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Two types of pole cells are present in the *Drosophila* embryo, one with and one without splicing activity for the third P-element intron

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SUMMARY

In *Drosophila*, it has been postulated that the third intron of the P-element is spliced only in germ-line cells. To test whether this postulate is applicable to pole cells, the progenitor cells of germ line, we carried out a histochemical assay to detect the splicing activity in embryos. The splicing activity was detected in pole cells and primordial germ cells. The activity increased to reach a maximum at 5-6 hours AEL (after egg laying), then decreased to an undetectable level by 8-9 hours AEL. The splicing activity showed a small second peak at 12-15 hours AEL. It was rather unexpected that not all pole cells were capable of splicing the third intron. Almost all pole cells that had the splicing activity at 5-6 hours AEL penetrated the embryonic gonads and differentiated into primordial germ cells. Our findings suggest that pole cells are selected to penetrate the gonads while they are migrating from the proctodeal cavity to the gonads. Furthermore, these results suggest that the machinery to splice the P-element is active in some pole cells, and that this activity is used for processing transcripts of genes that play important roles in the differentiation of pole cells into primordial germ cells.

Key words: transposon, P-element, splicing, pole cell, *Drosophila*

INTRODUCTION

P-elements are one of the families of transposable elements found in *Drosophila melanogaster* (for review see Rubin, 1983). They are responsible for P-M hybrid dysgenesis, a syndrome involving sterility, high rate of mutation, and chromosomal rearrangements (Engels, 1983; Bregliano and Kidwell, 1983). The autonomous 2.9 kb P-element has four exons, and encodes a transposase, which functions to excise or transpose P-elements in *trans* (Spradling and Rubin, 1982). High level transposition occurs only in germ-line cells (Engels, 1983). This tissue specificity is due to germ line specificity in splicing of the third P-element intron (Laski et al., 1986; Rio et al., 1986). A mutation that precisely deletes the third intron alters somatic cells to produce functional transposase (Laski et al., 1986). Laski and Rubin (1989) have revealed that *cis*-acting sequences that regulate the tissue specificity are mapped near, but not at, the splice junction. In P-elements that contain a point mutation in the region upstream of the 5′ splice site, third intron splicing occurs in all somatic tissues as well as in the germ line in vivo (Chain et al., 1991). It has therefore been postulated that splicing is prevented in somatic tissues by a *trans*-acting repressor that binds to the *cis*-acting regulatory sequence. The report by Siebel and Rio (1990) that splicing of the third intron occurs in human cell extracts but not in *Drosophila* somatic cell extracts supports this idea. A 97×10^3 M_r protein found in *Drosophila* extracts specifically binds to the *cis*-regulatory sequence. This binding may cause inhibition of splicing in vitro. Interestingly, the 97×10^3 M_r protein is detected in extracts from somatic cells that do not carry P-elements. This raises the possibility that pre-mRNAs from various genes in addition to the P-element are spliced with the same mechanism. Since the third intron is spliced only in the germ line, it is postulated that the intrinsic role of the splicing machinery acting on P-elements is to regulate the expression of genes whose function is specifically needed for germ-line differentiation. Consequently it is important for the study of germ-line differentiation to identify genes whose expression is regulated by splicing, and to determine how this splicing machinery regulates gene expression.

To characterize the role of the splicing machinery in normal development, it is necessary to determine precisely when and where splicing occurs in vivo. Laski and Rubin (1989) and Chain et al. (1991) reported that splicing activity is detected only in germ-line cells of adult flies. However, it is not known whether embryonic and larval germline cells share this splicing activity.

Pole cells, the progenitors of the germ line, are formed at the posterior pole of embryos at 90 minutes after egg laying (AEL; Zalokar and Erk, 1976; Foe and Alberts, 1983). Although pole cells can only contribute to the germ line, only a small fraction of all pole cells formed reach the gonads and become primordial germ cells. The remainder degenerate without reaching the gonads (Sonnenblick, 1950; Underwood et al., 1980; Hay et al., 1988a; Campos-Ortega and Hartenstein, 1985). At present, it is not known

how this diversity in the fate of pole cells is brought about, although a general belief is that pole cells reach gonads by chance and then differentiate into germ cells.

In this paper, we show that the capacity to splice the third P-element intron is present in pole cells during embryogenesis, and that this capacity changes during development. In addition, we report that pole cells with this capacity preferentially reach the embryonic gonads and differentiate into primordial germ cells.

MATERIALS AND METHODS

Plasmid constructions

A diagram of plasmid construction is shown in Fig. 1. The *PLH* fusion gene was constructed from pπ25.1. A *Sma*I-*Xba*I fragment from pπ25.1, containing 2.9 kb P-element, was inserted between *Sma*I and *Xba*I sites of pGEM-3 plasmid (Promega). The plasmid was digested with *Sal*I and was made blunt using DNA polymerase I Klenow fragment. Into this site, the 3103 bp *Sal*I fragment containing the *lacZ* gene, isolated from pMC1871 (Pharmacia), was inserted in frame to ORF3 of the P-element. This recombinant was partially digested with *Hin*dIII, and was treated with the Klenow fragment. Into the *Hin*dIII site at 40 bp from the 5′ end of the P-element, the 344 bp *Eco*RI-*Pst*I fragment containing a *hsp70* promoter, which was obtained from an Icarus-neo plasmid (Steller and Pirrotta, 1986), was inserted, following treatment with T4 DNA polymerase. This plasmid was cleaved with *Nae*I to release a 7.1 kb *hsp70*-P-element-*lacZ* fusion gene and the fragment was inserted into the *Sal*I site of Carnegie-20 (Rubin and Spradling, 1983).

*PLH*Δ23 was constructed by replacing an *Eco*RI-*Xma*I fragment of *PLH*, which contains the third P-element intron, with an *Eco*RI-*Sal*I fragment from a cDNA of poly(A)$^+$RNA from the P-element, which had no intron sequence. The *PLH* fusion gene was cleaved with *Xma*I and was made blunt using T4 DNA polymerase, followed by digestion with *Xba*I, and the 3.8 kb *Xma*I-*Xba*I fragment containing the *lacZ* gene and the 3′-region of the P-element was gel-purified. pT7P87 (a gift from M. Takahisa, Mitsubishi-kasei Institute of Life Sciences), which contains a cDNA sequence of P-element, was digested with *Sal*I and treated with T4 DNA polymerase. Following digestion with *Eco*RI, the 0.5 kb *Eco*RI-*Sal*I fragment containing the ORF2-ORF3 junction was isolated. The 2.8 kb *Sma*I-*Eco*RI fragment, containing the *hsp70* promoter and the 5′-half of the P-element was purified from the *PLH* fusion gene and was inserted between *Pvu*II and *Eco*RI sites of pGEM-3. The recombinant was digested with *Eco*RI and *Xba*I. Between these sites, the 0.5 kb *Eco*RI-*Sal*I and the 3.8 kb *Xma*I-*Xba*I fragments were inserted. The 6.9 kb fragment containing the *PLH*Δ23 fusion gene was isolated by cleavage with *Nae*I and was inserted into the *Sal*I site of Carnegie-20 to make the plasmid usable for transformation.

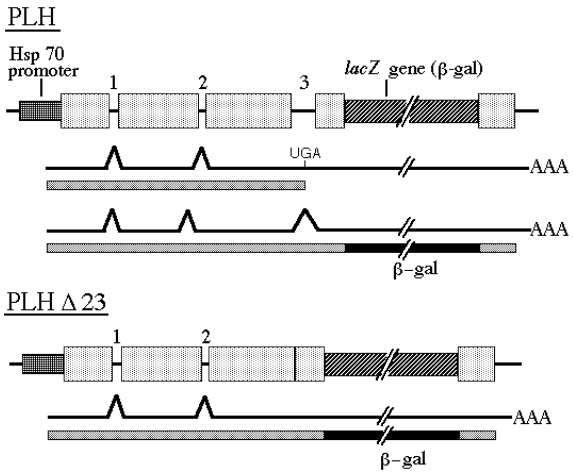

Fig. 1. Construction of the fusion genes used for histochemical assay of the activities of splicing the third P-element intron. Top, the structure of the *PLH* fusion gene. Shaded boxes represent the exons of the P-element, and the introns are numbered above the diagram. This construct expresses β-gal only when the *hsp70* promoter is activated and all three introns are spliced out. Bottom, *PLH*Δ23, the control fusion gene, which differs from *PLH* only in its third intron being precisely deleted.

P-element-mediated transformation

Transformation of *ry*^{506} M-strain embryos was carried out as described by Spradling (1986). Embryos were co-injected with 100 μg/ml of helper plasmid pπ25.7wc and 600 μg/ml of Carnegie-20 bearing the *PLH* or *PLH*Δ23 fusion gene. Independent *rosy*$^+$ transformants were inbred to establish homozygous stocks. The location of insertion sites of the fusion gene was examined by in situ hybridization to polytene chromosomes as described by Engels et al. (1986).

Heat treatment

Embryos were heat treated on a stainless steel mesh laid on a sheet of wet Kimwipe S-200 in a metallic Petri dish, which was equilibrated for 30 minutes in a water bath maintained at 31°C. The heat-treated embryos were stained with X-gal, following incubation at 25°C for 1 hour. Adult flies were heat treated in a depression slide covered with a coverslip. This was set in the Petri dish and incubated in a water bath as described above.

X-gal staining

Following heat treatment, the embryos were dechorionated in 3% sodium hypochlorite and were fixed in a 1:1 mixture of heptane and 4% formaldehyde in PBS (130 mM NaCl, 7 mM Na_2HPO_4, 3mM NaH_2PO_4) for 15 minutes on a rotary shaker. The embryos were washed for 5 minutes in staining buffer (10 mM sodium phosphate, pH 7.2, 150 mM NaCl, 1 mM $MgCl_2$, 3.1 mM potassium ferricyanide, 3.1 mM potassium ferrocyanide, 0.3% Triton X-100). Then, the embryos were incubated in the staining buffer containing a saturation of X-gal powder (1 mg per 0.2 ml of staining solution) for 10-12 hours at 37°C.

Ovaries or testes were dissected out of heat-treated adult flies in PBS and were transferred to the X-gal staining solution where these gonads were incubated for 10-12 hours at 37°C. The stained embryos or adult gonads were mounted in 7.6% gelatin, 54% glycerol, and were observed under a compound microscope equipped with Nomarski optics.

Double staining with X-gal and anti-vasa antibody

Double-staining with X-gal and anti-vasa antibody was carried out according to the previously described method (Kobayashi and Okada, 1993). We also carried out double-staining with anti-β-gal antibody and anti-vasa antibody, but found that the amount of β-gal in pole cells was not sufficient for the immunostaining with the antibody.

Embryos were processed for fixation, devitellinization and incubation with the first antibody (anti-vasa rat antibody, a gift from Dr G. Struhl, diluted 1:1000). The embryos were then incubated with the secondary antibody (biotinylated rabbit anti-rat antibody (Vector Laboratory)) and were subsequently incubated in the avidin and biotinylated horseradish peroxidase (HRP) solution

(ABC-Elite kit, Vector Laboratory). The embryos were incubated for 10-12 hours at 37°C in the staining buffer to which an excess amount of X-gal powder was added. Then, HRP staining was carried out on the X-gal-stained embryos with Histo Mark Orange (Kirkegaard & Perry Laboratories).

The stained embryos were post-fixed in 77% ethanol, 9.6% acetic acid, 3.8% formalin for 2 hours and processed for paraffin embedding. Serial sections were cut and were observed under a compound microscope equipped with Nomarski optics.

RESULTS

Histochemical detection of a splicing activity of P-element transcripts

To examine whether the splicing of the third intron of P-element transcripts occurs in pole cells during embryogenesis, we carried out a histochemical assay according to the method of Laski and Rubin (1989; see Materials and Methods). The bacterial *lacZ* gene, encoding β-galactosidase (β-gal) was inserted in frame into ORF3 downstream of the third intron, and the *Drosophila* heat shock protein 70 (*hsp70*) promoter was cloned into the promoter region of this modified P-element (Fig. 1). This fusion gene (*PLH*) only expresses β-gal when the *hsp70* promoter is activated and all three introns are spliced correctly. Laski et al. (1986) showed that the first and second introns are excised in somatic tissues as well as in the germ line. Therefore, in heat-shocked flies carrying the *PLH* fusion gene, only cells in which the third intron is spliced correctly should show β-gal staining. As a control, we constructed the *PLH*Δ23 fusion gene, which differs from *PLH* only in its third intron being precisely deleted (Fig. 1). This fusion gene should express β-gal in all somatic and germ-line cells where the *hsp70* promoter is activated and the first and second introns are excised. Both fusion genes were introduced into the germ line of flies by P-element mediated transformation, and three homozygous lines were established for each fusion gene. In situ hybridization to polytene chromosomes revealed that each transformant line carried the fusion gene at an independent locus (data not shown). Here, we concentrate on one of the three transformant lines, since no significant difference was detected among the three lines in the β-gal staining of pole cells.

First we repeated the experiments of Laski and Rubin (1989) showing that the P-element third intron was spliced out preferentially in the adult germ line in vivo. In contrast to the fusion gene they used (*hsp70*-P(1911-2183)-*lacZ* carrying the P-element third intron and its flanking sequence), the one we used included the full P-element sequence. They reported that β-gal was preferentially expressed in the ovaries and the tip of testes. In our study, in which transformant flies were heat treated at 31°C for 30 minutes, β-gal expression from the *PLH* fusion gene was detected only in the tip of ovaries as well as testes, whereas expression from the *PLH*Δ23 construct was detected in all somatic and germ-line tissues (data not shown). Although the staining in the gonads was confined to germ-line cells in *PLH* flies, some somatic tissues such as the gut and the oviducts were also stained but weakly. This staining seems to be due to endogenous β-gal as described by Glacer et al. (1986), since these tissues were also stained in flies without carrying *PLH* construct and in non-heat-treated flies carrying *PLH* (data not shown). These observations show that the *PLH* fusion gene expresses β-gal preferentially in the germ line in adults as reported for the *hsp70*-P-(1911-2183)-*lacZ* (Laski and Rubin, 1989).

As the next step, we heat treated embryos carrying the *PLH* fusion gene at defined stages, and stained them with X-gal. The β-gal activity was very conspicuous in the germ line. However, in the course of this experiment, we found that somatic tissues such as the primordia of the anterior and posterior midgut expressed β-gal at different embryonic stages depending on the tissues (Kitamura et al., 1993). Details of the splicing activity of pole cells and their derivatives during embryogenesis are given below.

Developmental changes in the splicing activity of pole cells during embryogenesis

To determine whether there was a particular stage when the third P-element intron was most efficiently spliced in pole cells, we heat treated (at 31°C for 30 minutes) embryos carrying the *PLH* fusion gene at defined stages. After incubation at 25°C for 1 hour, we stained the embryos with X-gal. In this experiment, β-gal expression should reveal a splicing activity at the stage of heat treatment. The degree of β-gal expression in the pole cells depends on the stage of embryogenesis (Figs 2, 3): β-gal expression from *PLH* was undetectable in pole cells when the embryos were heat treated during the period of 0-3 hours AEL. β-gal expression became detectable and started to increase at 3-4 hours AEL, and reached a maximum at 5-6 hours AEL. In 64.3% of the *PLH* embryos that were heat treated at 5-6 hours AEL, pole cells exhibited clear β-gal staining (Figs 2, 3C). When heat treated at stages after 5-6 hours AEL, β-gal expression in pole cells declined to become undetectable by 8-9 hours AEL. The percentage of embryos with stained pole cells increased gradually after 10-11 hours AEL. A small second peak in β-gal expression was noticed at 12-15 hours AEL (Fig. 2). However, the intensity of pole cell staining in embryos heat treated at 12-15 hours AEL was lower than that in pole cells of embryos heat treated at 5-6 hours AEL (Fig. 3C,G). We have not examined the β-gal staining in pole cells after this stage, since formation of the cuticle hinders the staining of whole-mount embryos with X-gal.

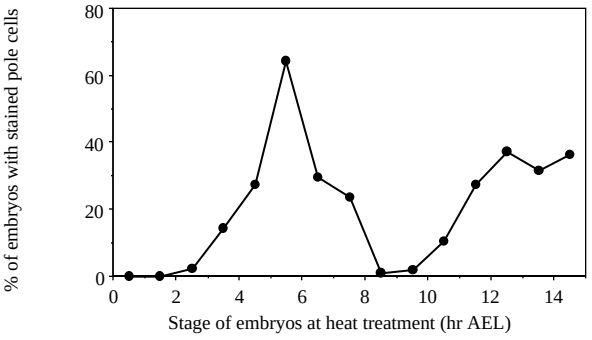

Fig. 2. Stage-dependent expression of β-gal in pole cells in *PLH* embryos. Embryos were heat treated at assigned stages and were stained with X-gal. The frequency of embryos with stained pole cells is plotted against the stage of the embryos when heat treated. Each point represents 30-150 embryos.

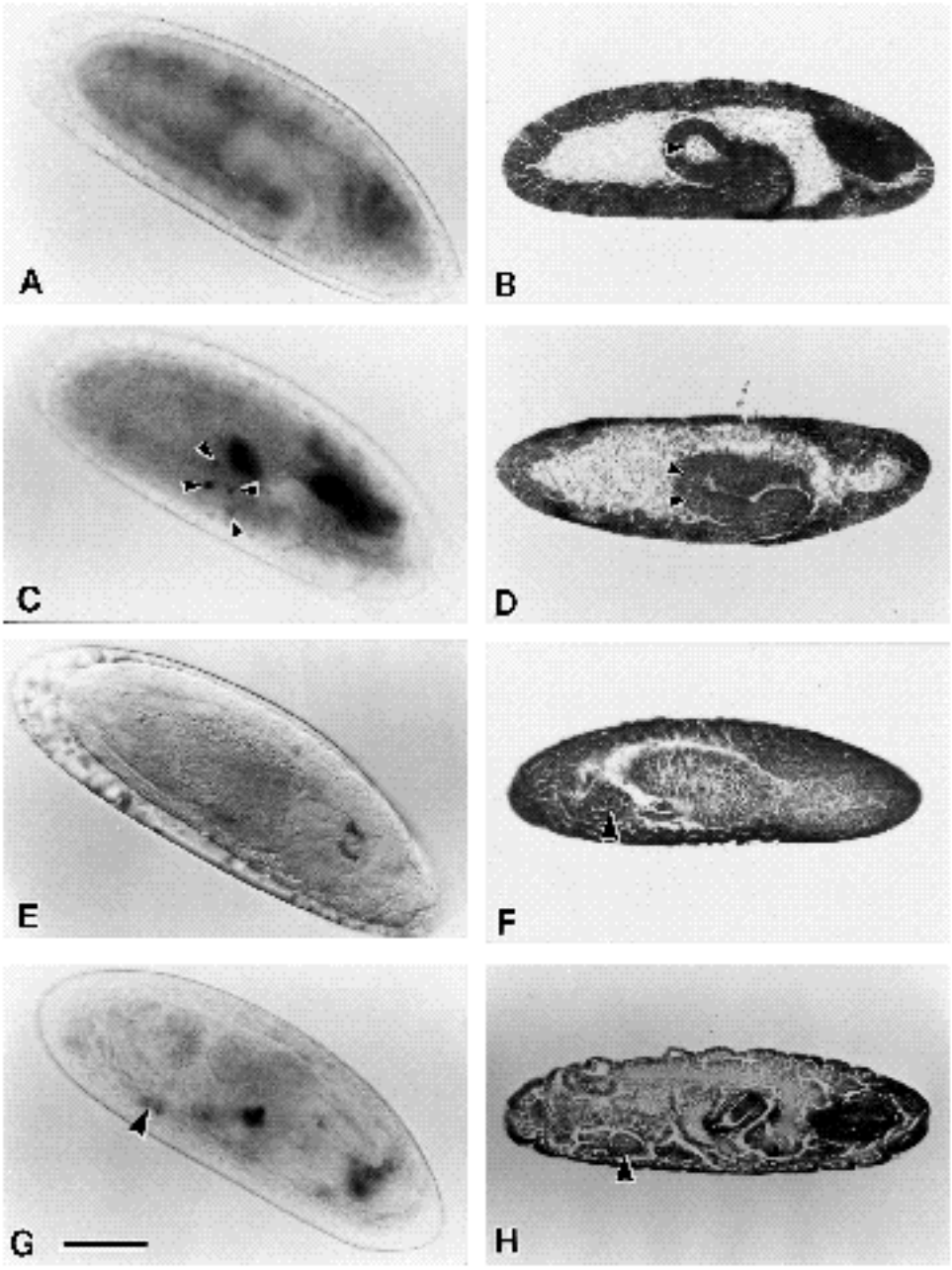

Fig. 3. Micrographs of the transformant embryos heat treated at various stages. Embryos carrying *PLH* (A, C, E, G), or *PLH*Δ23 (B, D, F, H) were heat treated at 2-3 hours AEL (A, B), 5-6 hours AEL (C, D), 8-9 hours AEL (E, F), or 14-15 hours AEL (G, H), then were stained with X-gal, following incubation at 25°C for 1 hour. In A, C, E and G, whole-mount embryos are shown. Somatic tissues are also stained in whole-mount preparations (see text). B, D, F and H are photomicrographs of sagittal sections of embryos through pole cells or embryonic gonads. Arrowheads in B-D point to pole cells. Arrowheads in F-H point to embryonic gonads. Bar (in G), 100 μm.

As a control, we stained *PLH* embryos which had not been heat treated and found no expression of β-gal in pole cells at any observed stages of embryogenesis (data not shown). When embryos with the control fusion gene, *PLH*Δ23, were heat treated at 31°C, β-gal was expressed at a high level in all tissues of the somatic- and germ-line, except for pole cells in embryos heat treated at 0-4 hours AEL (Fig. 3B). These results show that the ability of pole cells to splice the third intron changes during embryonic development with a remarkable peak at 5-6 hours AEL, and a lower second peak at 12-15 hours AEL. However, we cannot determine the stage at which splicing activity first appears in pole cells, since β-gal expression in pole cells was undetectable in embryos with *PLH*Δ23 before 2 hours AEL, and was at a low level in 2-4 hours old embryos (data not shown).

Heterogeneity among pole cells

At 5-6 hours AEL, when splicing activity in pole cells reaches the maximum, we noticed that only a small fraction of pole cells in each embryo was stained with X-gal. The embryos carrying the *PLH* gene were heat treated at 5-6 hours AEL and were double stained with X-gal and an antibody against *vasa* protein, a marker protein specific to pole cells (Hay et al., 1988a,b). The average number of immunoreactive cells was 36.1±6.4 (±s.d., n=18) in a *PLH* embryo that was ascertained to have X-gal-positive pole cells. Of these pole cells, which were identified for their staining with anti-vasa, 1-8 cells (X=4.0±1.6; n=18) were stained with X-gal. Fig. 4 clearly shows two types of pole cells, with and without X-gal staining. It was also clear that *PLH*Δ23 embryos expressed β-gal in almost all pole cells (Fig. 4C,D). These results suggest that the activity of splic-

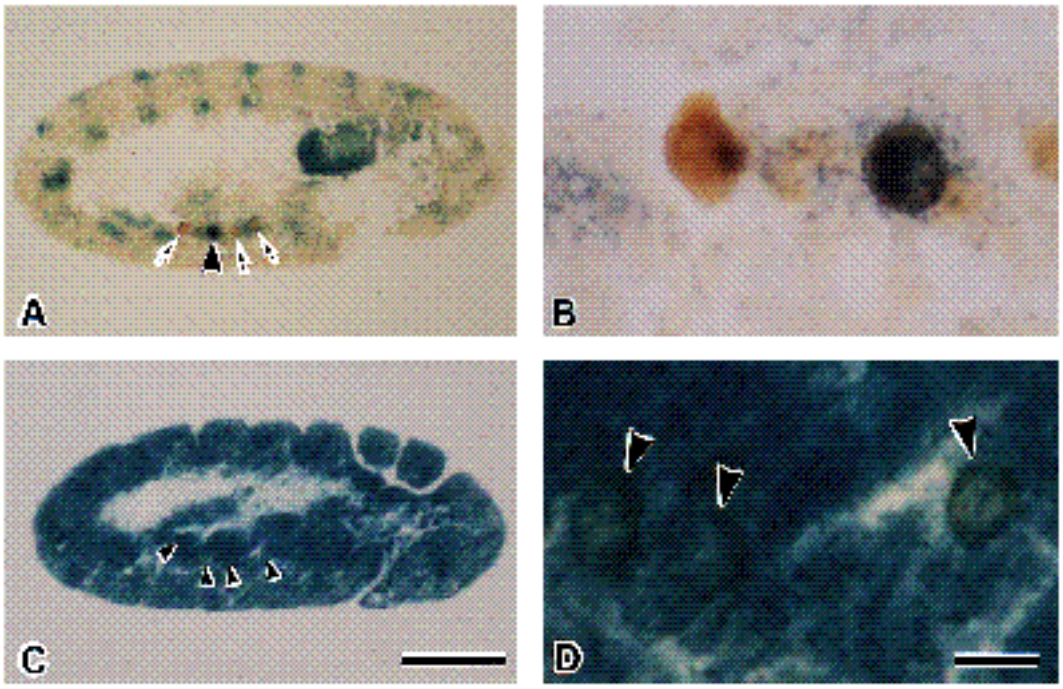

Fig. 4. Sections of embryos from the transformants heat treated at 5-6 hours AEL. Heat-treated embryos carrying *PLH* (A, B), or *PLH*Δ23 (C, D) were double-stained with X-gal (dark-blue) and anti-vasa antibody (orange). Sections through double-stained pole cells are shown at a low (A, C) and a high (B, D) magnification. Arrowheads in A-D point to double-stained pole cells. Arrows in A point to pole cells stained only with the antibody. Bars, 100 μm (C) and 10 μm (D).

ing the third intron is detected only in a subset of pole cells that stain with *vasa* antibody.

Difference in the developmental fate between pole cells with and without splicing activity

Several authors reported that not all pole cells penetrate gonads and become primordial germ cells, despite the fact that pole cells do not participate in any other cell fate (Sonnenblick, 1950; Underwood et al., 1980; Campos-Ortega and Hartenstein, 1985; Hay et al., 1988a). Pole cell that do not reach the gonad are trapped in the yolk or midgut wall, and finally degenerate (Underwood et al, 1980; Hay et al., 1988a). Thus, there are two developmental fates of pole cells; one is to become primordial germ cells and the other is to degenerate.

Our experiments also reveal two types of pole cells in 5-6 hours AEL embryos, one with and the other without the ability to splice the third P-element intron. In order to establish a potential correlation between the fate of pole cells and the splicing activity, we traced pole cells with the splicing activity throughout development. Since embryos have to be fixed in order to visualize splicing activity using X-gal, it is impossible to follow directly the fate of the pole cells using X-gal. To avoid this difficulty, we developed the following procedure. We heat treated embryos with the *PLH* fusion gene at 5-6 hours AEL, such that pole cells with splicing activity would express β-gal. The embryos were stained with X-gal at 13-14 hours AEL, when pole cells had been incorporated into the gonads. We reasoned that pole cells that possessed splicing activity at 5-6 hours AEL should contain β-gal activity at the later stages. To locate pole cells outside of the gonads, we performed double-staining with X-gal and an antibody against *vasa* protein. As summarized in Table 1, 71.2% of the embryos that had been heat treated at 5-6 hours AEL and developed to 13-14 hours AEL retained β-gal activity in the pole cells.

Table 1. Developmental fate of pole cells with the splicing activity at 5-6 hours AEL

No. of embryos observed*	No. of embryos with X-gal-stained pole cells (%)	No. of pole cells†			
		In gonads		Outside gonads	
		Total	Stained with X-gal (%)	Total	Stained with X-gal (%)
111	79 (71.2)	1581 [12.4 ± 2.8]‡	341 (21.6) [2.7 ± 2.0]§	125	4 (3.2)

*Embryos were heat treated at 5-6 hours AEL, and were allowed to develop for a further 8 hours at 25°C, then were double-stained with X-gal and anti-vasa antibody.

†Cells stained with anti-vasa antibody were identified as pole cells.

‡Mean ± s.d. of pole cells stained with the antibody was calculated per gonad.

§Mean ± s.d. of pole cells stained with both X-gal and the antibody was calculated per gonad.

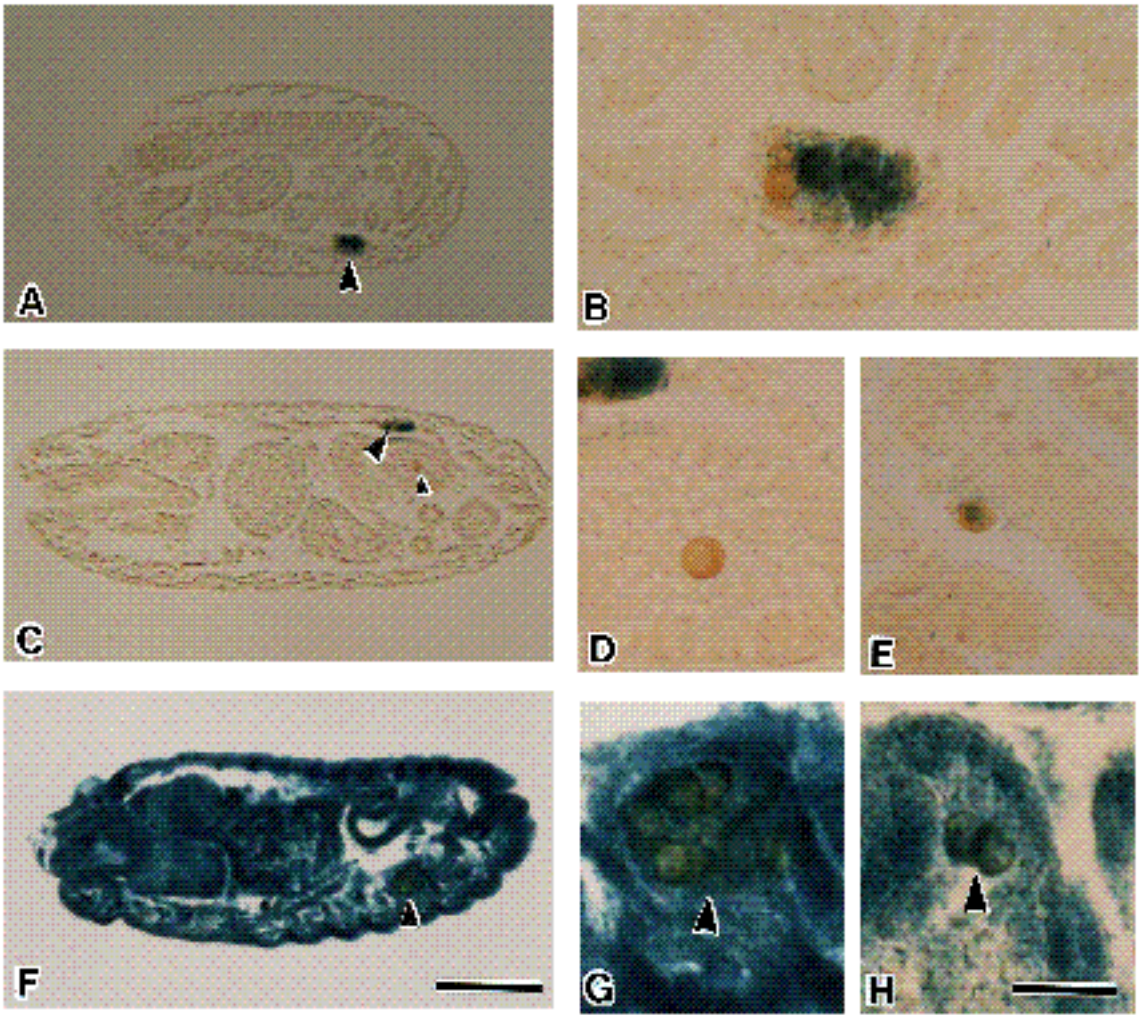

Fig. 5. Sections of the transformant embryos heat treated at 5-6 hours AEL, then incubated at 25°C for 8 hours. Embryos carrying *PLH* (A-E), or *PLH*Δ23 (F-H) were double-stained with X-gal and anti-vasa antibody. (A, B) Double-stained pole cells in embryonic gonads. (C-E), pole cells outside gonads. No pole cells outside gonads were stained with X-gal in most cases (C, D). However, one or two pole cells outside gonads were occasionally weakly stained with X-gal (E; see text and Table 1). (F-H), Double-stained pole cells in gonads (F, G) and outside gonads (H). Large arrowheads in A, C, F and G point to embryonic gonads. Small arrowhead in C points to pole cells outside gonads. Embryos in A, C and F, and those in B, D, E, G and H are all shown at the same magnification respectively. Bars, 100 μm (F) and 20 μm (H).

In these embryos, 21.6% of pole cells found in gonads were stained with X-gal. The average number of β-gal-positive cells per gonad was 2.7±2.0 (±s.d., n=128 gonads). An example is shown in Fig. 5 where β-gal-positive and -negative pole cells in an embryonic gonad are seen. In contrast, only 3.2% of pole cells outside the gonads were stained with X-gal (Table 1). In addition, these pole cells can be distinguished from those in the embryonic gonads due to apparently weaker X-gal staining (compare Fig. 5B and E). When the embryos carrying the *PLH*Δ23 control fusion gene were heat treated at 5-6 hours AEL and allowed to develop for an additional 8 hours, we found that all pole cells in the gonads were stained with X-gal. Furthermore there was no difference in the intensity of staining between pole cells within and outside of the gonads (Fig. 5G,H). These results show that practically all pole cells that have the capacity to splice the third intron at 5-6 hours AEL are able to reach the embryonic gonads and become primordial germ cells.

DISCUSSION

Previous reports (Laski and Rubin, 1989; Chain et al., 1991) have shown that the third P-element intron is spliced only in germ-line cells of adult flies. Using a histochemical assay we detect the same activity in adult germ cells in spite of the difference in the reporter gene construct.

To induce *PLH* fusion gene expression, we used a heat-shock promoter instead of a non-inducible constitutive promoter. The inducible promoter enables us to carry out a time course analysis of the splicing activity. One may argue that severe heat treatment inhibits pre-mRNA splicing (for review see Yost et al., 1990). However, it is unlikely that

the splicing of the third intron is inhibited by such a mild heat treatment as we used (31°C for 30 minutes), since the first and second introns are spliced out in the heat-treated *PLH*Δ23 embryos (Fig. 3). Furthermore, Laski and Rubin (1989) have reported that the third intron can be spliced in the germ line of flies heat treated at a higher temperature (37°C).

We have shown that the activity to splice P-element in pole cells changes during development with a maximum at 5-6 hours AEL, when pole cells are migrating from the proctodeal cavity toward the embryonic gonads. However, the percentage of embryos with stained pole cells did not reach 100%, even when *PLH* embryos were heat treated at 5-6 hours AEL (Fig. 2). This may be because the heat-treatment conditions were not optimal. Indeed, when 5-6 hour-AEL embryos were heat treated at a higher temperature (34°C), almost all (more than 90%) had stained pole cells, while the average number of stained pole cells per embryo was not different from that in embryos heat treated at 31°C (data not shown). In embryos heat treated at 34°C, however, we frequently observed β-gal activity in somatic tissues that obscured the detection of pole cell staining in whole-mount preparations. Therefore we heat treated embryos at 31°C.

We show that there are two types of pole cells, one with and the other without X-gal staining when embryos from females that carry the *PLH* construct were heat treated at 5-6 hours AEL. This heterogeneity among pole cells is unlikely to represent a heterogeneity in the efficiency of activation of the *hsp70* promoter, the splicing of the first and second introns, or translational activity, since the *PLH*Δ23 construct, which differs from *PLH* construct only in its complete deficiency of the third intron, expresses β-gal in all pole cells with equal intensity. Therefore, we conclude that splicing of the third P-element intron occurs only in some pole cells.

Furthermore, our data strongly support the idea that pole cells with splicing activity at the stage of 5-6 hours AEL are preferentially incorporated into embryonic gonads and have the developmental fate of primordial germ cells. Several authors have reported that only some pole cells reach the gonads and become primordial germ cells during normal development (Sonnenblick, 1950; Underwood et al., 1980; Hay et al., 1988a). We confirmed and extended these reports: 12 pole cells per gonad were observed in 13-14 hour-AEL embryos, whereas on average 36 pole cells were present in 5-6 hour-AEL embryos. Our experiments revealed that pole cells following heat treatment at 5-6 hours AEL kept β-gal activity at later stages after pole cells had migrated into the gonads. On average 22% (7-75%) of the pole cells that had reached the gonads stained with X-gal. In contrast, only 3% of the pole cells observed outside of the gonads stained. These results show that almost all pole cells that expressed β-gal at 5-6 hours AEL reach the gonads and become primordial germ cells. Since only cells with splicing activity express β-gal in *PLH* embryos, this result suggests that pole cells with the splicing activity of the third intron preferentially penetrate gonads.

One may argue that pole cells that express β-gal at 5-6 hours AEL but fail to reach the gonad later may have lost their β-gal activity. If so, the same loss of β-gal from pole cells outside gonads should be observed in embryos carrying *PLH*Δ23. However, this is not the case. We found that almost all pole cells in the control embryos were stained, irrespective of their location in the embryo. Furthermore, the number of stained pole cells in a *PLH* embryo heat treated at 5-6 hours AEL and stained 1 hour later was nearly identical to the number of stained pole cells in an embryo with the same heat treatment but stained 8 hours later. Considering that cell divisions are not observed in pole cells between 5 hours and 14 hours AEL (Sonnenblick, 1950), this result shows that pole cells with splicing activity do not degenerate during this period. Consequently selective degeneration cannot explain the difference in the proportion of β-gal-active pole cells inside and outside the gonads of 13-14 hours AEL embryos that have been heat treated at 5-6 hours AEL.

Wieschaus and Szabad (1979) have shown, using a clonal analysis, that only a small fraction of pole cells formed at blastoderm contribute to the germ cells in females. The number of pole cells at the blastoderm stage (3 hours AEL) destined to contribute to egg production was calculated to be seven, based on the relative clone size, and was 4.3 when deduced from the mosaic frequency. It is worthwhile to note that according to this study, the number of pole cells that contribute to egg production is approximately equal to that of pole cells with splicing activity in 5-6 hours AEL embryos (4.0 pole cells per embryo when heat treated at 5-6 hours AEL and stained 1 hour later, and 5.4 (2.7×2) pole cells in a pair of gonads in the embryo that received the same heat treatment and stained 8 hours later). We propose that only pole cells that have splicing activity are able to differentiate into germ-line stem cells. If this is true, about 80% of all pole cells in embryonic gonads, that have no splicing activity at 5-6 hours AEL, must degenerate later. Our preliminary observation supports this idea. Embryos heat treated at 5-6 hours AEL were allowed to develop for further 22 hours, and were then stained with X-gal. We found that in some of the resulting larvae all primordial germ cells (PGCs) in gonads were stained with X-gal. Considering that all observed gonads have two types of PGCs in embryos heat treated at 5-6 hours AEL and stained 8 hours later, selective degeneration of descendants from pole cells that are incapable of splicing the third P-element intron at 5-6 hours AEL probably occurs at around hatching. This is consistent with the observation that pole cell death in the gonads occurs following gonad formation during stages 14-16 (Hay et al., 1988a). We could not trace the developmental fate of pole cells with splicing activity to the second instar larvae or further, due to the loss of β-gal activity from the pole cells after the first instar.

It has been shown that polar plasm contains maternal factor(s) required for pole cell formation and subsequent differentiation of pole cells (Illmensee and Mahowald, 1974; Okada et al., 1974). Considering our finding that the activity of pole cells to splice the third intron has strong correlation with their developmental fate to differentiate as germ cells, maternal factor(s) in the polar plasm may possibly be involved in the regulation of pole cell differentiation through modulating the splicing machinery. Siebel and Rio (1990), Chain et al. (1991) and Tseng et al. (1991) have suggested that the splicing of the third intron is regulated by a specific

interaction between RNA binding proteins and the region upstream from 5′ splice site of the third intron: these proteins repress the splicing when binding to the site. Although the spatial distribution of the RNA binding proteins in early embryos has not been elucidated, it is conceivable that germ line-determining maternal factors in the polar plasm regulate splicing through inactivation of the RNA binding proteins. The possibility remains to be tested, however, that an unknown physiological condition causes some pole cells to be unable to splice the third P-element intron and at the same time unable to differentiate into germ cells.

Several previous reports noted the heterogeneity among pole cells. Sonnenblick (1950) reported that the distribution of polar granules, which are polar-plasm-specific structures, is variable among pole cells. Polar granules have been suggested to be associated with a factor necessary for germline development (Mahowald, 1968, 1971; Boswell and Mahowald, 1985; Schüpbach and Wieschaus, 1986; Lehmann and Nüsslein-Volhard, 1986). A component of polar granules has been identified as the product of a maternal-effect posterior group gene *vasa*. Mutations in *vasa* cause the grandchildless phenotype (Lasko and Ashburner, 1988; Hay et al., 1988b). Variation in the number of polar granules could cause a difference in the developmental fate of pole cells. Technau and Campos-Ortega (1986) have observed that the number of mitotic cycles that pole cells undergo before they migrate to gonads is not constant but varies from 0 to 2. Factors regulating the splicing in the polar plasm may be diluted at every mitotic cycle. Our findings together with these reports support the idea that pole cells are a set of heterogeneous cells. However, it awaits further studies to determine how the heterogeneity is brought about.

In this paper, we have provided evidence to suggest that the machinery for manipulating the splicing of the third P-element intron plays a crucial role in the differentiation of pole cells into germ cells. However, this does not mean that P-elements themselves are involved in germ-line development. In fact, embryos lacking P-elements can develop into fertile adults. Furthermore, the 97×10^3 M_r protein, which has been suggested to be a negative regulator of splicing of the third intron, is detected in extracts of somatic cells derived from a strain that does not carry P-elements (Siebel and Rio, 1990). This raises the possibility that this protein also modulates splicing of pre-mRNAs other than that of P-elements. Thus we propose that the splicing machinery present in pole cells is not only used for P-element splicing but is also required for expression of genes whose function is needed for germ-line development during early embryogenesis.

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