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germ cell-less Acts to Repress Transcription during the Establishment of the *Drosophila* Germ Cell Lineage

Judith L. Leatherman,[1,2] Lissa Levin,[2] Julie Boero,[2] and Thomas A. Jongens[2,3]

[1]Cell and Molecular Biology Graduate Program

[2]Department of Genetics

University of Pennsylvania School of Medicine

Philadelphia, Pennsylvania 19104-6100

Summary

Previously, it has been shown that, during early *Drosophila* and *C. elegans* development, the germ cell precursors undergo a period of transcriptional quiescence [1–4]. Here, we report that Germ cell-less (GCL), a germ plasm component necessary for the proper formation of "pole cells," the germ cell precursors in *Drosophila* [5, 6], is required for the establishment of this transcriptional quiescence. While control embryos silence transcription prior to pole cell formation in the pole cell-destined nuclei, this silencing does not occur in embryos that lack GCL activity. The failure to establish quiescence is tightly correlated with failure to form the pole cells. Furthermore, we show that GCL can repress transcription of at least a subset of genes in an ectopic context, independent of other germ plasm components. Our results place GCL as the earliest gene known to act in the transcriptional repression of the germline. GCL's subcellular distribution on the nucleoplasmic surface of the nuclear envelope [7] and its effect on transcription suggest that it may act to repress transcription in a manner similar to that proposed for transcriptional silencing of telomeric regions.

Results and Discussion

A period of transcriptional quiescence in the early germ cells is not only a conserved feature in *Drosophila* and *C. elegans*, but it also appears to be important for their development, as mutations that disrupt this quiescence affect the formation of the germline. The *pie1* gene encodes a protein that acts as a transcriptional repressor in the early germ cell precursors of *C. elegans* [8]. Embryos that lack *pie1* activity fail to repress transcription in the germ cell precursors and also fail to form a proper germline [4]. In *Drosophila*, the *nanos* and *pumilio* genes are required for transcriptional quiescence of the pole cells. In embryos that lack *nanos* or *pumilio* activity, the pole cells prematurely or inappropriately express genes and fail to develop into functional germ cells [9–11].

The Germ cell-less protein is a germ plasm component and specifically associates with those nuclei that enter the germ plasm, induce the formation of pole buds, and then are incorporated into the resulting pole cells [5]. In these nuclei, GCL is localized to the nucleoplasmic surface of the nuclear envelope [7], a localization necessary for its function [6]. Previously, examination of the *germ cell-less* null phenotype revealed that most embryos lacking maternally contributed *gcl* (hereafter called Δ*gcl* embryos) form no pole cells; the remaining embryos form a very small number of pole cells [6]. This failure to form pole cells does not appear to be due to a defect in germ plasm formation, maintenance, or levels. Analysis of known germ plasm components in Δ*gcl* embryos has failed to reveal any defects, suggesting that the failure to form pole cells in these embryos is due to a direct requirement for GCL in this process [6] (L.L. and T.A.J., unpublished data).

To investigate whether loss of *gcl* activity results in a failure to establish or maintain the state of transcriptional quiescence necessary for proper germ cell development, we stained both Δ*gcl* and control embryos with the H5 antibody. This monoclonal antibody recognizes a phosphorylated form of RNA polymerase II that is associated with active transcription [12], and it has been used as a reliable marker for the transcriptional quiescence of the germ cell precursors of *Drosophila* and *C. elegans* [13].

Interestingly, in the control embryos, we observed a difference in H5 staining in pole bud nuclei, and this occurs at an earlier stage than what was previously reported [13] (see the Experimental Procedures). Pole buds first appear during nuclear cycle 9 and pinch off at the end of nuclear cycle 10 to form the pole cells. In many cycle 9 embryos, a slight decrease in H5 staining was observed in the pole bud nuclei (identified by their association with Vasa staining) compared to the somatic nuclei, and by cycle 10, the pole bud nuclei displayed a dramatic reduction in H5 staining (arrowheads in Figure 1C), indicating that a state of transcriptional quiescence is being established prior to the formation of the pole cells.

In Δ*gcl* embryos, the pole bud nuclei fail to become transcriptionally quiescent. When stained with the H5 antibody, most pole bud nuclei in the Δ*gcl* embryos stained at levels comparable to the somatic nuclei (arrowheads in Figures 1F and 1I). However, in a few scattered Δ*gcl* pole bud nuclei, we observed a reduction in H5 staining similar to that seen in wild-type (asterisk in Figure 1F). Since the few successfully formed pole cells in Δ*gcl* embryos have dramatically reduced H5 staining similar to control embryo pole cells [6] (see the Supplementary Material available with this article online), we speculated that the few silenced pole bud nuclei we observed might be those that will become part of the few pole cells seen in Δ*gcl* embryos.

To further explore this possibility, we quantitated this loss of transcriptional quiescence by counting the pole bud nuclei in control and Δ*gcl* embryos at the pole bud stage (nuclear cycle 10, see the Experimental Procedures) and noted whether they had reduced H5 staining. We found between 7 and 14 pole bud nuclei per embryo in both control and Δ*gcl* embryos, as is expected [14]. In control embryos, nearly all nuclei had reduced H5 staining (99% of the total number of nuclei counted, n =

[3]Correspondence: jongens@mail.med.upenn.edu

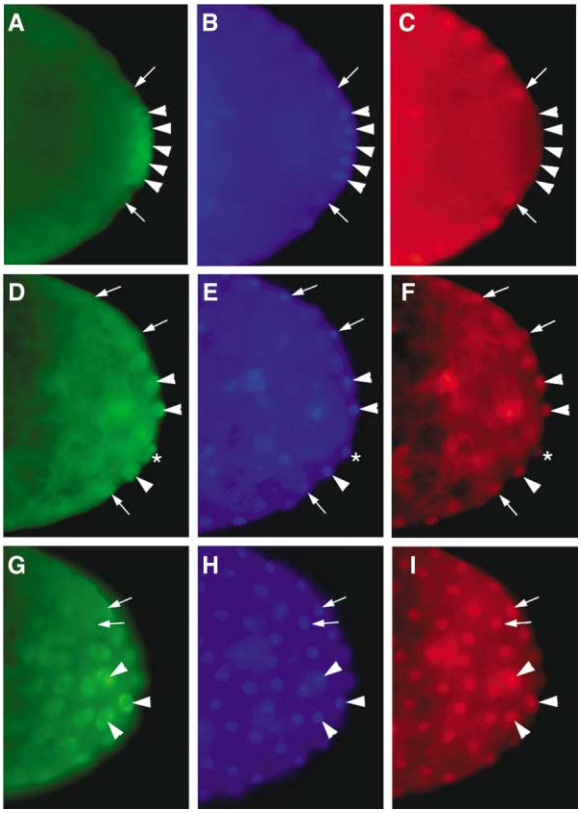

Figure 1. Staining of Embryos with the H5 Antibody Reveals Transcriptional Quiescence in the Pole Bud Nuclei of Control, but Not Δ*gcl*, Embryos

(I) (A)–(C) show a nuclear cycle 10 control (w^{1118}) embryo; (D)–(F) and (G)–(I) show a nuclear cycle 10 Δ*gcl* embryo at two different focal planes in order to give an accurate representation of the majority of the pole buds in an embryo, rather than those that appear only at the very posterior of the embryo. (A, D and G) Anti-vasa staining of the embryos marks the germ plasm that surrounds the pole bud nuclei. (B, E, and H) Høechst staining of embryos shows the positions of the nuclei. (C, F, and I) H5 staining of the embryos shows transcriptional activity. Arrows indicate somatic nuclei that are not incorporated into pole buds and have strong H5 staining. Arrowheads indicate nuclei incorporated into the pole buds. The control pole bud nuclei have dramatically reduced H5 staining, while most of the Δ*gcl* embryo pole bud nuclei have strong H5 staining, indicating a loss of transcriptional silencing. The asterisk in (F) indicates the position of one pole bud nucleus in a Δ*gcl* embryo showing reduction in transcriptional activity, as shown by a decrease in H5 staining.

145 nuclei). However, in 50% of the Δ*gcl* embryos, none of the pole bud nuclei displayed reduced H5 staining (n = 20 embryos). This correlates well with the observed 48% of Δ*gcl* embryos with no pole cells at the blastoderm stage [6]. Of the total number of Δ*gcl* pole bud nuclei counted, only 11.9% had reduced H5 staining (n = 194 nuclei), indicating transcriptionally silenced nuclei. After pole cell formation, each pole cell then divides between 0 and 2 times, so the total number of transcriptionally silenced pole bud nuclei cannot be compared directly to the number of pole cells observed in Δ*gcl* embryos. However, since 11.9% of the pole bud nuclei are silenced, we would therefore expect a reduction to a similar percent in the number of pole cells in Δ*gcl* embryos compared to control embryos. Since control embryos have an average of 23.4 pole cells at the blastoderm stage [6], we would predict an average of (23.4 × 0.119), or 2.8 pole cells, to be present in blastoderm-stage Δ*gcl* embryos based on the number of silenced pole bud nuclei. This number is identical to our previously observed average of 2.8 pole cells in Δ*gcl* embryos at the blastoderm stage [6].

This strong quantitative correlation, in addition to our failure to find any other defects, including defects in the germ plasm, suggests that those few nuclei that successfully silence transcription in the pole buds of Δ*gcl* embryos (as shown by loss of H5 staining) are those which will form the few functional pole cells. This implies that establishing transcriptional quiescence is a necessary step for pole cell formation; however, further experiments will be necessary to prove this type of causal relationship between transcriptional silencing and pole cell formation.

Since the H5 stainings indicated that pole bud nuclei in Δ*gcl* embryos fail to become transcriptionally silent, we speculated that we should therefore be able to see misexpression of specific gene transcripts in these nuclei. We examined the expression of two genes that are transcribed at this time, *sisterless A* (*sisA*) and *sisterless B* (*sisB*). These transcripts are ubiquitiously expressed in nuclei as early as nuclear cycle 8 but are repressed in pole bud nuclei [15] (Figures 2B and 2F). By using whole-mount in situ hybridization, we found that *sisA* (Figures 2D and 2H) and *sisB* (not shown) transcripts are present not only in somatic nuclei, but also in the majority of pole bud nuclei in Δ*gcl* embryos, and this finding independently verifies that Δ*gcl* embryos are deficient in transcriptional silencing in the pole bud nuclei.

The results described above indicate that *gcl* is required to repress transcription during the establishment of the germ cell lineage. To determine if this activity is dependent or independent of other germ plasm components, we examined the effect of ectopically localizing GCL on transcription. Previously, we had found that replacement of the 3′ UTR of the *gcl* transcript with the 3′ UTR of *bicoid* would result in the anterior localization of *gcl* mRNA and protein to the anterior pole of the embryo [7]. In these "hgb" embryos, we found a slightly variable but consistent decrease in the intensity of H5 staining in the anterior nuclei compared to control embryos throughout the syncytial blastoderm stage (Figure 3), and this decrease indicates that GCL is sufficient to repress transcription ectopically. However, the anterior expression of GCL clearly does not lead to complete silencing of the anterior nuclei, since some H5 staining persists.

The reduced H5 staining observed in the anterior of the hgb embryos could be due to global partial repression of all genes, or it could result from a specific subset of genes being severely repressed while others are unaffected. To distinguish between these possibilities, we examined the expression of specific genes whose expression pattern includes the anterior of the embryo, including *sisA*, *sisB*, *tailless*, *huckebein*, *hunchback*, and *knirps*. These genes are all independently activated by maternally contributed factors, so any effects on their transcription are likely to be direct rather than a consequence of an earlier defect. By using in situ hybridiza-

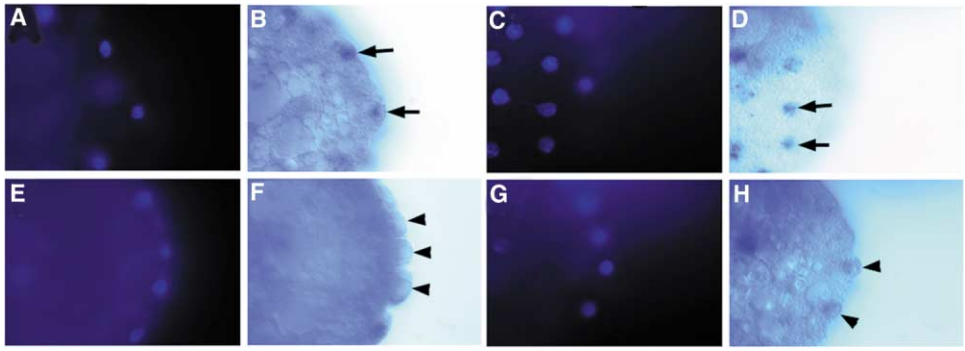

Figure 2. Ectopic Expression of *sisA* Is Observed in the Pole Bud Nuclei of Δ*gcl* Embryos

(A–H) Control and Δ*gcl* embryos were fixed and stained with Høechst dye to determine the nuclear cycle after probing by whole-mount in situ hybridization using a probe specific for the *sisA* transcript. (A), (C), (E), and (G) are Høechst stainings, and (B), (D), (F), and (H) are DIC images. (A) and (B) show a control (w^{1118}) embryo, and (C) and (D) show a Δ*gcl* embryo focused on nuclei that will be incorporated into somatic cells. Arrows indicate expression of *sisA* in these nuclei. (E) and (F) show a control (w^{1118}) embryo, and (G) and (H) show a Δ*gcl* embryo focused on pole bud nuclei. Arrowheads show the absence of *sisA* transcripts in control pole bud nuclei, but improper expression of *sisA* in the Δ*gcl* embryo pole bud nuclei.

tion, we found that the early anterior expression domains of *sisA*, *sisB*, *tailless*, and *huckebein* were severely repressed in all of the hgb embryos examined (Figure 4; see figure legend for expression details; *sisB* not shown), but no effect was seen on *hunchback* and *knirps* expression (not shown). These data suggest that the transcriptionally repressive effect of GCL is not global, but rather specific to a subset of genes. GCL is also present in a variety of tissues later in development [5], at times when transcription is active, which further suggests a non-global mode of silencing.

If GCL only silences selected genes, then the question arises as to how the pole cells accomplish what appears to be complete repression of mRNA transcription. Experiments from Van Doren et al. [16] have shown that

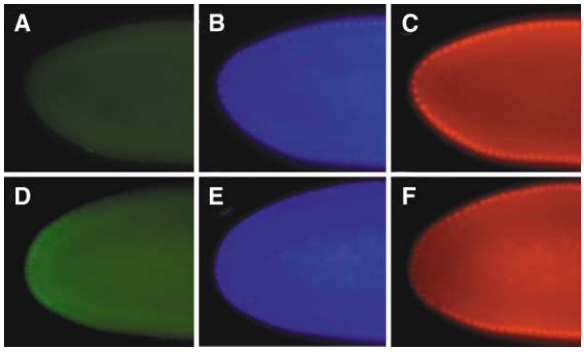

Figure 3. Transcriptional Activity Decreases in the Anterior of the Embryo during the Syncytial Blastoderm Stage When GCL Is Ectopically Localized to the Anterior, as Shown by a Decrease in H5 Staining

(A–F) (A)–(C) show a control (w^{1118}) embryo, and (D)–(F) show an hgb embryo in which GCL is ectopically localized to the anterior. Both are at the syncytial blastoderm stage. (A and D) GCL antibody staining shows where GCL is ectopically localized in (D) hgb but not (A) control embryos. (B and E) Høechst staining shows the position of the nuclei. (C and F) H5 staining shows transcriptional activity. (C) In the control embryo, all nuclei stain equally. (F) In the hgb embryo, H5 staining is specifically reduced in the nuclei that contain GCL. A representative embryo is shown.

reporter genes driven by the strong Gal4-VP16 activator cannot activate in the early germ cells. If only specific genes are repressed, it is unlikely that a novel transgene would be repressed by the same silencing mechanism, thereby arguing that there is widespread repression of transcription in early pole cells. Furthermore, observations of pole cell nuclear morphology, which we have shown to be mediated by GCL, suggest a more global mode of silencing than our current studies. Pole cell nuclei are normally round in shape and more compact than somatic nuclei [17], which is consistent with chromatin silencing. Compaction increases when GCL is overexpressed, and ectopic compaction occurs when GCL is ectopically localized [7]. It seems unlikely that GCL could accomplish such a global nuclear structure change just through the repression of transcription of a few genes.

One possible explanation for this discrepancy is the existence of other as yet unidentified factors that act in transcriptional silencing during pole cell formation which allow the formation of the few pole cells that form in Δ*gcl* embryos. These other factors, while less effective initially than GCL, might have a more global mode of action. This argument is supported by experiments that show that, when Oskar is ectopically localized to the anterior, which causes ectopic pole cell formation by ectopic localization of essential germ plasm components [18], *hunchback* transcription is repressed [16]. Since GCL does not repress hunchback transcription, there must be other factors that accomplish this. Another possibility is that general repression can be accomplished by silencing only a few genes. At the time of pole cell formation, there are very few genes known to be transcribed, so it is conceivable that specific mechanisms could exist for silencing all of them. Repression of these few genes could alter the developmental program in the pole bud nuclei, thereby resulting in more widespread silencing in pole cells.

Data on Nanos and its binding partner Pumilio support this view. These factors are also required for transcriptional silencing in *Drosophila* pole cells, although at a later stage than GCL is required. Deshpande et al. [10]

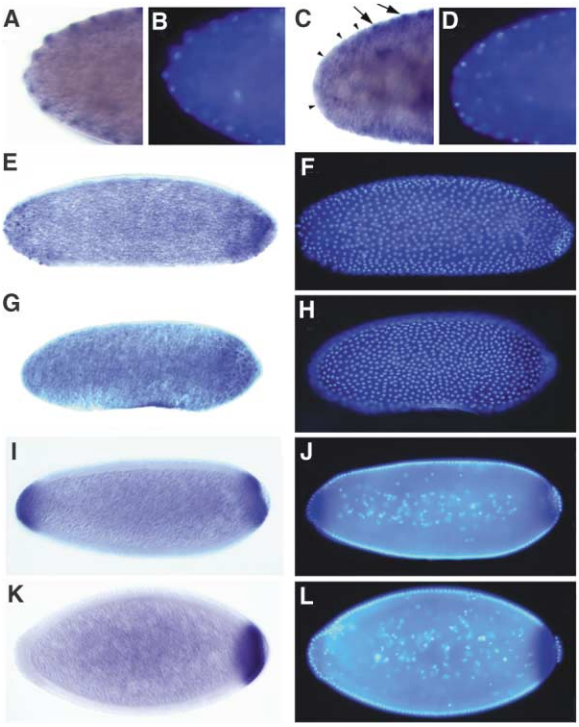

Figure 4. Ectopically Localized GCL in the Anterior of the Embryo Causes Repression of *sisterlessA*, *tailless*, and *huckebein* Transcripts in Their Anterior Expression Domains

(A–L) (A), (B), (E), (F), (I), and (J) show control (w^{1118}) embryos, and (C), (D), (G), (H), (K), and (L) show hgb embryos that have GCL ectopically localized to the anterior. Embryos were probed by whole-mount in situ hybridization with either (A–D) *sisterless A* (*sisA*), (E–H) *tailless* (*tll*), or (I–L) *huckebein* (*hkb*) probes, then stained with Høechst dye to show the nuclei. (A), (C), (E), (G), (I), and (K) show DIC images, and (B), (D), (F), (H), (J), and (L) show Høechst images that reveal the developmental stage of the embryo. All embryos are oriented with the anterior to the left. (A) shows *sisA* transcripts in a control (w^{1118}) embryo, where they are found in all nuclei in the anterior at nuclear cycle 9 [15]. (C) shows an hgb embryo at the same stage. *sisA* transcripts are repressed in anterior nuclei where GCL is ectopically localized (arrowheads), whereas transcripts are still present in more posterior nuclei along the side of the embryo (arrows). (E) shows *tll* transcripts in a control (w^{1118}) embryo when they first appear in a punctate pattern at the anterior and posterior ends of the embryo at nuclear cycle 12 [26]. (G) shows an hgb embryo at the same stage. The early punctate expression of *tll* is visible in the posterior of the embryo, but not in the anterior, where GCL is ectopically localized. (I) shows *hkb* transcripts in a control (w^{1118}) embryo soon after they first appear at the syncytial blastoderm stage at the anterior and posterior of the embryo [28]. (K) shows an hgb embryo at the same stage that has *hkb* transcripts in the posterior expression domain, but not in the anterior where GCL is present. Through the end of the syncytial blastoderm stage, these genes were consistently repressed in hgb embryos. *sisA* and *tll* were repressed to undetectable levels, whereas *hkb* occasionally had faint anterior staining (always less than control embryos), which is consistent with the variable levels of GCL in these embryos.

found that the pole cell migration defect in *nanos* mutant embryos was partially alleviated when *sex-lethal*, one improperly expressed transcript, was removed [10]. This suggests that the number of genes that *nanos* causes to be transcriptionally repressed is small, since removal of only one gene can cause rescue. Furthermore, transcriptional activation of *sex-lethal* is dependent on the two genes we have shown to be repressed in the germ plasm by GCL, *sisA* and *sisB* [15]; this finding hints that *sex-lethal* is a key gene that must be silenced in the early pole cells in order for them to achieve their proper developmental fate.

Work from our lab and others on a mouse homolog of *Drosophila gcl* further suggests a specific, rather than global, mode of repression for GCL. We found that *mgcl-1*, a functional homolog of *gcl*, is highly expressed in spermatocytes [19] at a time when transcriptional activity in these cells is high [20]. de la Luna et al. [21] found that mGCL physically interacts with the DP3α subunit of E2F. They show that mGCL can inhibit progression through the cell cycle by repressing the E2F transcription factor, possibly due to its sequestration at the nuclear envelope. This work demonstrates a mechanism of transcriptional repression for a specific set of genes — those that are transcriptionally controlled by E2F. However, it is unclear at this point whether this specific role for GCL occurs in *Drosophila*, since GCL does not bind to *Drosophila* DP (J.L.L. and T.A.J., unpublished data), the only apparent isoform of this protein in the *Drosophila* genome.

While we do not know the mechanism by which GCL accomplishes transcriptional silencing, interesting parallels in budding yeast, *S. cerevisiae*, suggest a possible mechanism whereby GCL could be working. In yeast, the nuclear periphery has been linked to transcriptional silencing activity, largely through studies of telomeric chromatin and the subtelomeric genes that are affected. MLP1 and MLP2, which are tethered to the nuclear envelope, provide the anchor by which the Yku70/Yku80 heterodimer, which binds to chromatin, brings telomeric chromatin into the perinuclear "silent domain". Once chromatin is at the nuclear periphery, silent information regulators 3 and 4 (Sir3 and Sir4) can gain access to the DNA and cause transcriptional silencing (for a review, see [22]). Since GCL localizes to the nuclear envelope [5], we speculate that it could be accomplishing transcriptional repression similarly to MLP 1 and MLP2 by anchoring chromatin to the nuclear periphery through protein binding partners. We are currently attempting to test this model by looking at the function of binding partners of GCL.

Experimental Procedures

Fly Stocks

The *gcl* mutant line has been described in [6]. The hgb line is described in [7].

Immunocytochemistry

Collections (0- to 3-hr) of embryos were fixed and immunostained as previously described [5]. The H5 antibody (Research Diagnostics) was used at 1:1000 dilution. The *vasa* antibody has been described by Hay et al. [23] and was preabsorbed with an equal volume of embryos at a 1:50 dilution, then used at a final dilution of 1:2500. The *gcl* antibody has been described in Jongens et al. [5] and was preabsorbed with an equal volume of embryos at a 1:20 dilution, then used at a final dilution of 1:2000. Embryos were stained with Høechst 33342 (Sigma) at 5 μg/ml. Secondary antibodies were purchased from Jackson Immunologicals.

Previously, we used the H5 antibody to stain Δ*gcl* embryos after pole cell formation, found no H5 staining in the pole cell nuclei,

and therefore at that time concluded that *gcl* was not involved in transcriptional silencing [6]. However, since then, better H5 antibodies have become available, allowing us to visualize nuclei in stages prior to pole cell formation with ease.

Pole Bud Nuclei Counts

Pole bud nuclei counts were done in fixed embryos from 0- to 3-hr collections stained with anti-Vasa, H5, and Høechst with a Leica DMR microscope with epifluorescence. Embryos at nuclear cycle 10 were selected by their nuclear density under Høechst staining, and pole bud nuclei were identified by their association with Vasa protein. Vasa protein and other germ plasm components associate with the nuclei that enter the germ plasm and induce the formation of pole buds. Pole bud nuclei in Δ*gcl* embryos were deemed transcriptionally silenced if the reduction in H5 staining was similar to that seen in control pole bud nuclei. The remaining pole bud nuclei were classified together as transcriptionally active even if some reduction in H5 staining was observed compared to somatic nuclei.

In Situ Hybridizations

Overnight or 0- to 3-hr collections of embryos were used for whole-mount in situ hybridizations as described by Tautz and Pfeifle [24], except antisense and control sense digoxigenin-labeled probes were made as described by Mlodzik et al. [25].

The *sisA* and *sisB* cDNAs were described in Erickson and Cline [15] and were provided by James Erickson. The *tailless* cDNA was described in Pignoni et al. [26] and was provided by Frank Pignoni. The *hunchback* cDNA was described in Bender et al. [27], and the *huckebein* cDNA was obtained from ResGen, clone LD25709.

Supplementary Material

Suplementary Material including Figure S1 is available at http://images.cellpress.com/supmat/supmatin.htm.

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