Overlapping mechanisms function to establish transcriptional quiescence in the embryonic *Drosophila* germline

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Summary

In *Drosophila melanogaster*, the germline precursor cells, i.e. pole cells, are formed at the posterior of the embryo. As observed for newly formed germ cells in many other eukaryotes, the pole cells are distinguished from the soma by their transcriptional quiescence. To learn more about the mechanisms involved in establishing quiescence, we ectopically expressed a potent transcriptional activator, Bicoid (Bcd), in pole cells. We find that Bcd overrides the machinery that downregulates transcription, and activates not only its target gene *hunchback* but also the normally female specific Sex-lethal promoter, Sxl-Pe, in the pole cells of both sexes. Unexpectedly, the terminal pathway gene *torso-like* is required for Bcd-dependent transcription. However, terminal signaling is known to be attenuated in pole cells, and this raises the question of how this is accomplished. We present evidence indicating that *polar granule component* (*pgc*) is required to downregulate terminal signaling in early pole cells. Consistently, pole cells compromised for pgc function exhibit elevated levels of activated MAP kinase and premature transcription of the target gene *tailless* (*tll*). Furthermore, *pgc* is required to establish a repressive chromatin architecture in pole cells.

Supplemental data available online

Key words: Transcriptional quiescence, Germ cells, *Drosophila melanogaster*

Introduction

The germline of *Drosophila melanogaster* is derived from a special group of cells called pole cells that are formed during early embryonic development (St Johnston, 1993) The *Drosophila* embryo initially develops as a syncytium of rapidly dividing nuclei that undergo multiple rounds of synchronized mitotic cycles. Prior to the tenth division cycle, several nuclei migrate into the specialized cytoplasm or pole plasm at the posterior of the embryo. These nuclei cellularize precociously and these newly formed cells divide two or three times to produce ~30-35 germline precursor cells. The remaining nuclei migrate to the surface of the embryo at nuclear division cycle 10-11. They then undergo several more synchronous divisions and cellularize at the end of nuclear cycle 14 to form the cellular blastoderm (Zalokar and Erk, 1976; Foe and Alberts, 1983; Williamson, and Lehmann, 1996).

In addition to their earlier cellularization and slower rate of mitosis, pole cells differ in their transcriptional activity. Somatic nuclei substantially upregulate RNA polymerase II transcription after they migrate to the surface of the embryo. The activation of zygotic gene expression is essential for these nuclei to respond appropriately to the maternal pathways that assign positional information along the axes of the embryo. By contrast, pole cell nuclei shut down RNA polymerase II transcription when they enter the pole plasm (Zalokar, 1976) and they then remain transcriptionally quiescent until much later stages of embryogenesis. Transcriptional quiescence is a hallmark of germline precursor cells in many organisms (Seydoux and Strome, 1999). For example, in *C. elegans*, RNA polymerase II transcription is repressed in the germ cell lineage by the product of the *pie-1* gene. Transcriptional inactivity appears to be crucial in establishing germ cell identity as mutations in *pie-1* switch the fate of these cells to that of a somatic lineage (Mello et al., 1996; Seydoux et al., 1996).

A number of maternally derived gene products are likely to contribute to transcriptional quiescence in the pole cells of *Drosophila*. One of these is Germ cell less (Gcl), a component of the germ plasm that is necessary for the formation of pole cells. *gcl* appears to be involved in the establishment of transcriptional quiescence and in embryos lacking *gcl* activity, newly formed pole buds are unable to silence the transcription of genes such as *sisterless-a* and *scute*. Conversely, when Gcl protein is ectopically expressed in the anterior of the embryo it can downregulate the transcription of terminal group genes such as *tailless* (*tll*) and *huckebein* (Leatherman et al., 2002). A second maternally derived gene product involved in transcriptional quiescence is Nanos. In the soma, Nanos, together with Pumilio, plays a key role in posterior determination by blocking the translation of maternally derived *hunchback* (*hb*) mRNA (Irish et al., 1989; Lehmann and Nüsslein-Volhard, 1991). Nanos (Nos) also plays a role in downregulating transcription in pole cells, and in embryos produced by *nos* mutant mothers, genes that are normally active only in somatic nuclei are inappropriately transcribed in pole cells (Kobayashi et al., 1996; Asaoka et al., 1998; Deshpande et al., 1999). These include the pair-rule genes *fushi*

tarazu and *even skipped*, and the somatic sex determination gene *Sex-lethal* (*Sxl*) (Deshpande et al., 1999).

The global effects of *nos* and *gcl* mutations on RNA polymerase II activity in pole cells are analogous to those seen in *pie-1* mutants in *C. elegans*. In *pie-1* mutants, genes that are normally expressed only in somatic lineages are turned on in the germ cell lineage. In wild-type *C. elegans* embryos, the inhibition of transcription in the germ cell lineage is correlated with a marked reduction in phosphorylation of the CTD repeats of the large subunit of RNA polymerase II (Seydoux and Dunn, 1997). The CTD repeats are phosphorylated when polymerase is transcriptionally engaged (reviewed by Dahmus, 1996). PIE-1 protein may prevent transcription by inhibiting this modification. As in *C. elegans*, the RNA polymerase II CTD repeats are underphosphoryted in the pole cells of wild-type *Drosophila* embryos. In the pole cells of *gcl* and *nos* mutant embryos, however, the level of CTD phosphorylation is elevated (Leatherman et al., 2002) (G.D., unpublished).

Previous studies have shown that when a heterologous transcriptional activator, GAL4-VP16 is expressed in pole cells, it is unable to activate transcription of target gene(s) (Van Doren et al., 1998). This finding suggested that even if a potent activator were to be produced in pole cells, it would not be able to overcome the inhibition of the basal transcriptional machinery by *gcl*, *nos* and other factors. However, as GAL4-VP16 is a chimera of a yeast DNA-binding domain and a mammalian activation domain, an alternative possibility is that co-factors essential for its activity may be absent or inactive in *Drosophila* pole cells. For these reasons, we decided to test whether a transcription factor that is normally present and active in the somatic cells of early *Drosophila* embryos can promote the transcription of target genes when inappropriately expressed in pole cells. We chose the homeodomain protein Bicoid (Bcd), which activates the zygotic transcription of *hb* and other genes specifying anterior development. A Bcd protein gradient is generated in precellular blastoderm embryos from the translation of maternal mRNA localized at the anterior pole (Driever and Nüsslein-Volhard, 1988; Driever and Nüsslein-Volhard, 1989; Struhl et al., 1989). Although Bcd is not present in the posterior of wild-type embryos, increasing the *bcd* gene dose results in expansion of the gradient toward the posterior and a concomitant change in the pattern of zygotic gene expression. This result suggests that co-factors crucial for Bcd function are likely to be ubiquitous.

We show that ectopic expression of Bcd in pole cells can induce the transcription of the *bcd* target gene *hb*. In addition to activating *hb* transcription, Bcd protein perturbs the migration of the pole cells to the primitive somatic gonad and causes abnormalities in cell cycle control. These effects on germ cell development resemble those observed in embryos from *nos* mutant females. Moreover, as in the case of *nos*$^{-}$ pole cells, the *Sxl* promoter *Sxl-Pe* is also turned on in pole cells by Bcd in a sex-nonspecific manner. Surprisingly, transcriptional activation in pole cells by Bcd requires the activity of the terminal signaling system. This observation is unexpected, as previous studies have established that the transcription of a downstream target gene of the terminal pathway, *tailless* (*tll*) is shut down completely in pole cells (Rudolph, et al., 1997). Moreover, the doubly phosphorylated active isoform of MAP kinase ERK, which serves as a sensitive readout of the terminal pathway, is nearly absent in pole cells (Gabay et al, 1997). Taken together, these findings argue that the activity of terminal signaling pathway in pole cells of wild-type embryos must be substantially attenuated, but not shut off completely. What mechanisms are responsible for downregulating terminal signaling in the presumptive germline? We present evidence indicating that *polar granule component* (*pgc*) functions to attenuate the terminal pathway in newly formed pole cells. *pgc* encodes a non-translated RNA that is localized in specialized germ cell-specific structures called polar granules (Nakamura et al., 1996). We demonstrate that loss of *pgc* function in newly formed pole cells results in the ectopic phosphorylation of ERK and the activation of the ERK dependent target gene *tll*. We also show that *pgc* is required to block the establishment of an active chromatin architecture in pole cells.

Materials and methods

Fly stocks

All fly stocks, unless otherwise noted, are referenced by Lindsley and Zimm (Lindsley and Zimm, 1992). Flies were grown on standard *Drosophila* medium and maintained at room temperature (22°C) unless otherwise specified. Transgenic flies carrying antisense *pgc* construct were kindly provided by Paul Lasko.

Transgene construction and germline transformation

To create the *bcd-nos3′UTR* hybrid gene, the 3′UTR of the *bcd* cDNA from the *bcd* TN3 plasmid (Driever et al., 1990) was truncated at the *Hpa*I site and then fused to an *Eco*RI-*Not*I fragment from pHSXgnosb^{R} (Gavis and Lehmann, 1992) that contains the *nos* 3′UTR and 3′ genomic DNA. The Nanos Response Element within the *bcd* 3′UTR is removed by the *Hpa*I truncation. Note that in *bcd* TN3, the *bcd* 5′UTR has been replaced with the *Xenopus* β-*globin* leader (Driever et al., 1990). The β-*globin-bcd-nos3′UTR* sequences were then fused to the *nos* promoter and 5′UTR at an *Nde*I site engineered at the *nos* translation start codon to create P_{nos}-β*bcd-nos3′UTR*. Finally, the P_{nos}-β*bcd-nos3′UTR* sequences were inserted into the P element vector pDM30 (Mismer and Rubin, 1987). Injection of the pDM30 P_{nos}-β*bcd-nos3′UTR* plasmid into ry^{506} embryos was carried out according to Spradling (Spradling, 1986). Analysis was carried out using two independent transgenic lines.

Histochemical analysis

Synthesis of digoxigenin-labeled *bcd* probe and whole-mount in situ hybridization to *bcd* RNA was carried out as described previously (Gavis and Lehmann, 1992). Whole-mount antibody staining using the Bcd monoclonal antibody 733.3 (gift of W. Driever) was performed as described previously (Gavis and Lehmann, 1992). All other antibody staining was carried out as described elsewhere (Deshpande et al., 1995). Anti β-galactosidase antibody (Promega) was used at 1:500. Anti p-H3 antibody (Upstate Biotechonology) was used at 1:1000. Anti ERK antibody was obtained from Sigma and used at 1:200. Anti-Vasa antibody (kind gift of P. Lasko) was used at 1:2000. For confocal analysis, secondary antibodies conjugated with different fluorophores, anti-rabbit ALEXA-546 (red) and anti rat ALEXA-488 (green) were used (Molecular Probes). All the secondary antibodies were obtained from Molecular Probes.

Results

Bcd can induce transcription of hb in pole cells

To determine if a potent transcriptional activator can overcome the machinery that imposes transcriptional quiescence in pole cells, Bicoid (Bcd) protein was ectopically expressed in pole cells using a transgene in which the sequences encoding the 3′

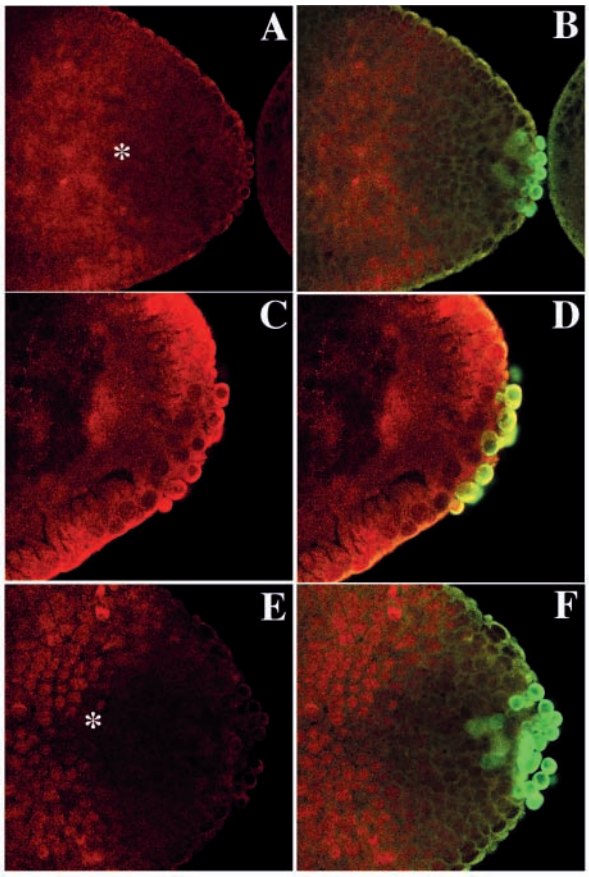

Fig. 1. Ectopic Bcd induces *hb* transcription in pole cells. Co-immunostaining of embryos with anti-β-galactosidase (red) and anti-Vasa (green) antibodies. The anti-Vasa antibody is used to mark pole cells. (A,C,E) Anti-β-galactosidase staining alone. (B,D,F) Merged images of anti-β-galactosidase and anti-Vasa staining. (A,B) Embryos produced by wild-type females mated to males carrying the *hb:lacZ* reporter transgene. β-galactosidase can be detected in somatic cells of wild-type blastoderm embryos in an anterior cap (not shown) and a posterior stripe (asterisk at posterior boundary); however, there is no evidence of β-galactosidase expression in the pole cells. (C,D) Embryos produced by *bcd-nos3′UTR* females mated with males carrying the *hb:lacZ* reporter transgene. Anti-β-galactosidase staining reveals expression of the *hb:lacZ* reporter in pole cells of *bcd-nos3′UTR* embryos (yellow in D). As is evident here, expression of *hb:lacZ* is also detected in somatic cells at the posterior in some *bcd-nos3′UTR* embryos. In this respect, the *hb:lacZ* transgene differs from the endogenous *hb* gene as little if any Hb protein is detected in the posterior soma of *bcd-nos3′UTR* embryos. It seems likely that we are able to detect a low level of β-galactosidase because this protein is much more stable than Hb. (E,F) Embryos produced by *tsl* mutant, *bcd-nos3′UTR* females mated with males carrying the *hb:lacZ* transgene. β-Galactosidase is no longer detected in pole cells in the absence of *tsl* function. Indicative of the *tsl* mutation, the posterior boundary of the posterior *hb* stripe (asterisk) shifts anteriorly.

untranslated region (UTR) of the *bcd* mRNA are replaced with the *nos* 3′UTR (a kind gift from L. Gavis). The *nos* 3′UTR directs mRNA localization to the posterior pole during oogenesis and ensures that only this localized message is translated in the early embryo (Gavis and Lehmann, 1994). In situ hybridization and antibody staining experiments have shown that the transgene *bcd-nos3′UTR* mRNA is localized and translated at the posterior pole of pre-blastoderm embryos (see Fig. S1 at http://dev.biologists.org/supplemental). However, there is less hybrid *bcd-nos3′UTR* mRNA at the posterior than the endogenous *bcd* mRNA at the anterior, and the level of Bcd produced at the posterior is low and for the most part restricted to the pole cells. Consistent with the low level of Bcd, transgenic embryos show no evidence of posterior segmentation defects as would be expected if Bcd inappropriately activated high levels of *hb* transcription in the soma or interfered with *nos* activity.

To determine whether Bcd can overcome quiescence, we examined the expression of the *bcd* target, *hunchback* (*hb*) in *bcd-nos3′UTR* transgenic embryos. By antibody staining, we found that Hb protein could be detected in pole cells of *bcd-nos3′UTR* embryos but not in pole cells of wild-type embryos (data not shown). As the accumulation of Hb protein in pole cells of transgenic embryos could be due to the inappropriate translation of maternally derived *hb* mRNA rather than the zygotic transcription of the *hb* gene, we compared the expression of a paternally derived *hb* promoter:*lacZ* reporter transgene (*hb:lacZ*) in wild-type and *bcd-nos3′UTR* embryos. In wild-type embryos, *hb:lacZ* expression is detected in an anterior domain (not shown) and a posterior stripe (Fig. 1A). However, like the endogenous *hb* gene, no expression of the *hb:lacZ* reporter is detected in the pole cells (Fig. 1B). By contrast, the *hb:lacZ* reporter is expressed in pole cells of *bcd-nos3′UTR* embryos (Fig. 1C,D). These findings indicate that the Bcd transcription factor can overcome the inhibition of the Pol II transcriptional machinery in pole cell nuclei and activate transcription of a known target gene.

CTD phosphorylation is altered in pole cells of *bcd-nos3′UTR* embryos

We reasoned that the presence of potent transcriptional activator might have effects on the transcriptional machinery in pole cells beyond simply activating the *hb* promoter. To explore this possibility, we examined a marker for transcriptional activity. Phosphorylation of a serine residue at position 2 in the seven amino acid CTD repeats of the large subunit of RNA polymerase II has been correlated with polymerase molecules actively engaged in transcription (Dahmus, 1996). Although Pol II with phosphorylated CTD repeats can be readily detected in somatic nuclei of wild-type blastoderm embryos, the Pol II in pole cell nuclei is largely unphosphorylated (Seydoux and Dunn, 1997) (see Fig. 2A). To determine whether transcriptional activation by Bcd in the pole cells of *bcd-nos3′UTR* embryos results in a change in CTD phosphorylation, we used the monoclonal antibody, H5, which specifically recognizes CTD repeats phosphorylated on serine 2. In wild-type embryos, the H5 antibody stains transcriptionally active somatic nuclei but not pole cell nuclei (Fig. 2A). In *bcd-nos3′UTR* embryos, by contrast, the antibody stains nuclei of both somatic cells and pole cells (Fig. 2B). This result indicates that ectopic expression of Bcd protein stimulates CTD phosphorylation in pole cells. As transcription of a single target gene is unlikely to account for this increase in CTD phosphorylation, we presume that other genes are also activated in *bcd-nos3′UTR* pole cells (see below).

In addition to changes in CTD phosphorylation, ectopic expression of Bcd in pole cells seems to increase the number

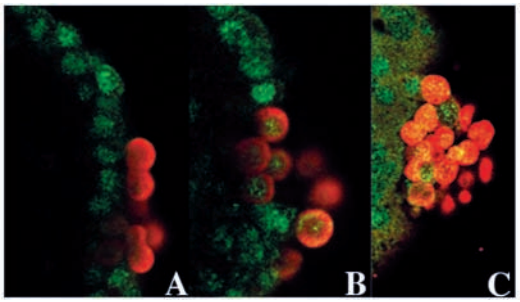

Fig. 2. The level of CTD phosphorylation is altered in pole cells of *bcd-nos3′UTR* embryos. Co-immunostaining of embryos with monoclonal antibody H5 (green) and anti-Vasa antiserum (red). The H5 antibody specifically recognizes RNA polymerase II CTD repeats phosphorylated on serine 2. (A) Wild-type embryo. H5 antibody staining is readily apparent in the transcriptionally active somatic nuclei but not in the transcriptionally quiescent pole cells. (B) *bcd-nos3′UTR* embryo. H5 antibody stains pole cell nuclei in addition to the adjacent somatic nuclei, indicating that the level of CTD phosphorylation is elevated in these pole cells. (C) *pgc* embryo. H5 antibody stains pole cell nuclei in early blastoderm embryos. Note that all the somatic nuclei have not yet reached the periphery.

of germ cells at the syncytial blastoderm stage. This may be due, at least in part, to a continued mitotic activity in a subset of the germ cells. At stages when wild-type germ cells stop dividing and do not express the p-H3 marker, *bcd-nos3′UTR* germ cells (in 4/21 embryos examined) express p-H3 (not shown). These cells also show changes in morphology. Although wild-type germ cells are round, with a smooth regular surface, *bcd-nos3′UTR* germ cells have irregular shapes, with blebbing of the cell membrane. Moreover, in contrast to wild-type germ cells, *bcd-nos3′UTR* germ cells seem to be somewhat variable in size (not shown).

Germ cell migration defects in *bcd-nos 3′UTR* embryos

In wild-type embryos, pole cells migrate along the dorsal surface of the embryo during germ band extension. At stage 10, they pass through the invaginated midgut epithelium to enter the interior of the embryo, where they migrate along the dorsal side of the endoderm. After reaching the dorsal mesoderm, the germ cells are segregated into two groups of 10-15 cells, located on either side of the ventral midline (Fig. 3A). These two groups of cells become encapsulated by the somatic gonadal mesoderm, forming the embryonic gonads (Jaglarz and Howard, 1995; Warrior, 1994; Moore et al., 1998).

Previous studies have implicated both cell autonomous and non-autonomous factors in germ cell migration. Two of the better characterized cell-autonomous factors are the Nanos (Nos) protein and its regulatory partner, Pumilio (Pum) (Asaoka et al., 1999; Deshpande et al., 1999). Pole cells in progeny of mothers lacking either *nos* or *pum* activity do not migrate properly and fail to associate with somatic gonadal precursor cells to form the primitive gonad. It is thought that the failure to establish/maintain transcriptional quiescence in germ cells, which normally occurs in the pre-cellular blastoderm, is likely to be the major cause of the migration defects in *nos* and *pum* embryos.

Interestingly, like *nos* mutant embryos, *bcd-nos3′UTR* embryos also exhibit defects in germ cell migration. Germ cell migration in *bcd-nos3′UTR* embryos is indistinguishable from wild-type until stage 10, after the germ cells migrate to the dorsal mesoderm. Although the germ cells appear to segregate into two clusters on either side of the ventral midline, they either fail to associate with the somatic gonadal mesoderm or sustain contact with the somatic gonad. At stage 13, many of the germ cells in *bcd-nos3′UTR* embryos are scattered through several segments rather than having coalesced in the somatic gonad (Fig. 3B).

Ectopic Bcd induces *Sxl* expression

In previous studies (Deshpande et al, 1999), we found that inappropriate expression of Sxl protein in pole cells induces mitotic and migration defects similar to those observed here for *bcd-nos3′UTR* embryos. As Duffy and Gergen (Duffy and Gergen, 1991) found that expression of Sxl protein from the *Sxl* establishment promoter, *Sxl-Pe*, is upregulated in female embryos by Bcd, one possible explanation for the mitotic and migration defects seen in *bcd-nos 3′ UTR* pole cells is that the *Sxl-Pe* promoter has been inappropriately activated in these germ cells by Bcd. Consistent with this possibility, we found that in marked contrast to the pole cells of wild-type embryos where Sxl protein is not expressed, Sxl is detected in the pole cells of blastoderm stage of female and male *bcd-nos3′UTR* embryos (data not shown). To show that the expression of Sxl protein in these cells is due to the transcriptional activation of *Sxl-Pe*, we compared the activity of a *Sxl-Pe:lacZ* reporter transgene which faithfully mimics the endogenous *Sxl-Pe* promoter in wild-type and *bcd-nos3′UTR* embryos (Keyes et al., 1992; Estes et al., 1995). In wild-type embryos, *Sxl-Pe:lacZ* expression is detected in somatic cells of female embryos but not in male embryos or in the pole cells of either sex (Fig. 4A; data not shown). By contrast, *Sxl-Pe:lacZ* expression is readily detected in both the somatic and pole cells of female *bcd-nos3′UTR* embryos (Fig. 4B). *Sxl-Pe:lacZ* is also expressed in pole cells of male *bcd-nos3′UTR* embryos that lack somatic expression (data not shown)

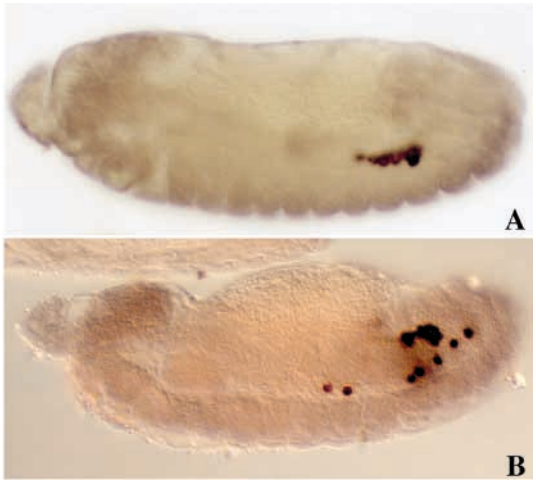

Fig. 3. Germ cell migration defects in *bcd-nos3′UTR* embryos. Immunostaining of stage 13 embryos with anti-Vasa antibody. (A) Wild-type embryo. Germ cells have coalesced into the somatic gonad. (B) *bcd-nos3′UTR* embryo. Many germ cells are found scattered over several segments.

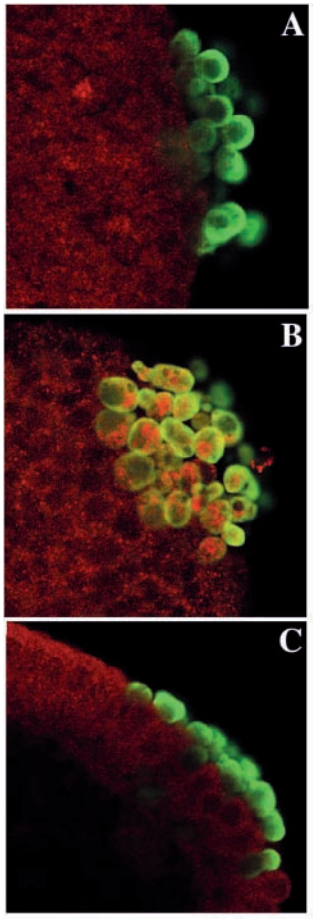

Fig. 4. Ectopic expression of *bcd* at the posterior pole induces Sxl expression. Merged images of embryos co-immunostained with anti-β-galactosidase (red) and anti-Vasa (green) antibodies. (A) Embryo produced by wild-type female mated to male carrying the *Sxl-Pe:lacZ* reporter transgene. β-galactosidase is detected in somatic cells but not in pole cells. (B) Embryo produced by *bcd-nos3′UTR* female mated to male carrying the *Sxl-Pe:lacZ* reporter transgene. β-galactosidase is detected in both somatic and pole cells. (C) Embryo produced by *bcd-nos3′UTR* female mutant for *tsl* mated to male carrying the *Sxl-Pe:lacZ* reporter transgene. Expression of the *Sxl-Pe:lacZ* reporter is no longer detected in pole cells when *tsl* function is removed. Embryos shown in A-C are judged to be female by the activation of *Sxl-Pe* in somatic cells.

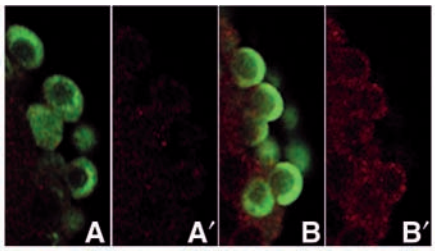

Fig. 5. *torso-nos3′UTR* can activate transcription of *tailless-lacZ* in early pole cells. Wild-type or *torso-nos3′UTR* females were mated to males carrying the *tll:lacZ* transgene. Embryos were co-immunostained with anti-Vasa (green) and anti-β-galactosidase antibodies (red). All images show embryos at cycle 10. Wild-type embryo labeled with (A) both the antibodies or with (A′) β-galactosidase antibody. *torso-nos3′UTR* embryo labeled with (B) both antibodies or (B′) only with β-galactosidase antibody. Little or no β-galactosidase is observed in the wild-type embryo, while a low but clearly detectable level of β-galactosidase is evident in the *torso-nos3′UTR* embryos.

tsl function is required for Bcd-dependent transcriptional activation in pole cells

In earlier studies on transcriptional quiescence, Van Doren et al. (Van Doren et al., 1998) induced pole cell formation at the anterior of the embryo by mis-localizing *oskar* (*osk*) mRNA during oogenesis (Ephrussi and Lehmann, 1992). Even though the amount of Bcd protein at the anterior pole is substantially higher than it is at the posterior pole in *bcd-nos3′UTR* embryos, zygotic *hb* expression was not detected in these ectopic pole cells. One potentially significant difference between our studies and those of Van Doren et al. is that the ectopic pole cells were induced in embryos deficient in the terminal pathway gene product *torso-like* (*tsl*) (Savant-Bhonsale and Montell, 1993; Martin et al., 1994), whereas the terminal pathway was wild type in the *bcd-nos 3′UTR* embryos.

The terminal signaling pathway is required for patterning of the anterior- and posterior-most regions of the embryo (Janody et al., 2000; Schaeffer et al., 2000). This pathway has been shown to have opposite effects on Bcd activity in different regions of the embryo. Close to the anterior pole where both terminal signaling activity and Bcd protein concentration is highest, the terminal pathway antagonizes transcriptional activation by Bcd (Ronchi et al., 1993). By contrast, near the middle of the embryo where the terminal signaling activity and Bcd protein concentration are much lower, the terminal pathway potentiates Bcd function (Janody et al., 2000; Schaeffer et al., 2000). These observations, together with the differences between our results and those of Van Doren et al. suggested that the terminal pathway may be critical for the Bcd-dependent activation of transcription in pole cells.

To test this possibility, we analyzed the expression of the *hb:lacZ* reporter in *bcd-nos3′UTR* embryos mutant for *tsl*. We found that unlike wild-type *bcd-nos3′UTR* embryos, *hb:lacZ* expression is not detected in pole cells from *bcd-nos3′UTR* embryos mutant for *tsl* (Fig. 1E,F). As the elimination of *tsl* function did not alter the amount of Bcd protein at the posterior in *bcd-nos3′UTR* embryos (not shown), we concluded that Bcd-dependent activation of *hb* in pole cells requires *tsl* function. Additionally, this observation suggests that the terminal pathway must be active, at least at some level, in pole cells.

As *bcd* activation of *hb* transcription in pole cells is dependent upon the terminal pathway, we wondered whether this was also true for the activation of *Sxl-Pe*. To test this possibility, the *Sxl-Pe:lacZ* reporter was introduced into *bcd-nos3′UTR* embryos mutant for *tsl* Like *hb*, the activation of *Sxl-Pe:lacZ* in pole cells by Bcd requires *tsl* function (Fig. 4C).

A ‘gain-of-function’ mutation in the terminal signaling pathway gene *torso* alters gene expression in the soma, but has only minimal effects on transcription in pole cells

The finding that *tsl* is required for Bcd-dependent activation of *hb* and *Sxl-Pe* transcription in pole cells prompted us to examine the functioning of the terminal pathway in pole cells in more detail. The terminal signaling pathway is known to activate the transcription of the *tailless* (*tll*) gene in the soma; however, it does not normally turn on *tll* transcription in pole cells (Rudolph et al, 1997). As the elevated levels of CTD phosphorylation seen in *bcd-nos3′UTR* pole cells suggested that Bcd probably turns on genes in addition to *hb* (and Sxl-Pe), we decided to determine whether one of these is *tll*. For this purpose we introduced a *tll:lacZ* reporter into bcd-nos3′UTR embryos. Although we could detect β-galactosidase in bcd-nos3′UTR pole cells, the level of antibody staining was only marginally above the background staining seen for the *tll:lacZ* reporter in wild-type pole cells (data not shown).

One reason why tll might show little response to ectopic Bcd

is that the terminal signaling pathway has to be fully activated in order to efficiently induce tll transcription. If the terminal signaling pathway is downregulated (but not completely off in the pole cells) the tll reporter would remain repressed. To explore this possibility, we asked whether it was possible to activate tll expression in pole cells by potentiating terminal signaling. We used two approaches to upregulate the terminal pathway. In the first, we used a gain-of-function allele of the terminal pathway receptor gene torso, torsoRL3, which is active independent of ligand. In the second, we increased the dose of the torso gene product in pole cells using a nos promoter transgene that drives the expression of torso protein coding sequences linked to the nos 3′UTR (Casanova and Struhl, 1993). As can be seen by comparing β-galactosidase expression from the *tll:lacZ* reporter in wild-type embryos and in torso-nos3′UTR transgene embryos, potentiating the terminal signaling pathway weakly activates transcription of the tll reporter in pole cells (Fig. 5). Similar results were obtained for the constitutively active torsoRL3 receptor. Although the expression of the tll reporter was upregulated in the soma of torsoRL3 embryos, there was only a very weak activation of the reporter in the pole cells (not shown).

For torsoRL3, we also determined whether upregulation of the terminal signaling pathway turned on the Sxl-Pe reporter in pole cells. It did not (not shown). However, we did find that the gain-of-function Torso receptor activates Sxl-Pe in the soma. In the progeny of wild-type mothers, the Sxl-Pe reporter is active only in female embryos and ~50% of the embryo population express β-galactosidase, while 50% do not (compare with Keyes et al., 1992; Estes et al., 1995). By contrast, in the progeny of torsoRL3/+ mothers, all of the embryos express readily detectable levels of β-galactosidase (not shown). Based on the staining pattern the torsoRL3 embryos could be divided into two equal classes. One class had a high level of staining, like wild-type females, while the other had much lower levels.

polar granule component (*pgc*) downregulates the activity of the terminal group genes in the pole cells

The finding that increasing *torso* activity induces only a low level of *tll:lacZ* expression in pole cells (and has no effect on *Sxl-Pe*) suggests that there may be special mechanisms to repress the terminal pathway in the germline of early embryos. With the aim of identifying factors that are involved in downregulating the terminal system in pole cells and blocking *tll* transcription, we asked if the three genes namely *nos*, *gcl* and *polar granule component* (*pgc*) that are known to function in pole cell differentiation have any effects on the activity of the *tll-lacZ* reporter.

Although we found that the *tll-lacZ* reporter is substantially upregulated in the posterior soma of embryos produced by *nos* mutant mothers, we failed to detect β-galactosidase expression in the pole cells (not shown). This observation would suggest that the loss of *nos* activity in itself is not sufficient to override the inhibition of the terminal signaling pathway in pole cells; however, as *tll-lacZ* is clearly upregulated in the nearby posterior soma, it would be reasonable to conclude that *nos* is likely to have a ‘redundant’ role in downregulating the transcription of the terminal pathway gene *tll* in pole cells. This possibility would be consistent with previous studies that showed that the transcription of *Sxl-Pe* as well as several segmentation genes is turned on in the pole cells of *nos* mutant embryos. In the case of *gcl*, we observed no strong effects on

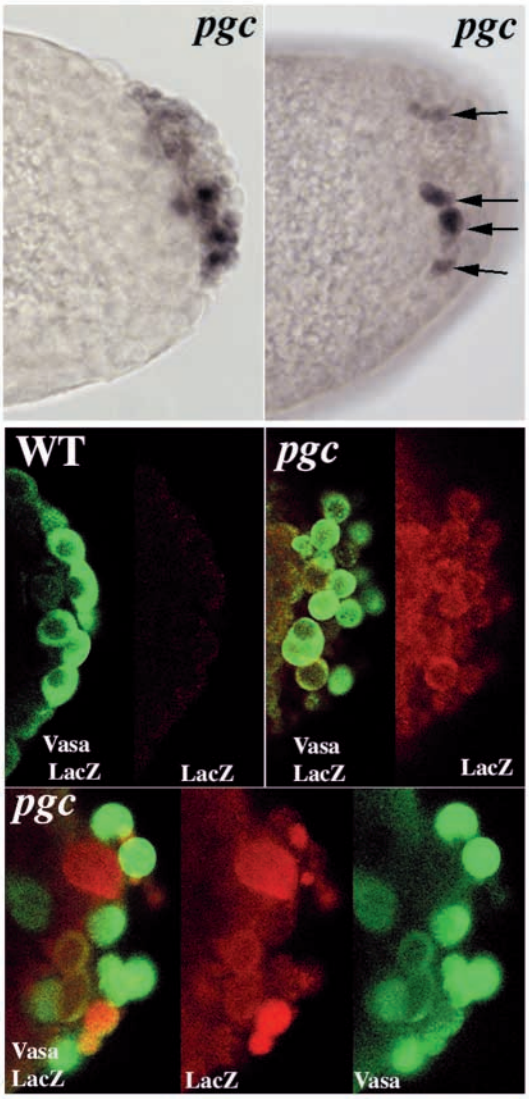

Fig. 6. *tailless-lacZ* reporter is turned on in the newly formed pole cells of embryos maternally compromised for *pgc* activity. Wild-type females or females carrying two copies of the antisense *pgc* transgene were mated to males carrying the *tll-lacZ* reporter. The two panels at the top show *pgc* embryos probed with β-galactosidase antibody and visualized by DAB staining. The panel on the left shows a cycle 10 embryo which has a higher number than normal pole cells. Several of these *pgc* pole cells express high levels of β-galactosidase, while others have little if any β-galactosidase. At this stage, wild-type *tll-lacZ* embryos probed with β-galactosidase antibody show no detectable DAB staining in either the pole cells or the soma. The panel on the right shows a cycle 10 *pgc* embryo that has β-galactosidase positive ‘Pole cells’ at unusual positions (marked with arrows). Some of these are in the interior of the embryo, while others are near the surface. The pole cells shown in the middle and lower panels were co-immunostained with anti-Vasa (green) and anti-β-galactosidase antibodies (red). All images show embryos at cycle 10. Note that some of the ‘pole cells’ in the *pgc* embryo in the bottom panels express very high levels of β-galactosidase but have little or no Vasa protein. Note also that some of the Vasa-positive pole cells are in the interior of the embryo rather than at the surface at the posterior pole.

tll-lacZ expression in the soma. Although β-galactosidase could be detected in the pole cells of *gcl* mutant embryos, the level was just above background (not shown). Thus, the loss of *gcl* activity would appear to have only a minor effect on the transcription of this terminal pathway gene. In addition, there were at most only small effects on the activity of *Sxl-Pe* (not shown).

A different result was obtained in embryos deficient for *pgc*. For *pgc* we used an antisense transgene that is thought to substantially reduce but not completely eliminate *pgc* activity (Nakamura et al., 1996). As shown in Fig. 6, we found that the *tll-lacZ* reporter is activated in pole cells of *pgc* embryos, and relatively high levels of β-galactosidase expression are observed. Strikingly, the expression of the *tll-lacZ* reporter in *pgc*$^-$ pole cells commences between nuclear cycle 9-10 at the time pole cells first begin to form. This is even prior to the activation of this reporter in somatic nuclei which normally occurs around cycle 12. As illustrated in the DAB stained embryos in Fig. 6, β-galactosidase expression is not always restricted to the newly budded pole cells at the very posterior of the embryo. Instead, some embryos have β-galactosidase positive 'cells' at positions that can be rather far from the posterior pole. Double staining with β-galactosidase and Vasa antibodies indicates that at least some of these unusual β-galactosidase positive 'cells' are also Vasa positive (Fig. 6). In addition in several instances, 'cells' that have high levels of β-galactosidase often have low levels of Vasa, while 'cells' that have high levels of Vasa have low levels of β-galactosidase (Fig. 6). Although this variability in *tll-lacZ* expression could reflect the action of other repressive factors, such as *nos*, an alternative possibility is that *pgc* is not completely inactivated by the antisense transgene. In this case, even more extreme defects in repressing *tll-lacZ* and pole cell formation might be expected in the absence of any *pgc* activity.

Though the *tll-lacZ* reporter is activated prematurely in *pgc* pole cells, our experiments indicate that the reporter is subsequently 'downregulated' after nuclear cycle 12-13, and by cellularization only little β-galactosidase is detected in the *pgc* pole cells (not shown). By contrast, high levels of β-galactosidase accumulate in the soma of both *pgc* and wild-type embryos during this same period. One plausible suggestion is that *pgc* is required to block *tll* transcription when pole cells first form, but that subsequently other repressive mechanisms become active in these cells. Although this is likely to be the case, it should be noted that the number of Vasa-positive cells at cellularization (average of 18 in 10 embryos) is typically less than the number that are present earlier at nuclear cycles 9/10 (average of 22 in 10 embryos). Because a subset of the pole cells in nuclear cycle 9/10 *pgc* embryos express only moderate to low levels of β-galactosidase (see Fig. 6), it is possible that only these cells remain at cellularization, while the pole cells that express the highest levels of β-galactosidase are selectively lost.

Finally, unlike *tll-lacZ*, the *Sxl-Pe:lacZ* reporter was not detectably activated at any point in the pole cells of *pgc* embryos (not shown). This finding suggests that *pgc* may influence the expression of a different set of genes than either *nos* or *gcl* (Asaoka et al., 1998; Deshpande et al., 1999; Leatherman et al., 2002).

The presence of activated MAP kinase correlates with *tll* transcription in pole cells

The signaling cascade activated by the *torso* receptor leads to the phosphorylation of MAP kinase (Gabay et al., 1997). To determine whether *pgc* is required to repress this signaling cascade in pole cells, we used a monoclonal antibody that recognizes the doubly phosphorylated, active form of MAP kinase, ERK. As reported previously by Gabay et al. (Gabay et al., 1997), we observed a graded activation of ERK in the soma at anterior and posterior of nuclear cycle 12-13 syncytial embryos (Fig. 7A). By contrast, the pole cells of these cycle 12-13 embryos have only a low level of ERK, indicating that the functioning of the terminal pathway is attenuated in these cells. We presume that this small amount of ERK is sufficient to cooperate with ectopically expressed Bcd to activate transcription in pole cells. The activation of MAP kinase in the soma of early embryos is only transient, and by cellular blastoderm formation anti-ERK specific staining is greatly diminished (Fig. 7B).

To visualize ERK phosphorylation in pole cells of wild-type and *pgc* embryos more precisely, we double stained the embryos with the ERK monoclonal antibody and Vasa antibody. As illustrated by a representative wild-type embryo in Fig. 7C, pole cells from nuclear cycle 10-11 wild type have a low level of the ERK specific signal. By contrast, much higher amounts of activated ERK are evident in the pole cells

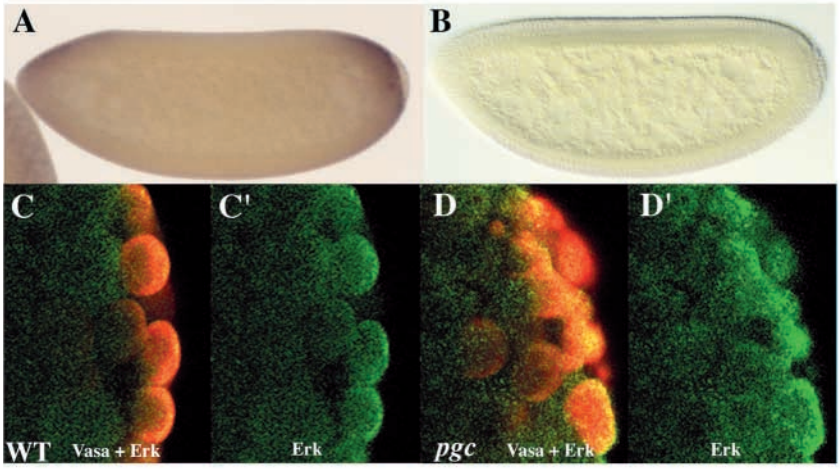

Fig. 7. MAP kinase signaling is elevated in the pole cells compromised for *pgc* function. (A,B) Wild-type embryos were stained with anti ERK antibodies and signal was visualized with DAB. (A) A syncytial blastoderm embryo displays increased levels of staining at both the termini. Very little, if any, signal is detected in the pole cells. (B) Signal specific for anti-ERK antibodies completely disappears by cellular blastoderm stage. (C,D) Pre-syncytial cycle 10 embryos co-immunostained with monoclonal anti-ERK antibody (green) and anti-Vasa antiserum (red). (C′,D′) The same embryos as in C and D labeled with only anti-ERK antibody. Pole cells from the wild-type embryo (C and C′) have only a low level of anti-ERK specific signal whereas pole cells compromised for *pgc* function (D,D′) have much higher levels.

of nuclear cycle 10-11 *pgc* embryos (Fig. 7D). This finding argues that *pgc* functions either directly or indirectly to attenuate the terminal signaling cascade.

pgc is required to block the formation of an active chromatin structure in pole cells

Although *Sxl-Pe* was not turned on in *pgc* pole cells, it seemed possible that the loss of *pgc* activity might have more widespread effects on transcription than just turning on the *tll* gene. To investigate this possibility, we determined whether markers for global transcriptional activity were present in *pgc* pole cells. One of these markers is the phosphorylation of ser 2 residues in the Pol II CTD repeats. As described above, high levels of phosphorylated CTD ser2 are present in transcriptionally active somatic nuclei of wild-type embryos, while in the transcriptionally quiescent pole cells, ser2 is largely unphosphorylated. Consistent with a more general upregulation of transcription in *pgc* pole cells, we found that CTD ser2 phosphorylation is elevated in *pgc* pole cells of presyncytial blastoderm embryos (Fig. 2C). Moreover, as was observed for the *tll* reporter, the level of CTD ser2 phosphorylation is greatly reduced in the pole cells of cellularized *pgc* embryos.

Another marker of global transcription is the methylation of lysine residue 4 in histone H3 (abbreviated as H3MeK4). Schaner et al. (Schaner et al., 2003) have shown that there is little if any H3 K4 methylation during the nuclear cycles preceding the migration of the nuclei to the periphery of the embryo, and even in nuclear cycle 10/11 embryos only a little H3 K43 methylation is detected in the somatic nuclei (Fig. 8A). However, by nuclear cycle 13/14, the level K4 methylation increases substantially (Fig. 8B), and at cellular blastoderm formation all somatic nuclei appear to have a high level of this methylation. By contrast, K4 methylation is not upregulated in wild-type pole cells during the syncytial blastoderm stage (see Fig. 8A,B) and there is little methylated H3 K4 in germ cells until much later in development when they begin migrating from the midgut towards the somatic gonad.

A different result is obtained for *pgc* pole cells. As illustrated in Fig. 8C, methylated H3 K4 can be detected in the pole cells of nuclear cycle 10 *pgc* embryos. This is even before methylated H3 K4 begins to accumulate in the somatic nuclei of wild-type embryos. The amount of methylated H3 K4 increases (Fig. 8D) through nuclear cycle 13. However, as was observed for CTD phosphorylation and *tll* transcription, the level of K4 methylation in the *pgc* pole cells present at cellularization is almost the same as in wild-type pole cells. This finding together with the elevated amounts of CTD ser2 phosphorylation suggest that the loss of *pgc* activity may have wide spread effects on the establishment of transcriptional quiescence in pole cells.

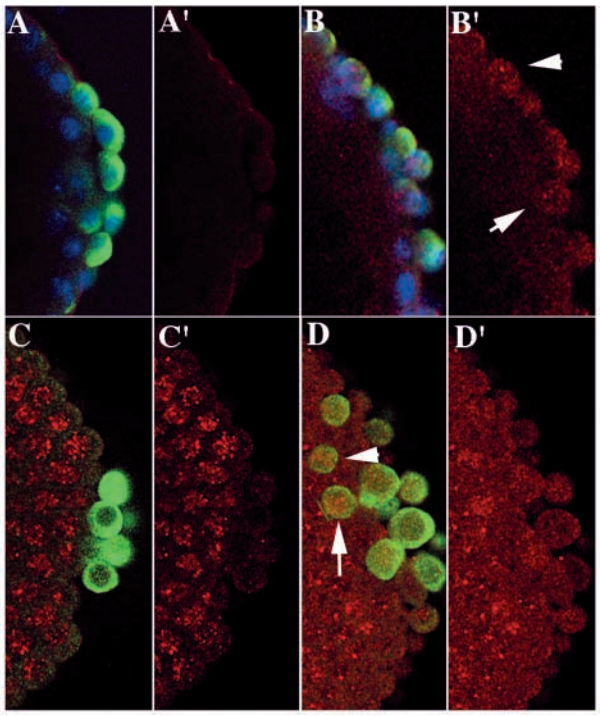

Fig. 8. H3meK4 is absent in newly formed wild-type *Drosophila* pole cells but is present in pole cells depleted for *pgc* function. Wild-type embryos (0-3 hours) and *pgc*- embryos (0-3 hours) were fixed and co-immunostained with anti-H3meK4 (red) and anti Vasa (green) antibodies. Nuclear density was estimated using a DNA dye, Hoechst (imaged in blue as seen in A,B). (A) Pre-syncytial wild-type blastoderm embryo probed with both antibodies and Hoechst dye. (A′) Same embryo showing very little H3meK4-specific signal either in the soma or in the newly formed pole cells. (B) Similar age embryo compromised for *pgc* function probed with both antibodies and Hoechst dye showing the presence of K4 signal in the newly formed pole cells. (B′) Same embryo. Pole cells stained with H3meK4 specific antibodies marked with an arrow and an arrowhead. (C) Wild-type syncytial blastoderm embryo probed with both antibodies. H3meK4 specific signal appears in the soma but pole cells are still devoid of the signal. (C′) Same embryo showing just the H3MeK4 staining. (D) A late syncytial blastoderm embryo compromised for *pgc* function probed with both the antibodies. H3MeK4 specific staining is reduced in almost all pole cells, except in two slightly internally positioned Vasa-positive cells that still show considerable level of signal (marked with an arrow and an arrowhead). (D′) Same embryo showing just H3MeK4-specific signal.

Discussion

Germ cells in *Drosophila* embryos remain transcriptionally quiescent until after they exit the midgut primordium and begin to move towards the somatic gonadal precursor cells. Although Nos and its partner Pumilio (Pum) as well as Gcl have been implicated in establishing/maintaining transcriptional quiescence, it is not known how they shut down RNA polymerase II activity (Parisi and Lin, 2000; Leatherman et al., 2002). In addition, other factors are likely to play a role in turning off transcription in the germ cells. To gain further insights into the mechanisms responsible for transcriptional quiescence in germ cells, we asked whether transcription can be activated in the pole cells by expressing the *Drosophila* homeodomain protein Bcd.

We show that Bcd protein expressed from a *bcd-nos3′UTR* transgene can activate the transcription of its target gene *hb* in pole cells, overcoming whatever mechanisms are responsible for transcriptional quiescence. In addition to activating transcription of *hb*, Bcd has other phenotypic effects. It prevents the pole cells from properly arresting their cell cycle and disrupts their migration to the somatic gonad. Because

similar defects in pole cell development can be induced by the inappropriate expression of Sxl protein in these cells (Deshpande et al., 1999), one plausible hypothesis is that Bcd not only activates the *hb* promoter, but also turns on the *Sxl* establishment promoter, *Sxl-Pe*. Consistent with this idea, the *Sxl-Pe:lacZ* reporter is turned on in the pole cells of male and female *bcd-nos* 3′ UTR embryos and Sxl protein accumulates in these cells. Although previous studies by Duffy and Gergen (Duffy and Gergen, 1991) indicate that *Sxl-Pe* is responsive to Bcd, it is somewhat surprising that *Sxl-Pe* is not only inappropriately turned on in pole cells by Bcd, but that it is activated in both sexes. This suggests that Bcd activation of *Sxl-Pe* in pole cells must proceed by a mechanism that bypasses the X/A chromosome counting system which controls *Sxl-Pe* activity in the soma. It is interesting to note that the activation of *Sxl-Pe* in pole cells in the absence of *nos* function also seems to depend upon a mechanism(s) that circumvents the X/A chromosome counting system.

The behavior of Bcd contrasts with that of a chimeric Gal4-VP16 protein, which does not activate transcription when expressed in pole cells, although it does function in the surrounding somatic cells (Van Doren et al., 1998). The difference in the activity of Bcd and Gal4-VP16 proteins could reflect a requirement for different co-factors to activate transcription of their target genes. For example, the VP16 activation domain has been shown to interact with the TAFs, $TAF_{II}40$ and $TAF_{II}70$ and with the TATA factor itself (Klemm et al., 1995; Nishikawa et al., 1997). If one of the TAFs crucial for VP16 function or some as yet unidentified co-factor is missing or inactive in pole cell nuclei, Gal4-VP16 protein would not be able to activate transcription in *Drosophila* germ cells. Alternatively, as the target enhancers/promoters for Bcd and Gal4-VP16 proteins are different, it is possible that distinct chromatin remodeling factors are required to access these sequences, and that factors required for GAL4-VP16 targets are absent in pole cell nuclei.

That Bcd protein depends upon other ancillary factors to turn on transcription in pole cells is demonstrated by the requirement for *tsl* function in the activation of both the *hb* and *Sxl-Pe* promoters. *tsl* is a component of the maternal terminal signaling pathway which activates the zygotic genes, *tll* and *huckebein* (*hkb*), at the poles of the embryo. In addition, the terminal pathway has opposing effects on the expression of *bcd*-dependent gap genes (Grossniklaus et al., 1994; Wimmer et al., 1995; Gao et al., 1996). At the anterior pole, where terminal signaling activity is highest, Bcd targets such as *hb* and *orthodenticle* (*otd*) are repressed (Driever and Nüsslein-Volhard, 1989; Finkelstein and Perrimon, 1990; Ronchi et al., 1993). At a distance from the anterior pole, where both the concentration of Bcd protein and the strength of the terminal signaling cascade is much lower, the terminal pathway has an opposite, positive effect on *hb* and *otd* expression (Janody et al., 2000; Schaeffer et al., 2000). Two mechanisms are thought to account for the positive effects of the terminal pathway on *bcd* target genes. First, Bcd is a direct target for phosphorylation by the terminal signaling cascade. Second, regulatory regions of *bcd* target genes have sites for other transcription factors whose activity can be directly modulated by the terminal system.

In our experiments, the concentration of Bcd protein produced by the *bcd-nos3′UTR* transgene in pole cells is much less than it is at the anterior pole. Similarly, the activity of the terminal signaling cascade in pole cells is much reduced compared with that in the somatic nuclei at the anterior and posterior poles. Thus, in both of these respects, the conditions in the *bcd-nos* 3′ UTR pole cells would appear to most closely approximate those in the region of the embryo where the terminal signaling cascade potentiates rather than inhibits Bcd activity. This would explain why activation of transcription in pole cells by Bcd depends on the terminal signaling pathway and why in this particular instance this pathway does not antagonize the activity of the ectopically expressed Bcd protein.

The fact that the terminal pathway can function in pole cells, yet does not turn on its target gene *tll* indicates that the activity of this pathway is attenuated in the germline. It seems likely that several different mechanism may be responsible for preventing pole cells from responding to the terminal pathway and turning on *tll* transcription. One mechanism appears to be an inhibition of the signaling cascade itself. In the posterior and anterior soma of pre-cellular blastoderm embryos the terminal signaling cascade directs the phosphorylation of the MAP kinase ERK. While phosphorylated ERK can also be detected in wild-type pole cells, the amount of activated kinase is much less than in the surrounding soma. Consistent with this observation, potentiating the terminal system using either a gain-of-function *torso* receptor mutant or by expressing elevated level of the receptor in pole cells using a *torso* transgene which has the *nos* 3′ UTR had only a small effect on the activity of a *tll-lacZ* reporter in the germline. By contrast, gain-of-function *torso* mutation substantially upregulates the *tll* reporter in the soma.

To identify factors that could be involved in repressing the terminal pathway in pole cells, we examined three genes, *nos*, *gcl* and *pgc*, that are known to play an important role in the early development of the germline and have been implicated in transcriptional quiescence. Of these three, only *pgc* appeared to have significant effects on the terminal signaling pathway in pole cells. We found that the expression of a *tll* reporter is turned on in pole cells of embryos deficient in *pgc* activity. That this is due at least in part to a failure to properly attenuate the terminal signaling pathway in the germline is suggested by the fact that the level of activated ERK is greatly elevated in *pgc* pole cells compared with wild type. Although these findings implicate *pgc* in downregulating the terminal pathway, how this is accomplished and whether *pgc* has a direct rather than an indirect role in this process remains to be determined. In addition, our studies indicate that *pgc* has functions in addition to attenuating this signaling cascade. First, we found that there are abnormalities in the formation of pole cells in *pgc* embryos and Vasa-positive ‘cells’ are observed in cycle 9-10 embryos at abnormal locations. Second, the loss of *pgc* activity may lead to the inappropriate activation of genes in addition to *tll*. We found that two markers for global transcriptional activity, CTD phosphorylation and histone H3 K4 methylation, are present in pole cells of *pgc* embryos.

Our results also suggest that multiple and interrelated levels of regulation are responsible for ensuring transcriptional quiescence in the pole cells. For example, *Sxl-Pe* can be upregulated by the terminal pathway in the soma and requires this pathway to be activated by Bcd in pole cells. However, this promoter is not activated in pole cells in the absence of *pgc*

function. Thus, the activation of the terminal signaling cascade in pole cells is not sufficient in itself to induce *Sxl-Pe*. This suggests that mechanisms are in place in *pgc* pole cells that would override any effects of activated ERK on *Sxl-Pe* activity. Similarly, although loss of *nos* activity leads to the activation of *Sxl-Pe* in pole cells, and the upregulation of *tll* in the posterior soma, the *tll* promoter is not turned on in *nos* pole cells. We presume that *tll* is not activated in pole cells because it requires the terminal system that still remains attenuated in *nos* pole cells. Redundancy is also suggested by the finding that although the loss of *gcl* leads to the expression of the X chromosome counting genes *sis-a* and *scute* in pole cells (Leatherman et al., 2002), *Sxl-Pe* is not activated. This suggests *nos* function is sufficient to keep *Sxl-Pe* off in *gcl* mutant pole cells even though several X chromosome counting genes are activated. Similarly, we have not observed an obvious effect of *nos* mutations on *scute* expression in pole cells (G.D, unpublished). This implies that *gcl* and *nos* may be responsible for repressing the transcription of different sets of genes.

Finally, although transcription is upregulated in *pgc* pole cells between nuclear cycles 9/10-13, a high level of transcriptional activity is not maintained in the pole cells that are present by the time the cellular blastoderm is formed. The *tll* reporter is turned off, and both CTD phosphorylation and histone H3 K4 methylation disappear. One possible interpretation of this finding is that *pgc* has an early function in establishing transcriptional quiescence, but is not required after nuclear cycle 13 because of the activity of other factors such *nos* or *gcl*. However, as the number of pole cells at cellularization is reduced compared with the number present earlier, it also possible that the only pole cells that remain are the ones in which the amount of *pgc* activity is sufficient to establish some degree transcriptional repression. Further studies with bona fide null alleles will be required to resolve this question, and understand how *pgc* functions during pole cell formation and germ cell determination

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