Novel Functions of nanos in Downregulating Mitosis and Transcription during the Development of the Drosophila Germline

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Summary

It has previously been shown that germ cells in embryos derived from nos mutant mothers do not migrate to the primitive gonad and prematurely express several germline-specific markers. In the studies reported here, we have traced these defects back to the syncytial blastoderm stage. We show that pole cells in nos embryos fail to establish/maintain transcriptional quiescence; the sex determination gene Sex-lethal (Sxl) and the segmentation genes fushi tarazu and even-skipped are ectopically activated in nos germ cells. We show that nos germ cells are unable to attenuate the cell cycle and instead continue dividing. Unexpectedly, removal of the Sxl gene in the zygote mitigates both the migration and mitotic defects of nos germ cells. Supporting the conclusion that Sxl is an important target for nos repression, ectopic, premature expression of Sxl protein in germ cells disrupts migration and stimulates mitotic activity.

Introduction

In Drosophila melanogaster and other insects, the germ line arises from a special group of cells, the pole cells, which are formed at the posterior of the embryo (reviewed in Williamson and Lehmann, 1996). After fertilization, the Drosophila embryo develops as a syncytium of nuclei, which undergo rapid and synchronous nuclear division (Foe and Alberts, 1983). Between the eighth and ninth division cycle, two to three nuclei migrate into a specialized cytoplasm at the posterior pole called the pole plasm. These nuclei undergo two more division cycles (mitotic cycle 10–11) and then become surrounded by cell membranes to form a small cluster of pole cells. These cells divide asynchronously, and by cellular blastoderm there are between 30–35 pole cells (Hay et al., 1988). The somatic nuclei migrate to the surface of the embryo between nuclear division cycle 9–10 and then go through several additional rounds of synchronous nuclear division before finally cellularizing at the end of nuclear cycle 14 (Zalokar and Erk, 1976). The pole cells remain segregated from the soma during gastrulation, migrating anteriorly along the dorsal surface of the embryo until they are carried inward by the posterior midgut invagination. Subsequently, the germ cells migrate through the developing gut and then dorsally over the surface of the gut to the overlying mesoderm. After reaching the mesoderm, the germ cells split into two clusters, which migrate laterally until they contact and coalesce with the somatic precursors of the gonad (Warrior, 1994; Jagnraz and Howard, 1995).

In addition to differences in the time of cellularization and the rate of mitosis, pole cells differ from the surrounding soma in regard to transcriptional competence. The onset of RNA polymerase II-dependent transcription in the soma coincides with the migration of nuclei to the embryo surface. Zygotic transcription of patterning genes sets in motion the regulatory pathways that ultimately assign specific identities to somatic cells in different regions of the embryo. In striking contrast, pole cell nuclei shut down RNA polymerase II (but not polymerase I) dependent transcription when they enter the pole plasm (Seydoux and Dunn, 1997; Van Doren et al., 1998). Moreover, once pole cells are formed, they remain transcriptionally quiescent until they associate with the primitive somatic gonad. Transcriptional quiescence is a characteristic feature of germ cells in other organisms besides Drosophila. In C. elegans, for example, RNA polymerase II transcription in the germ cell lineage is specifically repressed by the product of pie-1, a gene which is critical for maintaining germ cell identity (Seydoux et al., 1996).

Germ cell formation in the Drosophila embryo depends upon the assembly of the pole plasm during oogenesis (Ilmenese et al., 1976). Genes required for the formation of a functional pole plasm have been identified in genetic and molecular screens. One group of genes, the posterior group, is required for both pole cell formation and abdominal segmentation. Included in this group are oskar (osk), mago nashi (mago), vasa (vas), tudor (tud), valois (vlv), and staufen (stau) (reviewed in Lehmann, 1995). Mutations in this group of genes fail to assemble pole plasm and are unable to localize nanos (nos) mRNA at the posterior pole. Consequently, in embryos produced by mutant mothers, no pole cells are formed, and abdominal segmentation is disrupted because maternal hunchback mRNA is translated in the posterior half of the embryo in the absence of the Nos protein. Of the known genes in this group, osk seems to play a pivotal role (Lehmann, 1995). In wild-type oocytes, Osk protein is expressed at the posterior pole where it initiates the assembly of the pole plasm and recruits nos mRNA (Ephrussi et al., 1991; Kim-Ha et al., 1991). It can also assemble pole plasm and recruit nos when ectopically expressed at the anterior pole. This ectopically localized pole plasm can direct the formation of fully functional germ cells (Ephrussi and Lehmann, 1992). In addition to the posterior group genes, antisense experiments have implicated several other gene products in pole cell formation and/or pole cell identity. These include mitochondrial 18s rRNA (mtr), germ cell-less (gcl), and polar granule component (pgrc) (reviewed in Williamson and Lehmann, 1996).

The nos gene product differs from other posterior group genes in that it is not required for pole plasm assembly in the egg or for pole cell formation in the...
embryo (Lehmann, 1995); however, the germ cells produced in embryos from nos mutant mothers are abnormal (hereafter referred to as nos− embryos or pole/germ cells). These nos− germ cells exhibit striking defects in migration. These migration defects are most clearly evident in progeny derived from nos hb− germ line clones in which the development of the soma is normal because maternal hb− is removed (Hulskamp et al., 1989; Irish et al., 1989; Struhl, 1989; Forbes and Lehmann, 1998). When the nos− pole cells begin exiting the posterior midgut, these cells remain clustered along the distal tip, instead of migrating along the dorsal surface of the midgut. Only a few of the nos− pole cells associate with the mesoderm, but these usually fail to migrate laterally or make contact with the somatic gonad. Pole cell transplantation experiments argue that these migration defects arise because the nos gene product is required within the pole cells (Kobayashi et al., 1996). In addition to migration defects, Kobayashi et al. (1996) showed that nos− germ cells prematurely activate several germ-line-specific enhancer traps. In wild-type embryos, these enhancer traps are turned on only after the germ cells coalesce with the somatic gonad; however, in nos− embryos, the enhancer traps are prematurely expressed during the midgut invagination (Asaoka et al., 1998).

Results

The premature activation of several germline-specific enhancer trap lines in nos− pole cells (Kobayashi et al. 1996) raised the possibility that Nos is involved in either the establishment or maintenance of transcriptional quiescence. If this were true, genes, which are normally active in the soma, but not in the germline of early embryos, might be inappropriately expressed in nos− pole cells. To test this idea, we first examined the expression of the Sxl gene, which is known to be quiescent in early germ cells, in nos− embryos.

The transcription of Sxl in precellular blastoderm embryos is controlled by a system that measures the X chromosome to autosomal ratio (X/A) (Keyes et al., 1992). When the X/A ratio is 1 (female), the Sxl establishment promoter, Sxl-Pe, is activated in all somatic nuclei. In contrast, when the X/A ratio is 0.5 (male), this promoter remains off. The X/A counting system does not operate in the germline, and the Sxl-Pe promoter is not turned on in the pole cells of either sex (Keyes et al., 1992). In fact, expression of Sxl protein in the germline cannot be detected until much later in embryogenesis, after the primitive gonad is formed. As in the soma, Sxl is found only in the female germline; however, it is not known how the gene is activated (Cline and Meyer, 1996).

To test whether Sxl is prematurely turned on in pole cells in the absence of nos, we stained 0–4 hr embryos laid by either wild-type or homozygous nos− mothers with Sxl antibodies. Shown in the top panel of Figure 1 is the Sxl protein expression pattern in wild-type male, wild-type female, and nos− female blastoderm embryos. As expected, Sxl antibody heavily stains the somatic cells of the female embryos, while the male embryo is unstained. The nos− female embryo differs from wild type in that we detect a low level of staining in the pole cells. Like the nos− female embryos, Sxl antibody staining is also observed in the pole cells of nos− male embryos (data not shown, see below). (We also typically detect a low level of Sxl antibody staining in the posterior soma of nos− male embryos; data not shown.) Sxl protein was also observed in the pole cells of male and female embryos from nosRC/nosRC mothers.

To confirm that Sxl is ectopically expressed in the pole cells of nos− embryos, we used confocal microscopy to examine 0–4 hr embryos double labeled with Sxl (imaged in red) and Vasa (imaged in green) antibodies. As expected, we found that Vasa antibody labels the pole cells of wild-type embryos (Hay et al., 1990; Lasko and Ashburner, 1990), while Sxl antibody does not (data not shown). A different result was obtained for nos− embryos. As illustrated by the double labeled male nos− embryo in the middle panel in Figure 1, we detect both Vasa and Sxl protein in the pole cells. Precisely the same result was obtained for female nos− embryos; however, in this case a high level of Sxl antibody staining was also observed in the soma. Confocal microscopy was also used to examine Sxl protein expression in germ cells later in embryonic development during stages 10–12. This is still prior to the time when Sxl is normally activated in the female germline, and no Sxl protein could be detected in the wild-type control (data not shown). We found variable levels of Sxl protein in the Vasa-positive germ cells of nos− embryos (bottom row). Moreover, Sxl protein was seen in the nos− germ cells irrespective of the sex of the embryo (left, female; right, male).

Sxl Establishment Promoter (Sxl-Pe) Is Activated in the Pole Cells of Embryos That Lack nos Activity Maternally

Two different mechanisms could potentially account for the accumulation of Sxl protein in pole cells of nos− embryos. Since nos is known to block the translation and promote the turnover of maternally deposited hb mRNA, it is possible that maternal Sxl mRNA is inappropriately translated in the absence of the Nos protein. Alternatively, if nos is required to establish/maintain transcriptional quiescence, then Sxl protein might be produced in nos− pole cells by the inappropriate transcriptional activation of Sxl-Pe.

To distinguish between these possibilities, we used a Sxl-Pe:β-galactosidase reporter (Keyes et al., 1992). In an otherwise wild-type background, the Sxl-Pe promoter drives β-galactosidase expression in female but not male embryos. Males carrying the Sxl-Pe:β-galactosidase transgene were mated to either wild-type or nos− females, and the pattern of β-galactosidase expression was assayed by antibody staining. β-galactosidase is expressed in the soma but not in the pole cells of wild-type female embryos (Figure 2A). A different result is obtained for nos− embryos. As illustrated by the female nos− embryo in Figure 2A, β-galactosidase is observed not only in the somatic cells, but also in the pole cells. Similar results were obtained for male nos− embryos (data not shown). These findings argue that the transcriptional activation of Sxl-Pe is likely to account for the presence of Sxl proteins in the pole cells of nos− embryos. However, it does not exclude the possibility that nos also plays some role in the translational regulation of maternal or zygotic Sxl mRNAs.
Novel Functions of nanos

Figure 1. Sxl Protein Is Ectopically Expressed in the Pole Cells of nos Embryos

Syncytial blastoderm embryos stained with Sxl antibody. Embryos (0–4 hr old) laid by white and nos mothers were stained with monoclonal antibodies to Sxl and visualized by immunohistochemical detection. (Top, left to right) white male embryo showing complete absence of anti-Sxl antibody staining in both the somatic nuclei and the pole cells; white female embryo showing somatic nuclei stained with Sxl antibody while pole cells are devoid of Sxl protein; nos female embryo showing presence of Sxl protein in somatic nuclei as well as in the pole cells. These results were subsequently confirmed as follows. Anti-Sxl staining was repeated by mixing nos and wild-type embryos. The mixed embryos were fixed together and stained in the same staining solution (data not shown).

(Middle) Embryos (0–3 hr old) were double labeled with Sxl antibody (imaged in red) and Vasa antibody (imaged in green). The staining was visualized by subsequent treatment with secondary antibodies conjugated with different fluorophores, anti-mouse alexa 543 (red) and anti-rat alexa 488 (green). Shown in this panel is a nos syncytial blastoderm stage male embryo. Sxl (red) and Vasa (green) protein can be detected in the pole cells. This can be seen by the yellow color of the pole cells in the merged image.

(Bottom) A similar double staining was performed on 0–12 hr old embryos using Sxl and Vasa antibodies. Shown are female (left) and male (right) nos embryos. In both embryos, the yellow color indicates that both Sxl and Vasa protein are present in the germ cells. As expected, Sxl protein is present in the female somatic cells but is absent in the somatic cells of male embryo.

Somatic Genes Involved in Segmentation Are Expressed in the Pole Cells of nos Embryos

The inappropriate activation of Sxl-Pe in pole cells of both female and male nos embryos supports the idea that Nos protein may be more generally responsible for downregulating transcription in germ cells. If this is the case, removal of nos function might result in ectopic activation of genes that are not normally expressed in the germline. To explore this possibility, we examined the expression of two pair-rule segmentation genes, ftz and eve. Shown in Figure 2C is the Ftz expression pattern in embryos from wild-type and nos mothers. Strikingly, Ftz protein can be detected in nos pole cells but is not seen in wild-type pole cells. The severe disruption in the Ftz stripe pattern in the medial region of the embryo confirms that these are indeed nos embryos. Similar results were obtained for another segmentation gene, eve (data not shown).

To test whether the Ftz protein seen in nos pole cells is due to the transcriptional activation of ftz, we crossed wild-type or nos mutant females to males carrying a ftz-LacZ reporter. Expression of β-galactosidase from this reporter is under the control of the ftz upstream regulatory elements. In wild-type embryos, the reporter drives β-galactosidase expression in the characteristic seven-stripe pattern; however, no β-galactosidase protein is observed in the pole cells. In contrast, in nos embryos β-galactosidase is detected in the pole cells (see Figure 2B).

We also tested tailless and twist. tailless is normally expressed at the very posterior of the embryo in the region of the soma immediately adjacent to the pole cells, while twist is expressed along the ventral side of the embryo. Unlike ftz and even-skipped, we were unable to detect the expression of either of these genes in nos pole cells. This result suggests that transcriptional derepression in nos pole cells may be restricted to a subset of the genes which are active in the soma of the early embryo. Consistent with this idea, Van Doren et al. (1998) found that bicoid failed to activate the β-galactosidase reporter in nos pole cells formed ectopically at the anterior of the embryo by mislocalized Oskar protein.

nos− Pole Cells Fail to Arrest the Cell Cycle

Pole cells differ from the surrounding soma not only in their transcriptional activity, but also in their mitotic activity. Soon after the pole cell nuclei enter the pole plasm, their mitotic cycle lengthens, and the newly
formed pole cells divide asynchronously until the formation of the cellular blastoderm. At this stage, wild-type embryos have slightly more than 30 pole cells (see Table 1). The pole cells then cease dividing and remain mitotically inactive until after they coalesce with the somatic gonadal precursor cells to form the primitive gonad. The germ cells appear to be arrested at the G2-M transition; their DNA content indicates that they have completed S phase, and they have high levels of the mitotic cyclins A and B (Su et al., 1998b).

Since nos seems to be required in pole cells to inhibit RNA polymerase II transcription and block the expression of somatic genes, an obvious question is whether it also plays a role in regulating the cell cycle. To address this question, we compared the number of pole cells in wild-type and nos mutant embryos at different stages of development.

Differences in the number of pole cells in wild-type and nos embryos are already apparent in nuclear cycle 12-13 syncytial blastoderm embryos. At this stage, wild-type embryos have, on average, ~19 pole cells (n = 14), while nos embryos have 24 (n = 15). Just after cellular blastoderm formation, wild-type embryos have about 33 pole cells (n = 10), while nos embryos have about 44 pole cells (n = 10). These findings indicate that the nos gene product could be important for slowing down cell cycle progression in the pole cells of precellular blastoderm embryos. Our results are in agreement with Smith et al. (1992), who observed a similar increase in the number of germ cells with a completely different nos mutant allele.

Wild-type pole cells cease dividing after the cellular blastoderm stage. Hence, we wondered whether the pole cells of nos embryos are able to stop cell division after this stage or whether they continue to divide. While

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n, total number of embryos; SD, standard deviation.
Figure 3. Mitotic Markers Are Expressed in nos– Pole Cells

Embryos of different genotypes were double labeled with anti-Vasa (green) and anti-phosphorylated histone H-3 antibodies (red). After subsequent treatment with secondary antibodies conjugated with different fluorophores, anti-rabbit alexa 543 (red) and anti-rat alexa 488 (green), the staining was visualized by standard confocal analysis. The age of the embryos varies between stage 12 to stage 13. (A–C) Wild-type embryos. (D–H) nos– embryos. (I–L) Sx-FL embryos. (M and O) Unrescued embryos from nos,hb clonal analysis. (N and P) Rescued embryos from nos,hb clonal analysis. Embryos of different genotypes were double labeled with anti-Vasa (green) and anti-cyclin antibodies (red). After subsequent treatment with secondary antibodies conjugated with different fluorophores, anti-rabbit alexa 543 (red) and anti-rat alexa 488 (green), the staining was visualized by standard confocal analysis. (Q) Wild-type embryos. (R and S) nos– embryos.

it is possible to count nos– germ cells during the early stages of germband extension, counting becomes difficult at later stages because the mutant germ cells clump and stain irregularly with Vasa antibody. For this reason, we sought alternative strategies for examining the mitotic activity of pole cells in germband-extended embryos.

When the pole cells of wild-type embryos stop dividing around cellular blastoderm formation, they arrest at the G2-M transition. Wild-type germ cells blocked at this stage in the cell cycle do not express mitotic markers like phosphorylated histone H-3 (p-H3) (Wei and Allis, 1998). We therefore used an antibody to p-H3 to determine whether the pole cells of nos– embryos were appropriately arrested in the cell cycle. Although we readily detected cells staining with the p-H3 antibody in wild-type embryos, these cells did not stain with Vasa antibody (Figures 3A–3C). In fact, in no case did we observe a germ cell in a wild-type germband-extended embryo that expressed the p-H3 mitotic marker (n = 20). In contrast, as illustrated in Figures 3D–3H, virtually every nos– embryo examined had at least one Vasa-positive cell that also stained with the p-H3 antibody, indicating that it had entered mitosis (n = 17). These findings suggest that nos– germ cells continue dividing after the cellular blastoderm stage.

To provide further evidence that Nos protein is required to maintain the G2-M cell cycle block in pole cells after cellular blastoderm formation, we examined the expression of cyclin B. In wild-type embryos, cyclin B accumulates in germ cells from the time they are first formed until after they coalesce in the somatic gonad (Su et al., 1998b). This is illustrated in Figure 3 by the wild-type germband-extended embryo double stained with cyclin B and Vasa antibodies (Figure 3Q). In contrast, little or no cyclin B can be detected in germ cells from nos– germband-extended embryos (Figures 3R and 3S). These findings indicate that nos is required in germ cells not only to attenuate the cell cycle in precellular blastoderm embryos but also to maintain the G2-M cell cycle block after the onset of gastrulation.

Sxl Is One of the Critical Targets of nos– Mediated Repression

Why do germ cells in nos mutant embryos fail to regulate cell cycle progression and exhibit defects in their migration to the somatic gonad? Since germ cells become transcriptionally active in the absence of nos function, one mechanism that might give rise to these mutant phenotypes would be the inappropriate expression of a gene(s) in the newly formed pole cells that alters their identity or behavior. As described above, we identified
three genes, Sxl, ftz, and eve, whose transcription is turned on in the pole cells of precellular blastoderm embryos from nos− mothers. Of these three, Sxl is a good candidate, as it is known to function in the germ-line, and consequently germ cells might have regulatory targets that are susceptible to Sxl action.

Migration Defects

If the inappropriate expression of Sxl contributes to some of the defects seen in nos− germ cells, it should be possible to alleviate these defects by removing the Sxl gene in the zygote. To test this idea, we crossed nosBN mothers that are heterozygous for a Sxl deletion mutant, Sxl7BO, to Sxl7BO mutant fathers. In this cross, 50% of the embryos will have a wild-type Sxl gene, while the other 50% will not. We then stained 0-12 hr embryos from this cross with either Vasa antibody or Vasa and Sxl antibody and determined the percentage of embryos that had two lateral clusters of germ cells. Bilateral germ cell clusters are never observed in nos− embryos (>100); instead the germ cells clump prematurely and remain attached to the end of the gut (see Figures 4A-4C). In contrast, from a cross between Sxl7BO/+; nosBN/nosBN females and Sxl7BO males, 11 out of 60 embryos (or about 15%) had bilateral germ cell clusters (see Figures 4D-4F).

The presence of the embryos with bilateral germ cell clusters in this cross suggests that the removal of the Sxl gene can modify the migration defect of germ cells from nos− embryos. However, bilateral germ cell clusters were observed in only 15% of the embryos, while approximately half of the embryos are expected to lack the Sxl gene. There could be several explanations for this discrepancy. One possibility is that the rescue is incomplete because of the extensive segmentation defects in the posterior half of the nos− embryo (see below). Another, not necessarily mutually exclusive, possibility is that the migration defects in nos− embryos arise from the misexpression of not only Sxl, but also of one or more additional genes.

The segmentation defects of nos− embryos arise from a failure to repress the translation of maternally derived hunchback (hb) mRNA, and these defects can be eliminated by generating germline clones that lack a functional hb gene. We generated germline clones that are mutant for both hb and nos in a female that is also heterozygous for the Sxl7BO mutation. Females carrying these germine clones were then mated to Sxl7BO males, and we scored germ cell migration after staining with Vasa antibody (data not shown). In the experiment in which we stained only with Vasa antibody, 20 of the 50 germ-band-extended embryos examined had bilateral germ cell clusters like those seen in wild-type embryos, while the other 50% and suggests strongly that suppression of the nos− migration defects results from the removal of the Sxl gene. The experiment in which we stained with both Vasa and Sxl antibody, 7 of the 20 embryos examined had bilateral germ cell clusters. While the germ cells in these embryos stained with Vasa, they did not stain with Sxl antibody. The remaining embryos did not have bilateral germ cell clusters. Of these, all but two showed Sxl protein. These findings suggest that the misexpression of Sxl is a significant contributing factor to the migration defects of nos− germ cells.

Cell Cycle Defects

We next asked whether the cell cycle defects of nos− germ cells can be suppressed by the removal of the Sxl gene. As before, nos− hb− germine clones were generated in females heterozygous for Sxl7BO, and the females were then mated to Sxl7BO males. The progeny
Figure 5. Removal of Sxl Can Modify the Germ Cell Migration Phenotype Observed in nos hb Embryos

Sxf279/Bin virgin females carrying nos hb germ line clones were mated with males of the genotype SxI279/Y. The embryos generated from this cross were fixed and stained with Vasa antibody. Staining was visualized using anti-rat alexa 488 (green). Embryos could be divided into two classes based on the staining pattern. (A) and (C) show top view, while (B) and (D) show lateral view. In the first class (see example in [A]), the germ cells clumped prematurely and stayed in the middle of the embryo. In the second class (see example in [C]), the germ cells migrate laterally forming two distinct clusters. (B) shows lost and scattered germ cells in the posterior of the embryo, while a similar stage embryo with properly positioned and coalesced gonad is shown in (D).

Ectopic Expression of Sxl Protein in Pole Cells

The results in the preceding section suggest that the inappropriate activation of Sxl in nos- germ line cells contributes to the defects in their development. If this is the case, it should be possible to induce at least some of these defects by ectopically expressing Sxl protein in pole cells. For this purpose, we took advantage of a transgene, Sx.IFL, which expresses a modified female Sxl mRNA under the control of the constitutive hsp83 promoter. We discovered that eggs derived from mothers carrying the Sx.IFL transgene differ from wild type in that they contain low but detectable quantities of Sxl protein. This is illustrated in the Western blot of extracts similar to those seen in the absence of Nos protein. In all cases, germ cell-specific expression of the p-H3 mitotic marker was observed (Figures 3M and 3O). We conclude from these findings that the cell cycle defects of nos- germ cells can be alleviated by the removal of Sxl.

Unlike nos- mutant embryos, the embryos derived from the nos- hb- germ line clones develop normally and can reach adulthood. If the removal of Sxl suppresses all the defects of nos- germ cells, then the Sxf279 males from this cross (the females are dead) should be fertile. However, all of the surviving Sxf279 males were sterile (~50).

Figure 6. Sx.IFL Transgene Product Is Maternally Deposited and Is Present in the Pole Cells of Syncytial Blastoderm Embryos

(Top) Shown is a Western blot probed with Sxl antibody. Lanes from left to right: WF, white1 female extract. UF, unfertilized eggs collected from white1 virgin females. Sx.IFLUF, unfertilized eggs collected from Sx.IFL transgene virgin females. As a loading control, we probed the blot with antibodies against the snRNP protein Snf.

(Bottom) Embryos laid by Sx.IFL females mated with white1 males were double stained with Sxl and Vasa antibody. Embryos (0-3 hr old) were double stained with Sxl antibody (imaged in red) and Vasa antibody (imaged in green). The staining was visualized by subsequent treatment with secondary antibodies conjugated with different fluorophores, anti-mouse alexa 543 (red) and anti-rat alexa 488 (green). From left to right, is a male embryo in which the Vasa, Sxl, and Vasa + Sxl staining pattern is visualized.
Differences in germ cell migration were observed, and in On the other hand, nos (wild type) (data not shown), both presumably male embryos of the equivalent age. (A–D) Embryos laid by wild-type embryos. While p-H3 can be detected in the pole cells of germband-extended embryos, we counted the number of pole cells in wild-type embryos, while there were nearly 45 (n = 10) germ cells in hsp83:Sx.FL.

We next stained 0–12 hr embryos with antibodies against p-H3. As illustrated in Figure 3, this mitotic marker is found in germ cells of Sx.FL embryos, but not wild-type embryos. While p-H3 can be detected in the pole cells of germband-extended Sx.FL embryos, the frequency is much lower (~15%) than seen in nos embryos of the equivalent age.

Germ Cell Migration

Like nos embryos, the germ cells of Sx.FL exhibit migration defects. Migration defects first become apparent around stage 10, and by stage 12–13 the distribution of germ cells in the Sx.FL embryos is quite abnormal. Early on, defects include a failure to establish contact with the presumptive gonadal mesoderm and occasional premature clumping (data not shown). Subsequently, by stage 13 several embryos show a distinctly abnormal pattern of migration (Figures 7A–7D). Some of the phenotypes include lack of sustained contact with the presumptive gonadal mesoderm, mispositioning with respect to gonadal tissue, and failure to coalesce. Although bilateral primitive gonads are ultimately formed double-labeled embryos. The overlap between the two different classes of embryos the number of embryos exhibiting migration defects was similar.

Embryos Are Sex Nonspecific

The Germ Cell Migration Defects Seen in hsp83-Sxl Embryos Are Sex Nonspecific

We also determined whether the germ cell migration defects were produced in both male and female embryos by staining with antibodies against both Vasa and Sxl. Based on the Sxl antibody staining pattern, the embryos could be classified as either strongly stained and presumably female embryos (Figures 7E and 7F), or weakly stained (Sx.FL−) (Figure 7G) and unstained (wild type) (data not shown), both presumably male embryos. We then examined the migration of germ cells in these embryos using the Vasa antibody. No sex-specific differences in germ cell migration were observed, and in each class of embryos the number of embryos exhibiting migration defects was similar.

Discussion

In early Drosophila embryos, the germline precursors, the pole cells, exhibit characteristics that distinguish them from the soma. Most of these characteristics depend upon the functioning of maternal gene products. Thus far only a small number of genes involved in pole cell determination have been identified. Mutations in most of these genes interfere with the formation of pole cells, and no pole cells are observed. Included in this class are the posterior group genes, which are required not only for pole cell formation, but also for the development of the posterior segments. While mutations in nos also disrupt posterior development, nos differs from other posterior group genes in that pole cells are formed.

On the other hand, nos− pole cells are abnormal and...
exhibit a number of defects at later stages of embryogenesis, including the premature expression of germ-line-specific enhancer traps and a failure to migrate to the presumptive gonad. In studies reported here, we trace the developmental defects of nos− germ cells back to the blastoderm stage and show that they are in processes that are likely to be fundamental to the establishment of pole cell identity. Unlike wild-type pole cells, nos− pole cells are transcriptionally active at the blastoderm stage, and several RNA polymerase II-dependent genes that are normally expressed only in the soma are ectopically activated. In addition, nos− pole cells fail to control the cell cycle and instead continue dividing. Finally, we show that the Sxl gene is an important target for repression by nos.

Transcriptional Quiescence

Probably the most striking defect of nos− pole cells is their failure to properly establish and/or maintain transcriptional quiescence. Soon after formation, wild-type pole cells in Drosophila downregulate RNA polymerase II transcription until they have been incorporated into the primitive gonad. Kobayashi et al. (1996) have reported that several germ cell-specific enhancer trap lines are prematurely activated in nos− germ cells while they are migrating through the gut. Our data suggest that the premature activation of these germ-line-specific genes is likely to reflect a more general defect in transcriptional regulation that arises early in embryogenesis, soon after the pole cells are formed. Instead of shutting off RNA polymerase II transcription, nos− pole cells inappropriately transcribe several somatic genes.

Why do nos− germ cells fail to regulate RNA polymerase II transcription? The only known regulatory target for nos in the embryo is the hb transcription factor. Nos together with the Pumilio protein is thought to bind to maternally derived hb mRNA and block its translation (Wharton and Struhl, 1991). Since Hb protein is produced throughout much of the posterior in the absence of Nos, one possibility is that this gap gene protein activates transcription in the pole cells. However, this explanation does not seem likely. Although hb regulates eve and ftz in the soma, it is not clear that the ectopic expression of only the Hb protein would be sufficient to activate either of these genes in the absence of other factors. In addition, hb has no known role in controlling the activity of Sxl-Pe. In fact, ectopic expression of Hb in the soma seems to repress rather than activate Sxl-Pe (G. D., unpublished data). Finally, germ cells derived from nos− germ cells exhibit a similar set of developmental defects as nos− germ cells (Forbes and Lehmann, 1998). Conversely, these defects are not induced when hb is ectopically expressed in pole cells (Kobayashi et al., 1996).

A more likely possibility is that nos− germ cells have a defect in the system responsible for attenuating RNA polymerase II activity. If this is true, there must be additional target(s) for nos regulation besides maternal hb mRNA. In this context it is interesting to note that the failure to establish/maintain transcriptional quiescence in nos− pole cells is reminiscent of the defects seen in the germ cell lineage of C. elegans pie-1 mutants. In pie-1 mutants, the germ cells transcribe many genes that are normally expressed only in the soma assuming an inappropriate identity. The Pie-1 protein contains two copies of a C6H zinc finger motif found in proteins implicated in pre-mRNA metabolism. Antibody staining indicates that Pie-1 is restricted to germ cells and is localized preferentially in the nucleus.

A plausible explanation for why pie-1 mutants fail to repress transcription comes from studies on the phosphorylation of the RNA polymerase II large subunit carboxy-terminal domain (CTD). The CTD contains tandem repeats of a seven-amino acid sequence that contains two serine residues (2 and 5) that are targets for phosphorylation. Phosphorylation is thought to play an important role in polymerase elongation and in the recruitment of pre-mRNA modifying enzymes (Dahmus, 1996).

In wild-type C. elegans, RNA polymerase II phosphorylated in the serine-2 residues of the CTD is detected in somatic cells but is not observed in the germline. In contrast, in pie-1 mutants, phosphorylated CTD serine-2 is detected in both cell types. The available evidence suggests that Pie-1 may directly inhibit CTD serine-2 phosphorylation, perhaps by interfering with a protein that recognizes the CTD (Seydoux and Dunn, 1997; Batchelder et al., 1999).

Like the C. elegans germline lineage, pole cells in Drosophila also have greatly reduced levels of phosphorylated CTD serine-2, which could be responsible for the inhibition of RNA polymerase II in the fly germline. However, it is not clear at this point whether the failure to establish/maintain transcriptional quiescence in nos− pole cells is due to a defect in the system that regulates serine-2 phosphorylation. Seydoux and Dunn (1997) have reported that the level of CTD serine-2 phosphorylation in nos− pole cells is not greatly different from that seen in wild type. It is possible that the changes in the level of CTD serine-2 phosphorylation in nos− pole cells were too small to detect. Alternatively, RNA polymerase II activity in the germline might be controlled not only by CTD phosphorylation, but also by some other unknown mechanism which is the target for the Nos protein. Supporting this later possibility is the finding that only a subset of the genes expressed in the soma of early embryos are ectopically activated in nos− pole cells. By contrast, changes in the status of CTD phosphorylation might be expected to have quite global effects on transcription. Further studies will be required to resolve this question.

Cell Cycle

Another feature that distinguishes germline precursors from the soma is the cell cycle. Since hb has no known role in cell cycle regulation, we suspect that the cell cycle defect is due to a failure in the regulation of some other target gene. One possible candidate is cyclin B. Cyclin B mRNA is specifically localized to the posterior pole of early embryos, where it is incorporated into the newly formed pole cells (Dalby and Glover, 1992). Moreover, high levels of cyclin B protein are present in pole cells from the syncytial blastoderm stage until after the formation of the primitive gonad. Consistent with a failure in cyclin B regulation, we have found that nos− germ cells in stage 11-15 embryos have little or no cyclin B. However, we were unable to detect any obvious abnormalities in cyclin B accumulation in nos− pole cells at
earlier stages of embryogenesis. Since cell cycle defects are evident in nos− pole cells as early as the syncitial blastoderm stage, the reduction in cyclin B midway through embryogenesis might be simply a consequence of continued cycling rather than arresting the cycle in G2 at the blastoderm stage. Another possible candidate is suggested by the recent studies of Su et al. (1998a). They have presented evidence indicating that germ cells are blocked in G2 because cdc2 is inhibited by the phosphorylation of two amino acid residues, Thr-14 and Tyr-15. Since phosphorylated cdc2 is normally reactivated by the string phosphatase, cdc25H, it is possible that Nos prevents the translation of maternally derived stg mRNA in germ cells (Edgar and Lehner, 1996).

Sxl Is a Target for nos

In wild-type embryos, transcription factors, such as Runt, Sisterless-a, and Scute, are responsible for activating the Sxl establishment promoter, Sxl-Pe. The genes encoding these positive regulators are on the X chromosome, and they are expressed in the early precellular zygote in direct proportion to the number of gene copies. Sufficient quantities of the X-linked activators are produced by 2X/2A nuclei to activate Sxl-Pe, while quantities produced by 1X/2A nuclei are insufficient to activate Sxl-Pe (Deshpande et al., 1995). Pole cells differ from the surrounding soma in that these activators are not expressed at detectable levels in the germline precursors, and Sxl-Pe remains off in both sexes. The failure to express these activators most likely reflects the global downregulation of RNA polymerase II transcription in wild-type pole cells. Thus, one mechanism that might account for the inappropriate activation of Sxl-Pe in nos− pole cells would be a general derepression of somatically active genes. As a consequence, the genes encoding the X-linked activators would be expressed, and these in turn would activate Sxl-Pe. Although it seems reasonable to believe that the ectopic expression of the X-linked activators could contribute to the activation of Sxl-Pe in nos− pole cells, it does not readily explain why Sxl-Pe is turned on not only in 2X but also in 1X pole cells. Moreover, when we examined Scute protein expression in nos− embryos, we found that the level of Scute protein in pole cells was less than that seen in 1X/2A somatic nuclei (unpublished data). For this reason, we suspect that Sxl-Pe may be activated in nos− pole cells by a mechanism that, at least in part, bypasses the normal regulation of this promoter by the X/A counting system.

Although Nos protein is likely to control Sxl-Pe activity by an indirect mechanism, a number of lines of evidence indicate that Sxl is an important nos regulatory target. In wild-type, Sxl proteins are normally not expressed in the germline until after the formation of the primitive gonad, and at this stage expression is restricted to the female germline. As a consequence of the ectopic activation of Sxl-Pe, Sxl proteins are present in nos− pole cells at the blastoderm stage. It would appear that the premature appearance of Sxl proteins in the pole cells is an important contributing factor to the nos− phenotype. We have found that the migration and cell cycle defects of nos− germ cells can be alleviated by the elimination of the Sxl gene. Conversely, it is possible to induce both of these defects in wild-type germ cells by ectopically expressing Sxl protein. While the removal of Sxl mitigates some of the defects of nos− germ cells, it should be noted that these cells are still abnormal. They fail to establish/maintain transcriptional quiescence, and they cannot form a functional adult germline. This finding indicates that Sxl is not the only target for nos regulation.

Why does ectopic expression of Sxl protein (either in the absence of nos or in the presence of the Sxl transgene) disrupt germ cell migration and induce cell cycle defects? Sxl encodes an RNA-binding protein that functions in the soma as both a splicing and translational regulator (Cline and Meyer, 1996). Since the Sxl protein is predominantly localized in the cytoplasm of nos− pole cells, we imagine that it also functions to regulate the translation of mRNAs encoding proteins critical to migration or cell cycle control. An important goal for future study will be the identification of these Sxl targets.

Experimental Procedures

Fly Stocks

All fly stocks, unless otherwise noted, are referenced by Lindsley and Zimm (1992). Flies were grown on standard Drosophila medium and maintained at room temperature (22°C) unless otherwise specified. White1 files were used as wild-type control for embryo staining experiments and as recipients for p element trans formations. nos8R, nostn, and D(3R) Di-FX3, which is a deficiency spanning nos locus, have been described previously (Forbes and Lehmann, 1998). ywSxl2R is a Sxl deficiency chromosome.

Histochemical Analysis

Antibody stainings of embryos were performed as described previously (Bopp et al., 1991). Anti-Sxl antibody, m-18, is a mouse monoclonal and was used at 1:10 dilution. Anti-β-galactosidase antibody (Promega) was used at 1:500. Anti p-H3 antibody (Upstate Biotechnology) was used at 1:1000. Anti-Vasa (a gift from Paul Lasko) and anti-cyclin-B antibodies were used at 1:2000. For confocal analysis, secondary antibodies conjugated with different fluorophores, anti-rabbit alexa-543 (red) and anti-rat alexa-488 (green), were used (Molecular Probes). All the secondary antibodies were obtained from Jackson Immunoresearch Laboratories. Anti-Sxl and anti-Ftz antibody stainings were repeated by mixing nos− and wild-type embryos. The mixed embryos were fixed together and stained in the same staining solution.

Germ Cell Counts

Syncytial blastoderm stage or gastrulating embryos were collected, fixed, and double labeled using anti-Vasa antibody and nuclear dye (Hoechst; 1:100). Germ cells were marked and counted by taking serial sections through the embryo. Total number of germ cells from 10–15 embryos of each genotype were counted and the counts averaged. For the detailed statistical analysis, see Table 1.

Germline Clones

Maternal clones were made by FLP-mediated recombination as described in Chou and Perrimon (1992). yw; FRThb8 nos8R and ywHSFLP; FRTovo stocks were a kind gift from Claude Desplan. Homozygous germline clones of the genotype hbnos8R were generated as described in Forbes and Lehmann (1998). Similar protocol was used with modification to obtain clones that were simultaneously heterozygous for Sxl. Females carrying such clones were mated with males of the genotype Sxl2R/Y to generate embryos that lacked Sxl completely.

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