gcm2 Promotes Glial Cell Differentiation and Is Required with glial cells missing for Macrophage Development in Drosophila

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INTRODUCTION

The glial cells missing gene (gcm) is the primary regulator of glial cell determination in Drosophila. It encodes a transcription factor that is transiently expressed in all embryonic glia, except for the midline/mesectoderm-derived glia (Akiyama et al., 1996; Hosoya et al., 1995; Jones et al., 1995; Schreiber et al., 1997; Vincent et al., 1996). gcm loss-of-function mutant embryos lack nearly all lateral glial cells, and presumptive glial cells are transformed into neurons (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). Conversely, when gcm is ectopically expressed, presumptive neurons are transformed into glia (Hosoya et al., 1995; Jones et al., 1995). Thus, within the nervous system, gcm acts as a genetic switch, with Gcm-positive cells becoming glia and Gcm-negative cells becoming neurons. Moreover, nonneural cells may adopt a glial cell fate when forced to express gcm (Akiyama-Oda et al., 1998; Bernardoni et al., 1998).

In addition to acting as a regulator of glial cell differentiation, gcm has been found to have an instructive role in the development of the plasmatocyte/macrophage lineage of blood cells (Bernardoni et al., 1997; Lebestky et al., 2000). Embryonic blood cells, or hemocytes, are derived from the procephalic mesoderm (Tepass et al., 1994). Hemocyte precursors give rise to two populations of hemocytes in the embryo: plasmatocytes and crystal cells (Mathey-Prevot and Perrimon, 1998). Plasmatocytes constitute the majority of circulating hemocytes. They migrate throughout the embryo in the hemolymph space, where they convert into macrophages that are responsible for the phagocytosis of apoptotic cells (Abrams et al., 1993; Tepass et al., 1994). Early plasmatocytes, first seen in stage 10 embryos, express the extracellular matrix protein Per oxidatin. Per oxidatin expression is maintained throughout embryogenesis (Abrams et al., 1993; Nelson et al., 1994; Tepass et al., 1994). In stage 11, plasmatocytes begin to express Croquemort, a CD36-related macrophage receptor (Franc et al., 1997).
1996), and they convert into macrophages. Croquemort expression is required for the efficient phagocytosis of apoptotic cells (Franc et al., 1999). By the end of embryogenesis, nearly all plasmatocytes are macrophages that express the Croquemort receptor.

Before gcm is activated in glial progenitors, gcm is expressed in the primordium of hemocyte precursors (Bernardoni et al., 1997; Jones et al., 1995). gcm continues expression in hemocyte precursors as they develop into plasmatocytes through stage 11, after which it rapidly disappears (Bernardoni et al., 1997). As plasmatocytes migrate out of the procephalon and convert into macrophages, gcm expression can no longer be detected.

gcm has been proposed to promote the differentiation of plasmatocytes (Bernardoni et al., 1997; Lebestky et al., 2000). Early misexpression of gcm induces an increase in circulating plasmatocyte numbers in the embryo (Bernardoni et al., 1997). Ectopic expression of gcm in the crystal cell lineage of hemocytes induces Croquemort expression and the adoption of plasmatocyte characteristics (Lebestky et al., 2000). gcm mutant embryos are deficient by 40% in plasmatocyte numbers (Bernardoni et al., 1997). These results indicate an instructive role for gcm in plasmatocyte/macroglial development, and gcm has been proposed to be a master regulator of the plasmatocyte/macroglial lineage (Bernardoni et al., 1997). However, since mutation of gcm causes only a decrease in plasmatocyte numbers (Bernardoni et al., 1997), without changing their ability to differentiate into macrophages (Jones et al., 1995), gcm cannot be the sole determinant of plasmatocyte/macroglial cell fate.

In this paper, we describe the characterization of a homolog of the gcm gene in Drosophila, gcm2. gcm2 was previously identified as a PCR fragment containing a gcm-motif (Akiyama et al., 1996), the highly conserved DNA-binding domain (Akiyama et al., 1996; Schreiber et al., 1997) that is shared with gcm’s two mammalian homologs (Akiyama et al., 1996; Altschuller et al., 1996; Kim et al., 1998). We found gcm2 located 27.9 kb proximal to gcm, and show that gcm2 has partially redundant functions with gcm. gcm2 is expressed in a pattern similar to gcm, but at lower levels, in glial cells and in hemocyte precursors. Ectopic expression of gcm2 is sufficient to induce glial cell differentiation. Moreover, a deletion of both genes completely removes all glial cell development, apart from the midline glia. While this paper was in preparation, similar results were reported (Kammerer et al., 2001); however, without a mutation in gcm2, its exact role in development was not clear. Here, we describe a null mutation of gcm2 and its effects on both glial cell and hemocyte differentiation.

gcm2 loss-of-function has only a mild effect on glial cell differentiation. More significant is its role in plasmatocyte/macrophage differentiation. Like the gcm mutation, gcm2 loss-of-function results in a deficit in plasmatocytes. Deletion of both gcm and gcm2 results in the striking absence of Croquemort expression. However, Peroxidasin-expressing hemocytes are still present. These Peroxidasin-expressing hemocytes are reduced in numbers by over half, fail to undergo normal migration, and do not exhibit the morphology associated with their complete conversion into macrophages. We conclude that gcm and gcm2 are not required for the initial specification of the plasmatocyte lineage but are necessary for the proliferation of plasmatocyte precursors, the expression of Croquemort protein, and the ability of plasmatocytes to convert into macrophages.

**MATERIALS AND METHODS**

**Identification and Analysis of gcm2 Locus and cDNA**

Database searches for gcm homologs using the BLAST program (Altschul et al., 1997) identified an open reading frame (ORF) with homology to gcm located on a Drosophila melanogaster genomic library clone BACR24A22 (GenBank Accession No. AC007257), which had been generated and sequenced by the Berkeley Drosophila Genome Project (BDGP). A pair of oligonucleotide primers flanking the ORF (forward, AGTACGCAATTGCCTGGCATCC; and reverse, ACTGTTGTGTTGGCGTGC) was used to amplify a 0.7-kb PCR product from the BACR24A22 DNA template. This PCR product was used to screen a 9- to 12-H. D. melanogaster embryonic cDNA library (Zinn et al., 1988). A single gcm2 cDNA was isolated; the 3-kb insert was subcloned into pBluescript SK(+) (Stratagene) and sequenced (GenBank Accession No. AF461416).

**In Situ Transcript Localization**

In situ hybridization in whole-mount embryos was performed by using digoxigenin-labeled riboprobes as described previously (Jones et al., 1995). gcm antisense probes were generated from a full-length gcm cDNA (Jones et al., 1995). thioredoxin probe was generated from a thioredoxin cDNA, GH24203, identified by the BDGP (Rubin et al., 2000), and obtained from ResGen.

**Immunohistochemistry**

Horseradish peroxidase (HRP) immunohistochemistry and embryo dissection were done as previously described (Patel, 1994). Anti-Repo monoclonal antibody (Ab) (MAB 8D12; B.W.J., B. Blankemeier, and C. S. Goodman, unpublished observations) was used at a 1:10,000 dilution. Rabbit anti-Croquemort antibody (Ab) (Franc et al., 1996) was used at a 1:1,000 dilution, mouse anti-Peroxidasin Ab (Nelson et al., 1994) at 1:500, mouse anti-β-galactosidase (β-gal) MAb (Roche) at 1:500, and rabbit anti-β-gal Ab (Cappel) at 1:10,000. Secondary antibodies conjugated to HRP or Biotin (Jackson Immunoresearch) were used at 1:300 dilutions. Biotinylated secondary antibodies were detected by using Vectastain Elite ABC and HRP/ diaminobenzidine (DAB) reaction. DAB reactions were enhanced to give a black color by addition of 0.067% NiCl2.

**D. melanogaster Stocks and Genetics**

Wild-type embryos were w1118. The gcm null allele gcm2 (Fba10045751) and UAS-gcm reporter lines P[UAS-gcm]2 and P[UAS-gcm]3 (Fba10045765) were described previously (Jones et al., 1995). Df(2L)200 (Fba10022176) was described by Lane and Kalderon (1993). Df(2L)200 and gcm2(1) were balanced over CyO,
P{w^mC-Act5C-lacZ} (FBba0000031) for the identification of mutant embryos.

USA-gcm2 lines were generated by inserting the gcm2 cDNA into pUAST, a UAS reporter P element vector (Brand and Perrimon, 1993), to generate P{UAS-gcm2}. Several lines carrying P {UAS-gcm2} insertions were generated by using P element-mediated transformation into w^{1118} flies using standard methods.

Ectopic expression of gcm2 or gcm in neuroblasts was achieved by crossing P{UAS-gcm2} or P{UAS-gcm2}J with sca-Gal4 (FBa10040466) (Klaes et al., 1994). Ectopic expression of gcm in a Df(2L)200 background was achieved by using daughterless-Gal4 (da-Gal4) (FBr0013991) (Wodarz et al., 1995), by crossing Df(2L)200/Cyo-P {UAS-gcm2}J with Df(2L)200/Cyo-da-Gal4. Expression of a Iz reporter gene (Iz-lacZ) in a Df(2L)200 background was achieved by crossing Iz-Gal4;Df(2L)200/+ males with Df(2L)200/+-UAS-lacZ/+ females. Iz-Gal4 (FBr0003702) and UAS-lacZ (FBt0002140) have been described previously (Brand and Perrimon, 1993; Crew et al., 1997; Lebestky et al., 2000).

Deletions at the gcm2 locus were generated by imprecise excision of a P element as previously described (Jones et al., 1995), using the P element P(EP)2018 (FBt0010775) (Liao et al., 2000; Rorth et al., 1998). Several hundred w- lines were generated and analyzed by Southern blot to detect deletions; one of these excision lines, Df(2L)gcm2, has a deletion that removes the entire ORF of gcm2 as well as the thioredoxin promoter (see Fig. 5).

Molecular Characterization of Df(2L)200 and Df(2L)gcm2

The deletion breakpoints of Df(2L)200 and Df(2L)gcm2 were determined by Southern analysis using probes generated from genomic DNA walks of the region spanning 308–30C (Jones et al., 1995; Lane and Kalderon, 1993), as well as probes generated from the gcm2 and thioredoxin cDNAs. The extent of the Df(2L)gcm2 deletion was determined by amplifying a 0.9-kb PCR product using primers flanking the deletion (forward, CAAAGTGAGACGC-TATCAGC; and reverse, CCTGTACACCATGTGGATT), with genomic DNA from homzygous Df(2L)gcm2 flies as a template. The PCR product, cloned into pCR2.1 (Invitrogen), was sequenced.

RESULTS

Identification and Characterization of the gcm2 Locus

A second gcm-motif gene in D. melanogaster, named gcm2, was originally identified from a 137-bp PCR product amplified from D. melanogaster genomic DNA using degenerate primers (Akiyama et al., 1996). Using BLAST searches (Altschul et al., 1997), we found a sequence with significant homology to gcm on a D. melanogaster genomic library clone. This sequence has an ORF with a gcm-motif and identity to the predicted sequence of the gcm2 PCR fragment. BLAST searches with the near complete sequence of the D. melanogaster genome (Adams et al., 2000) failed to identify additional gcm-motif genes. We concluded that we had identified gcm2.

A 0.7-kb PCR product was amplified by using primers flanking the gcm2 ORF. This PCR product was used as a probe to isolate a single 3-kb clone from an embryonic cDNA library. The cDNA clone was sequenced. It has an ORF that encodes a protein of 613 amino acids (Fig. 1B), a 928-bp 5’ UTR, and a 171-bp 3’ UTR with poly(A) tail. Comparison with the published genomic sequence (GenBank Accession No. AE003625; Adams et al., 2000) revealed the gcm2 transcription unit to have 3 exons and a location 27.9 kb 5’ to the gcm gene, in opposing orientation (Fig. 1A). Located between gcm2 and gcm are two predicted genes encoding for carboxylesterases (CG3841 and CG4382). Immediately 3’ of gcm2 is the thioredoxin gene, one of several Thioredoxin-like genes in Drosophila.

The predicted amino acid sequence and exon structure of gcm2 are in agreement with the prediction of the Genome Project (GenBank Accession No. AAFS2793), differing in amino acid sequence at only six residues (bold, underlined in Fig. 1B). However, our cDNA differs from that of Kammerer et al. (2001) at the 5’ end. Their cDNA (GenBank Accession No. AF184664) has a fourth, alternative 5’ exon, located upstream, that replaces the first 11 residues of our predicted protein with four different residues. These results suggest that gcm2 has two separate promoters with alternative splicing at the 5’ end.

Gcm2 protein is similar in structure to other Gcm family members, sharing a highly conserved N-terminal gcm-motif of 156 amino acids (boxed in black in Fig. 1B). While all Gcm family members are very similar in the gcm-motif, Gcm2 and Gcm are more similar to each other (69% identity; 83% similarity) than to their two vertebrate counterparts Gcm1/GcmA and Gcm2/Gcmb (55–64% identity; 70–78% similarity to human Gcm1 and Gcm2) (Fig. 1C).

Expression of gcm2 Transcripts

We followed gcm2 expression in embryos using in situ hybridization to mRNA. Similar results were obtained whether we used a full-length probe for gcm2 or a probe made from the first 930 bp of the gcm2 cDNA that ensured no cross-hybridization with gcm mRNA. Cross-hybridization with gcm was of concern as the expression profiles of the two genes overlap, though gcm2 is expressed at much lower levels. With gcm2 and gcm probes of similar lengths under identical hybridization conditions, gcm2 transcripts were first detected after 3 h of reaction, while gcm transcripts were detected after only 5 min of reaction.

The expression profiles of gcm2 transcripts compared with gcm are shown in Fig. 2. Like gcm, gcm2 is first detected in an anterior ventral region in stage 5 embryos (Fig. 2A). During gastrulation, these cells invaginate at the end of the ventral furrow just anterior to the cephalic furrow, in the primordium of presumptive hemocyte precursors (Fig. 2C). Expression in hemocyte precursors persists through stage 11, after which it rapidly fades. At late stage 9, gcm2 is first detected in the neuroectoderm in each hemisegment in a single cell at the lateral edge of the CNS (Fig. 2E). At stage 11, gcm2 continues to be expressed in a single cell per CNS hemisegment, which is now in the position of neuroblast N B1-3 or its progeny (Fig. 2G). N B1-3
gives rise to several CNS and peripheral glial (Schmidt et al., 1997). By stage 12, gcm2 is detected in longitudinal glia precursors, as well as other CNS glia, and in a stripe of ectodermal cells of the lateral body wall in each hemisegment (Fig. 2). Through stage 15, gcm2 continues to be detected in the longitudinal glia, in other CNS glia at very low levels, and in the lateral ectoderm (Figs. 2K and 2M). After stage 15, gcm2 expression rapidly fades.

In summary, the expression pattern of gcm2 in part mirrors that of gcm, but expression is at very low levels compared with that of gcm. gcm2 expression is highest in the hemocyte primordia and in the longitudinal and nerve root glia.

**Ectopic Expression of gcm2 Induces Glial Cell Differentiation**

The similarity between Gcm2 and Gcm proteins, especially in the gcm-motif, suggested they have redundant functions. Ectopic expression of gcm is sufficient to promote glial cell differentiation, transforming presumptive neurons into glia (Hosoya et al., 1995; Jones et al., 1995). To test whether ectopic expression of gcm2 is sufficient to promote glial cell differentiation, we used the Gal4/UAS binary system (Brand and Perrimon, 1993) to generate embryos that express gcm2 in all presumptive neuroblasts. UAS-gcm2 flies were crossed with sca-Gal4 (Klaes et al., 1994), which drives expression throughout the neurogenic ectoderm and developing neuroblasts. In sca-Gal4/UAS-gcm2 embryos, there is a striking increase in the number of glial cells as revealed by anti-Repo antibody (Fig. 3C), a glial-specific marker (Campbell et al., 1994; Halter et al., 1995; Xiong et al., 1994). Many of these Repo-positive cells exhibit the elongated or irregular cell shapes typical of glial cells. This phenotype is similar to that of ectopically expressed gcm (Fig. 3B). Similar results were also shown by Kammerer et al. (2001); in addition, they induced ectopic gcm2 in neuroblasts in gcm null mutant embryos and demonstrated that gcm2 is sufficient, in the absence of gcm, to induce gliogenesis.

**gcm2 Is Necessary for Gliogenesis**

gcm mutant embryos retain a low level of glial cell development (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). gcm<sup>−/−</sup> is a null allele that deletes the transcription unit. In gcm<sup>−/−</sup> mutant embryos, isolated single Repo-positive cells, as well as occasional small clusters of Repo-positive cells can be seen in many segments (Fig. 4C). These glial cells are typically, but not always, in the positions longitudinal glia and nerve root glia—cells in which gcm2 expression is strongest. The number of Repo-positive cells per hemisegment may vary from 0 to as many as 8. We counted an average of 2 Repo-positive cells per hemisegment in the ventral nerve cord (n = 180 hemisegments) (Fig. 4C), compared with the wild-type number of 30 glial cells per hemisegment (Fig. 4B). To ask whether gcm2 is required for the glial cell development in gcm mutants, we analyzed homozygous Df(2L)200 embryos. Southern blot analysis established that Df(2L)200 is a deletion removing approximately 120 kb that includes both gcm and gcm2, as well as 15 other predicted genes, none of which are known to affect glial cell differentiation (Fig. 4A). In Df(2L)200 homozygous embryos, Repo staining is completely abolished (Fig. 4D). These results implicate gcm2 in promoting glial cell differentiation.

To gain further evidence for a role for gcm2 in gliogenesis, we isolated a deletion allele of gcm2. A P element inserted into the 5′ UTR of the thioredoxin gene, located 584 bp 3′ of the gcm2 polyadenylation site, was mobilized to recover small deletions at the gcm2 locus. We recovered one deletion, Df(2L)gcm2, that removes the entire gcm2 ORF as well as the thioredoxin promoter (Fig. 5A). This mutation is homozygous viable. Homozygous Df(2L)gcm2 embryos lack gcm2 expression (data not shown). Df(2L)gcm2 embryos also show altered thioredoxin expression. Thioredoxin transcripts are normally expressed at high levels in the gut and at low levels throughout the embryo (data not shown). In Df(2L)gcm2 embryos, the normal pattern of thioredoxin expression is abolished. But a low level of thioredoxin expression is detected in the pattern of gcm2, reflecting a fusion of thioredoxin with the gcm2 promoter (data not shown). Smaller deletions removing just the thioredoxin promoter are homozygous viable but sterile, suggesting that the low level of thioredoxin expressed in Df(2L)gcm2 rescues the sterility of thioredoxin mutation.

The pattern of Repo expression in Df(2L)gcm2 homozygous embryos is normal (Fig. 5B), demonstrating that gcm2 alone is not necessary for gliogenesis. However gcm2's role in gliogenesis is revealed in a background where gcm expression is reduced by half. When Df(2L)gcm2 is crossed with Df(2L)200 embryos, removing one copy of gcm and two copies of gcm2, the resulting transheterozygotes are lethal, and glial cell deficiencies are consistently observed. Glial cell deficiencies are most notable in the longitudinal glia tracts, but are also observed with other CNS glial cell types (Figs. 5C–5E). To assay the penetrance of this glial cells missing phenotype, we counted longitudinal glia (LG) in Df(2L)gcm2/Df(2L)200 embryos. In wild-type and in Df(2L)gcm2 homozygous embryos, we counted an average of 8.5 LG per hemisegment (n = 656 767, respectively). In Df(2L)gcm2/Df(2L)200 embryos, we counted an average of 6.4 LG per hemisegment (n = 1106). Thus, lowering the dosage of gcm and completely removing gcm2 reveals the contribution of gcm2 to glial cell differentiation, especially in the LG lineage.

**Both gcm2 and gcm Are Required Together for the Terminal Differentiation of Plasmatocytes**

Both gcm and gcm2 are expressed in the primordia of embryonic hemocytes and in plasmatocytes through stage 11. In gcm mutant embryos, there is a decrease by 40% in the number of plasmatocytes (Bernardoni et al., 1997).
FIG. 1. Map of the gcm/gcm2 locus, Gcm2 sequence, and comparison to human homologs. (A) Map of genomic region 30B-30C, showing locations of gcm2, gcm, and three other predicted genes, derived from sequences and gene predictions from the Drosophila Genome Project (GenBank Accession No. AE003625; Adams et al., 2000) and from experimental data. Arrows indicate direction of transcription. Predicted transcript structures are shown below the line, with rectangles representing exons; black regions within the rectangles represent predicted ORFs. Distal to proximal orientation is from left to right. (B) Predicted amino acid sequence of Gcm2 protein. Gcm-motif is boxed in black. The six amino acid residues that differ from the predicted sequence of the Genome Project are underlined in bold. (C) Sequence alignments of gcm-motifs of D. melanogaster Gcm2, D. melanogaster Gcm (GenBank Accession No. AAC46912), H. sapiens hGcm1 (GenBank Accession No. BAA13651), and H. sapiens hGcm2 (GenBank Accession No. AAC33792). Sequence identities are boxed in black. Conserved cysteine and histidine residues are marked underneath. The percentage sequence identity is shown in the lower half, and the percentage sequence similarity is shown in the upper half of the inserted table. Sequence similarities are based on BLAST analysis. Note that Gcm2 and Gcm are more similar to each other than to their human homologs.

FIG. 2. In situ localization of gcm2 and gcm transcripts in whole-mount embryos at different stages, viewed ventrally, with anterior to the left. gcm2 expression (A, C, E, G, I, K, M) is shown after 6 h of staining reaction. gcm expression (B, D, F, H, J, L, N) is shown after 1 h of staining reaction. (A, B) Stage 5: gcm2 (A) and gcm (B) are expressed in a ventral anterior patch of cells in presumptive procephalic mesoderm (arrowheads). (C, D) Stage 6: gcm2 (C) and gcm (D) expressing cells invaginate into the ventral furrow anterior to the cephalic furrow (arrowheads). (E, F) Stage 9: gcm2 (E) is expressed in procephalic mesoderm (arrowhead) and in a single cell per hemisegment at the lateral edge of the neuroectoderm (arrow); gcm (F) is expressed in a patch of cells at the same position as gcm2 at the lateral edge of the neuroectoderm (arrow), as well as in procephalic mesoderm (arrowhead). (G, H) Stage 11: gcm2 (G) continues to be expressed in a single cell per CNS hemisegment (arrow) and in hemocyte precursors (arrowhead); gcm (H) is expressed in the same cell as gcm2 in the CNS (arrow) as well as other glial precursors and in hemocyte precursors (arrowhead). (I, J) Stage 12: gcm2 (I) is expressed in migrating longitudinal glia (arrow) at low levels in other CNS glia; gcm (J) is expressed at high levels in all developing glia, including the longitudinal glia (arrow). Both gcm2 and gcm begin expression in a stripe of lateral ectodermal cells (thin arrows). Note that gcm2 and gcm are no longer expressed in hemocytes at stage 12. (K, L) Stage 13: gcm2 (K) is detected in longitudinal glia (arrow), as well as in other glia; gcm (L) is expressed in all glia. (M, N) Stage 15: gcm2 and gcm expression fades in the ventral nerve cord. Scale bar, 50 μm.
Given that gcm2 may have redundant functions with gcm in the plasmatocyte lineage, we counted Croquemort-positive cells in Df(2L)gcm2 embryos. At stage 15, we found an average of 219 per half embryo in Df(2L)gcm2 mutants (n = 6; 226, 202, 223, 196, 210, 259), as compared with 294 per half embryo in wild type (n = 6; 303, 314, 292, 305, 325, 222).
283, 310). This constitutes a 25% reduction in the number of Croquemort-positive hemocytes in Df(2L)gcm2 embryos compared with wild-type. We also looked at Peroxidasin protein expression, which is an early marker of the plasmatocyte lineage (Abrams et al., 1993; Nelson et al., 1994; Tepass et al., 1994). At stage 15, we counted an average of 234 Peroxidasin-labeled hemocytes per half embryo in Df(2L)gcm2 mutants (n = 6; 205; 240; 248; 204; 257; 251), as compared with 314 per half embryo in wild type (n = 6; 306; 341; 297; 314; 322; 301). This constitutes a 26% reduction in Peroxidasin-labeled hemocytes in Df(2L)gcm2 embryos compared with wild type.

In Df(2L)200 mutant embryos, deleting both gcm and gcm2, we found a complete absence of Croquemort expression (Fig. 6B). To ascertain the fate of hemocytes in Df(2L)200 embryos, we also looked at Peroxidasin expression. In Df(2L)200 embryos, Peroxidasin-labeled hemocytes are still present (Fig. 7); however, they are reduced in number and have aberrant morphologies and migration behavior. In wild-type stage 13 embryos, plasmatocytes migrate from the head along different routes: midventrally between the ventral epidermis and ventral nerve cord (Fig. 7C), between the dorsal surface of the nerve cord and the mesoderm, along the dorsal boundary of the epidermal...
By stage 16, 90% of Peroxidasin-labeled hemocytes in wild-type embryos have converted into macrophages that are enlarged due to phagocytic activity (Tepass et al., 1994). Macrophages are typically 8–15 microns in diameter and contain large vacuoles filled with dark inclusions (Figs. 8A and 8C). In Df(2L)200 embryos, the majority of Peroxidasin-labeled hemocytes are small, rounded, but irregular in shape, and less than 5 microns in diameter; some contain small vacuoles and occasionally enlarge up to 8 microns (Figs. 8B and 8D).

Given these phenotypes, we were curious to test whether expression of gcm in gcm/gcm2-deficient embryos was sufficient to rescue Croquemort expression in hemocytes. We used daughterless-Gal4 (da-Gal4) (Wodarz et al., 1995) to drive ubiquitous expression of UAS-gcm throughout embryogenesis in Df(2L)200 embryos. In Df(2L)200;UAS-gcm/da-Gal4 embryos, there is a partial rescue of Croquemort expression in hemocytes (Fig. 6C). We counted an average of 68 Croquemort-positive hemocytes per half stage 15 embryo (n = 6; 54, 63, 57, 76, 85, 72), which is 23% of the normal complement of Croquemort-positive hemocytes. These Croquemort-positive hemocytes are distributed throughout the embryo and have the enlarged characteristics of wild-type macrophages. These results demonstrate that expression of gcm alone is sufficient to rescue both Croquemort expression and the migration and morphological defects associated with hemocytes that lack endogenous gcm and gcm2 genes.

Hemocyte precursors generate two populations of embryonic hemocytes: plasmatocytes (approximately 600–700) that migrate throughout the embryo, and crystal cells (approximately 40–60) that are clustered around the proventriculus (Lebestky et al., 2000; Mathey-Prevot and Perrimon, 1998; Tepass et al., 1994). Crystal cell differentiation is promoted by the lozenge (lz) gene, and gcm expression is excluded from the crystal cell population (Lebestky et al., 2000). Lz expression was found not to be altered in gcm mutants (Lebestky et al., 2000). To test whether the number of crystal cells is altered in gcm/gcm2-deficient embryos, we looked at lz expression as monitored by a UAS lacZ reporter driven by a lz-Gal4 driver (lz-lacZ) in Df(2L)200 embryos. We detected no change in the number or position of lz-lacZ-expressing cells (data not shown). These results exclude the possibility that some presumptive plasmatocytes had been transformed into crystal cells in embryos that lack both gcm and gcm2.

**DISCUSSION**

gcm2 Functions Overlap with gcm in Both Glial Cell and Hemocyte Development

gcm has previously been shown to be the primary regulator of lateral glial cell fate in Drosophila. In addition, gcm has been implicated in promoting the differentiation of the plasmatocyte/macrophage lineage of blood cells. In this paper, we describe a second gcm-like gene, gcm2, which has...
functions that overlap those of gcm. We have shown that gcm2 has a minor role in gliogenesis, and a more significant role in hemocyte differentiation. Analysis of a deletion of both genes has allowed us to clarify the role of these two transcription factors in plasmatocyte development. We present evidence that gcm and gcm2 are required for the proliferation and maturation of plasmatocytes, but not their initial specification.

The sequence of Gcm2 protein is closely related to Gcm, especially in the highly conserved gcm-motif DNA-binding domain, where it shares 69% identity. While the degree of similarity between the gcm-motifs of all Gcm family members is close. D. melanogaster Gcm2 and Gcm are more similar to each other than to their two mammalian counterparts Gcm1/GCMa and Gcm2/GCMb, suggesting that independent duplications produced these two gene

**FIG. 7.** gcm/gcm2-deficient embryos show aberrant plasmatocyte differentiation. (A–H) Whole-mount embryos at different stages stained with anti-Peroxidasin antibody (anterior to left). (A, C) Wild-type stage 13. Lateral view (A) shows plasmatocytes located in the head and migrating dorsally along the edge of the epidermis and ventrally along the developing CNS. Ventral view (C) shows plasmatocytes migrating along the ventral surface of the CNS (arrows). (B, D) Df(2L)200, gcm/gcm2-deficient embryos, stage 13. Lateral view (B) shows fewer plasmatocytes, most located in the head; some have migrated out of the head, but are clustered at the dorsal edge of the epidermis (arrow). Ventral view (D) shows that plasmatocytes do not migrate along the ventral surface of the CNS past the second thoracic segment (arrow). (E, G) Wild-type stage 15. Lateral view (E) shows plasmatocytes distributed throughout the hemolymph. Ventral view (G) shows plasmatocytes distributed around the ventral surface of the CNS. (F, H) Df(2L)200, gcm/gcm2-deficient embryos, stage 15. Lateral view (F) shows fewer plasmatocytes; most are still located in the head, though some plasmatocytes are scattered in the hemolymph of the dorsal half of the embryo. Ventral view (H) shows very few plasmatocytes on the ventral surface of the CNS. Scale bar, 50 μm.
pairs. Gcm and its mammalian homologs have all been shown to bind the same octamer-binding site sequence with similar affinities (Akiyama et al., 1996; Schreiber et al., 1997, 1998; Tuerk et al., 2000). Expression of rat or mouse Gcm1/GCM a in Drosophila is sufficient to activate glial cell development (Kim et al., 1998; Reifegerste et al., 1999). Given these results, it is likely that D. melanogaster Gcm2 has similar DNA-binding and functional specificities.

gcm2 Promotes Gliogenesis

The presence of a second factor promoting gliogenesis explains why a small number of glial cells still develop in gcm null mutants. gcm2 is expressed at very low levels in lateral glial cells. The complete deletion of both gcm and gcm2 results in the elimination of all lateral glial cell development. Ectopic expression of gcm2 induces gliogenesis, and its effectiveness is indistinguishable from gcm (Fig. 3). These results suggest that Gcm and Gcm2 proteins have redundant biochemical capabilities, which are likely to be mediated through the similar gcm-motif DNA-binding domains.

Mutation of gcm2 alone is viable and has little effect on glial cell differentiation, confirming that the presence of normal gcm expression is sufficient to carry out gliogenesis in the absence of gcm2. However, when gcm2 mutation is crossed with a deletion removing both gcm and gcm2, the resulting transheterozygote is lethal, and glial cell deficiencies are visible. Thus, a twofold reduction of gcm and the complete removal of gcm2 reveal a small contribution of gcm2 to glial cell differentiation, especially in the LG lineage, where gcm2 expression is highest. This phenotype shows that glial cell differentiation is sensitive to the dosage of gcm and gcm2 gene products. Kammerer et al. (2001) demonstrated that in gcm mutant embryos there is a

**FIG. 8.** In gcm/gcm2-deficient embryos, plasmatocytes do not convert into normal macrophages. (A–D) Magnified views of anti-Peroxidasin-labeled plasmatocytes at stage 16. (A, C) Wild-type viewed from the dorsal surface (A) and on the ventral anterior surface of the CNS (C). Nearly all wild-type plasmatocytes have become enlarged macrophages that are typically 8–15 microns in diameter and contain large vacuoles filled with dark inclusions (arrows). (B, D) Df(2L)200, gcm/gcm2-deficient embryos viewed from the dorsal surface (B) and on the ventral anterior surface of the CNS (C). In Df(2L)200, gcm/gcm2-deficient embryos, most Peroxidasin-labeled hemocytes remain small and irregular in shape (arrow heads). An occasional Peroxidasin-labeled hemocyte may contain vacuoles and enlarge up to 8 microns in diameter. (D, arrow). Scale bar, 20 μm.
reduction in the glial expression of gcm2 transcripts; in addition, they showed that ectopic gcm expression induces gcm2 expression, and vice versa. These results suggest that cross-regulation between the two genes may contribute to the phenotypes observed.

Dosage sensitivity and the regulation of gcm2 by gcm may explain why gcm2 has a weak effect on glial cell differentiation in gcm mutants. gcm2 is expressed at such low levels that Gcm2 protein may be at concentrations below a threshold that triggers glial cell differentiation. Sporadically it surpasses this threshold in some neural progenitors, triggering occasional longitudinal glia and nerve root glia differentiation in the absence of gcm. It has been proposed that a fairly high threshold for Gcm protein is required to trigger glial cell differentiation in neuroglioblasts that give rise to both neurons and glia. In these neuroglioblasts, low levels of Gcm expression are not sufficient to trigger glial cell fate, but may be necessary to confer glial potential when upregulated in daughter cells (Freeman and Doe, 2001).

gcm and gcm2 Are Required for the Proliferation of Plasmatocyte Precursors but Not Their Initial Specification

It is clear that both gcm and gcm2 are required for the proper differentiation of the plasmatocyte lineage. Mutation of either gcm or gcm2 results in deficits in plasmatocyte numbers (Bernardoni et al., 1997; this study), and the ectopic expression of gcm leads to extra plasmatocytes (Bernardoni et al., 1997). Previous reports had suggested that gcm specifies the plasmatocyte lineage, and that perhaps the existence of a second gcm-motif gene in Drosophila accounted for the presence of only reduced number of plasmatocytes in gcm mutants rather than their elimination (Bernardoni et al., 1997).

In light of this, we were surprised to find, in embryos deficient for both gcm and gcm2, plasmatocyte-like cells still developing and expressing the early plasmatocyte marker Peroxidasin. However the number of Peroxidasin-labeled hemocytes in gcm/gcm2-deficient embryos is reduced by 60% compared with wild type. This reduction roughly corresponds to the combined reduction in plasmatocytes in gcm and gcm2 mutant embryos together (40% for gcm mutants plus 25% for gcm2 mutants). It has previously been observed that the procephalic mesoderm, from which plasmatocytes develop, forms a mitotic domain that undergoes four divisions during embryonic stages 8–11 (Tepass et al., 1994). After the final division, most procephalic mesoderm cells are recognizable as plasmatocytes and undergo no further divisions (Tepass et al., 1994). These cell divisions are coincident with the highest levels of gcm and gcm2 expression. These results suggest that gcm and gcm2 promote the proliferation of plasmatocyte precursors, rather than their initial specification.

We believe that the increased number of plasmatocytes observed when embryos receive an overexpression of gcm under control of the heat shock promoter (Bernardoni et al., 1997) is due to an over-proliferation of plasmatocytes in the procephalic mesoderm, which subsequently migrate throughout the embryo. Bernardoni et al. (1997) observed that ectopic expression of gcm in the nervous system leads to an increase in Peroxidasin-labeled hemocytes clustered around the CNS. They ascribed this phenotype to a transformation of CNS to hemocyte cell fate. We counted the number of Peroxidasin-labeled hemocytes in sca-Gal4; UAS-gcm embryos, where gcm is ectopically expressed in all neuroblasts, and found that the number of plasmatocytes in stage 15 embryos is not significantly increased (average of 320 Peroxidasin-labeled hemocytes per half embryo; n = 6; 292, 339, 287, 309, 358, 337), even though their distribution has changed, with more macrophages observed around the CNS (data not shown). We believe this phenotype arises from the recruitment of macrophages to the CNS due to increased apoptosis, rather than transformation of neurons into macrophages. Our results, and those of others, show that ectopic expression of gcm transforms presumptive neurons into glial cells (Hosoya et al., 1995; Jones et al., 1995) but not into plasmatocytes, consistent with combinatorial models of transcription factor action.

Hemocyte precursors in the embryo give rise to two populations of blood cells, crystal cells and plasmatocytes (Mathey-Prevot and Perrimon, 1998), promoted by the GATA transcription factor Serpent (Rehorn et al., 1996). Lack of Serpent results in the complete absence of hemocytes (Rehorn et al., 1996). Crystal cells development is promoted by the AML-1 transcription factor homolog Lozenge (Lebestky et al., 2000). In the absence of both gcm and gcm2, the number and location of crystal cells remains the same. We conclude that gcm and gcm2 do not act as genetic switches between plasmatocyte and crystal cell fate. However, ectopic expression of Gcm in the crystal cell lineage causes them to express Croquemort and assume plasmatocyte-like morphology (Lebestky et al., 2000) showing that there is some plasticity between hemocyte lineages.

The persistent expression of Serpent suggests that it may continue to have a role in plasmatocyte development after the initial specification of hemocyte precursors (Rehorn et al., 1996). In addition, a second factor, U-shaped, is expressed in plasmatocytes (Fossett et al., 2001). U-shaped acts to limit the proliferation of crystal cells. In the absence of U-shaped, there is an increase in the crystal cell population, and forced expression of U-shaped reduces the crystal cell population (Fossett et al., 2001). We suggest that a combination of Serpent, U-shaped, lack of Lozenge expression, and perhaps unidentified factors promotes the initial specification of plasmatocytes and Peroxidasin expression.

gcm and gcm2 Promote Macrophage Development

Our results are consistent with a requirement of gcm and gcm2 for the conversion of plasmatocytes into macro-
Deletion of both \( gcm \) and \( gcm2 \) results in the complete absence of Croquemort expression in hemocytes. These mutant hemocytes retain Peroxidasin expression and have some migratory properties characteristic of plasmocytes; some move out of the procephalon and are ultimately distributed in the hemolymph. However, many of these mutant hemocytes remain in the head; those that do migrate, migrate dorsally, and do not follow stereotypic ventral paths along the surface of the CNS. Normal plasmocytes migrate to prominent sites of programmed cell death (Abrams et al., 1993; Tepass et al., 1994). The distribution of plasmocytes in embryos lacking \( gcm \) and \( gcm2 \) functions suggests that they are not attracted to cells undergoing programmed cell death or that their ability to migrate is impaired.

In addition, the majority of mutant plasmocytes fail to enlarge, nor do they contain large vacuoles filled with dark inclusions. This phenotype suggests that most mutant plasmocytes lack phagocytic activity. We cannot rule out that they are incapable of phagocytosis; the fact that a small number of mutant plasmocytes enlarge slightly may indicate that they are competent for phagocytosis. Plasmocytes mutant for Croquemort protein are impaired in their ability to engulf apoptotic cells, but will still recognize and engulf bacteria (Franc et al., 1999). Whether \( gcm/gcm2 \)-deficient plasmocytes will convert into macrophages in response to bacterial invasion or other insults remains to be explored. The morphology of these cells and high level of Peroxidasin expression suggest characteristics of plasmocytes just before their conversion to macrophages (Tepass et al., 1994). We suggest a model (Fig. 9) whereby \( gcm \) and \( gcm2 \) promote the expansion and differentiation of a Peroxidasin-expressing population of hemocytes, or “proplasmocytes.” Expression of \( gcm \) and \( gcm2 \) is required for the differentiation of these proplasmocytes into plasmocytes that are competent for macrophage conversion, and they do so by initiating the expression of the macrophage receptor Croquemort and other genes that promote macrophage morphogenesis and function.

**FIG. 9.** Model for \( gcm \) and \( gcm2 \) functions in plasmocyte differentiation. (A) Wild-type. Hemocyte precursors differentiate into a population of proplasmocytes that express \( Gcm \), \( Gcm2 \), and Peroxidasin. \( Gcm \) and \( Gcm2 \) stimulate the proliferation and differentiation of proplasmocytes into plasmocytes that are competent to become macrophages by activating Croquemort and other macrophage genes. After stage 11, \( Gcm \) and \( Gcm2 \) expression disappears. Macrophage competent plasmocytes convert into macrophages in response to signals from cells undergoing apoptosis. (B) \( gcm^-/gcm2^- \). In the absence of \( Gcm \) and \( Gcm2 \) proteins, proplasmocytes fail to proliferate. They express Peroxidasin and partially differentiate into cells with characteristics of plasmocytes. However, these plasmocytes are not competent to convert into macrophages in response to signals from apoptotic cells.
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