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Maternal RNAs encoding transcription factors for germline-specific gene expression in *Drosophila* embryos

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ABSTRACT In early *Drosophila* embryos, germ plasm is localized to the posterior pole region and is partitioned into the germline progenitors, known as pole cells. Germ plasm contains the maternal factors required for germline development. It has been proposed that germline-specific gene expression is initiated by the function of maternal factors that are enriched in the germ plasm. However, such factors have remained elusive. Here, we describe a genome-wide survey of maternal transcripts that encode for transcription factors and are enriched in the germ plasm. We isolated pole cells from blastodermal embryos by fluorescence-activated cell sorting (FACS) and then used these isolated cells in a microarray analysis. Among the 835 genes in the Gene Ontology (GO) category "transcription regulator activity" listed in FlyBase, 68 were found to be predominantly expressed in pole cells as compared to whole embryos. As the early pole cells are known to be transcriptionally quiescent, the listed transcripts are predicted to be maternal in origin. Our *in situ* hybridization analysis revealed that 27 of the 68 transcripts were enriched in the germ plasm. Among the 27 transcripts, six were found to be required for germline-specific gene expression of *vasa* and/or *nanos* by knockdown experiments using RNA interference (RNAi). The identified transcripts encode a transcriptional activator *(ovo)*, components of the transcriptional initiation complexes *(Trf2, bip2* and *Tif-IA)*, and the Ccr4-Not complex *(CG31716* and *l(2)NC136)*. Our study demonstrates that germ plasm contains maternal transcripts encoding transcriptional regulators for germline-specific gene expression in pole cells.

KEY WORDS: *germ line, germ plasm, pole cell, transcription factor, Drosophila*

Introduction

Identifying the mechanisms underlying germline establishment is a century-old issue in developmental biology. In the animal species, there are at least two distinct modes of germline establishment (Extavour and Akam, 2003). In certain animal species, germline progenitors are characterized by inheriting a specialized ooplasm, or germ plasm, that contains the maternal factors required for and sufficient for germline development (Beams and Kessel, 1974; Eddy, 1975; Rongo and Lehmann, 1996). In other animal species, the germline is specified by inductive signals from the surrounding tissues (Lawson *et al.*, 1999). It has been known that, irrespective of the modes of germline establishment, *vasa* (*vas*) and *nanos* (*nos*) genes are commonly expressed in the germline progenitors (Extavour and Akam, 2003). Thus, it is possible that the mechanism underlying *vas* and *nos* expression is conserved throughout the animal

Abbreviations used in this paper: GFP, green fluorescent protein; eGFP, enhanced GFP.

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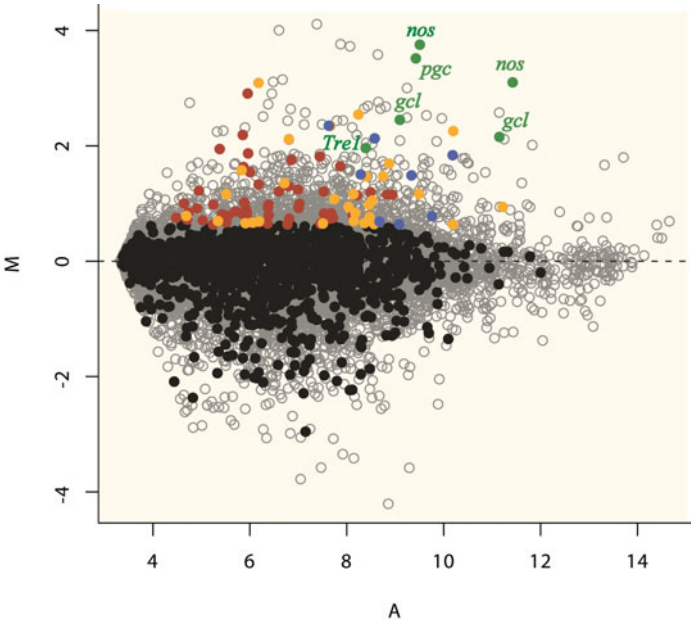


Fig. 1. MA plot of microarray signal intensities. *The MA plot uses M as the y-axis and A as the x-axis where:* $M = \log_2(P/W)$, $A = \log_2\sqrt{PW}$, *P = pole-cell-signal intensity, W = whole-embryo-signal intensity. The filled circles (black, brown, orange and blue) represent the transcripts encoding a transcription regulator (GO:0030528). The brown, orange and blue circles show the transcript expression examined by* in situ *hybridization. Orange and blue: the transcripts enriched in the germ plasm (Table 1). Blue: the transcripts with dsRNA that caused a decrease in pole cell* vas *and/or* nos *expression (Table 3). Green: the transcripts which have been identified to be enriched in the germ plasm and in pole cells. Open circles: the remaining protein-coding transcripts encoded by the* Drosophila *nuclear genome.*

species, and that their expression is a marker for germline establishment. However, how the germline-specific gene expression is activated within the germline progenitors has remained unclear.

In *Drosophila*, the germ plasm is localized to the posterior pole region of the cleavage embryos (stage 1-2) and is partitioned into pole cells (stage 3). The pole cells move during midgut rudiment gastrulation, pass through the midgut epithelium to associate with the somatic gonadal precursors (stage 6-12), and then aggregate with the somatic cells to form the embryonic gonads by stage 15. Expression of *vas* begins in the pole cells immediately after gastrulation and is the earliest example of gene expression in the germline progenitors (Van Doren *et al.*, 1998). Once initiated, *vas* expression continues in the germline cells (Hay *et al.*, 1988a; Hay *et al.*, 1988b; Lasko and Ashburner, 1990). Later, zygotic expression of *nos* becomes detectable in the pole cell within the embryonic gonads. Although the role of zygotic *vas* and *nos* in pole cells remains obscure, their expression makes them ideal markers to analyze how germline-specific gene expression is initiated in the pole cells.

Germline-specific gene expression is thought to be activated by maternal factors localized in the germ plasm. To date, several maternal factors have been identified whose functions are required for early events in germline development (Mahowald, 2001). For example, the mitochondrial large ribosomal RNA and the BTB/POZ protein Germ cell-less (Gcl) are both involved in pole cell formation (Iida and Kobayashi, 1998; Robertson *et al.*, 1999). Likewise, the maternal RNA binding protein Nos is essential to repress mitosis, apoptosis, and somatic differentiation of pole cells (Asaoka-Taguchi *et al.*, 1999; Hayashi *et al.*, 2004; Sato *et al.,* 2007). *polar granule component* (*pgc*) RNA is required for pole cell survival (Nakamura *et al.*, 1996; Martinho *et al.*, 2004). Pole cells also require *trapped in endoderm-1* (*Tre1*), a maternal RNA encoding a G-protein-coupled receptor that is required for their transepithelial movement through the midgut wall (Kunwar *et al.*, 2003). Furthermore, several maternal transcripts enriched in germ plasm have been additionally identified by *in situ* hybridization screens (Szuperák *et al.*, 2005; Sato *et al.*, 2007). However, neither genetic nor molecular screens have yet identified any maternal factors that encode for transcriptional regulators involved in germline-specific pole cell gene expression.

Here, we describe the identification of maternal RNAs that are enriched in the germ plasm and encode for transcription factors. We utilize RNAi knockdown to investigate transcription factor function for germline-specific *vas* and *nos* expression in pole cells. Six transcripts were found to be required for *vas* and/or *nos* gene expression. These transcripts encode for a transcriptional activator (*ovo*), for transcriptional initiation complex components (*Trf2*, *bip2* and *Tif-IA*), and for the Ccr4-Not complex (*CG31716* and *l(2)NC136*). This study provides an important first step toward understanding gene regulation in pole cells and suggests that these identified maternal factors may be involved in germline development by regulating pole cell gene expression.

Results and Discussion

A screen for candidate transcripts enriched in early pole cells

To identify novel maternal transcripts enriched in the *Drosophila* germ plasm, we compared the transcriptome from pole cells and from whole embryos at stage 4 by microarray analysis. First, fluorescence-activated cell sorting (FACS) was used to isolate pole cells from embryos carrying the transgene EGFP-*vasa*, which express GFP specifically in the germline. RNA was extracted from the isolated pole cells and from the whole embryos. These RNA isolates were amplified and labeled with Cy3 and Cy5, and hybridized to a microarray containing oligo probes for all of the predicted protein-coding genes in *Drosophila* genome. After filtering out any probe sets with low quality results, e.g. low signal intensity and high variance between the dye-swap experiments (see Materials and Methods for details), we identified candidate transcripts enriched in pole cells (Fig. 1). In this screen, maternal transcripts known to be enriched in the germ plasm and in pole cells were identified. RNA transcripts for *nos*, *gcl, Tre1* and *pgc* were detected at 13.7-, 5.5-, 4.1- and 11.5-fold higher levels respectively in pole cells when compared to whole embryos (Fig. 1). These results suggest that maternal transcripts enriched in pole cells were successfully identified by the microarray analysis, even though only a single RNA sample was screened in the microarray analysis due to the technical difficulty of obtaining RNA from the sorted pole cells.

In this study, we focused on the genes encoding for transcription factors because they represent probable candidates for maternal regulators of pole-cell gene expression. Among the 835 genes in the Gene Ontology (GO) category “transcription regula-

tor activity" (GO:0030528) in FlyBase (http://flybase.bio.indiana.edu/), 68 were selected on the basis of their higher pole cell to whole embryo ratios and signal intensities in the microarray analysis (Fig. 1, see Materials and Methods for details).

Distribution of the selected transcripts in early embryos

Because RNA polymerase II-dependent transcription is globally repressed in early pole cells (Seydoux and Dunn, 1997), the 68 transcripts selected from the microarray analysis were predicted to be maternal in origin and present in the germ plasm. As a next step, whole-mount *in situ* hybridization (WISH) analysis was performed to select for the transcripts that are enriched in germ plasm. We obtained useful expression data for the 65 transcripts (see Materials and Methods), and we found that the transcripts from 27 genes were enriched in the germ plasm of cleavage embryos (Table 1, Fig. 2). Of the selected 27 genes, 63% (17/27) showed high signal intensities in microarray hybridization (A-value ≥ 8.0; $A = \log_2\sqrt{PW}$, P = pole-cell-signal intensity, W = whole-embryo-signal intensity), while the majority (89%) of the 38 genes not enriched in germ plasm showed low signal intensities (A-value < 8.0) (Fig. 1). Thus, at low expression, differential microarray hybridization levels were influenced by background noise in our microarray analysis.

To validate our WISH data, we compared the expression pattern of the 27 genes with the expression annotation and the representative images in the Fly-FISH (http://fly-fish.ccbr.utoronto.ca/) and BDGP Gene Expression Report (http://www.fruitfly.org/cgi-bin/ex/insitu.pl) which include expression data for 3370 and 6138 genes, respectively (Tomancak *et al.*, 2002; Lécuyer *et al.*, 2007). Among the 27 genes, 10 and 15 have been analyzed for their expression by Fly-FISH and BDGP, respectively. Seven out of the 10 and 6 out of the 15 are reported to be expressed in the germ plasm and/or pole cells at stage 4-5, and the remaining genes were classified as either "ubiquitously expressed" or "undetectable expression" (Supplementary Table S2). In contrast, among the 38 genes that were identified in our microarray analysis but not selected by our WISH screen, none (0/18 in Fly-FISH and 0/16 in BDGP) were reported as enriched in either the germ plasm or pole cells (data not shown). These observations suggest that our WISH analysis is almost compatible with those previously performed. Additionally, we succeeded to identify novel transcripts that are enriched in the germ plasm and/or pole cells (Fig. 2, Supplementary Table S2).

Among the identified 27 transcripts, 7 [*Arc 70, sd, ovo, mei-P26, CG10462, Trf2* and *Dsp1*] were detectable in pole cells until at least the end of embryogenesis. In contrast, the remaining 20 transcripts [*CG10445, bip2, EP2237, CG3136, Tif-1A, Zyx102EF,*

TABLE 1

GENES ENCODING THE TRANSCRIPTS ENRICHED IN GERM PLASM

Genes	Distribution of hybridization signal*				GO categories (excerpt)	FlyBase ID
	GP	PC	mPC	gPC		
CG10445	+	+	–	–	RNA polymerase II transcription termination factor activity	FBgn0037531
Arc70	+	+	+	±	RNA polymerase II transcription mediator activity	FBgn0039923
sd	+	±	±	+	specific RNA polymerase II transcription factor activity	FBgn0003345
bip2	+	+	+	–	general RNA polymerase II transcription factor activity	FBgn0026262
EP2237	+	+	–	–	transcriptional activator activity	FBgn0043364
ovo	+	+	+	+	RNA polymerase II transcription factor activity	FBgn0003028
CG3136	+	±	–	–	transcription factor activity	FBgn0033010
Tif-IA	+	±	–	–	RNA polymerase I transcription factor activity	FBgn0032988
Zyx102EF	+	±	–	+	transcription regulator activity	FBgn0052018
rgr	+	±	–	–	transcription factor activity	FBgn0033310
mei-P26	+	+	+	+	transcription regulator activity	FBgn0026206
Tra1	+	+	–	–	transcription cofactor activity	FBgn0033013
CG10462	+	+	+	+	transcription regulator activity	FBgn0032815
Rel	+	–	–	–	specific RNA polymerase II transcription factor activity	FBgn0014018
NC2alpha	+	–	–	–	transcriptional repressor activity	FBgn0034650
ref(2)P	+	+	+	–	transcription regulator activity	FBgn0003231
Pcl	+	+	–	–	transcription regulator activity	FBgn0003044
CG31716	+	+	–	–	transcriptional repressor activity	FBgn0051716
Hcf	+	+	+	–	transcription coactivator activity	FBgn0039904
H	+	±	+	–	transcription corepressor activity	FBgn0001169
zfh1	+	+	–	+	RNA polymerase II transcription factor activity	FBgn0004606
Trf2	+	+	±	+	specific RNA polymerase II transcription factor activity	FBgn0026758
Dsp1	+	+	+	+	transcription corepressor activity	FBgn0011764
Smox	+	–	–	–	transcription regulator activity	FBgn0025800
CG5640	+	±	–	–	transcriptional repressor activity	FBgn0032207
l(2)NC136	+	+	–	–	transcription regulator activity	FBgn0033029
ttk	+	–	–	–	specific RNA polymerase II transcription factor activity	FBgn0003870

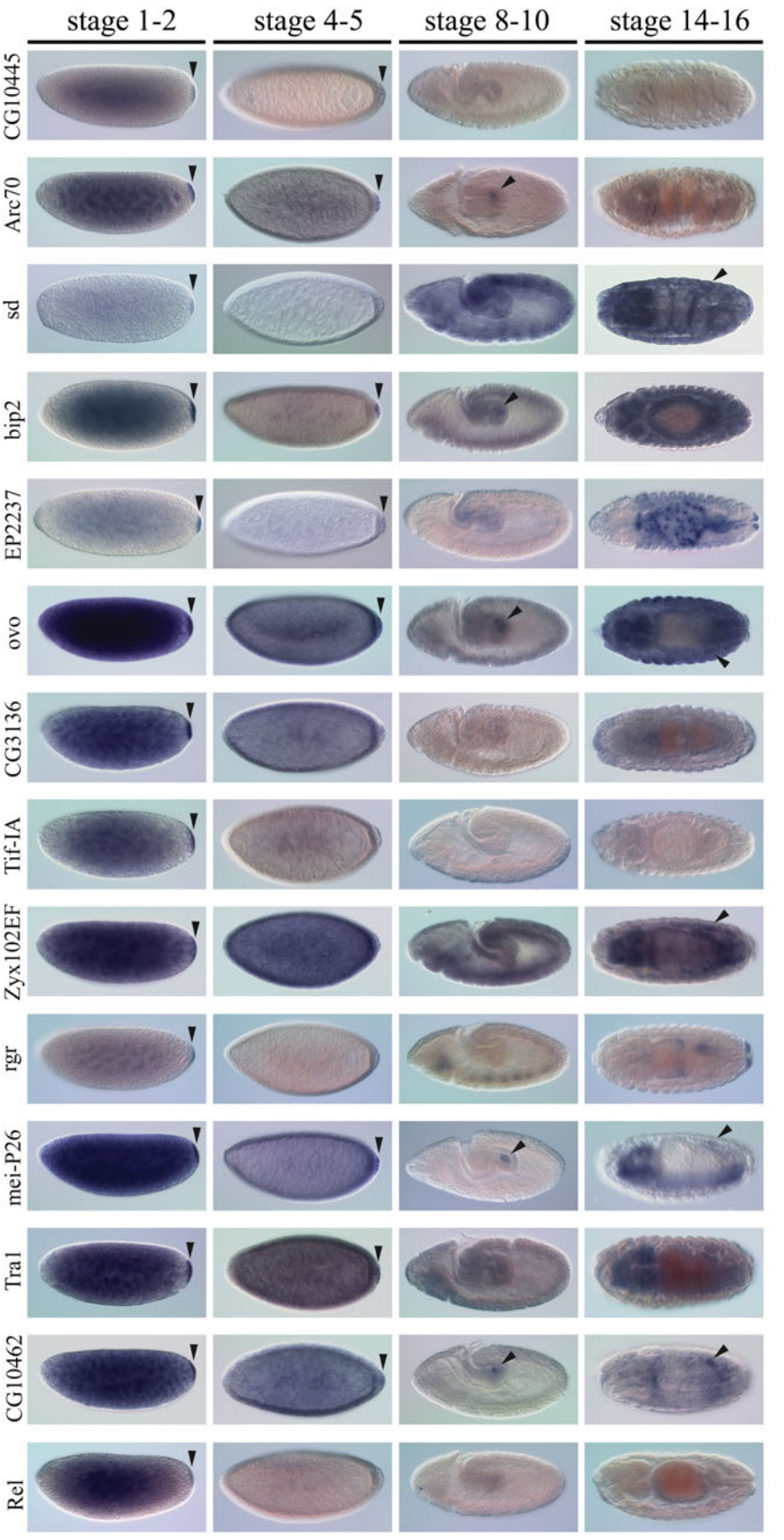
* The embryos were *in situ* hybridized with the probes for the listed genes. Hybridization signals were observed at a high (+), a low (±) and an undetectable level (-) in the germ plasm (GP), pole cells at stage 4/5 (PC), migrating pole cells (mPC) and pole cells within the gonads (gPC).

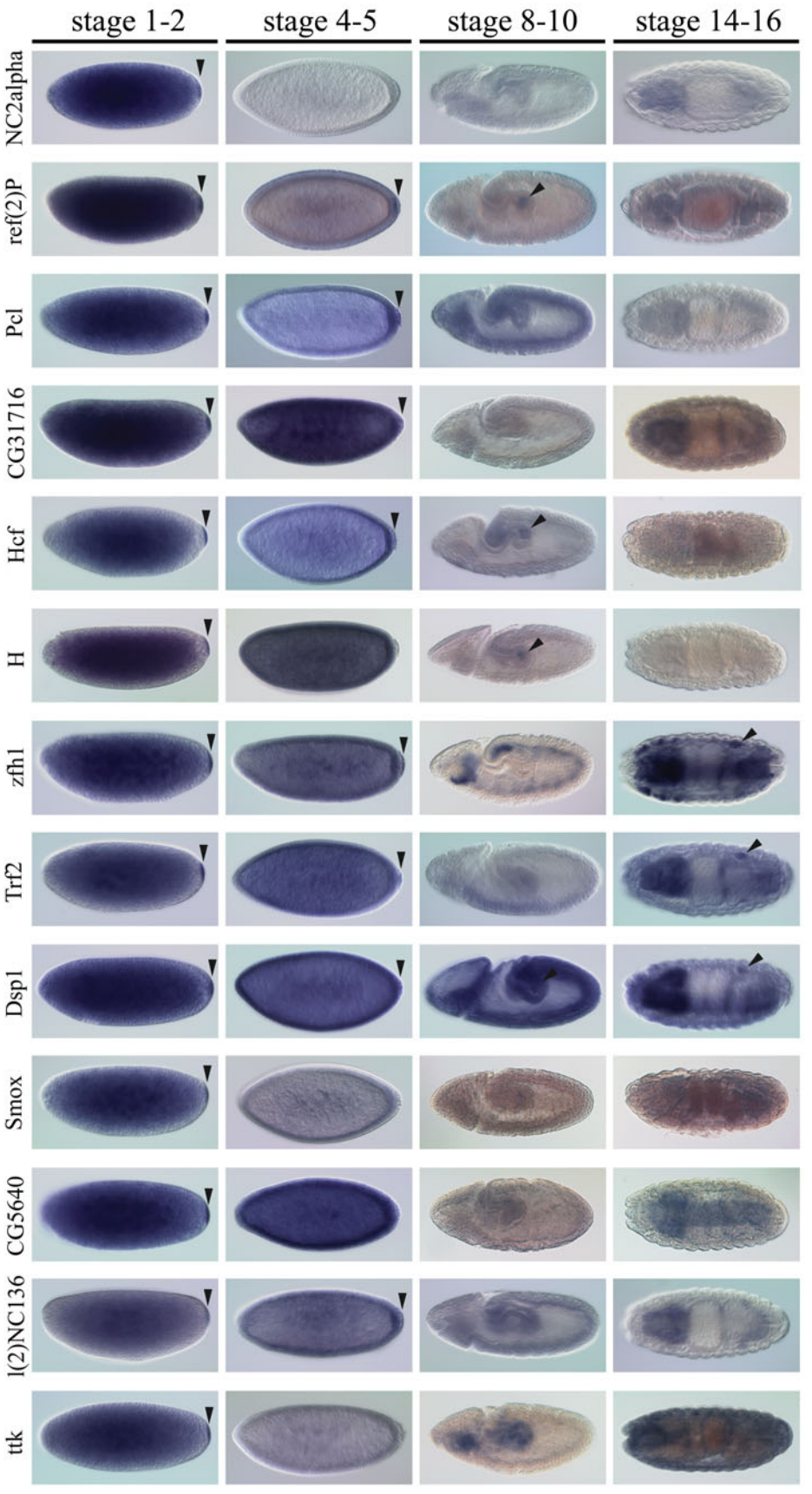
rgr, Tra1, Rel, NC2alpha, ref(2)P, Pcl, CG31716, Hcf, H, zfh1, Smox, CG5640, l(2)NC136 and *ttk*] were no longer detectable by *in situ* hybridization in pole cells during their migration to the embryonic gonads. However, *Zyx102EF* and *zfh1* were again detected in pole cells within the embryonic gonads, suggesting that these two genes were expressed zygotically within the gonads. All of the genes, except for *CG10445* and *Arc70*, were also expressed zygotically in the somatic tissues, such as the midgut, mesoderm, and the central nervous system (Fig. 2).

RNAi screen to determine maternal transcript requirement for germline-specific gene expression

The 27 maternal transcripts were further screened by RNAi for their possible function in germline-specific gene expression. RNAi has been increasingly exploited to knockdown target RNA levels and to explore gene function *in vivo* and in cultured cells (Kobayashi *et al*., 2006). In *Drosophila*, transgenes expressing double-stranded RNA (dsRNA) are used to knockdown gene function *in vivo*, and *UAS-RNAi* lines which target many *Drosophila* genes are now readily available (Akasaka *et al*., 2006; Kobayashi *et al*., 2006; Yang *et al*., 2006; Dietzl *et al*., 2007). Here, however, dsRNA targeting each maternal transcript was injected into cleavage embryos to diminish the activity from each maternal transcript. The germline-specific expression of *vas* and *nos* genes in pole cells was then examined. Instead of expressing dsRNAs later in pole cells, this "injection" method takes advantage of the opportunity to target maternal transcripts immediately after oviposition and thus effectively reduce their function (Sato *et al*., 2007). In normal embryos, zygotic *vas* and *nos* expression is initiated in pole cells approximately at stages 9 and 15, respectively. Their expression continues to be detectable until at least the end of embryogenesis. Hence, *vas* and *nos* gene expression in dsRNA-injected embryos was examined at stage 15-16 [26 hr after egg laying (AEL) at 18ºC].

DsRNA-injected embryos were allowed to develop at 18°C until 26 hr AEL, and then RNAi-based screen in two steps. First, *vas* and *nos* expression was examined promptly and without regard to their developmental status in order to identify required candidate transcripts (Table 2). Second, a detailed analysis of the dsRNA-induced phenotype against the candidate transcripts was performed with regard to embryonic development, pole cell development, pole cell transcriptional ability, and expression of the germline-specific genes in pole cells (Tables 3-5). Taken together, these two steps identified six transcripts whose dsRNAs significantly decreased the per-





centage of the stage 15-16 embryos expressing *vas* and/or *nos* in pole cells (Table 3, Fig. 3).

A significant reduction in the amount of the six transcripts were evident, when their dsRNAs were injected into embryos (Supplementary Fig. S1). Injection of dsRNAs targeting the transcripts of *bip2, ovo, CG31716* and *Trf2* repressed both *vas* and *nos* expression. *l(2)NC136* dsRNA repressed *vas* expression, while *Tif-IA* dsRNA suppressed *nos* expression in pole cells. None of the six dsRNAs against these transcripts except *CG31716* and *Trf2* affected embryonic development (Table 4), including pole cell formation, pole cell migration, and gonad formation. The expected population of pole cells was incorporated within the embryonic gonads of the injected embryos at stage 15-16 (Fig. 3, data not shown). Table 4 shows that the injection of *CG31716* dsRNA slightly reduced the percentage of embryos developing to stage 15-16, but, in the surviving embryos, the pole cells colonized normally within the gonads (Fig. 3, data not shown). Although *Trf2* dsRNA injection retarded embryonic development during germ band shortening in 60-90% of the embryos (Table 3-5), the embryos that survived to stage 15-16 showed no defects in pole cell development (Fig. 3, data not shown). Pole cells in the stage 15-16 embryos that were injected with each of the six dsRNA had retained a normal ability to express the *UAS-lacZ* transgene under maternally supplied Gal4 (Table 5). The above data suggest that repression of the germline-specific gene expression is not caused by an indirect effect of the injected dsRNAs on the embryonic development or on the transcriptional machinery.

Overview of the identified transcripts

The above data suggest that *vas* and *nos* expression is regulated by the combination of transcription factors encoded by the germ plasm-enriched maternal transcripts. The identified transcripts encode for a transcriptional activator (*ovo*), for components of the transcriptional initiation complexes (*Trf2*, *bip2* and *Tif-IA*), and for the

Fig. 2. Expression of the 27 transcripts in embryos. *Embryos were probed by* in situ *hybridization for the gene shown on the left. Developmental stages are shown at the top. Arrowheads show the germ plasm and pole cells with a strong signal (+ in Table 1). Anterior is to the left.*

TABLE 2

EXPRESSION OF *VAS* AND *NOS* RNA IN POLE CELLS OF THE dsRNA-INJECTED EMBRYOS

	No. of embryos expressing *vas* in pole cells[$]				No. of embryos expressing *nos* in pole cells[$]				
dsRNA*	**No. of embryos examined**	**+ (%)**		**± (%)**	**- (%)**	**No. of embryos examined**	**+ (%)**		**± (%)**	**- (%)**
- (DW)	208	176 (84.6)		14 (6.7)	18 (8.7)	172	134 (74.9)		21 (12.2)	17 (9.9)
CG10445	25	21 (84.0)	#	4 (16.0)	0 (0)	29	23 (79.3)	#	0 (0)	6 (20.7)
Arc70	21	15 (71.4)	#	5 (23.8)	1 (4.8)	24	18 (75.0)	#	3 (12.5)	3 (12.5)
sd	32	25 (78.1)	#	6 (18.8)	1 (3.1)	37	26 (70.3)	#	8 (21.6)	3 (8.1)
bip2	31	16 (51.6)	###	14 (45.2)	1 (3.2)	28	11 (39.3)	###	17 (60.7)	0 (0)
EP2237	35	32 (91.4)	#	2 (5.7)	1 (2.9)	33	29 (87.9)	#	4 (12.1)	0 (0)
ovo	23	7 (30.4)	###	8 (34.8)	8 (34.8)	28	13 (46.4)	###	4 (14.3)	11 (39.3)
CG3136	20	18 (90.0)	#	2 (10.0)	0 (0)	33	24 (72.7)	#	5 (15.2)	4 (12.1)
Tif-IA	30	22 (73.3)	#	8 (26.7)	0 (0)	32	17 (53.1)	###	11 (34.4)	4 (12.5)
Zyx102EF	25	23 (92.0)	#	1 (4.0)	1 (4.0)	17	16 (94.1)	#	1 (5.9)	0 (0)
rgr	36	32 (88.9)	#	0 (0)	4 (11.1)	22	19 (86.4)	#	3 (13.6)	0 (0)
mei-P26	29	22 (75.9)	#	4 (13.8)	3 (10.3)	14	9 (64.3)	#	2 (14.3)	3 (21.4)
Tra1	19	17 (89.5)	#	1 (5.3)	1 (5.3)	21	16 (76.2)	#	0 (0)	5 (23.8)
CG10462	28	27 (96.4)	#	1 (3.6)	0 (0)	31	26 (83.9)	#	0 (0)	5 (16.1)
Rel	28	28 (100)	##	0 (0)	0 (0)	39	34 (87.2)	#	2 (5.1)	3 (7.7)
NC2alpha	29	24 (82.8)	#	5 (17.2)	0 (0)	30	24 (80.0)	#	3 (10.0)	3 (10.0)
ref(2)P	30	22 (73.3)	#	6 (20.0)	2 (6.7)	26	19 (73.1)	#	3 (11.5)	4 (15.4)
Pcl	34	30 (88.2)	#	3 (8.8)	1 (2.9)	26	21 (80.8)	#	2 (7.7)	3 (11.5)
CG31716	27	16 (59.3)	###	5 (18.5)	6 (22.2)	23	6 (26.1)	###	5 (21.7)	12 (52.2)
Hcf	31	29 (93.5)	#	1 (3.2)	1 (3.2)	27	25 (92.6)	#	1 (3.7)	1 (3.7)
H	31	26 (83.9)	#	5 (16.1)	0 (0)	31	26 (83.9)	#	1 (3.2)	4 (12.9)
zfh1	32	26 (81.3)	#	1 (3.1)	5 (15.6)	30	21 (70.0)	#	2 (6.7)	7 (23.3)
Trf2	30	0 (0)	###	0 (0)	30 (100)	14	6 (42.9)	###	2 (14.3)	6 (42.9)
Dsp1	32	28 (87.5)	#	0 (0)	4 (12.5)	20	13 (65.0)	#	3 (15.0)	4 (20.0)
Smox	34	26 (76.5)	#	7 (20.6)	1 (2.9)	25	19 (76.0)	#	4 (16.0)	2 (8.0)
CG5640	31	20 (64.5)	###	10 (32.3)	1 (3.2)	21	19 (90.5)	#	0 (0)	2 (9.5)
l(2)NC136	35	14 (40.0)	###	13 (37.1)	8 (22.9)	34	31 (91.2)	#	2 (5.9)	1 (2.9)
ttk	38	30 (78.9)	#	8 (21.1)	0 (0)	30	27 (90.0)	#	3 (10.0)	0 (0)

* *y w* embryos were used as recipients for dsRNA microinjection.

[$] The injected embryos were stained for *vas* or *nos* RNA. No. of the embryos expressing *vas* or *nos* RNA in pole cells at a high (+), a low (±) and an undetectable level (-) are shown.

[#] $P > 0.05$, [##] $0.05 > P > 0.01$, [###] $P < 0.01$; Significance was calculated vs. DW-injected control by using Fisher's exact probability test.

Ccr4-Not complex (*CG31716* and *l(2)NC136*). It is worthwhile to note that this screen identified at least two genes, *ovo* and *Trf2*, that have been reported to be required for oogenesis. Genetic screens have identified many maternally acting genes required for early germline development (Mahowald, 2001). However, these screens could only recover mutations that did not affect oogenesis, and therefore failed to identify many maternal genes that play a critical role both in oogenesis and embryonic germline development. In this study, we injected dsRNA into early embryos to examine the role of maternal transcripts in germline-specific gene expression. This method enabled us to circumvent the oogenesis defects and screen for phenotypes in embryos. Detailed functions of the identified six genes are discussed below.

A transcriptional activator, ovo

The maternal *ovo* transcript was enriched in the germ plasm and was required for *vas* and *nos* expression in pole cells. The *ovo* gene encodes for a developmentally regulated, DNA-binding, C_2H2 Zn-finger protein that is involved in oogenesis (Oliver *et al.*, 1987; Mével-Ninio *et al.*, 1991; Staab and Steinmann-Zwicky, 1996) and in denticle formation in the epidermal cells (Wieschaus *et al.*, 1984). The *ovo* gene produces at least three alternate isoforms. Ovo-A and Ovo-B function as a negative and a positive transcriptional regulator in the germline, respectively (Andrews *et al.*, 2000). Ovo-Svb is expressed in the epidermal cells and is required for their differentiation (Payre *et al.*, 1999; Delon *et al.*, 2003). Ovo-B protein activates transcription from the *ovarian tumor* (*otu*) and *ovo-B* promoters in a germline-specific manner via binding to the short regions proximal to their transcriptional start site (Lü and Oliver, 2001; Bielinska *et al.*, 2005). Ovo-B binding to the core promoter region suggests that this protein could interact with the initiation complex TFIID at the same region, either directly or indirectly (Lü and Oliver, 2001). Thus, we postulate that Ovo-B, along with TFIID components, initiates germline-specific expression of *vas* and *nos* in pole cells.

The role of maternal *ovo* in germline development remains elusive, since *ovo* dsRNA injection caused lethality at the end of embryogenesis due to the defect in epidermal differentiation (data not shown) which requires zygotic *ovo* function (Oliver *et al.*, 1987). Embryos injected with *ovo* dsRNA did not show any deleterious defects in pole cell development during embryogenesis. Thus, we speculate that the pole cells with reduced maternal *ovo* activity may be impaired in their post-embryonic development. Future work is needed to test this consideration by transplanting the pole cells with reduced *ovo* activity into normal hosts.

Components of the transcriptional initiation complex

In the fly, frog, and mouse, *Trf2* is predominantly expressed in the germline cells during gametogenesis in both females and males (Kopytova *et al.*, 2006; Xiao *et al.*, 2006). *Trf2* expression is required for proper *Drosophila* development of both oocytes and spermatocytes (Kopytova *et al.*, 2006) and for spermatogenesis in mice (Zhang *et al.*, 2001). Although *Trf2* is additionally required for somatic development in *Drosophila* embryos (Kopytova *et al.*, 2006), its function in the germline is evolutionarily conserved.

TABLE 3

EXPRESSION OF *VAS* AND *NOS* RNA IN POLE CELLS OF THE dsRNA-INJECTED EMBRYOS

Expression of *vas*

dsRNA*	No.of injected embryos	No.of surviving embryos$	No. of embryos expressing *vas* in pole cells$$			
			+ (%)		± (%)	- (%)
GFP	62	55	47 (85.5)		6 (10.9)	1 (3.6)
bip2	54	41	19 (46.3)	##	17 (41.5)	5 (12.2)
ovo	77	61	22 (36.1)	##	17 (27.9)	22 (36.1)
Tif-IA	65	55	42 (76.4)	#	10 (18.2)	3 (5.5)
CG31716	62	40	12 (30.0)	##	14 (35.0)	14 (35.0)
Trf2	102	20	5 (25.0)	##	3 (15.0)	12 (60.0)
l(2)NC136	78	72	12 (16.7)	##	24 (33.3)	36 (50.0)

Expression of *nos*

dsRNA*	No.of injected embryos	No.of surviving embryos$	No. of embryos expressing *nos* in pole cells$$			
			+ (%)		± (%)	- (%)
GFP	57	48	37 (77.1)		5 (10.4)	6 (12.5)
bip2	47	40	19 (47.5)	##	10 (25.0)	11 (27.5)
ovo	68	59	21 (35.6)	##	15 (25.4)	23 (39.0)
Tif-IA	57	43	13 (30.2)	##	10 (23.3)	20 (46.5)
CG31716	64	36	9 (25.0)	##	3 (8.3)	24 (66.7)
Trf2	104	18	5 (27.8)	##	2 (11.1)	11 (61.1)
l(2)NC136	82	64	47 (73.4)	#	10 (15.6)	7 (10.9)

* *y* w embryos were used as recipients for dsRNA microinjection.

$ No. of the injected embryos normally developing to stage 15/16 were scored.

$$ The injected embryos (stage 15/16) were stained for *vas* or *nos* RNA. No. of the embryos expressing *vas* or *nos* RNA in pole cells at a high (+), a low (±) and an undetectable level (-) are shown.

P > 0.05, ## P < 0.01; Significance was calculated vs. *GFP* control by using Fisher's exact probability test.

Trf2 encodes a TATA box-binding protein (TBP)-related factor (Rabenstein *et al*., 1999). TBP and TBP-associated factors (TAFs) are known to be components of an initiation complex that recognizes the core promoter sequences (Tjian and Maniatis, 1994; Bell and Tora, 1999). Recent data suggest that *Trf2* is involved in the formation of a TFIID-like complex to regulate the expression of a set of genes in a cell-type-specific manner (Hochheimer *et al*., 2002). Trf2 has been shown to associate with restricted loci on chromosomes at sites different from those of TBP (Rabenstein *et al*., 1999). Furthermore, the TRF2-containing complex includes components of the chromatin-remodeling complex, NURF, and other proteins that might be involved in chromatin reorganization (Hochheimer *et al*., 2002). Considering that the *trf2* transcript is enriched in germ plasm and is required for *vas* and *nos* expression in pole cells, it is reasonable to speculate that Trf2 may regulate germline-specific genes via the rearrangement of chromatin organization and/or the formation of distinctive TFIID-like complexes.

This idea is supported by the observation that the transcript encoding one of the TAFs, Bric-a-brac interacting protein 2 (BIP2, also called as TAFII155), is enriched in the germ plasm (Fig. 2) and is required for *vas* and *nos* expression in pole cells (Fig. 3, Table 3). TAFs have been known to act as specific coactivators by interacting with transcriptional activators, and TAFs contribute to promoter recognition and selectivity (Verrijzer *et al*., 1994; Burke and Kadonaga, 1997). BIP2 was first identified as a partner of the Bric à brac 2 (Bab2) protein (Pointud *et al*., 2001). BAB2 interacts with BIP2 via BTB/POZ domains, and these two proteins colocalize at transcriptionally active regions of polytene chromosomes (Pointud *et al*., 2001). BAB2 acts as a developmentally regulated transcriptional activator involved in the morphogenesis of the somatic components of adult ovaries (Couderc *et al*., 2002). Although the transcriptional activators interacting with BIP2 in early pole cells remain unknown, the above observations suggests that the TFIID-like complex containing TRF2 and BIP2 is involved in the initiation of germline-specific gene expression.

TABLE 4

VIABILITY OF THE EMBRYOS INJECTED WITH dsRNAS

dsRNA*	No.of injected embryos	No.of surviving embryos$		
GFP	119	103	(86.6)	
bip2	101	81	(80.2)	#
ovo	145	120	(82.8)	#
Tif-IA	122	98	(80.3)	#
CG31716	126	76	(60.3)	##
Trf2	206	38	(18.4)	##
l(2)NC136	160	136	(85.0)	#

* *y w* embryos were used as recipients for dsRNA microinjection.

$ No. of the injected embryos normally developing to stage 15/16 were scored.

P > 0.05, ## P < 0.01; Significance was calculated vs. *GFP* control by using Fisher's exact probability test.

Components of Ccr4-Not complex

Here, we show that transcripts from *CG31716* and *l(2)NC136* are both enriched in the germ plasm and required for *vas* expression in pole cells. *CG31716* and *l(2)NC136* encode for the proteins NOT4 and NOT3/5, respectively, and both are components of the Ccr4-NOT complex (Collart, 2003). The Ccr4-Not complex is evolutionarily conserved from yeast to human. By potentially interacting with many different proteins, the Ccr4-NOT complex regulates various cellular functions, including transcriptional initiation, RNA metabolism, and protein modification (Collart, 2003). In yeast, NOT5 of the Ccr4-NOT complex can associate with promoters and is involved in transcriptional regulation by interacting with TAF1 (Deluen *et al*., 2002). Although transcriptional regulation by the Ccr4-NOT complex remains unclear in *Drosophila*, it is possible that this complex may be directly responsible for *vas* transcription. Alternatively, it is also possible that the Ccr4-NOT complex is involved in RNA turnover or in the translational regulation that is required for *vas* expression in pole cells, because the Twin/Ccr4-NOT complex is required for these molecular events during *Drosophila* development (Morris *et al*.,

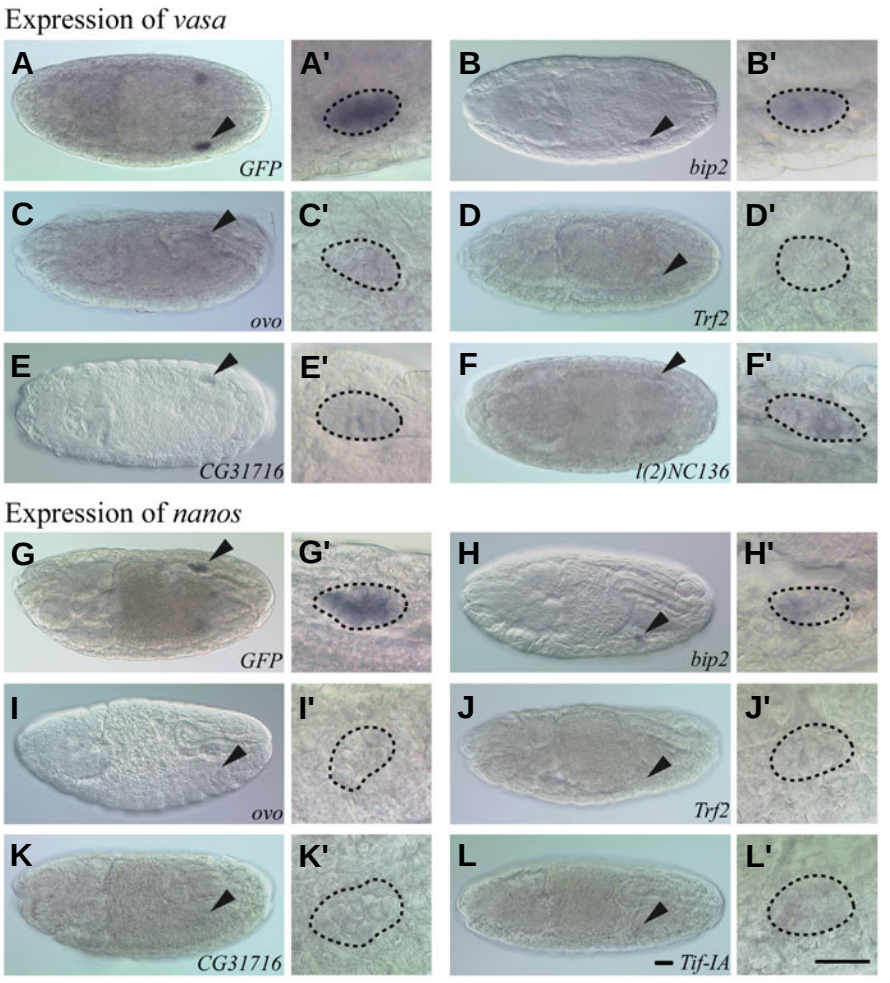


Fig. 3. *vas* and *nos* expression in dsRNA-injected embryos. (A,G) vas *(A) and* nos *expression (G) in the stage-15/16 control embryos injected with dsRNAs for* GFP. **(B–F and H-L)** *Expression of* vas *(B-F) and* nos *(H-L) in the stage-15/16 embryos injected with dsRNAs for* ovo **(B,H)**, CG31716 **(C,I)**, bip2 **(D,J)**, Trf2 **(E,L)**, l(2)NC136 **(F)** *and* Tif-IA **(K)**. **(A'-L')** *Higher magnification images of the embryonic gonads shown in (A-L). Anterior is to the left. Arrowheads in (A-L) point to the gonads. Scale bars, 20 μm.*

2005; Wharton and Aggarwal, 2006; Kadyrova *et al.*, 2007). In this regard, it is interesting to note that the maternal RNA binding protein Nos recruits the Ccr4-NOT complex in order to repress the translation of specific mRNAs (Kadyrova *et al.*, 2007). We have previously reported that maternal Nos represses translation of *cyclin-B* and *head involution defective* (*hid*) RNAs in pole cells to suppress their mitosis and apoptosis (Asaoka-Taguchi *et al.*, 1999; Sonoda and Wharton, 2001; Sato *et al.*, 2007). Moreover, maternal Nos is required for *vas* expression in pole cells, suggesting that Nos could prevent the production of a protein which otherwise inhibits *vas* expression in pole cells (Sano *et al.*, 2001). Thus, it is likely that the Ccr4-NOT complex regulates *vas* expression via Nos-dependent translational repression.

Our results show that injection of the dsRNA for *CG31716* but not for *l(2)NC136*, also affects *nos* expression in pole cells (Table 3). Perhaps *CG31716* has another role in *nos* expression that is independent of Ccr4-NOT function. Alternatively, it is also possible that pole cells express a gene which shares a redundant function with *l(2)NC136* in order to regulate *nos* expression.

A transcriptional initiation factor for RNA polymerase I

We found that *Tif-IA* RNA is enriched in pole cells and is required for *nos* expression but not *vas* expression. *Tif-IA* encodes a *Drosophila* ortholog of yeast Rrn3p and mammalian TIF-IA, both of which are the initiation factor for RNA polymerase I (RNA polI) (Moss and Stefanovsky, 2002; Grummt, 2003). TIF-IA acts as the key regulator for rRNA transcription by recruiting RNA polI into the rRNA promoter pre-initiation complex, and the interaction between TIF-IA and RNA polI is regulated by various pathways linking biosynthetic activities (Moss and Stefanovsky, 2002; Grummt, 2003). In early pole cells, RNA polII-dependent transcription is globally inhibited by maternal *Pgc* (Martinho *et al.*, 2004), but RNA polII-dependent transcription remains active (Seydoux and Dunn, 1997). Furthermore, the observed rate of protein synthesis is higher in pole cells than in the rest of somatic

TABLE 5

EXPRESSION OF *β-GAL* RNA IN POLE CELLS OF THE dsRNA-INJECTED EMBRYOS

dsRNA*	No. of injected embryos	No. of surviving embryos	No. of embryos expressing β-gal in pole cells$			
			+ (%)		± (%)	- (%)
GFP	42	36	26 (72.2)		3 (8.3)	7 (19.4)
bip2	32	26	22 (84.6)	#	4 (15.4)	0 (0)
ovo	30	26	21 (80.8)	#	2 (7.7)	3 (11.5)
Tif-IA	37	34	24 (70.6)	#	2 (5.9)	8 (23.5)
CG31716	37	28	21 (75.0)	#	2 (7.1)	5 (17.9)
Trf2	39	15	12 (80.0)	#	3 (20.0)	0 (0)
l(2)NC136	42	36	27 (75.0)	#	2 (5.6)	7 (19.4)

* The embryos produced from *nosGal4:VP16* females crossed with *UAS-lacZ* males were used as recipients for dsRNA microinjection.

$ The injected embryos (stage 15/16) were stained for *β-gal* RNA. No. of the embryos expressing *β-gal* RNA in pole cells at a high level (+), a low level (±) and an undetectable level (-) are shown.

P > 0.05; Significance was calculated vs. *GFP* control by using Fisher's exact probability test.

region in blastodermal embryos (Zalokar, 1976). Thus, we suggest that the pole cell-enriched *Tif-IA* RNA could then upregulate rRNA transcription and protein synthesis in these cells. It is possible that zygotic expression of *nos* requires a higher rate of protein synthesis than that of *vas*.

Conclusion

In this study, we performed a genome-wide survey of the maternal transcripts that are enriched in the germ plasm and that encode for transcriptional regulators. We identified 27 maternal transcripts based on microarray and *in situ* hybridization data. These results will allow us to understand the molecular basis for germ plasm function, although elucidation of specific processes regulated by each of the identified genes awaits future genetic analyses. We further utilized RNAi knockdown to investigate the role of the identified transcripts in germline-specific gene expression. Six transcripts were found to be required for *vas* and/or *nos* expression. This study demonstrates that the germ plasm contains maternal RNAs encoding for transcription factors that are required for germline-specific gene expression in pole cells.

Materials and Methods

Fly stocks

Pole cells were isolated from *EGFP-vas* embryos by FACS. This method efficiently enables us to obtain fractions highly enriched for pole cells (Shigenobu *et al*., 2006). For *in situ* hybridization and RNAi analyses, *y w* files were used. To examine *lacZ* expression in pole cells, the *nosGal4:VP16* and *UAS-lacZ* lines were used (Van Doren *et al*., 1998). Flies were raised on a standard *Drosophila* medium at 25°C. Embryos were staged according to Campos-Ortega and Hartenstein (1997).

Microarray analysis

Total RNA was extracted from pole cells isolated from the stage 4 syncytial-blastoderm embryos as described (Shigenobu *et al*., 2006). As a reference, total RNA was also extracted from stage 4 whole embryos using an RNeasy Mini kit (QIAGEN). Transcript profiles between these RNA samples were then compared by microarray analysis which was performed as described (Sato *et al*., 2007). Extracted RNA was amplified as cRNAs labeled with cyanine-3 CTP (Cy3) and cyanine-5 CTP (Cy5) using Agilent Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies, Palo Alto, CA). Custom-made 22K 60-mer oligo microarrays (Agilent) that contain 21,925 probes representing almost all of the predicted *Drosophila* transcripts were used (Sato *et al*., 2007) (GPL4336). Each array was hybridized with 1 μg of both Cy3-labeled and Cy5-labeled cRNA and washed according to the Agilent protocol. The dye-swap experiment was also carried out. Subsequently, the arrays were scanned with a G2565BA Microarray Scanner System (Agilent). Data were analyzed using the Feature Extraction 7.1 software (Agilent). LOWESS (20%) normalization was applied to the data using GeneSpring software (Agilent). The probe sets with low signal intensity (normalized value < 10) or with high variance between the dye-swap experiments (variance between Cy3/Cy5 and Cy5/Cy3 ratios >75%) were excluded, and data for 18,205 probes was used for the subsequent analysis. The data will be deposited in GEO under Accession No. GSE9679.

We selected 735 as "candidate transcripts enriched in pole cells "based on their differential microarray hybridization levels (pole cell to whole embryo ratios ≥ 1.56; when dual microarray probes were designed to hybridize single transcripts, the higher ratios were used for the selection). Among them, 68 were classified in the GO category "transcriptional regulator activity" in FlyBase (Release 3.1). GO annotation was based on the gene association file (version 2004-7) provided by GO consortium (http://www.geneontology.org/). These selected genes showed a wide variety of pole cells to whole embryo ratios ranging from 1.56 to 8.60.

In situ *hybridization*

Whole-mount *in situ* hybridization was essentially performed as described (Mukai *et al*., 2006). DIG-labeled antisense RNA probes were synthesized with T3 RNA polymerase from flanked PCR products amplified from pBluescript SK(+) containing the cDNA fragments and with T7 and T3 primers. The cDNA fragments corresponding to the identified 68 transcripts were amplified from an embryonic cDNA library (Brown and Kafatos, 1988) by using the primer pairs listed in Supplementary Table S1. Each primer pair was designed to amplify approximately 1 kb of cDNA fragment corresponding to a 3'-portion of the open reading frame. Under the conditions used, we failed to amplify the cDNAs corresponding to *Doc1, CG3711, and CG31782*. Thus, we examined the distribution of the other 65 transcripts by whole-mount *in situ* hybridization.

The probe for lacZ (1.1 kb) was synthesized as previously described (Fatchiyah Zubair *et al*., 2006).

Signal detection was carried out using an alkaline phosphatase-conjugated anti-DIG Fab fragment (Roche). Color development was performed using 0.45 mg/ml NBT and 0.18 mg/ml BCIP at 25ºC for 1-6 hr in the dark.

Microinjection of dsRNAs

Template DNA fragments flanked by T7 and T3 promoter sequences at each end were amplified from the same cDNA clones used for *in situ* hybridization using the primer pair
5'-TAATACGACTCACTATAGGG-3' and
5-GTAATACGACTCACTATAGGG**CTCACTAAAGGG**AACAAAAGC-3'. The T7 promoter sequence is underlined and the T3 promoter is in bold. dsRNA was synthesized from the templates with T7 RNA polymerase (MEGAscript Kit, Ambion). As a control, we also synthesized dsRNA from a *GFP* cDNA fragment [679-1398 of pEGFP-N1 vector (Clontech) (accession No. = U556762)]. Approximately 0.1 nl of dsRNA solution (3 ng/nl in distilled water) was injected into the poster region of cleavage embryos (stage 2). The injected embryos were allowed to develop until stage 15-16 (26 hr AEL at 18ºC) and were fixed for *in situ* hybridization.

RT-PCR analysis

The dsRNA-injected embryos were allowed to develop until stage 3-4 (2 hr AEL at 18ºC). Total RNA was isolated from 30 of the injected embryos using the RNeasy Mini Kit (QIAGEN). First-strand cDNA was synthesized from the RNA using oligo dT primer and SuperScript III RT (Invitrogen). Pairs of primers used for detecting *bip2, ovo, Tif-IA, CG31716, Trf2*, *l(2)NC136* and *RpL32* are listed in Supplementary Table S3. The thermal cycling condition was as follow: 1 cycle of 94˚C for 5 minutes, then 30 cycles of 94˚C for 15 seconds, 60˚C for 30 seconds, 68˚C for 1 minute. *RpL32* was used as a control.

β-gal expression in pole cells of dsRNA-injected embryos

The embryos produced from *nosGal4:VP16* females (Van Doren *et al*., 1998) crossed with *UAS-lacZ* males were used as recipients for dsRNA microinjection. Microinjection of dsRNA and *in situ* hybridization using the *β-gal* probe were carried out as described above.

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