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Embryonic fat-cell lineage in *Drosophila melanogaster*

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SUMMARY

The *Drosophila* adipose tissue, or fat body, and the body-wall muscle are two major tissues derived from the mesoderm. Although much is known about the lineage of muscle cells, little is known about the development of the fat body. Using known genes and an enhancer trap (29D), we have begun to trace the lineage of the cells comprising the fat body.

The genes *Adh* (alcohol dehydrogenase) and *DCg1* (type IV collagen) code for gene products involved in fat-cell metabolism and therefore serve as terminal fat-cell differentiation markers. The expression of these genes was used to identify the fat body at stage 17 and to identify the start of terminal fat-cell differentiation at stage 15. We found that the steroid-hormone receptor gene, *svp* (*seven-up*), was expressed transiently within the fat-cell lineage from stages 12 to 14. We suggest that stage 12 marks the beginning of early fat-cell differentiation and that the *svp*-positive cells within the mesoderm are early precursor fat cells. To confirm the identity of these cells and to establish the role of *svp* in the developing fat cell, we examined *svp* mutant embryos for alterations in the expression of the two terminal fat-cell differentiation markers, *Adh* and *DCg1*. Loss of *svp* function resulted in the loss of *Adh* transcript and a reduction of *DCg1* expression specifically in the fat body. Thus, *svp* plays a role in fat-body-specific expression of at least two terminal fat-cell differentiation genes. In contrast to *svp*, we found no evidence that the steroid receptor *HNF-4(D)* gene was expressed in the fat body nor that it was involved in the development of this tissue.

Using an enhancer-trap line (29D), we further traced the fat-cell lineage to nine bilateral clusters of cells within the mesoderm at germ-band extension. We suggest these 29D-positive cells represent the progenitor fat cells. In stage-12 embryos, the 29D-positive cell clusters can be identified within the mesoderm internal to *nautilus*-expressing cells. These data suggest that the precursor fat cells may be derived from the inner mesoderm, or spanchnopleura. Embryos deficient for the DNA region surrounding the site of the 29D enhancer trap lack most, if not all, of the cells in the fat-cell lineage. These embryos exhibit the loss of *svp*-positive precursor fat cells and concomitant loss of fat-body-specific expression of *Adh* and *DCg1*. The muscle-cell lineage, however, appears unaffected in embryos lacking the region containing the 29D enhancer trap. Both the *nautilus*-expressing cells, which are progenitors to body-wall muscle, and the visceral mesoderm were present in these embryos. These data suggest the presence of a gene(s) near the site of the 29D enhancer trap that is important in fat-cell determination. It is likely this *Drosophila* gene is associated with the 29D enhancer.

Key words: adipogenesis, determination, differentiation, fat body, mesoderm

INTRODUCTION

In *Drosophila*, the mesoderm gives rise to several tissues and cell types: the fat body, the visceral and somatic muscle, the dorsal vessel, gonad-sheath cells, pericardial cells and peritracheal cells (Technau, 1987; Hartenstein et al., 1992). Genes involved in the cascade of events leading to the formation of the mesoderm have been identified (for review see Nüsslein-Volhard, 1991; Ferguson and Anderson, 1991; Thisse and Thisse, 1992). Little is known, however, about the mechanism by which cell identity is determined within the mesoderm.

At stage 11 (stages are those of Campos-Ortega and Hartenstein, 1985), the mesoderm undergoes its third cell division and separates into the splanchnopleura and somatopleura. The visceral mesoderm is derived from the splanchnopleura and will give rise to the visceral musculature while the somatopleura will give rise to the fat body, somatic musculature and other cell types (Campos-Ortega and Hartenstein, 1985). Few genes have been identified that may be involved in specification within the mesoderm. Before the invagination of the mesoderm primordium, *tinman (tin*, formally *msh-2* and *NK-4*) is activated within the developing mesoderm. Subsequently, *tin* becomes restricted to the visceral mesoderm and heart-muscle primordium (Bodmer et al., 1990). *bagpipe* (*bap*), in contrast, is transiently expressed (Azpiazu and Frasch, 1993) in the mesoderm in the region that gives rise to the midgut muscle. Loss of *tin* or *bap* function does not affect the formation of the mesoderm but results in the absence of the

heart muscle (Bodmer et al., 1990; Azpiazu and Frasch, 1993; Bodmer, 1993) and disrupts the development of the midgut visceral mesoderm (Azpiazu and Frasch, 1993). These data suggest *bap* and *tin* may be involved in specifying cell fate within the mesoderm.

Part of the mesoderm gives rise to the *Drosophila* fat body. Little is known about the development of this tissue, much less the commitment of mesodermal cells to a fat-cell fate. Based on histological studies (Poulson, 1950), it is assumed that fat cells arise from the somatopleure after stage 13, and are small inconspicuous cells that do not appear to undergo differentiation until just before hatching. Recently, the identification of enhancer traps with multiple patterns of expression has allowed a closer examination of the mature fat body (Hartenstein and Jan, 1992).

To begin to understand the genetic and molecular events that are involved in fat-cell fate decisions and that may trigger fat-cell differentiation, we have sought to reconstruct the cell lineage of the fat body. To identify genes expressed in the developing fat cell that could be used as fat-cell markers, we examined enhancer-trap lines from several different collections. We have identified several lines that display fat-cell-specific enhancer activity in adults, larvae and embryos (Lunz et al., 1992; Hoshizaki, 1994; Hoshizaki, unpublished results). One enhancer trap, 29D, exhibits a highly restricted pattern of expression confined mainly to the developing embryonic fat cell.

Here, we describe the use of this enhancer trap and the genes *alcohol dehydrogenase* (*Adh*), *collagen IV* (*DCg1*) and *seven-up* (*svp*) to reconstruct the fat-cell lineage from the mature fat body to the progenitor fat cells in the mesoderm. To begin to understand the genetic hierarchy involved in fat-cell differentiation and to confirm the identity of the cells comprising the fat-cell lineage, we examined *svp* mutations and a deficiency for the region containing the 29D enhancer trap for effects upon fat-cell development. The fat-cell-specific expression of *Adh* and *DCg1* is dependent upon *svp* function. In contrast, the establishment of the fat-cell lineage requires the region containing the 29D enhancer.

The identification of the cells of the fat-body lineage has allowed us to establish a system in which genetic events in fat-cell specification and differentiation can now be examined. One gene involved in terminal fat-cell differentiation is the steroid hormone receptor gene *svp*. Another gene(s) involved in fat-cell development, most likely in fat-cell determination, lies in the region of the 29D enhancer trap and may be associated with the 29D enhancer.

MATERIALS AND METHODS

Immunohistochemical staining of whole-mount embryos

Embryos were collected and immunohistochemical staining was carried out as previously described (Hoshizaki, 1994). The anti-β-galactosidase was obtained from Organon Tekika Corporation and the anti-Fasciclin III was a gift from N. Patel. Both secondary antibodies (goat anti-rabbit IgG conjugated to horseradish peroxidase and horse anti-mouse IgG conjugated to horseradish peroxidase) were obtained from Vector Laboratories.

In situ hybridization to whole-mount embryos

Whole-mount in situ hybridization (Tautz and Pfeifle, 1989) was used to detect cell-specific expression of *Drosophila* genes of interest (see below) and the *lacZ* transgene. Embryos were collected as previously described (Hoshizaki, 1994). RNA probes were synthesized using a digoxigenin-labeled uracil analogue essentially as described by the manufacturer (Boehringer Mannheim, Germany). Hybridizations, washes, treatment with anti-digoxigenin antibody and histochemical staining were carried out as described by Jiang et al. (1991). Double labelling with anti-β-galactosidase was carried out as described by Lloyd and Sakonju (1991).

Synthesis of RNA probes

Adh anti-sense RNA was prepared from 1.3 kb of *Adh* coding sequence subcloned from P13E-3 (Herberlin et al., 1985) inserted into pGem1 at the *Sal*I-*Bam*HI sites (kindly provided by K. Hales). The plasmid was linearized by digestion with *Sal*I and transcribed using T7 RNA polymerase (Promega). The *DCg1* clone (Mirre et al., 1988) contains 4.7 kb of coding sequence inserted into pTZ18U (BioRad) between the *Not*I and *Xba*I sites (generously provided by Y. Le Parco). The plasmid was linearized by digestion with *Xba*I and anti-sense RNA synthesized by transcription with T7 RNA polymerase. The 3.2 kb *svp* cDNA (Mlodzik et al., 1990) was a gift from Y. Hiromi, and contains an *Eco*RI insert in pBluescriptSK$^-$ (Stratagene). The *svp* anti-sense RNA was made by linearizing the plasmid with *Not*I and transcribing with T7 RNA polymerase. Anti-sense *lacZ* RNA probe was synthesized from a 1.2 kb *lacZ* cDNA subclone of pWATGlac1 (Kuhn et al., 1988) inserted into pBluescript at the *Eco*RI site (J. Meridith, unpublished). The plasmid was digested with *Not*I and transcribed using T3 RNA polymerase. The *HNF-4(D)* cDNA contains the entire coding sequence (Zhong et al., 1993) and was a gift from W. Zhong. The plasmid was linearized with *Sal*I and anti-sense *HNF-4(D)* was synthesized by transcription with T3 RNA polymerase. The *Dmyd* or *nau* clone (Paterson et al., 1991) contains the entire coding sequence inserted into the *Eco*RI site of BluescriptKS$^+$ (Stratagene). Anti-sense *nau* RNA was synthesized by linearizing the plasmid by digestion with *Bam*HI and transcribed using T3 RNA polymerase.

Drosophila stocks

Enhancer-trap lines 2E2/TM3 and 29D/*Bc Elp* were two of six lines from a 3,000-line collection that exhibited transgene expression in the embryonic fat body (Bier et al., 1989) (generously provided by Y. N. Jan). These lines contain the modified P element P*lacw*$^+$. The β-galactosidase produced from the reporter gene is localized to the nucleus due to the presence of a nuclear localization sequence within the *lacZ* reporter-gene construct. 29D contains P*lacw*$^+$ integrated into the second chromosome balancer, CyO, at cytological position 58DE (data not shown) and the line 2E2/*CxD* has a lethal insertion into the third chromosome.

The expression pattern in 2E2 embryos (results to be published elsewhere) is similar to that of enhancer traps located within *svp*: *svp*$^{w+}$/TM3β, *svp*^{AE127}/TM3, and *svp*^{rA28}/TM3 (generously provided by Y. Hiromi). The lethal insertion in the 2E2 chromosome does not complement *svp*^{e22} (results to be published elsewhere) and we conclude that the P-element insertion in 2E2 is within the *svp* gene and that the expression pattern mainly reflects activity of the *svp* enhancer. We have designated this mutation *svp*^{2E2}. The TM3β balancer chromosome contains the *lacZ* reporter gene driven by the *ftz* promoter (Nambu et al., 1990). The *HNF-4(D)* deficiency TE196X1 was provided by T. Schupbach. Homozygous mutant TE196X1 embryos were recognized by the lack of *HNF-4(D)* expression.

The 29D deficiency stocks *y*/*y*$^+$ Y; Df(2R)X58-1 [cytological breakpoints 58D6-8; F3-5], *pr cn M(2)58F*/SM5 and *y*/*y*$^+$ Y; Df(2R)X58-3 [cytological breakpoints 58C3-7;D6-8], *pr cn*/CyO, *bw* were obtained from T. Kerremack. Based on genetic criteria, Df(2R)X58-1 may not remove M(2)58F because hemizygous Df(2R)X58-1 flies are viable although their fertility is somewhat reduced. Homozygous and *trans*-heterozygous (Df(2R)X58-1/Df(2R)X58-3) deficiency-bearing

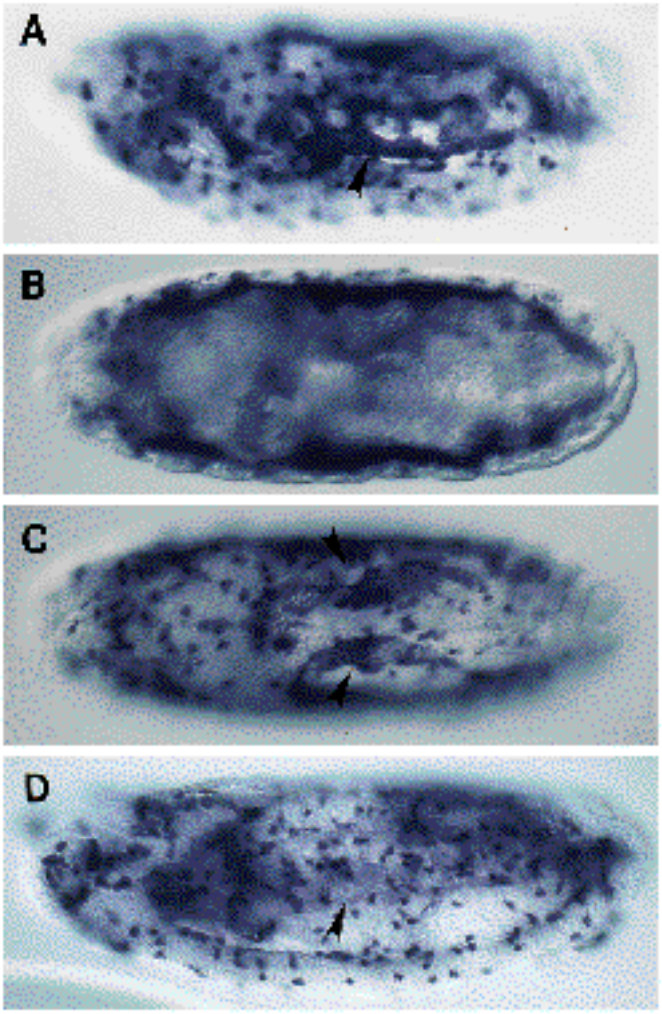

Fig. 1. Localization of *DCg1* transcripts by in situ hybridization to whole-mount embryos. (A) Lateral and (B,C) dorsal views of the same stage-16 wild-type embryo. *DCg1* transcripts were detected in the scattered fibroblast-like cells and in the fat body [arrowhead] that appears as a ribbon of cells in A (lateral view) and as a bilateral band along the periphery of the embryo in B (dorsal view). The dorsal fat body is out of the plane of focus in B but is readily visualized in C as two fingers of cells [arrowheads] extending from the posterior region of the main body of fat cells. (D) Lateral view of stage-15 wild-type embryo. *DCg1* transcript was first detected in the fat body [arrowhead] at this stage.

embryos were recognized at stage 12 by an aberrant segmentation pattern and at stage 15 by failure of the midgut to undergo its normal constrictions.

The wild-type stock was Canton-S. Further details concerning the mutations and chromosomes used in this study can be found in Lindsley and Zimm (1992).

RESULTS

Identification of the embryonic fat body

To begin the reconstruction of the lineage of cells that constitute the embryonic fat body, we have localized the transcripts of two genes: *Adh* (Lockett and Ashburner, 1989) and *DCg1* (Mirre et al., 1988). Both *Adh* and *DCg1* are expressed mainly in the embryonic fat cells of stage-17 embryos, and thus serve as cell markers for developing fat cells during late stages of embryogenesis. The *DCg1* gene codes for a basement membrane type IV collagen chain (Cecchini et al., 1987), and is expressed in the fat body and scattered fibroblast-like cells that have been described as circulating mesodermal cells (Mirre et al., 1988). Using *DCg1* as a cell marker, the cells of the fat body were readily identified in stage-16 embryos as a single-celled sheet, lying in a lateral ribbon sandwiched between the developing body-wall muscle and the visceral muscle (Fig. 1A,B). In the posterior region of the embryo, the fat body was shown to extend dorsally from the lateral ribbons (Fig. 1C). Holes within the ribbon are formed by the intrusion of various organs. In a dorsal view, the main portion of the fat body appeared as a bilateral band extending from the gonads to the thoracic region (Fig. 1B). The developing dorsal fat body was visible as it extended medially from the lateral bands anterior to the gonads (Fig. 1C). In the developing fat body, *DCg1* transcripts were first detected at stage 15 (Fig. 1D).

In late-stage embryos (stage 16), *Adh* is expressed in the fat body and in the gut (Lockett and Ashburner, 1989). We first detected *Adh* transcripts in stage-14 embryos in the anterior wall of the developing midgut (presumptive gastric caeca), the gonads and the atrium of the posterior spiracles (Fig. 2A,B). *Adh* transcripts were first detected in the cells of the developing fat body in stage-14/15 embryos (Fig. 2C,D). In stage-16/17 embryos, *Adh* transcripts were readily detected in the cells of the main portion of the fat body but were only faintly detected in the cells of the dorsal fat body. At stage 16, *Adh* transcripts were also localized to the hindgut and the developing gastric caeca but were absent in the gonads (Fig. 2E,F).

These data are in basic agreement with those of Lockett and Ashburner (1989), who examined the spatial and temporal expression of *Adh* by in situ hybridization using single-stranded ^{35}S-labeled DNA to sectioned embryos. Their inference of *Adh* expression in the fat body of the posterior region at early stage 14, however, most likely corresponds to expression in the gonads or the atrium of the posterior spiracles. We have found that the expression of *Adh* in the developing fat body was not easily detected until early stage 15.

Both *Adh* and *DCg1* code for gene products involved in fat-cell metabolism and are terminal fat-cell genes. Thus, the accumulation of *Adh* and *DCg1* transcripts at early stage 15 marks the beginning of terminal fat-cell differentiation.

The steroid-hormone-receptor gene *svp* is expressed in the fat-cell lineage

To observe earlier events in fat-cell differentiation, we examined the expression pattern of the steroid-hormone-receptor gene *svp*. The *svp* gene is involved in photoreceptor-cell-fate decisions (Mlodzik et al., 1990) and is likely to be the *Drosophila* homologue of the mammalian transcription factor, COUP (Mlodzik et al., 1990). *svp*, however, also plays an essential role in embryonic development because mutations in *svp* result in embryonic lethality (Gauss et al., 1981; Lindsley and Zimm, 1992). We obtained evidence that *svp* is expressed in the developing fat-body cells by staining several stocks carrying P-element enhancer-traps within the *svp* locus. In these lines, reporter-gene activity in the embryo was detected

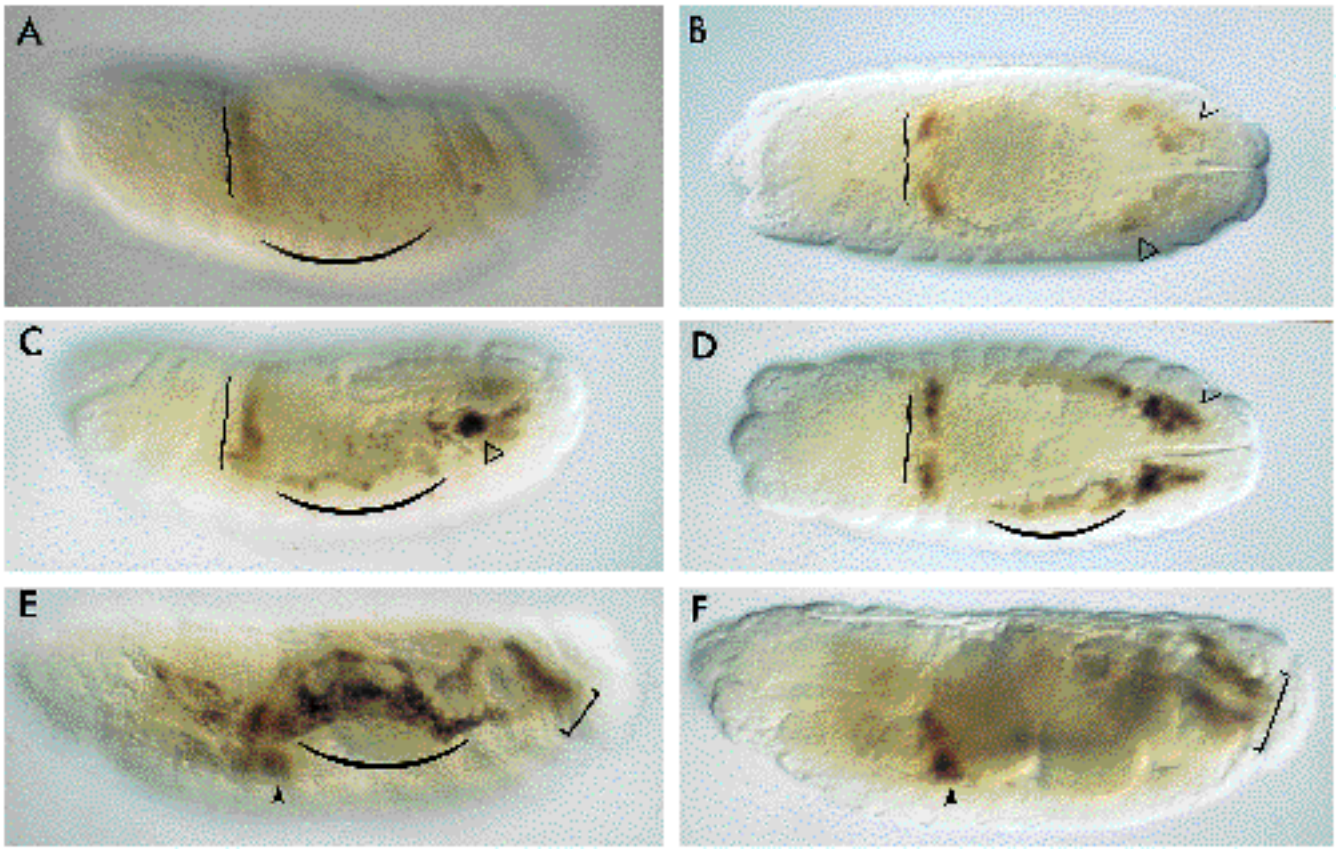

Fig. 2. Localization of *Adh* transcripts by in situ hybridization to whole-mount embryos. (A,B) Lateral and dorsal views, respectively, of stage-14 wild-type embryo. *Adh* transcripts were first detected at stage 14 in the anterior wall of the midgut [bracket], the atrium of the posterior spiracle [open arrowhead] and in the gonads [open triangle]. *Adh* transcripts in precursor fat cells might also be present [arc]. (C,D) Lateral and dorsal view, respectively, of stage-15 wild-type embryo. *Adh* transcripts were present in the cells of the fat body [arc] and continued to be present in the anterior wall of the midgut [bracket], in the gonads [open triangle] and in the atrium of the posterior spiracles [open arrowhead]. (E) Lateral view of stage-16 wild-type embryo. *Adh* expression was readily detected in the fat body [arc] and in the base of the gastric caeca [out of the plane of focus, solid arrowhead] and surrounding the posterior region of the hindgut [out of the plane of focus, square bracket]. (F) Lateral view of the same embryo as in E, but with a more internal plane of focus. Base of the gastric caeca [solid arrowhead]. Posterior region of the hindgut [square bracket].

in the fat body, oenocytes, central nervous system and in several other cell types (data not shown).

To determine whether the β-galactosidase staining pattern mirrors the expression pattern of the *svp* gene, we localized *svp* transcripts during embryogenesis. We found that *svp* was transiently expressed in the precursor fat cells at stages 12 through 14 (Fig. 3), but not in later stages of fat-cell development (Fig. 3A). In late stage-14/early stage-15 embryos, *svp* transcript was barely detected in the developing fat body (Fig. 3B). At stage 14, *svp* transcript was easily detected in the developing fat body in a bilateral band of cells located between the body wall and the developing gut (Fig. 3C). This bilateral band extended from thoracic segment t2 to abdominal segment a7 and was medial to the oenocytes in the epidermis that were also positive for *svp* expression. At stage 12, just prior to fusion of the anterior and posterior midgut, low levels of *svp* transcript were detected in cells that formed disjointed lateral bands located between the midgut and body wall (Fig. 3D). From a lateral view, the cells making up the disjointed bands appeared as small groups of cells medial to the epidermis in t2 and t3; in the abdominal segments these cells were located medial to the oenocytes (Fig. 3E,F). Thus, *svp* is expressed only in the early stages of embryogenesis (stages 12-14), prior to the beginning of terminal fat-cell differentiation at stage 15. This suggests that the *svp*-positive cells in the fat-cell lineage are the early precursor fat cells.

To examine further the identity of these *svp*-positive cells and to investigate the possible role of *svp* in fat-cell differentiation, embryos carrying one of three different mutant alleles of *svp* were examined for the presence of the fat body. Stage 16/17 embryos homozygous for svp^{e22}, svp^{w+}, or svp^{2E2} were examined for the expression of *Adh* and *DCg1*. All three mutants exhibited the loss of *Adh* expression within the fat body (Fig. 4B) and an apparent reduction in *DCg1* transcripts within this same tissue (Fig. 4D). We currently can not distinguish between a loss of a subset of fat cells and a loss of *DCg1* expression within these cells. Fat cells that expressed *DCg1* appeared to have lower levels of transcript. The expression of *Adh* and *DCg1* in other cell types, however, remained unaffected. *svp* function appears to be necessary for tissue-specific expression of *Adh* in the fat body and may be involved in modulating the levels of *DCg1* transcript specifically within the fat body. These data provide further evidence that the *svp*-positive cells are precursor fat cells and suggest that *svp* plays a role in

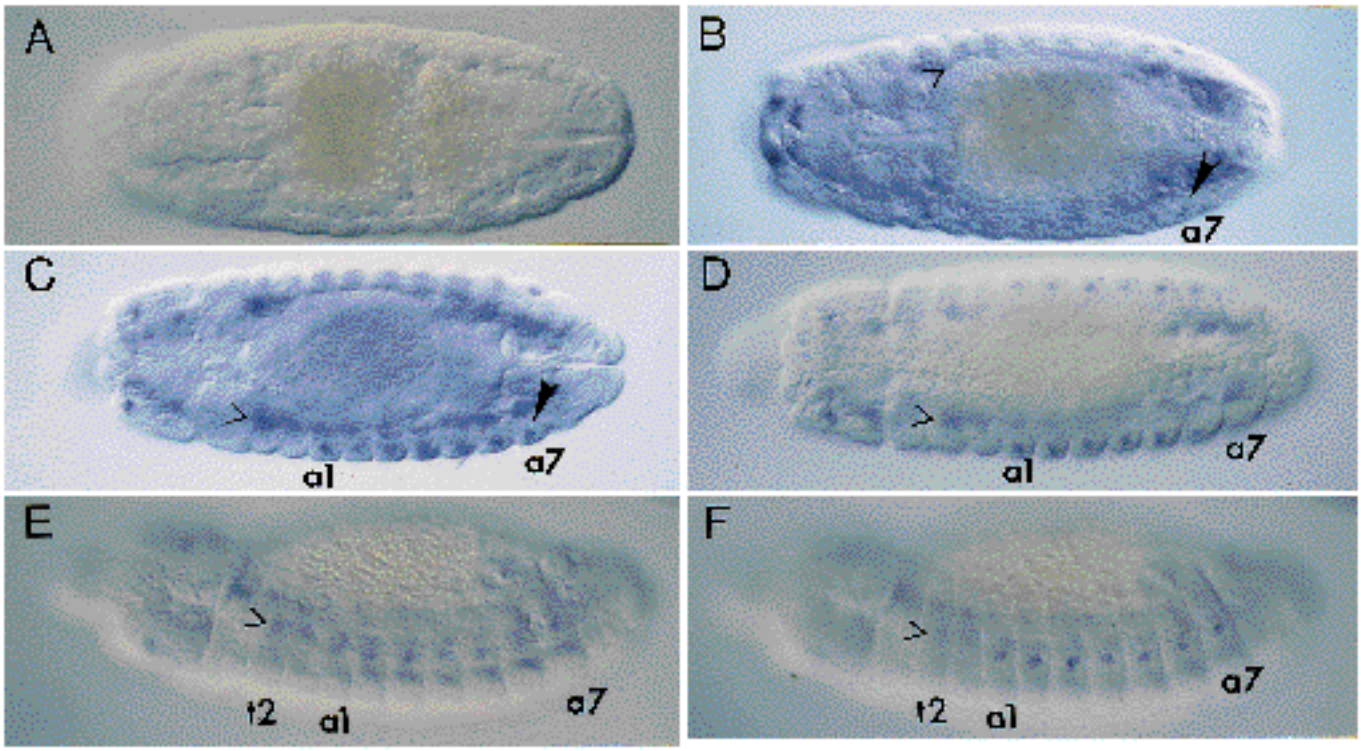

Fig. 3. Localization of *svp* transcript to precursor fat cells by in situ hybridization to whole-mount embryos. (A) Dorsal view stage-15 wild-type embryo. Note absence of *svp* transcript in the oenocytes and developing fat body. The oenocytes are located in each of the abdominal segments, a1 through a7. (B) Dorsal view of early stage-15 wild-type embryo. *svp* transcript was detected in the oenocytes [e.g., in a7, arrowhead] and faintly in the developing fat body [open arrowhead] in a bilateral band located between the body wall and gut and extending from thoracic segment t2 to abdominal segment a7. (C) Dorsal view of stage-14 wild-type embryo. *svp* transcript was detected in the oenocytes [e.g., in a7, arrowhead] and in the precursor fat cells [open arrowhead]. (D) Ventral view of late stage-12 wild-type embryo. svp transcript was detected in the oenocytes and in the precursor fat cells in a disjointed band of cells [open arrowhead]. (E,F) Lateral view of same embryos as in D at two different planes of focus. In E the *svp*-positive precursor fat cells were detected as a diffuse cluster of cells in t2 [open arrowhead] and t3 and in a1 though a7. In a more surface plane of focus (F), the oenocytes were readily detected immediately ventral but more external to the a1 through a7 precursor fat cells.

the regulation of at least two genes expressed during terminal fat-cell differentiation.

The *Drosophila* steroid receptor *HNF-4(D)* gene is the homologue to the mammalian liver transcription factor, *HNF-4* (Zhong et al., 1993). *HNF-4(D)* is reported to be expressed in the fat body, salivary glands, anterior and posterior midgut primordia and clusters of cells possibly related to the peripheral nervous system or endocrine system (Zhong et al., 1993). We localized *HNF-4(D)* transcripts within the developing embryo in anticipation of using *HNF-4(D)* as a cell marker to trace the fat-cell lineage. Surprisingly, we did not detect *HNF-4(D)* transcripts in the fat body or in any cells of the fat-cell lineage. In stage-14 and 15 embryos, we detected *HNF-4(D)* transcripts in the anterior and posterior region of the developing midgut (Fig. 5) as described by Zhong et al.(1993). *HNF-4(D)* transcripts were also detected in 6-cell clusters in each abdominal segment. These cells are oenocytes and not cells of the peripheral nervous system or endocrine system as previously described (Zhong et al., 1993). In an attempt to detect possible weak expression of *HNF-4(D)* in the developing fat body, we overstained embryos. We did not detect *HNF-4(D)* expression in the developing fat body even though expression in the anterior and posterior midgut and in the oenocytes was clearly evident (data not shown). Thus, we find no evidence to suggest that *HNF-4(D)* is expressed in the developing fat body. We can not eliminate the possibility, however, that *HNF-4(D)* is expressed at very low levels in this tissue.

To examine further the possible role of *HNF-4(D)* within the fat body, we examined the development of the fat body in TE196X1 homozygous embryos which carry a deletion spanning the *HNF-4(D)* locus (Zhong et al., 1993). We found in mutant embryos that the fat body was still present as shown by the expression of *Adh* and *DCg1* within the fat body (data not shown). Thus, we conclude that *HNF-4(D)* is not essential for the expression of at least two genes characteristic of terminal fat-cell differentiation.

Fat cells are derived from cells of the mesoderm

The genes *Adh* and *DCg1* have been used to identify the fat body in late stages of embryonic development. We have shown that *svp* is also expressed in the developing fat cells. These data have allowed the reconstruction of the fat-cell lineage from the fat body in stage-17 embryos to the early precursor fat cells at stage 12. We have identified a P-element enhancer-trap line, 29D, in which the reporter-gene activity is limited to the embryonic stages and mainly to the cells of the developing fat body. This line has allowed us to trace unambiguously the fat cell to its very progenitors in the developing mesoderm.

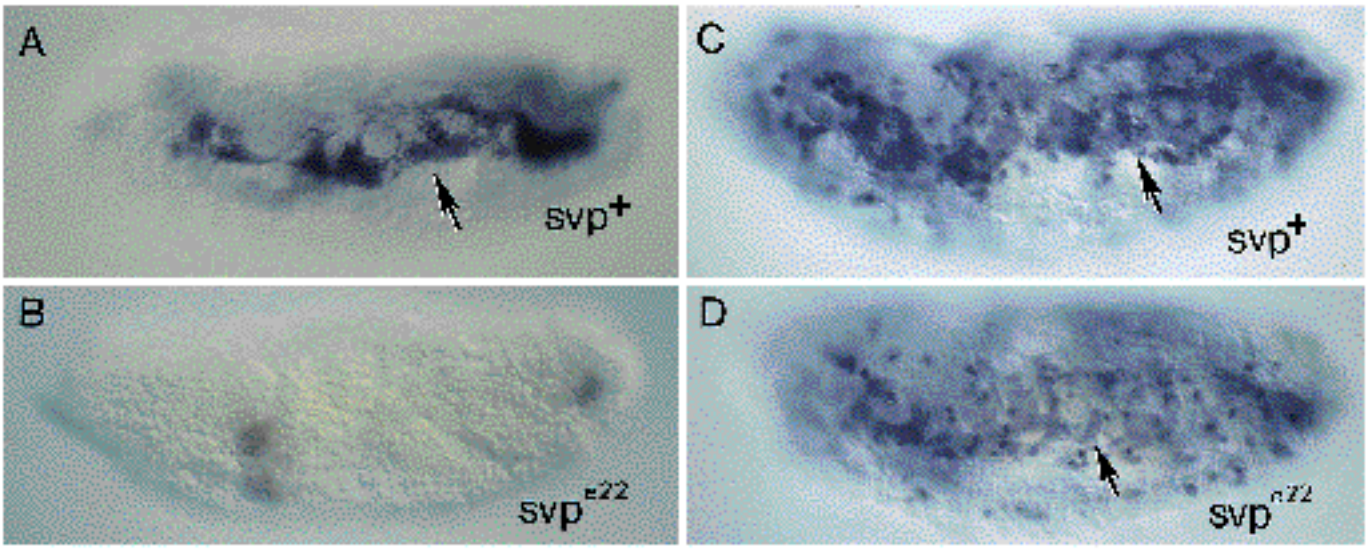

Fig. 4. Expression of terminal fat-cell differentiation genes *Adh* and *DCg1* in wild-type and *svp* mutant embryos. (A) Lateral view of svp^+ stage-16/17 embryo. Fat body [arrow]. (B) Lateral view of svp^{e22} stage-16/17 embryo. Note loss of *Adh* transcript in the location of the fat body. (C) Lateral view of svp^+ stage-16 embryo. Fat body [arrow]. (D) Lateral view of svp^{e22} stage-16 embryo. Low levels of *DCg1* transcripts were present in the fat body [arrow].

By immunohistochemical localization of β-galactosidase protein, transgene activity in the 29D line was detected in the developing fat cells in stage-15 embryos (Fig. 6A,B). By late stage 14, transgene activity was found in two lateral bands of precursor fat cells (Fig. 6C,D) that correspond to the *svp*-positive precursor fat cells. At stage 12, β-galactosidase protein, albeit at low levels, was detected in a disjointed bilateral band made up of nine bilateral sets of cells (Fig. 6E). Transgene transcripts, however, were easily detected in these cells (Fig. 6F). In stage-11 embryos, immediately ventral to the position of the lateral *nautilus* (*nau*)-expressing cells, transgene transcripts were detected in the nine bilateral clusters of cells. *nau* is the mammalian homologue of *MyoD* (Michelson et al., 1990; Paterson et al., 1991) and is expressed in a complex and dynamic pattern during embryogenesis. *nau* is restricted to cells of the muscle lineage. The 29D-positive cell clusters were located in the mesoderm in the regions corresponding to parasegments 4 through 12 (Fig. 6E,H); within each cluster there are approximately 8-12 cells (D. K. H. and C. P., unpublished results). In stage-12 embryos the cluster of cells expands dorsally; some of the cells overlap the lateral *nau*-expressing cells and others meet the most dorsal *nau*-expressing cells (Fig. 7). Based on examination of dissected embryos, it appears that the precursor fat cells are present in a mesodermal layer internal to the *nau*-expressing cells (Fig. 7B,C).

Using the 29D enhancer-trap line and the genes *Adh*, *DCg1* and *svp*, we have unambiguously reconstructed the lineage of the fat cell from the fat body to nine bilateral clusters of cells in the emerging mesoderm. These nine bilateral clusters detected by 29D-transgene activity were found to appear in the mesoderm at stage 11, the same developmental stage in which progenitor or founder muscle cells are recognized (Dohrmann et al., 1990; Michelson et al., 1990; Patterson et al., 1991). The appearance of the 29D-positive cells coincides with the establishment of muscle progenitor cells and, by analogy, the 29D-positive cells may represent the progenitors to the fat cells.

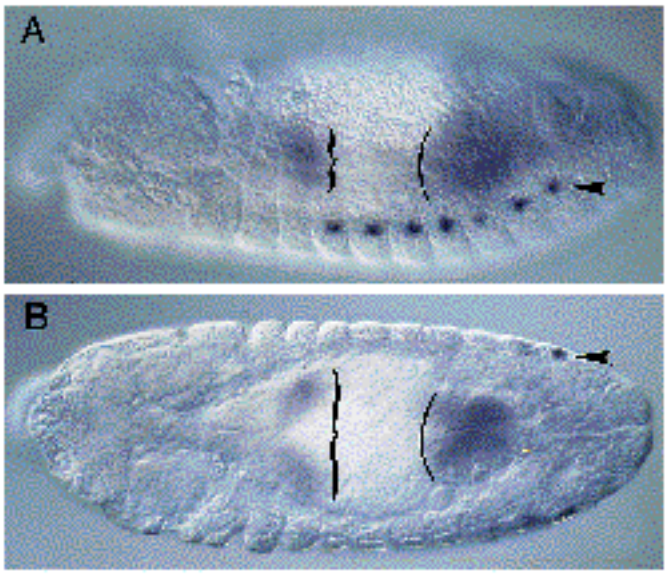

Fig. 5. Localization of *HNF-4D* transcript to anterior and posterior midgut and to the oenocytes by in situ hybridization to whole-mount embryos. (A) Lateral view of stage-14 wild-type embryo. *HNF-4D* transcript was detected in the oenocytes [e.g., in a7, arrowhead] and in the developing anterior [bracket] and posterior [arc] midgut. There were no *HNF-4(D)*-positive precursor fat cells located in a bilateral band between the body wall and the midgut. (B) Dorsal view of same embryo in A. Oenocytes [in a7, arrowhead], anterior [bracket] and posterior [arc] mid gut. *HNF-4(D)* transcripts were not detected in the precursor fat cells located in the thoracic segment t2 and t3 nor those associated with abdominal segments a1 through a7.

The region containing the 29D enhancer trap is necessary for fat-cell determination

The 29D transgene is expressed only in the embryo and is sensitive to a fat-cell lineage enhancer. It is possible that the

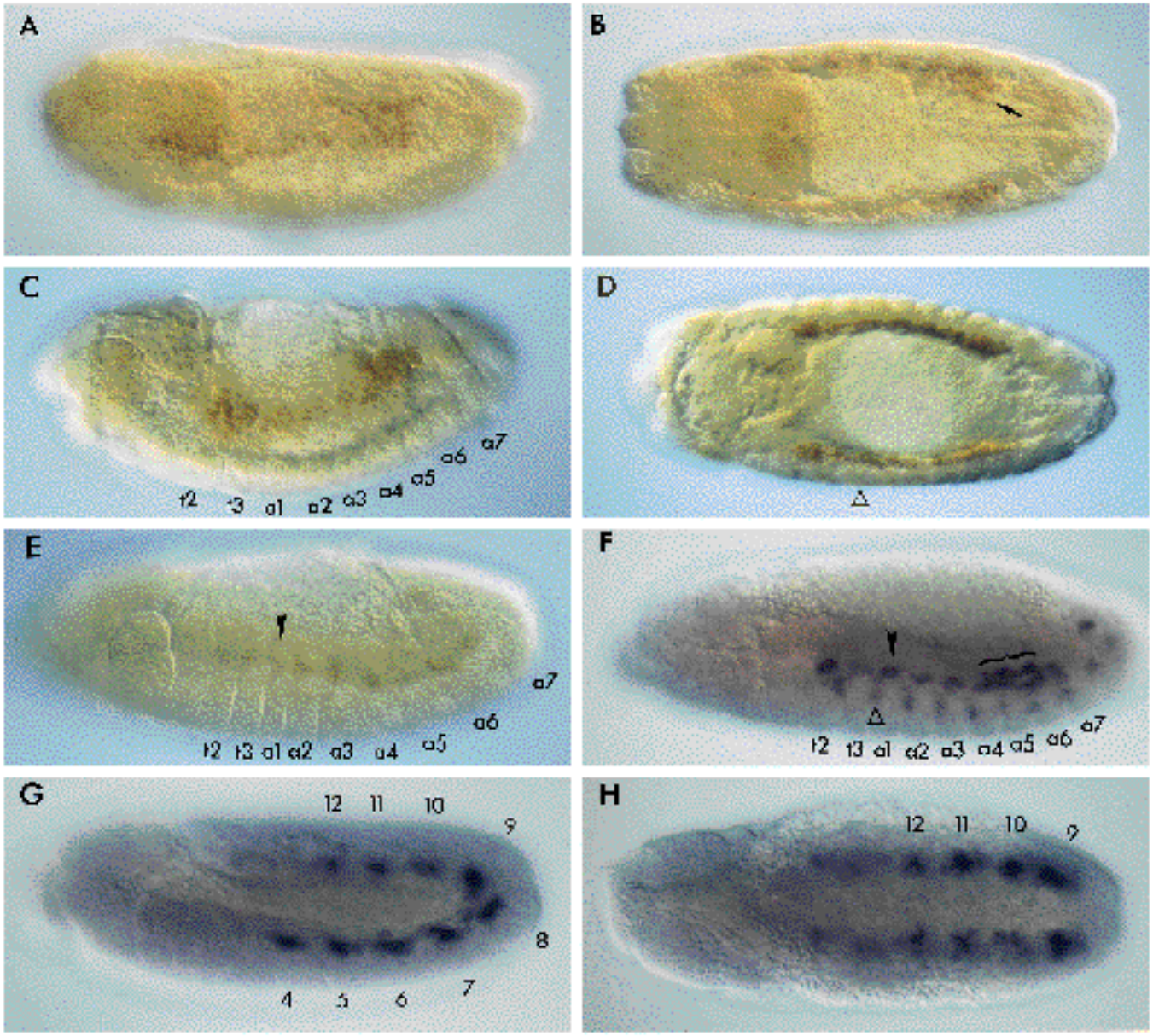

Fig. 6. Localization of *lacZ* reporter gene activity in whole-mount embryos from enhancer-trap line 29D. (A,B) Lateral and dorsal view, respectively, of same stage-15 embryo. β-galactosidase protein was detected in the nuclei of the cells of the fat body by immunohistochemical staining. Dorsal fat body is visible in B [arrow]. (C,D) Lateral and ventral view, respectively, of a stage-14 embryo. Precursor fat cells were located in thoracic segments t2 and t3 and abdominal segments a1 through a7 and were faintly detected in a8. Weak antibody cross-reactivity to β-galactosidase was also detected in the intersegmental grooves [e.g., between t3 and a1, open triangle in D]. (E) Lateral view of a late stage-12 embryo. Nine bilateral clusters of precursor fat cells were detected within thoracic segments t2 and t3 and abdominal segments a1 [solid arrowhead] through a7. (F) Lateral view of slightly older stage-12 embryo. *lacZ* transcripts were detected in the nine bilateral clusters of precursor fat cells associated with thoracic segments t2 and t3 and abdominal segments a1 [solid arrow head] through a6. In the more posterior segments (a4, a5 and a6) the number of precursor fat cells has expanded and extended across the a4-a5 and a5-a6 segment boundaries [F, bracket]. *lacZ* transcript was also detected in the intersegmental grooves [i.e., between t3 and a1, open triangle in F]. The transgene expression in the ectodermal cells was due to an enhancer associated with a gene distinct from the gene expressed within the fat-cell lineage (unpublished data). (G,H) Lateral and dorsal view, respectively, of same stage-11 embryo. *lacZ* transcript was detected primarily in nine bilateral clusters of cells associated with parasegments 4-12. (Parasegment 4 is equal to the posterior compartment of t1 and the anterior compartment of t2.) In slightly older stage-11 embryos, *lacZ* transcript was not detected in parasegment 13 and 14 (data not shown).

Drosophila gene associated with this enhancer plays a role in establishing the progenitor fat cell. If this gene is involved in fat-cell determination, then embryos lacking this gene might exhibit a loss or alteration in their fat-cell lineage. To begin an analysis of this putative fat-cell determination gene, we have examined embryos lacking the region containing the 29D enhancer trap. We mapped the 29D P-element insertion to cytological position 58DE by in situ hybridization to polytene chromosomes. Because the P-element insertion in the 29D line does not disrupt an essential gene, two deficiencies,

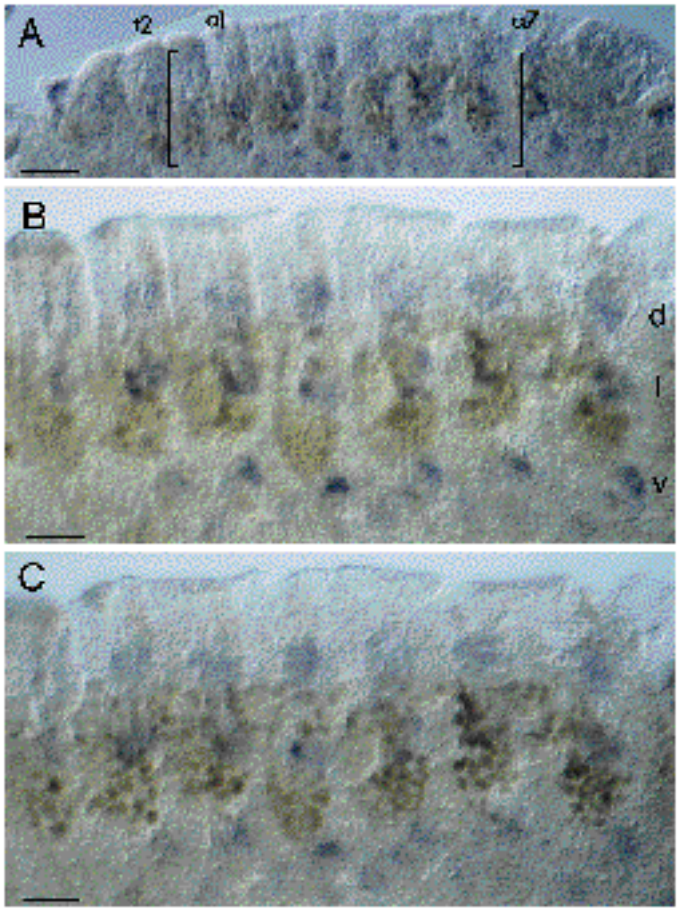

Fig. 7. Location of the precursor fat cells relative to *nau*-expressing cells. (A) Dissected late stage-12 29D embryo, internal side up. Brown-stained cells represent transgene activity in precursor fat cells. Blue-stained cells represent *nau*-expressing cells. Magnification bar represents 0.04 mm. (B) Higher magnification of A. Dorsal (d), lateral (l) and ventral (v) *nau*-expressing cells are in focus. Magnification bar represents 0.02 mm. (C) Same as B but a more internal (relative to the embryo) plane of focus. Precursor fat cells are in focus. *nau*-expressing cells and precursor fat cells are on different focal planes. Magnification bar represents 0.02 mm.

Df(2R)X58-1 and Df(2R)X58-3, were identified which span the site of P-element insertion (data to be presented elsewhere). To test whether a gene residing in this region is involved in fat-cell determination, *trans*-heterozygous [Df(2R)X58-1/Df(2R)X58-3] deficiency-bearing embryos were examined by in situ hybridization to whole-mount embryos. *Adh* and *DCg1* were used as cell markers for terminally differentiated fat cells, and *svp* as a cell marker for precursor fat cells. The *trans*-heterozygous deficiency-bearing embryos lacked *DCg1* (Fig. 8B) and *Adh* (data not shown) transcripts in the location of the fat body. These results are consistent with the loss of most, if not all, fat cells. Furthermore, these mutant embryos also lack *svp*-positive precursor fat cells (Fig. 8D). The loss of the region (58D6-8) as defined by the *trans*-heterozygous deficiency caused the alteration or loss of the fat-cell lineage. In contrast, the muscle-cell lineage appeared unaffected in embryos lacking 58D6-8. *nau*-expressing cells were detected in embryos lacking 58D6-8, although the expression pattern is slightly disordered (Fig. 8E,F). The visceral mesoderm also appeared to be unaffected based on the expression of Fasciclin III within this tissue (Patel et al., 1987) (data not shown). These data suggest that within the region containing the 29D enhancer trap (58D6-8) lies at least one gene necessary for the establishment of the fat-cell lineage and that this gene is not involved in the establishment of the muscle-cell lineage. It is likely that this gene is associated with the 29D enhancer.

DISCUSSION

Fat-cell lineage, adipogenesis and progenitor fat cells

The embryonic fat body is composed of a single layer of cells organized into a morphologically distinct tissue that forms a lateral ribbon with dorsal fingers extending from the posterior region. Using known genes (*Adh*, *DCg1* and *svp)* and an enhancer-trap line (29D), we have traced the fat-cell lineage from nine bilateral clusters of cells within the mesoderm to the fat body (summarized in Fig. 9). Our results are basically in concordance with recent results of Abel et al. (1993), which suggest that the fat body develops from segmentally repeated clusters of mesoderm cells, and with the description of late stages of fat-body development by Hartenstein and Jan (1992). However, we suggest that the precursor fat cells occupy the cell layer described as the inner mesodermal layer or splanchnopleura and not the somatopleura. The splanchnopleura also contains the visceral mesoderm and the progenitors of the dorsal vessel. The development of the fat body from nine bilateral clusters of cells appears to result from the expansion of the clusters and extension of cells arising from the a5/a6 region to form the dorsal fat body. The fat body does not appear to have a metameric structure. The organization of the progenitor fat cells, however, suggests that these cells may be segmentally arranged and thus may be regulated by genes involved in segment identity as first suggested by Rizki and Rizki (1978).

Progenitor fat cells, as represented by 29D-positive cells, are first detected at stage 11 during the specification of the mesoderm. Within the somatic mesoderm, single cells expressing the homeobox domain containing factor S59 can be identified (Dohrmann et al., 1990). These S59-containing cells may serve as founder muscle cells for lateral-oblique and ventral-transverse somatic musculature. Later, during germ-band retraction, *nau*-expressing cells which represent a set of progenitor muscle cells are detected in a segment-specific pattern in the dorsal, lateral and ventral positions (Michelson et al., 1990; Paterson et al., 1991). We have detected clusters of 29D-positive cells prior to germ-band retraction. By analogy to muscle development, we conclude that these 29D-positive cells represent the fat-cell progenitors. Loss of the region containing the 29D enhancer trap (albeit it includes several loci) resulted in the loss of the fat-cell lineage but appeared not to affect the myogenic lineage. Most, if not all, *nau*-expressing cells were still present although the pattern of expression is slightly disorganized, probably due to the absence of another locus (loci) mapping to 58D6-8. These results are consistent with the expression of the 29D transgene specifically in the progenitor fat cells.

Do these clusters of cells represent the entire complement of

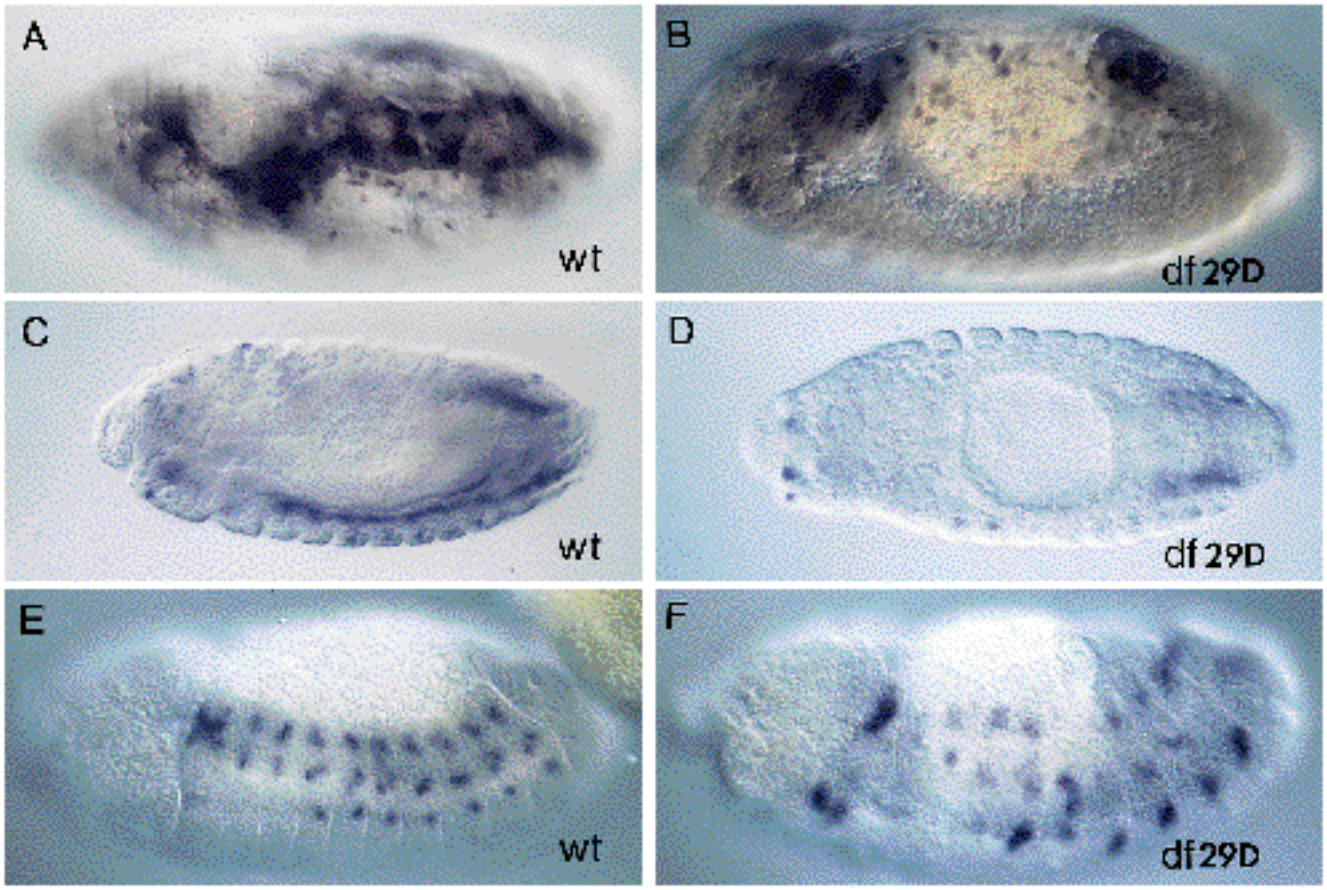

Fig. 8. Expression of the terminal fat-cell differentiation marker, *DCg1*, precursor fat-cell marker, *svp* and progenitor muscle-cell marker, *nau*, in wild-type embryos and embryos lacking the region containing the 29D enhancer trap. (A) Lateral view of wild-type (wt) stage-16/17 embryo. Fat body [arrow]. (B) Lateral view of stage-16/17 embryo lacking the region containing the 29D enhancer trap (df 29D). Note loss of expression of *DCg1* in the location of the fat body [arrow]. (C) Dorsal view of wild-type stage-13/14 embryo. Precursor fat cells [arrow]; oenocytes [arrowhead]. (D) Dorsal view of stage-13/14 embryo lacking the region containing the 29D enhancer trap. Note loss of expression of *svp* in the location of the precursor fat cells and the presence of *svp* transcript in the oenocytes [arrowhead]. (E) Lateral view of wild-type stage-12 embryo. *nau* expression in dorsal, lateral and ventral clusters of progenitor muscle cells. (F) Lateral view of stage-12 embryo lacking the region containing the 29D enhancer trap. *nau*-expression is detected in dorsal, lateral and ventral clusters of progenitor muscle cells. Note the slight disorder in the expression pattern.

progenitors to the fat body? Estimations based on cell numbers in the larval fat body and the assumption that the larval fat body like other larval tissues grows by cell expansion, suggest the embryonic fat body contains 2470 (female) or 2160 (male) cells (Rizki, 1978). In contrast, direct estimations using embryonic fat-cell markers suggest the fat body is made up of about 1600 cells (Hartenstein et al., 1992). Based on the number of embryonic fat cells, each progenitor fat-cell cluster should contain, on average, 89 cells. At stage 11, however, each 29D-positive cluster contains significantly fewer cells (approximately 8-12 cells). It is possible that these cells represent 'founder' cells that serve as focal points for recruiting cells into the fat-cell lineage analogous to founder cells proposed for muscle development (Bates, 1990). Alternatively, these 29D-positive cells may represent only a subset of the progenitor fat cells.

When does fat-cell differentiation begin? Based on histological studies, terminal fat-cell differentiation is not recognized until the accumulation of lipid just before hatching (Poulson, 1950; Campos-Ortega and Hartenstein, 1985). In a manner similar to defining the stages of adipogenesis in mouse (Ailhaud et al., 1992), we have taken advantage of the temporal expression patterns of *Adh*, *DCg1* and *svp* to define stages of fat-cell differentiation within the developing embryo and to name the cells in the fat-cell lineage (Fig. 9). Both *Adh* and *DCg1* were expressed in the developing fat body as early as stage 15. Because *Adh* and *DCg1* are involved in the metabolic function of the fat cell, we have used the initial expression of these genes at stage 15 to mark the beginning of terminal fat-cell differentiation and to identify late precursor fat cells.

Similarly, we have used *svp* as a marker for early precursor fat cells. The *svp* gene was transiently expressed in the fat-cell lineage and was first detected at stage 12 in nine bilateral sets of cells that form two disjointed lateral bands. *svp* is expressed before either *Adh* or *DCg1* and is a fat-cell-specific regulator of *Adh* and *DCg1* expression (discussed below). We have identified stage 12 as the beginning of fat-cell differentiation and the *svp*-positive cells at this stage as early precursor fat cells.

svp is the first gene to be identified that may be involved in embryonic fat-cell differentiation. Loss of *svp* function resulted

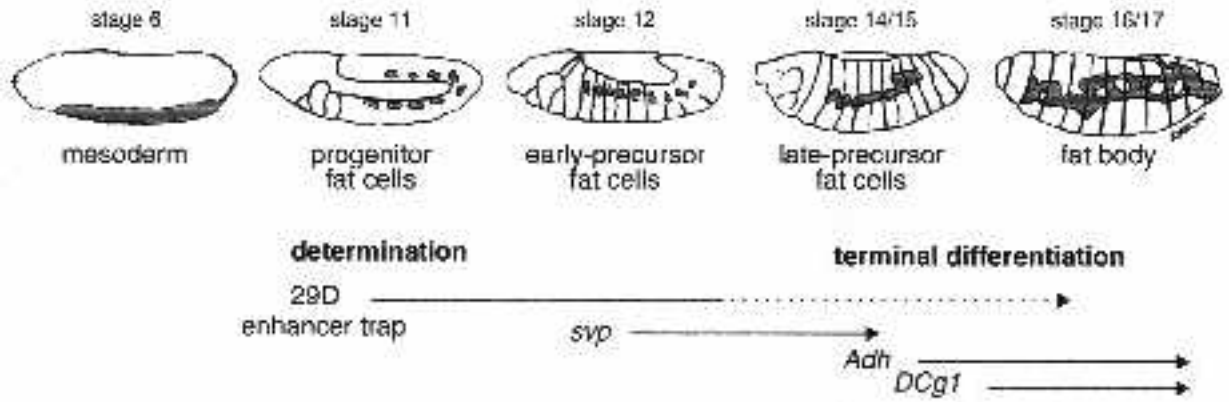

Fig. 9. Summary of the fat-cell lineage and its proposed relationship to fat-cell differentiation.

in the tissue-specific loss of *Adh* expression and a reduction of *DCg1* transcripts in the fat body. Thus, *svp*, plays a role in the fat-cell-specific expression of at least two genes. *svp* codes for a putative steroid-hormone receptor gene. It may function in the transcriptional activation of certain fat-cell metabolic genes during terminal fat-cell differentiation.

We have inspected the embryonic expression of the *Drosophila* homologue of a steroid-hormone receptor gene, *HNF-4*, a mammalian transcription factor involved in the activation of a wide array of liver-specific genes (Costa et al., 1989, Sladek et al., 1990; Mietus-Snyder et al., 1992). The *Drosophila HNF-4(D)* has been reported to be expressed in the embryonic fat body, salivary glands, and the anterior and posterior midgut primordium (Zhong et al., 1993). We found, however, that *HNF-4(D)* is not detected in the fat body nor does the lack of *HNF-4(D)* affect the formation of the embryonic fat body.

The gene *ABF* codes for a factor which is a member of the GATA family of transcriptional regulators (Abel et al., 1993). ABF is implicated in the regulation of *Adh* and is expressed in the embryonic fat body (Abel et al., 1993). In the cellular blastoderm, *ABF*, is detected in several regions, including the anterior and posterior midgut primordia and the primordium of the cephalic mesoderm. At germ-band retraction, *ABF*-positive mesodermal cells are detected in segmentally repeating clusters and later expand to form the fat body. These *ABF*-expressing cells in the mesoderm are most likely identical to cells comprising the fat-cell lineage as described here. *ABF* maps to 89B and does not correspond to the gene associated with the 29D enhancer. Whether *ABF* is necessary for expression of *Adh* in the fat body or the development of the fat cell is not known.

A gene necessary for establishing the fat-cell lineage is most likely located in the region containing the 29D enhancer trap. 29D-positive cells were detected immediately before full germ-band extension (D. K. H. and C. P., unpublished results) in the progenitor fat cells. Embryos lacking the region containing 29D enhancer trap no longer produced *svp*-positive early precursor fat cells and concomitantly did not exhibit *Adh* or *DCg1* expression in the fat body. Loss of this region specifically affects the fat-cell lineage and appears not to affect the myogenic lineage. It is likely, therefore, that the 29D enhancer is associated with a gene necessary for the correct establishment of the fat-cell lineage.

We have identified the embryonic fat-cell lineage and provide genetic evidence that the fat-cell lineage is derived from nine bilateral clusters of cells that represent the fat-cell progenitors. We hope that by characterizing the fat-cell lineage we have established a system that will enable the identification and analysis of the genetic hierarchy of events that take place during fat-cell determination and differentiation.

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