. To define the sequence involved more precisely, we then tested the first 190 bp from the N-terminal part of U3 (1–190) fused to LacZ (pZ190). The established transgenic line obtained with such a transgene expressed the LacZ reporter in the follicle cells located at the posterior pole of the oocyte. This indicated that the enhancer specific to this patch of cells is contained within the 190 bp of U3. The transgenic approaches used in this experiment demonstrate that the tissue-specific expression of *ZAM* is only dependent on sequences present in the U3 region of *ZAM* LTR and more precisely in its first 190 bp.

The EGF receptor signaling pathway controls the tissue-specific expression of *ZAM*

The identity of the terminal follicle cells where *ZAM* expression is detected, is determined by an oocyte signal transmitted to the follicle cells by the EGF receptor localized at the surface of the follicle cells (18). We therefore investigated whether the EGF receptor pathway is also responsible for the tissue specificity of *ZAM*. For this purpose, we tested the responsiveness of the U3 region through the pZ310 transgenes to signaling molecules of the EGF pathway, and searched whether mutations affecting this signaling also affect the expression of the transgenes.

In the first set of experiments, we generated small marked mosaic clones homozygous for top^{CO} [a null allele of the EGF receptor (*Egfr*) gene, also called *torpedo* in *D.melanogaster*] in the posterior follicle cells during oogenesis, and followed their *ZAM* control by staining for the β-galactosidase activity of the pZ310 transgene. The generation of $Egfr^{-/-}$ follicle cell clones were induced through *FLP* mitotic recombination in females *trans*-heterozygous for hs-*myc* and top^{CO}. One daughter of a recombinant follicle cell carries two copies of *myc*, whereas the other lacks *myc* and is homozygous for top^{CO} (Figure 2A).
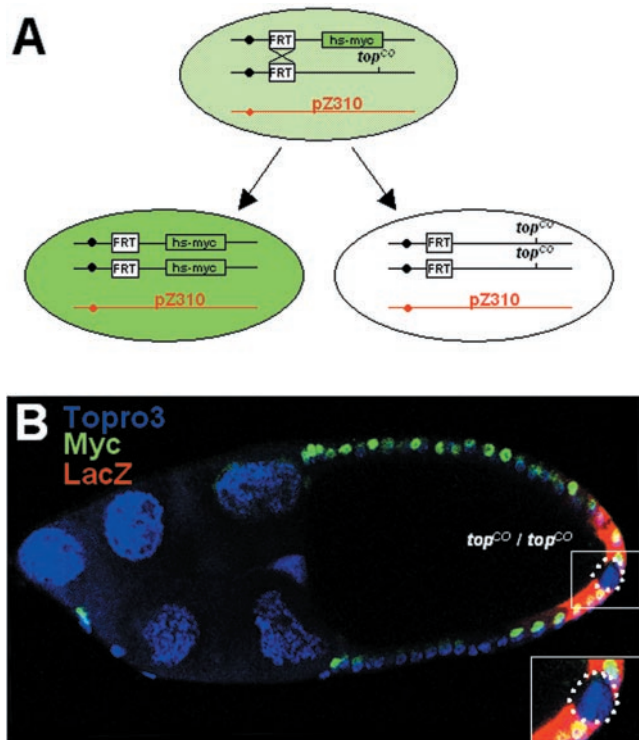
Figure 2. The tissue-specific expression of *ZAM* depends on the EGF receptor pathway. (**A**) Scheme for the generation of $Egfr^{-/-}$ follicle cell clones. *Flp*-mediated recombination was induced in females *trans*-heterozygous for hs-*myc* and top^{CO}, a null allele of the EGF receptor of *Drosophila, torpedo*. One daughter of a recombinant follicle cell carries two copies of *myc*, whereas the other lacks *myc* and is homozygous for top^{CO}. (**B**) A stage 10 egg chamber carrying mutant top^{CO} follicle cell clones at the posterior pole of the oocyte. In these mutant clones highlighted by broken lines, no *LacZ* expression (red) is detected. The wild-type clones express the myc marker (green) and *LacZ* at the posterior pole. In the whole structure, nuclei are in blue due to the ToPro3 marker.

In homozygous top^{CO} clones at the posterior follicle cells of stage 9 to 10 of oogenesis, we found that *ZAM* expression is abolished (Figure 2B). The *LacZ* staining is specifically absent in the mutant top^{CO} cells whereas it is detected in the surrounding cells expressing *myc*. This pattern of expression has been found in all the four clones generated in this experiment. Since removal of the *Egfr* causes an absence of *ZAM* expression, we conclude that *ZAM* expression is dependent on the EGF signaling pathway.

The Pointed transcription factor activates *ZAM* expression

In order to identify the transcription factor involved in *ZAM* expression, we next investigated whether a transcription factor acting downstream of the EGF receptor signaling pathway could be involved in transducing the EGF-type signal to the *ZAM* promoter (17,18). Candidate factors potentially involved are encoded by the *pointed* gene that is transcribed as *ZAM* in the follicle cells at the posterior pole of the oocyte. *Pointed* (*pnt*) encodes two transcription factors, PntP1 and PntP2, containing the same ETS domain that mediates sequence-specific DNA binding (24,25).

In flies bearing the pZ190 transgene, we generated marked mosaic clones, homozygous for $pnt^{\Delta 88}$, a null allele for both
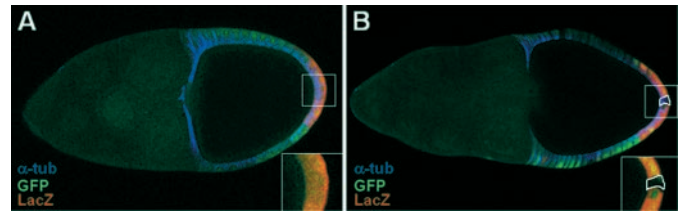

Figure 3. A loss of Pointed expression results in a loss of *ZAM* expression in the posterior follicle cells. (**A**) Wild-type follicle cells at the posterior pole of the oocyte. The wild-type cells express the GFP marker (green) and *LacZ* at the posterior pole. (**B**) In a $pnt^{\Delta 88}$ mutant clone (highlighted by a broken line) at the posterior pole of the oocyte, no *LacZ* expression (red) is detected. The overall structure of the ovariole is labelled for α-tubulin (blue).

PntP1 and PntP2 (19), in the posterior follicle cells. A wild-type chromosome was marked with the *GFP* gene controlled by a *ubiquitin* promotor which directs expression in all the follicle cells. The chromosome bearing the $pnt^{\Delta 88}$ null allele does not carry the *GFP* marker. Thus $pnt^{\Delta 88}$ homozygous mutant cells can be easily detected due to a lack of GFP expression. On another hand, *ZAM* expression is followed by staining for the β-galactosidase activity of the pZ190 transgene. Eleven mosaic clones were generated in posterior follicle cells. *LacZ* staining was never detected in these 11 clones, indicating that pZ190 is only expressed in cells in which Pointed is expressed (Figure 3). Thus, we conclude that *ZAM* expression depends on the Pointed transcription factors.

To further investigate the implication of *Pointed* in the restricted expression of *ZAM* in follicle cells with a posterior fate, we ectopically expressed *pointed* in another cell lineage owing to the GAL4/UAS system (26) and examined the pZ190 expression. The GAL4 driver present in line *55B* was used to induce expression of UAS-target genes in the anterior follicle cells at the ventral and dorsal sides of oocytes in stages 8 to 10, which is illustrated by staining for GFP expressed from an *UAS-GFP* target transgene [Figure 4 and (15)]. The Gal4 line *55B* carrying the *UAS-GFP* transgene as a marker, was then crossed to lines bearing Gal4-responsive *pntP1* or *pntP2* genes: *UAS-pntP1* or *UAS-pntP2*. This cross led to two independent lines with the following genotypes: [*UAS-GFP/CyO*; *UAS-pntP1/55-Gal4*] and [*UAS-GFP/CyO*; *UAS-pntP2/55-Gal4*] in which GFP and PntP1 or PntP2 are co-expressed in the anterior follicle cells. Submitted to the Gal4 driver, expression of these transgenes is thus driven in the same cells as the *UAS-GFP* transgene. Their expression is then indirectly followed through staining for GFP (Figure 4). In a final set of crosses, pZ190 was introduced within the genome leading to flies with the following genotypes: [*UAS-GFP/pZ190*; *UAS-pntP1/55-Gal4*] and [*UAS-GFP/pZ190*; *UAS-pntP2/55-Gal4*]. This allowed testing of the effects of ectopic expression of PntP1 and PntP2, respectively, on *ZAM* transcription by staining for β-galactosidase activity.

When ovaries dissected from females [*UAS-GFP/pZ190*; *UAS-pntP1/55B-Gal4*] were examined no expression of pZ190 over 10 independent clones examined could ever be detected in the anterior follicle cells where PntP1 is ectopically expressed (Figure 4A). In contrast, in ovaries dissected from females with the [*UAS-GFP/pZ190*; *UAS-pntP2/55B-Gal4*] genotype, *LacZ* staining due to an ectopic expression of pZ190 generated was clearly detected in numerous cells
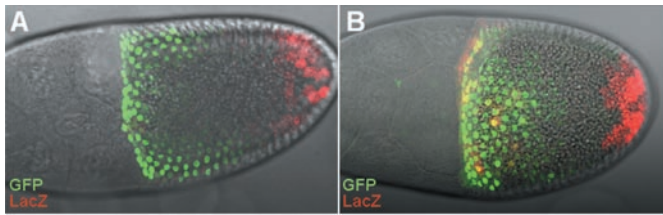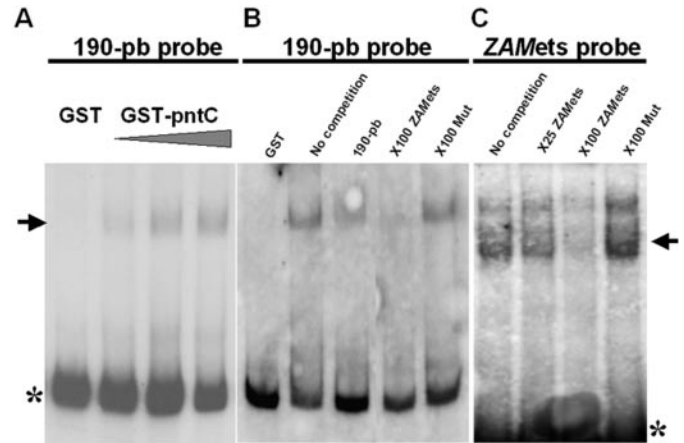
Figure 4. Ectopic expression of Pointed P2 results in ectopic expression of *ZAM*. (**A**) and (**B**) display X-Gal staining (red) resulting from the expression of the pZ190 transgene when Pointed P1 and Pointed P2, respectively, are expressed in the anterior follicle cells using the 55B-Gal4 driver. The ectopic expression of UAS-Pointed P1 and UAS-Pointed P2 is followed due to an UAS-GFP transgene used as a marker. PntP1 and pZ190 are never co-expressed in the anterior follicle cells, while a clear expression of pZ190 is detected in numerous cells of this lineage when PntP2 is expressed.

Figure 5. The ETS-transcription factor, Pointed, binds to an ETS-binding site located 79 bp within *ZAM* LTR. The DNA binding domain of Pointed fused to GST (GST–PntC) was used in these assays. (**A**) The GST–PntC protein specifically binds the first 190 bp of *ZAM*. Increasing amounts of GST-PntC used in this gel-shift assay are 44, 88 and 264 ng. In comparison, no shift is observed with 250 ng of GST protein alone. (**B**) Competition experiments: binding is tested with a 264 ng of unlabeled GST–PntC, 100-fold excess of ZAMets and 100-fold excess of Mut an oligonucleotide mutated for the EBS (see Materials and Methods). (**C**) Competition experiments: the GST–PntC specifically binds the ZAMets oligonucleotide (from nucleotides 72 to 86 on *ZAM* sequence) which contains the presumptive EBS. A 100-fold excess of unlabelled ZAMets competes with the labelled oligonucleotide for the binding. A 100-fold excess of unlabelled oligonucleotide Mut, mutated for the EBS, is unable to compete. The asterisk and the arrow indicate the free probe and the gift, respectively.

located at the anterior pole of the oocyte, and expressing PntP2 (Figure 4B). As illustrated in Figure 4B, *LacZ* staining due to the expression of pZ190 is not observed in all the cells expressing GFP. Several reasons can be considered. First, the PntP2 transgene may not be expressed in all the cells in which the *UAS-GFP* transgene is present. Second, the incomplete rescuing of *LacZ* expression could be due to a lower level of PntP2 expression compared to its endogenous expression within the posterior follicle cells. Third, PntP2 is active as a phosphorylated protein (27). When ectopically expressed, PntP2 may be less phosphorylated and therefore less efficient. Fourth, the transcriptional activation of *ZAM* by PntP2 may require a specific co-factor expressed at a limited rate in these anterior cells. Nevertheless, these experiments clearly show that activation of *ZAM* transcription may be partially recovered, in follicle cells ectopically expressing PntP2. These data indicate a functional requirement of PntP2 for proper patterning of *ZAM* expression in follicle cells with a posterior fate.

Pnt interacts with an ETS binding site located within the first 190 bp of *ZAM*

As Pointed is a transcription factor, there remains the question of whether it binds to the 190 bp of *ZAM* directly or indirectly. We expressed the DNA binding domain of Pointed tagged with GST in bacteria, and performed gel-shift mobility assays using the 190 bp fragment as a probe (see Materials and Methods). We have found that the GST–PntC fusion protein does indeed bind the 190 bp long probe of *ZAM* (Figure 5A) while a control lysate programmed with a GST peptide alone, with no Pointed sequence, is not. Additionally, the ability of an unlabeled 190 bp fragment to outcompete the DNA binding complex confirmed this result (Figure 5B).

We then proceeded to determine which site within the 190 bp is responsible for this binding, and scanned the *ZAM* promoter region *in silico* for putative ETS binding sites (EBS). One site potentially recognized by a *Drosophila* ETS transcription factor (5′-GGAA-3′) was found at position 79 according to *ZAM* sequence (28). If the ETS transcription factor is Pointed, then it should associate directly with this ETS-binding site, and one would expect to see competition when an excess of an oligonucleotide encompassing the domain is used. This is indeed what has been obtained. As shown in Figure 5B, the ZAMets oligonucleotide (from

nucleotides 72 to 86 on *ZAM* sequence, see Material and Methods) efficiently competed with the complex. Additionally, an oligonucleotide such as Mut identical to ZAMets but with a mutated ETS binding site (see Materials and methods) is then unable to compete with the complex (Figure 5B). These results indicate that the transcription factor Pointed directly binds an ETS-binding site present at position 79 within the *ZAM* sequence.

If Pointed associates with the ETS-binding site of *ZAM*, it should bind the ZAMets oligonucleotide in gel-shift assays. We performed a gel-shift assay using the ZAMets oligonucleotide as a probe. We have found that the GST–PntC fusion protein does indeed bind ZAMets (Figure 5C), while a control lysate with a GST peptide alone does not. Additionally, an unlabeled ZAMets primer added as a 25- or 100-fold excess is able to outcompete the DNA binding complex while Mut, the mutated primer for the EBS is not (Figure 5C). Overall, these results indicate that Pointed associates with an ETS binding site located at positions 79 to 82 within the *ZAM* LTR.

DISCUSSION

Oogenesis involves direct interaction between germline cells and somatic follicle cells for the successful development of fertile eggs (29). We have found that such intercellular communications from germ line to somatic line in the first instance, and from somatic line to germ line in the second also play crucial roles in the replication cycle of an LTR retrotransposon *ZAM* from *D.melanogaster*. We previously demonstrated the necessity of a somatic cell to germ cell communication for the

transport of *ZAM* particles. Indeed, *ZAM* particles formed within the follicle cells take benefit of the vittelogenin traffic to sort out of the follicle cells and enter the closely apposed oocyte (1). Here, we show that in the first steps of its replication cycle, *ZAM* requires another signal transmitted by the germ line and received by the somatic line.

The somatic transcription of *ZAM* depends on the Pointed factor acting downstream of the EGF receptor pathway

We previously reported that *ZAM* transcripts are detected in a group of somatically derived cells, which are the follicle cells surrounding the posterior part of the oocyte (1). This transcription of *ZAM* implies that a tissue-specific promoter controls its expression during oogenesis. By analyzing the long terminal repeat and specifally introducing the U3 region (5′ part of the LTR) upstream of the reporter gene *LacZ* into flies (leading to the so-called pZ310 transgenic flies), we demonstrate that this region contains the tissue-specific enhancer of ZAM. Furthermore, a similarly restricted pattern of expression was found in another series of transgenic flies called pZ190 that we have generated using 190 bp of the 5′ end of U3. *LacZ* expression was found to be similar to that observed when the full-length LTR or the U3 region was fused to *LacZ*. These transgenic lines indicate that the essential *cis*-regulatory elements for the transcriptional regulation of the *ZAM* promoter are included within its first 190 bp.

It has been well described that the EGF receptor pathway is necessary for the differentiation of these very specific follicle cells where *ZAM* is expressed. Through a genetic approach, we further demonstrated that the first 190 bp of *ZAM* are also the direct target of the EGF receptor pathway. Mosaic clones of cells homozygous for a null allele of the *torpedo* gene, the *Drosophila* EGF receptor, were generated within the posterior follicle cells. *LacZ* staining due to the pZ310 (U3–*LacZ*) or pZ190 (190 bp–*LacZ*) transgene expression was undetected in the clones, whereas it was present within surrounding cells with a wild-type genotype.

Various factors have been shown to be involved in mediating a transcriptional response upon the reception of signals from the EGF receptor pathway. Two of them are the isoforms encoded by the *pointed* genes: Pointed1 and Pointed2. Ectopic expression of Pointed2 in a subset of follicle cells with an anterior fate of differentiation (instead of a posterior fate as necessary for *ZAM* expression) has shown that the presence of Pointed2 causes an ectopic expression of the pZ190 transgene in these anterior follicle cells. This result contrasts with what is observed in a wild-type background or with ectopic expression of Pointed1 in the anterior follicle cells, which does not allow *ZAM* expression within those follicle cells with an anterior fate. Our data therefore indicate that Pointed2 is a necessary positive effector in *ZAM* expression. Pnt2 is required for proper cell determination during various stages of *Drosophila* development: in the eyes or during the development of renal tubules (30–32). Nevertheless, expression of endogenous *ZAM* elements has never been detected in these tissues. Similarly, when we ectopically expressed Pointed2 in the eyes under control of a Glass-Gal4 driver, no LacZ staining resulting from the ectopic expression of pZ190 could be detected (data not shown). These results indicate that the expression

of PntP2 is necessary but not sufficient to activate *ZAM*. Additional co-factors are almost certainly required for a refined regulation of its transcription in the specific tissue, i.e. the ovaries. Such co-factors have not been identified yet, however, we have to stress the fact that, at this time of our study, Pointed1 cannot be definitely discarded as a potential candidate. Indeed, if PntP2 acts in parallel or downstream of PntP1, our experiments would not have allowed us to detect its involvement in *ZAM* transcription.

Whatever the multiple factors involved, we have provided further evidence that Pointed, and its ETS-binding site common to PntP1 and PntP2, is able to activate *ZAM* transcription via its direct binding to *cis*-regulatory elements present in the responsive target of 190 bp. *In silico* we have found that an ETS binding site (5′-GGAA-3′), located within the 190 bp at position 79 according to *ZAM* sequence, is indeed the precise target DNA sequence recognized by Pointed. Overall, these data indicate that Pointed binds *ZAM* LTR and is a key factor in determining the strict expression of *ZAM* in the follicle cell layer in response to a signal emitted by the oocyte.

An outcome of the present investigation is that genes that are regulated by the Pointed factor and participate in differentiation of the posterior lineage of follicle cells could be identified by using the characterized target DNA sequence of *ZAM* as an easily detectable marker. Thus it provides an entry point to monitor the EGF receptor pathway and study mechanisms of posterior follicle cell induction and patterning in *Drosophila* at the transcriptional level. Additionally, our study provides an experimental tool, the *ZAM* promoter, which can be specifically used to direct expression of any transgene to the posterior pole of the oocyte.

Biological significance of a retroviral cycle starting within a somatic tissue, whereas its ultimate insertion occurs within the germ line

At the biological level, an intriguing question yet to be answered is why *ZAM* expression takes place in a somatic tissue in response to a signal (Gurken) transmitted by the oocyte while novel insertions are precisely known to occur in the germ line (7,33). We can hypothesize that it is advantageous for the retrotransposon to produce its transcripts in somatic cells to circumvent transcriptional arrest of the oocyte during meiosis. Alternatively, a high expression of *ZAM* occurring directly within the germ line might generate too many proviral copies of *ZAM* and be deleterious for the next generation. In the course of evolution, an adaptive strategy to limit the extent of *ZAM* insertion within the germ line could have led the element to be expressed outside of the germ line. Expression within a somatic lineage and its subsequent transfer to the germ line could decrease the efficiency of its insertion and confer a selective advantage. Alternatively, some defence mechanism may specifically protect the germ line from the presence of any retroviral/retrotransposon RNA. This constraint may have led *ZAM* to adopt a novel strategy to invade the germ line. It starts its replication cycle and assembles viral-like particles (VLP) in a somatic tissue of the reproductive apparatus. While encapsidated in particles, the RNA genome is converted into a cDNA. When VLPs enter the closely apposed germ line by virtue of retroviral infection, DNA copies of the retroelement are no more recognized by the

host defence selectively addressed against RNA invasions. The retroviral element can then complete its replication cycle leading to insertion within the host germ-line.

Recent studies implicate the biological role of dsRNA-mediated silencing as a transposon repression and antiviral mechanism (34–37). This host surveillance system is known to be active in the germ line (38,39) and could certainly account for such a protection against LTR retrotransposons. However, numerous questions remain to be addressed to demonstrate whether this mechanism could have acted as an evolutionary constraint, resulting in *ZAM*, and potentially some related elements being transcribed in the somatic lineage.

This expression of *ZAM* within a somatic tissue apposed to the germ line is reminiscent of what is observed for some mouse or chicken viruses (40,41), or for endogenous retroviral particles which are massively expressed in the human placenta (42,43). A question raised by our data is: could some HERVs be expressed in placental tissues because of a signal emitted by the fetus? The fetal tissue would then be responsible for the acquisition of specific trophoblastic functions that have been attributed potentially to HERVs, such as protection against retroviral infection, protection of the fetus against maternal immune system, or placenta morphogenesis through fusogenic effects (44,45). Although the question is open, it would be worth studying signals coming from the fetus, that could ultimately be responsible for endogenous retroviruses activation in the placenta.

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REFERENCES

1. Leblanc,P., Desset,S., Giorgi,F., Taddei,A.R., Fausto,A.M., Mazzini,M., Dastugue,B. and Vaury,C. (2000) Life cycle of an endogenous retrovirus, ZAM, in *Drosophila melanogaster*. *J. Virol.*, **74**, 10658–10669.

2. Pelisson,A., Song,S.U., Prud'homme,N., Smith,P.A., Bucheton,A. and Corces,V.G. (1994) Gypsy transposition correlates with the production of a retroviral envelope-like protein under the tissue-specific control of the *Drosophila* flamenco gene. *EMBO J.*, **13**, 4401–4411.

3. Matyunina,L.V., Jordan,I.K. and McDonald,J.F. (1996) Naturally occurring variation in copia expression is due to both element (*cis*) and host (*trans*) regulatory variation. *Proc. Natl Acad. Sci. USA*, **93**, 7097–7102.

4. Lammel,U. and Klambt,C. (2001) Specific expression of the *Drosophila* midline-jumper retro-transposon in embryonic CNS midline cells. *Mech. Dev.*, **100**, 339–342.

5. Vogel,A.M. and Gerster,T. (1999) Promoter activity of the zebrafish bhikhari retroelement requires an intact activin signaling pathway. *Mech. Dev.*, **85**, 133–146.

6. Cavarec,L., Jensen,S., Casella,J.F., Cristescu,S.A. and Heidmann,T. (1997) Molecular cloning and characterization of a transcription factor for the copia retrotransposon with homology to the BTB-containing lola neurogenic factor. *Mol. Cell. Biol.*, **17**, 482–494.

7. Leblanc,P., Desset,S., Dastugue,B. and Vaury,C. (1997) Invertebrate retroviruses: ZAM a new candidate in *D.melanogaster*. *EMBO J.*, **16**, 7521–7531.

8. Desset,S., Meignin,C., Dastugue,B. and Vaury,C. (2003) COM, a heterochromatic locus governing the control of independent endogenous retroviruses from *Drosophila melanogaster*. *Genetics*, **164**, 501–509.

9. Gonzalez-Reyes,A. and St Johnston,D. (1994) Role of oocyte position in establishment of anterior–posterior polarity in *Drosophila*. *Science*, **266**, 639–642.

10. Gonzalez-Reyes,A., Elliott,H. and St Johnston,D. (1995) Polarization of both major body axes in *Drosophila* by gurken-torpedo signalling. *Nature*, **375**, 654–658.

11. Roth,S., Neuman-Silberberg,F.S., Barcelo,G. and Schüpbach,T. (1995) *Cornichon* and the EGF receptor signalling process are necessary for both anterior–posterior and dorsal–ventral pattern formation in *Drosophila*. *Cell*, **81**, 967–978.

12. Neuman-Silberberg,F.S. and Schupbach,T. (1993) The *Drosophila* dorsoventral patterning gene gurken produces a dorsally localized RNA and encodes a TGF alpha-like protein. *Cell*, **75**, 165–174.

13. Neuman-Silberberg,F.S. and Schupbach,T. (1996) The *Drosophila* TGF-alpha-like protein Gurken: expression and cellular localization during *Drosophila* oogenesis. *Mech. Dev.*, **59**, 105–113.

14. Lu,X., Melnick,M.B., Hsu,J.C. and Perrimon,N. (1994) Genetic and molecular analyses of mutations involved in *Drosophila* raf signal transduction. *EMBO J.*, **13**, 2592–2599.

15. Brand,A.H. and Perrimon,N. (1994) Raf acts downstream of the EGF receptor to determine dorsoventral polarity during *Drosophila* oogenesis. *Genes Dev.*, **8**, 629–639.

16. Schnorr,J.D. and Berg,C.A. (1996) Differential activity of Ras1 during patterning of the *Drosophila* dorsoventral axis. *Genetics*, **144**, 1545–1557.

17. Morimoto,A.M., Jordan,K.C., Tietze,K., Britton,J.S., O'Neill,E.M. and Ruohola-Baker,H. (1996) Pointed, an ETS domain transcription factor, negatively regulates the EGF receptor pathway in *Drosophila* oogenesis. *Development*, **122**, 3745–3754.

18. Gonzalez-Reyes,A. and St Johnston,D. (1998) The *Drosophila* AP axis is polarised by the cadherin-mediated positioning of the oocyte. *Development*, **125**, 3635–3644.

19. Scholz,H., Deatrick,J., Klaes,A. and Klambt,C. (1993) Genetic dissection of pointed, a *Drosophila* gene encoding two ETS-related proteins. *Genetics*, **135**, 455–468.

20. Klaes,A., Menne,T., Stollewerk,A., Scholz,H. and Klambt,C. (1994) The Ets transcription factors encoded by the *Drosophila* gene pointed direct glial cell differentiation in the embryonic CNS. *Cell*, **78**, 149–160.

21. Clifford,R.J. and Schupbach,T. (1989) Coordinately and differentially mutable activities of torpedo, the *Drosophila melanogaster* homolog of the vertebrate EGF receptor gene. *Genetics*, **123**, 771–787.

22. Xu,T. and Rubin,G.M. (1993) Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development*, **117**, 1223–1237.

23. Kauffmann,R.C., Li,S., Gallagher,P.A., Zhang,J. and Carthew,R.W. (1996) Ras1 signaling and transcriptional competence in the R7 cell of *Drosophila*. *Genes Dev.*, **10**, 2167–2178.

24. Klambt,C. (1993) The *Drosophila* gene pointed encodes two ETS-like proteins which are involved in the development of the midline glial cells. *Development*, **117**, 163–176.

25. Kodandapani,R., Pio,F., Ni,C.Z., Piccialli,G., Klemsz,M., McKercher,S., Maki,R.A. and Ely,K.R. (1996) A new pattern for helix–turn–helix recognition revealed by the PU.1 ETS-domain-DNA complex. *Nature*, **380**, 456–460.

26. Brand,A.H. and Perrimon,N. (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development*, **118**, 401–415.

27. Baonza,A., Murawsky,C.M., Travers,A.A. and Freeman,M. (2002) Pointed and Tramtrack69 establish an EGFR-dependent transcriptional switch to regulate mitosis. *Nat. Cell. Biol.*, **4**, 976–980.

28. Macleod,K., Leprince,D. and Stehelin,D. (1992) The ets gene family. *Trends Biochem. Sci.*, **17**, 251–256.

29. Goode,S., Wright,D. and Mahowald,A.P. (1992) The neurogenic locus brainiac cooperates with the *Drosophila* EGF receptor to establish the ovarian follicle and to determine its dorsal–ventral polarity. *Development*, **116**, 177–192.

30. Brunner,D., Ducker,K., Oellers,N., Hafen,E., Scholz,H. and Klambt,C. (1994) The ETS domain protein pointed-P2 is a target of MAP kinase in the sevenless signal transduction pathway. *Nature*, **370**, 386–389.

31. O'Neill,E.M., Rebay,I., Tjian,R. and Rubin,G.M. (1994) The activities of two Ets-related transcription factors required for *Drosophila* eye development are modulated by the Ras/MAPK pathway. *Cell*, **78**, 137–147.

32. Sudarsan,V., Pasalodos-Sanchez,S., Wan,S., Gampel,A. and Skaer,H. (2002) A genetic hierarchy establishes mitogenic signalling and mitotic competence in the renal tubules of *Drosophila*. *Development*, **129**, 935–944.

33. Desset,S., Conte,C., Dimitri,P., Calco,V., Dastugue,B. and Vaury,C. (1999) Mobilization of two retroelements, ZAM and Idefix, in a novel unstable line of *Drosophila melanogaster*. *Mol. Biol. Evol.*, **16**, 54–66.

34. Aravin,A.A., Naumova,N.M., Tulin,A.V., Vagin,V.V., Rozovsky,Y.M. and Gvozdev,V.A. (2001) Double-stranded RNA-mediated silencing of genomic tandem repeats and transposable elements in the *D. melanogaster* germline. *Curr. Biol.*, **11**, 1017–1027.

35. Mourrain,P., Beclin,C., Elmayan,T., Feuerbach,F., Godon,C., Morel,J.B., Jouette,D., Lacombe,A.M., Nikic,S., Picault,N. *et al.* (2000) Arabidopsis SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. *Cell*, **101**, 533–542.

36. Waterhouse,P.M., Wang,M.B. and Lough,T. (2001) Gene silencing as an adaptive defence against viruses. *Nature*, **411**, 834–842.

37. Plasterk,R.H., Izsvak,Z. and Ivics,Z. (1999) Resident aliens: the Tc1/mariner superfamily of transposable elements. *Trends Genet.*, **15**, 326–332.

38. Kennerdell,J.R., Yamaguchi,S. and Carthew,R.W. (2002) RNAi is activated during *Drosophila* oocyte maturation in a manner dependent on aubergine and spindle-E. *Genes Dev.*, **16**, 1884–1889.

39. Sijen,T. and Plasterk,R.H. (2003) Transposon silencing in the *Caenorhabditis elegans* germ line by natural RNAi. *Nature*, **426**, 310–314.

40. Lock,L.F., Keshet,E., Gilbert,D.J., Jenkins,N.A. and Copeland,N.G. (1988) Studies of the mechanism of spontaneous germline ecotropic provirus acquisition in mice. *EMBO J.*, **7**, 4169–4177.

41. Di Stefano,H.S. and Dougherty,R.M. (1966) Mechanisms for congenital transmission of avian leukosis virus. *J. Natl Cancer Inst.*, **37**, 869–883.

42. Villarreal,L.P. and Villareal,L.P. (1997) On viruses, sex, and motherhood. *J. Virol.*, **71**, 859–865.

43. Harris,J.R. (1998) Placental endogenous retrovirus (ERV): structural, functional, and evolutionary significance. *Bioessays*, **20**, 307–316.

44. Blaise,S., de Parseval,N., Benit,L. and Heidmann,T. (2003) Genomewide screening for fusogenic human endogenous retrovirus envelopes identifies syncytin 2, a gene conserved on primate evolution. *Proc. Natl Acad. Sci. USA*, **100**, 13013–13018.

45. Mallet,F., Bouton,O., Prudhomme,S., Cheynet,V., Oriol,G., Bonnaud,B., Lucotte,G., Duret,L. and Mandrand,B. (2004) The endogenous retroviral locus ERVWE1 is a bona fide gene involved in hominoid placental physiology. *Proc. Natl Acad. Sci. USA*, **101**, 1731–1736.