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Original Article

Germ-line expression of a functional LINE from *Drosophila melanogaster:* fine characterization allows for potential investigations of *trans*-regulators

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ABSTRACT The I factor (IF) is a functional non-viral retrotransposon, or LINE, from *Drosophila melanogaster*. It is mobilized in the germ-line of dysgenic SF females during I-R hybrid dysgenesis. In previous papers (Lachaume *et al., Development 115:* 729-735, 1992; Lachaume and Pinon, *Mol. Gen. Gen. 240:* 277-285, 1993) we used a transgenic fusion between the 5' part of the IF and the *lacZ* gene to characterize IF expression and its regulation. This *I-lacZ* transgenic fusion expresses ß-galactosidase activity during oogenesis. We established a *Drosophila* line bearing four transgenic insertions (the *4I-lacZ* line) and got new insights about IF expression: (1) *I-lacZ* expression is proportional to the copy number of transgenes present in the genome, (2) the expression occurs just before or when meiosis begins, (3) this expression seems to be subjected to a variegation effect within the germ-line cells, (4) the transgenic activity is mainly directed toward the decondensed chromatin of nurse cells. The close relationship between I factor expression and oogenesis led us to investigate the role played by genes expressed during oogenesis on I factor expression. We present recent data indicating that mutants which interfere with oogenesis can also affect I factor expression. From this data we propose an original screen using the *4I-lacZ* line to detect identified mutations which also affect I factor expression.

KEY WORDS: *I factor, Drosophila, oogenesis, meiosis, addressing, regulation*

Introduction

LINEs (Long Interspersed Nuclear Elements) are repetitive elements of less than 10 kb long, dispersed in the genomes of plants, protists and insects as well as mammals (Finnegan, 1989). So far the transposition cycle of these elements and the way they are regulated are not well defined. I factor (IF) from *Drosophila melanogaster* has been extensively studied since it is the only LINE which can be mobilized by a simple cross (Bucheton, 1990). In *Drosophila*, inducer (I) strains carry functional IFs on their chromosomes whereas reactive (R) strains do not. However R strains are characterized by a particular cellular state maternally transmitted, called reactivity (Picard *et al.*, 1972), and probably involved in IF mobilization (Lachaume and Pinon, 1993). Crosses between I males and R females give dysgenic females, called SF females, which lay eggs that usually do not hatch (reviewed by Bregliano and Kidwell, 1983). In the germ-line of SF females, the IFs transpose at a high frequency producing chromosomal rearrangements and insertional mutagenesis in the progeny (Busseau *et al.*, 1989).

The active IFs are 5.4 kb long and possess two open reading frames (ORF) (Fawcett *et al.*, 1986; Abad *et al.*, 1989). ORF1 (1287 bp) encodes a putative polypeptide with zinc fingers homologous to the nucleic acid binding motif found in retroviral "gag" protein. ORF2 (3675 bp) encodes a polypeptide showing similarities to viral reverse transcriptase and to RNase H (Finnegan, 1988). A full length transcript has been proposed as the transposition intermediate. This transcript is found in ovaries of SF females (Chaboissier *et al.*, 1990) and its synthesis is driven by the IF itself via an internal promoter (McLean *et al.*, 1993).

We have constructed a translational fusion between the 5' part of the IF and *lacZ* from *E. coli* (Lachaume *et al.*, 1992). P-mediated transformation of the strong reactive *Charolles* strain was per-

Abbreviations used in this paper: IF, I factor; LINE, long interspersed nuclear element; SF, stérilité femelle; I, inducer; R, reactive; ORF, open reading frame; gag, group-specific antigen; RNaseH, Ribonuclease H; X-gal, 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside; ONPG, ortho-nitrophenyl ß-D-galactopyranoside; A, absorbance; l.m., light microscope; e.m., electron microscope.

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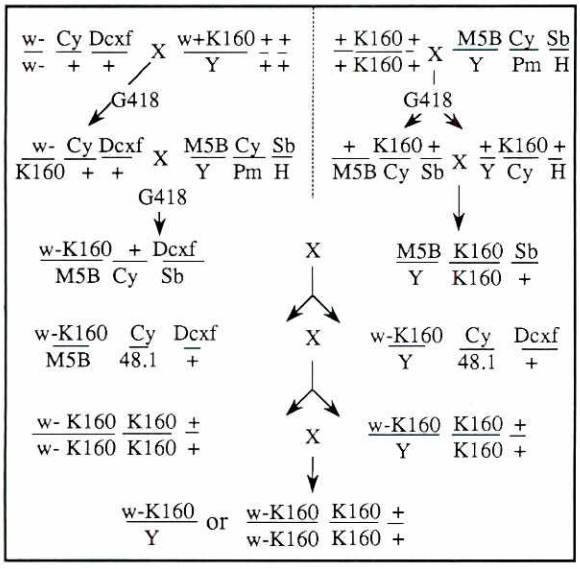


Fig. 1. Establishment of the *4l-LacZ* reactive line. *Balancer chromosomes Müller 5 Bar (M5B), Curly O(Cy) and Dcxf were used as well as homozygous lethal markers stubble (Sb) and hairless (H). 53.1 and 48.1 lines were homozygous for K160 insertion respectively on chromosome X and II. K160 transgene bore the l-lacZ fusion and conferred G418 resistance (Lachaume et al., 1992). Recombination events between K160 and the w^K allele of the white gene was obtained by selecting G418 resistant line associated with the white⁻ phenotype. The 4l-LacZ line harbored 4 insertions of K160, a white⁻ genetic background and was reactive in the I-R system of hybrid dysgenesis.*

formed with this construct. Expression of such a transgenic fusion was observed only in the germ-line of females from reactive mothers as is the case for the 5.4 kb RNA. Furthermore parameters which modify the I-R hybrid dysgenesis (i.e. reactivity level, ageing, heat shock) also modify the expression of the *l-lacZ* fusion (Lachaume and Pinon, 1993). This demonstrates that all signals necessary for the specificity of expression during development are located on the short segment of the IF used in this fusion and that this construct is an efficient marker to follow IF expression.

This work describes new points on IF expression and addressing: (1) a significant expression of the IF is detected before/when meiosis begins, (2) this expression could be subjected to a variegation effect and (3) gives products which are specifically addressed to decondensed chromatin of germ-line cells, as might be expected from a putative retrotransposition complex.

Furthermore, taking advantage of this well defined expression pattern, we searched for mutations that modify I factor expression using the *l-lacZ* fusion as a marker. This founded the basis of a new approach to study the germ-line regulation of I factor expression.

Results

Expression level in ovaries is dependent upon the copy number of transgenes

In a previous study we showed that the pattern of expression of transgenic *l-lacZ* fusions was similar during oogenesis in four different transgenic lines (Lachaume *et al.*, 1992). This expression was low and detectable by X-gal staining only when the insertion was homozygous.

In order to increase the *l-lacZ* expression we decided to construct a strain homozygous for two insertions: the *4l-lacZ* line. We used the *53.1* and *48.1* lines, homozygous for a functional transgenic insertion: the first on chromosome X, the later on chromosome II. Both exhibited similar levels of transgenic ß-galactosidase activities in ovaries.

We constructed the *4l-lacZ* line using balancer chromosomes to avoid crossing over except on the X chromosome where recombination events between K160 and the w^K allele (Lüning, 1981) of the *white* gene were selected (Fig. 1). Thus we obtained the *4l-lacZ* line: a *white*⁻ strong reactive line homozygous for both insertion sites of the *48.1* and *53.1* lines, and therefore harboring 4 copies of the *l-lacZ* fusion per genome.

We tested the effect of copy number (0 to 4 copies of the transgenic fusion) on ß-galactosidase expression. We found that the higher the copy number of transgenes, the higher the accumulation of transgenic activity (Fig. 2). This clearly shows that expression of *l-lacZ* fusions detected in *48.1* and *53.1* strains was not the highest level of IF expression we can detect (Lachaume *et al.*, 1992; Lachaume and Pinon, 1993). Thus, by using the *4l-lacZ* line and its increased expression level, it may be possible to detect new characteristics of IF expression.

Significant expression of IF is detected before/when meiosis begins

The peak of expression for transgenic *l-lacZ* fusions was described between stage 4 and 9 of oogenesis (Lachaume *et al.*, 1992; stages according to King, 1970; Fig. 3A to 3C). By that time

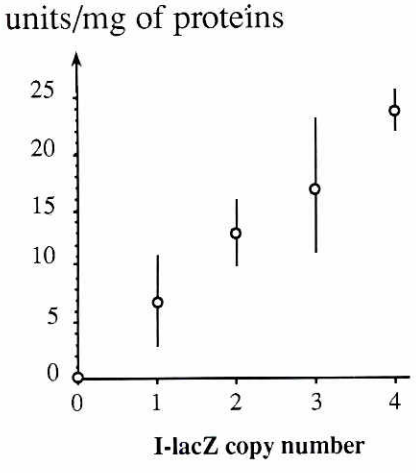


Fig. 2. ß-galactosidase activity as a function of *l-LacZ* copy number. *Ovaries were dissected from females between 4 and 7 days after hatching and analyzed as described in Materials and Methods. Mean values and standard deviation error bars for 0 copies of l-LacZ (Charolles line; 5 different extracts) 1 copy of l-LacZ (females issued from the cross between 53.1 females and Charolles males; 3 different extracts) 2 copies of l-LacZ (females issued from the cross between 4l-LacZ females and Charolles males; 9 different extracts) 3 copies of l-LacZ (females issued from the cross between 4l-LacZ females and 53.1 males; 4 different extracts) and 4 copies of l-LacZ (4l-lacZ line; 2 different extracts) are given in units/mg of proteins.*

Unscheduled DNA Synthesis (UDS), and probably recombination, are still active (Spradling, 1993). X-gal staining performed as usual but on isolated ovaries from 4I-lacZ females (Fig. 3D) disclosed 2 new facts:

First, a significant expression of the transgenic activity was recorded in the germarium (Fig. 3F and G). Most cysts were colored at stage 1 of oogenesis as well as 50% of the germarium in region 2b (Fig. 3F to 3H). However no coloration was detected in the stem cells located at region 1 of the germarium.

Second, discontinuous patterns of coloration (i.e. cyst(s) with no coloration between two colored ones (Fig. 3E)) were present in the *4I-lacZ* line, despite the high level of ß-galactosidase expression recorded. This suggests that discontinuity which was a characteristic of the *48.1* strain was not due to a threshold of coloration but truely reflects some variegation effect within the germ-line cells.

Nuclear addressing of the I-lacZ fusion protein

Both the large size of nurse cell nuclei and their clear compartmentalization made them ideal for localizing the transgenic proteins inside these nuclei. This was done between stages 4 and 9 of oogenesis, when transgenic activity culminates.

X-gal colored ovaries were fixed, embedded, cut and analyzed by electron microscope (e.m.) as described in Materials and Methods. Ovaries from the *Charolles* strain were used as a negative control. They were not stained after X-gal coloration (Fig. 3A) and cristals caracteristic of ß-galactosidase activity could not be detected by e.m. studies (Fig. 4A). In contrast, nurse cells from *4I-lacZ* flies were heavily stained when observed under l.m. (Fig. 3D), and exhibited numerous crystals of various length under e.m. (Fig. 4B). Such crystals were easily recorded within X-gal colored ovaries even on non-contrasted sections (Fig. 4C) and were mainly nuclear. In nurse cells most of these crystals were found in decondensed chromatin, in some cases overlapped slightly with condensed chromatin, but were never present in the nucleolus.

Mutants with abnormal pattern of expression

Since the expression level of each *I-lacZ* transgenic copy is cumulative and linear from 0 to 4 copies (see Fig. 2), the *2I-lacZ* lines *(48.1 or 53.1)* can be used to monitor either an increase or a decrease in I factor expression. This is a strinking point because

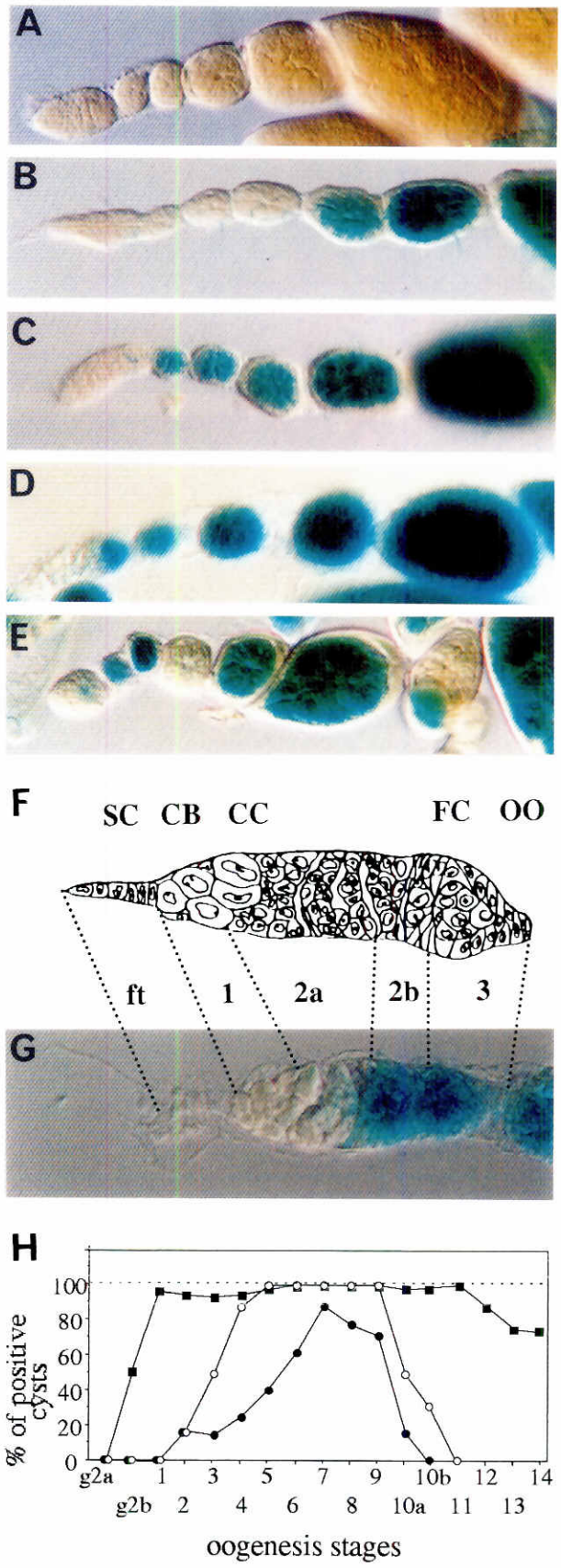
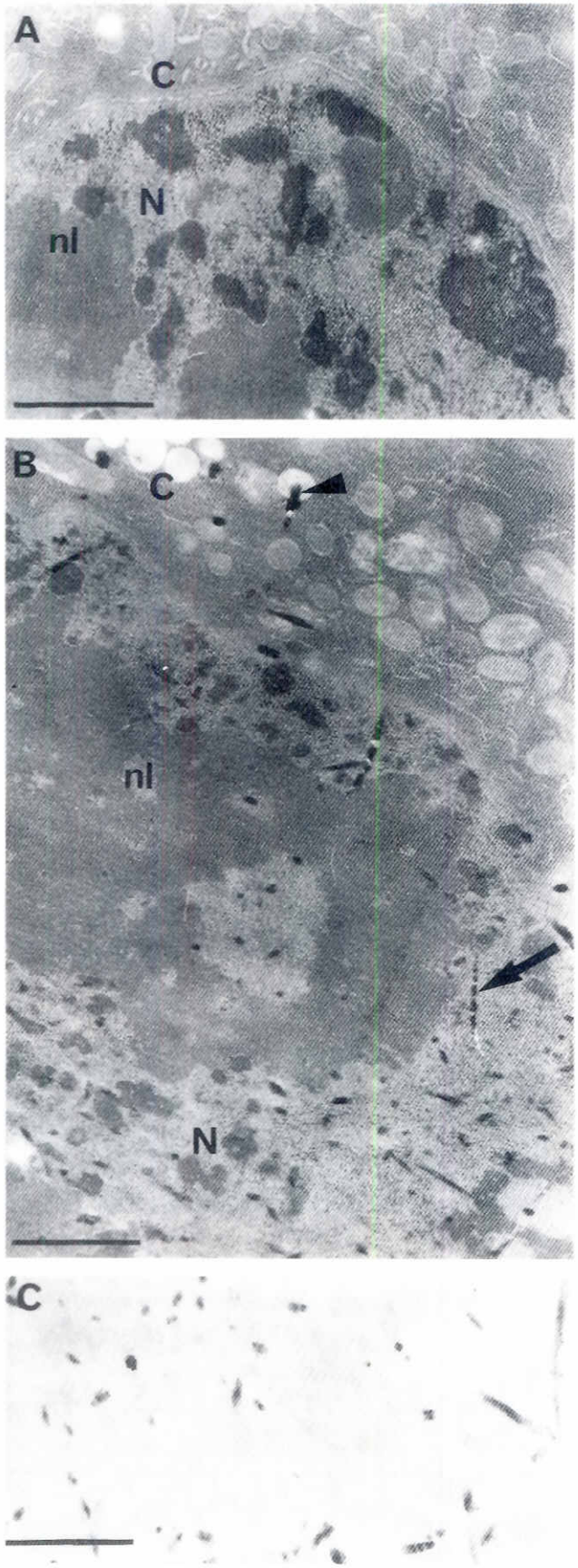


Fig. 3. Detection by X-gal staining of the ß-galactosidase expression in ovaries from *Drosophila* lines transgenic for K160. *X-gal staining of ovaries was done as previously described (Lachaume et al., 1992).* **(A)** *Charolles line used as a negative control.* **(B)** *Transgenic-line 48.1 harboring one homozygous insertion of K160 on the second chromosome.* **(C)** *Transgenic-line 53.1 harboring one homozygous insertion of K160 on the X chromosome.* **(D,E)** *Transgenic-line 4I-lacZ, homozygous for K160 on chromosomes II and X.* **(D)** *In most of the ovarioles tested expression was detected from region 2b of the germarium.* **(E)** *But in 29% of the ovarioles tested one non-colored cyst was flanked by two strongly stained cysts.* **(F-G)** *Germarium details of the 4I-lacZ line:* **(F)** *schematic representation of the different areas of the germarium (adapted from King, 1970): terminal filaments (ft), regions 1, 2a, 2b and 3 (region 3 is also the stage 1 of oogenesis). Stem cell (SC), cystoblasts (CB), cystocytes (CC), follicular cells (FC) and oocyte (OO) are also positioned.* **(G)** *ß-gal expression was recorded in regions 2b and 3 of the germarium.* **(H)** *Evolution of the percentage of germinal cysts expressing K160 during oogenesis. Percentage of positive egg chambers at different stages were estimated in ovaries of 48.1 (black circles), 53.1 (open circles) and 4I-lacZ (black squares). Magnifications were x240 for A to F and x480 for G.*



the I-lacZ fusion could then be used to detect mutations affecting I factor expression. Because I factor is expressed during oogenesis, we suspected that mutations which interfere with oogenesis could also deregulate I factor expression.

To test this hypothesis we screened for EMS (ethyl methyl sulfonate)-induced mutations of recessive female sterility (fs) on the X chromosome. The mutagenesis was carried out in a reactive strain (the screenning and characterization of these *trans*-acting mutations will be described elsewhere, Pinon *et al.*, in preparation). Some of them have an effect on *I-lacZ* expression. In fact, three classes of abnormal expression of the *I-lacZ* fusion were detected: under-, over- and/or extended expression (Fig. 5).

The establishment of the *4I-lacZ* line offered a simpler alternative to this approach. In fact, known dominant mutants could be assayed for their deregulatory effect on I factor expression by a simple cross with the *4I-lacZ* line. It should be noted that all available mutants belong to the inducer class: their effects on *I-lacZ* fusion can be monitored only in dysgenic context. To initiate this screen, males bearing a dominant female sterile mutation (Fs) on the X chromosome (Fs(1); Komitopoulou *et al.*, 1983) or on the third chromosome (Fs(3), Erdélyi and Szabad, 1989) were crossed with *4I-lacZ* reactive females. The hybrid females were dysgenic and bore 2 copies of *I-lacZ* fusion. For all the mutations tested (see material and methods), the pattern of expression of ß-galactosidase was similar to the pattern observed in control females from the same cross but without the Fs mutation.

Discussion

Expression of *I-lacZ* transgenic fusions is restricted to the germline of females from reactive mothers during oogenesis (Lachaume *et al.*, 1992). From this study we can add that a significant surge of expression is observed in region 2b of the germarium. In this region follicle cells migrate from the wall of the germarium to surround cystocytes and may provide a necessary signal to initiate I factor expression. Each cyst being formed contains either 8 cystocytes where meiosis has not begun (i.e. premeiotic cells) or 16 cystocytes (one of which enters meiosis) from which a single gamete will arise (see Spradling, 1993). Therefore even if IF transposes actively in premeiotic cells (8 cystocytes stage), each gamete is unique with regard to every new insertion of IF in the genome. This temporal expression still coincides with the fact that "cluster of transpositions" (several gametes identical for the same transposition event) have rarely if ever been observed after I-R hybrid dysgenesis whereas they are the general case for P-element transposition (Engels, 1989).

At the end of pachytene, when *I-lacZ* expression culminates, ultrastructural studies showed that most of the ß-galactosidase activity is localized in the decondensed chromatin of germ-line cells. As IF appears devoid of integrase activity, it has been

Fig. 4. Micrographs of nurse cells from stage 10 egg chambers. (A) *Charolles strain used as a control, no ß-gal crystals were present in the nucleus (N) nor in the cytoplasm (C).* **(B,C)** *4I-lacZ strain: ß-gal was abundant inside the nucleus especially in decondensed chromatin (arrows), but was absent in the nucleolus (n); deposite was also present in cytoplasmic vacuoles (arrowhead).* **(B)** *Ultra-thin section contrasted by uranyl acetate and lead citrate.* **(C)** *Nucleoplasm of the cell in an uncontrasted section. (magnification: x19000, Bar, 1 μm).*

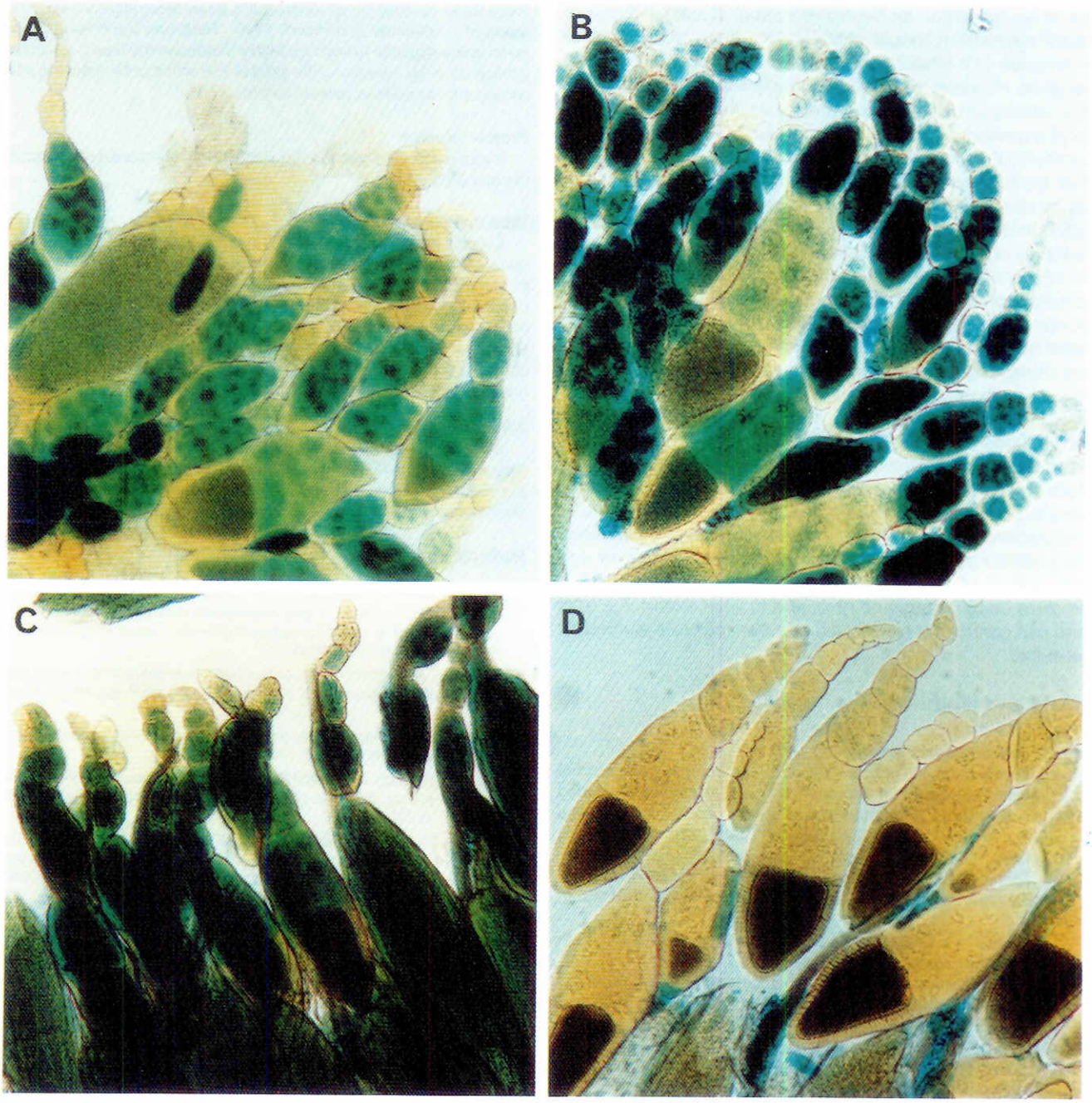


Fig. 5. Detection by X-gal staining of the ß-galactosidase expression in ovaries from *53.1* line transgenic for K160 and homozygous for an fs(1) mutation. (A) *53.1 line (see Fig. 2C for details of expression).* **(B)** *53.1 line homozygous for fs(1)608e: this particular mutation overexpresses the I-lacZ fusion without any significant modification of the pattern.* **(C)** *53.1 line homozygous for fs(1)74d: this mutation induces a prolongation of the ß-galactosidase expression which is detected from stage 2 to stage 14 of oogenesis.* **(D)** *53.1 line homozygous for fs(1)185b: this mutation induces strong down-regulation of K160 expression.*

proposed that its insertion relies on the availability of chromosomal double strand breaks (Bucheton, 1990) common during meiosis (see Hawley and Arbel, 1993). Thus this subtle nuclear localization of the fusion protein is exactly what we expected since it could mimic the compartimentalization of a retrotransposition complex which has been suggested for the transposition cycle of other LINEs (Deragon *et al.*, 1990). Furthermore this confirms again that all signals necessary for the specificity of expression and addressing during development are located on our *I-lacZ* fusion.

We also found that, for a given level of reactivity, expression of *I-lacZ* fusions is directly proportional to the number of transgenic copy number (0 to 4) meaning that the pool of transcription factors

is not a limited condition for the expression of at least 4 copies of IF. Thus IF expression should be considered more as the cumulative expression of a small population than as the expression of a unique gene. However we have previously shown that there is a positive correlation between the maternal level of reactivity and the level of IF expression and proposed the I-lacZ fusion as a new tool for quantifying the reactivity level (Lachaume and Pinon, 1993). All together, these results seem to indicate that the level of IF expression could be regulated not only by the reactivity level but also by the copy number of functional IF. An attractive explanation could be that reactivity controls the level of expression of each individual IF, maybe by controlling chromatin structure and the availability of transcription complexes to each transgenic copy. The variegated expression of *I-lacZ* fusion protein which we observed in the 4*I-lacZ* line could be the reflection of this phenomenon.

Also interesting is the fact that mutations which affect I factor expression during oogenesis can now be screened using the *4I-lacZ* line. Until now we have not succeeded, but the negative results obtained with the Fs mutants could be considered as valuable results: none of the tested mutations significantly affected the I factor expression. Other dominant mutations will be tested in this simple screen. Using this approach, we will not be able to detect mutations acting on I factor transposition but only mutations affecting either I factor transcription or translation. However, the characterization of mutants deregulated for I factor expression should allow us to understand how, and to what extent, the host genome can control the expression of a transposable element in the germ-line.

Materials and Methods

Drosophila melanogaster strains

All the *Drosophila* strains used in this study are from the stocks of the "Laboratoire de Génétique" of Clermont-Ferrand and were described elsewhere, excepted for Fs(1) mutants: ovo^{D1}, ovo^{D2}, ovo^{D3} (Komitopoulou *et al.*, 1983), and Fs(3) mutants: Apc^{10d}, $Avar^{2a}$, $Damasa^{16b}$, $Damasa^{21m}$, $Gerec^{15e}$, $Horka^{12h}$, $Huba^{8/4b}$, $Keve^{17d}$, $Levente^{3f}$, $Tevel^{4a}$, $Tomaj^{29d}$, $Tomaj^{4091}$, $Tonuz^{5a}$, $Varas^{5b}$, $Zerind^{15d}$, $Zolta^{20e}$ (Erdélyi and Szabad, 1989). All the strains are maintained in axenic medium from David (1959) at 20°C.

ß-galactosidase activity

For *in situ* detection isolated ovaries were stained with X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside) as described in Lachaume *et al.* (1992). For quantitative assays on ovarian extracts we performed as follows: 10 pairs of ovaries were dissected in buffer A (10 mM Na phosphate pH 7, 0.1% Triton X100). They were crushed in 100 ml of buffer A using small pestles specifically adapted for 1.5 ml microtubes. After centrifugation, the supernatant was collected. ß-galactosidase activity of this extract was measured twice at each pH as follows. 75 mM citrate-phosphate buffer (from pH 4.0 to pH 6.2) and phosphate buffer (from pH 6.6 to pH 8) were obtained at various pH after adjustment to 10 mM KCl, 1 mM $MgSO_4$, 50 mM ß-mercaptoethanol. 40 µl of ovarian extract were added to 227 µl of pH adjusted buffer and 40 µl of a 4 mg/ml o-nitrophenyl ß-D-galactopyranoside (ONPG) solution in water. After 6 to 8 h of incubation at 37°C, 100 µl of 1 M Na_2CO_3 solution were added to stop the reaction and reveal the reaction product. The microtubes were centrifuged and the supernatants collected. Optical density at 420 nm and 550 nm (A_{420}, A_{550}) were measured. The reference used as zero was a reaction mixture in which the reaction was stoped at t= 0. The A_{550} reading gives an estimate of the light scattering effects and was used for correction of the A_{420} reading as: A_{420}-1,75xA_{550}. Units of ß-galactosidase expressed as nanomoles of ONPG transformed/min were computed for each sample according to the formula: DO_{420}/[0.0045xt (min) x volume assayed (ml)]. Using this modified extraction procedure, we could get as much as fifty times more activity than previously reported (Lachaume and Pinon, 1993). Reducing the number of ovaries during the extraction procedure highly increased the final ß-galactosidase activity either by increasing the yield of extraction or by reducing inhibitory compounds present in ovarian extract.

Protein titration

It was performed with the Bio-Rad Protein Microassay as described by the manufacturer.

Ultrastructural studies

Ovaries were fixed for 1 h at 4°C in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2); rinsed 1 h in the same buffer 0.2 M, dehydrated through alcohol, propylene oxyde, and embedded in Epon Araldite (E. Fullam; NY, USA). Semi-thin sections were observed under light microscope (l.m.), unstained or stained either with toluidine blue or Nil's blue (Trump *et al.*, 1961). Ultra thin sections were observed, uncontrasted or contrasted with uranyl acetate followed by lead citrate (Reynolds, 1963), under a Jeol 100B electron microscope at 80 kV.

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