headcase, an imaginal specific gene required for adult morphogenesis in Drosophila melanogaster

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SUMMARY

The majority of adult organs of a holometabolic insect like Drosophila melanogaster are derived from specific imaginal cells. These cells differ from their larval equivalents in many important cellular characteristics, ranging from the nature of the cell cycle to the timing and pattern of cellular differentiation. Here we describe the cellular, molecular and genetic characterization of a gene, headcase (hdc), which is required for imaginal cell development. hdc is the first gene to be described which is specifically expressed in all imaginal cells; this has allowed us to identify many imaginal primordia in the embryo and follow their morphogenesis throughout embryonic and larval development. The Hdc protein is an extremely basic (pI 9.6) cytoplasmic protein with no obvious sequence similarities or conserved motifs. Interestingly, the spatial-temporal pattern of hdc expression prefigures imaginal cell re-entry into the mitotic cell cycle and persists until the final cell divisions. hdc null alleles have been isolated and found to cause pupal lethality, with dead pharate adults exhibiting defects in the differentiation of many adult tissues, most notably in head development. Ectopic expression of hdc, provided by a hdc-lacZ minigene, rescues the pupal lethality. Imaginal disc morphology in null mutants appears normal, therefore loss of hdc expression does not affect imaginal cell growth, but instead interferes with the ability of the imaginal primordia to differentiate properly during pupal development, suggesting that hdc may be involved in hormonal responsiveness during metamorphosis.

Key words: Drosophila, imaginal primordia, adult morphogenesis, proliferation, hormone, headcase

INTRODUCTION

During the life cycle of a holometabolic insect like Drosophila melanogaster the body plan is laid down in two forms, one larval and one adult (imago). The vermiform larval plan is established during embryogenesis and, for the most part, does not change significantly in cell number or overall organization through three successive larval instars. In contrast, the majority of adult organs are derived from distinct lineages of imaginal cells, best seen in the mature third instar (Fig. 1A). These cells undergo a strict program of proliferation and patterning throughout postembryonic stages and, through hormonal signalling, are instructed to initiate a series of defined morphogenetic processes that result in the histolysis of larval organs and the genesis of the adult body shape. (For reviews of imaginal development see Bodenstein, 1950; Bryant, 1978; Cohen, 1993; Fristrom and Fristrom, 1993.)

What are the embryonic origins of imaginal tissue? Classic transplantation studies in the embryo have shown that the imaginal lineage is allocated sometime between cellular blastoderm (Chan and Gehring, 1971; Simcox and Sang, 1983) and the onset of gastrulation (Meise and Janning, 1993). Morphological studies for the presence of the imaginal cells showed that the thoracic imaginal disc primordia can be detected at precise positions in the early embryo. For example, each leg disc primordium first emerges as a cluster of cells which invaginate underneath the larval epidermis, adjacent to the CNS and towards the middle of each thoracic hemisegment (Bate and Martinez-Arias, 1991); whereas the wing and haltere disc primordia are located more dorsally, and invaginate deeper underneath the epidermis (Anderson, 1963; Bate and Martinez-Arias, 1991). Morphological criteria alone are insufficient to identify the other imaginal primordia. Therefore, gene(s) or P-lacZ enhancer trap lines expressed in particular imaginal tissues have been used as molecular markers to trace the embryonic origins of some primordia including adephelial cells, histoblasts and imaginal discs (reviewed in Cohen, 1993; Martinez-Arias, 1993).

One of the most important aspects of the imaginal developmental program is the regulation of the cell cycle. Following the 16th embryonic mitosis, both imaginal and larval cells (with the exception of certain neural lineages) undergo G1 arrest (reviewed in Foe et al., 1993). Importantly, larval cells never re-enter the mitotic cycle, but instead follow a strict spatiotemporal pattern of endoreplication, leading to polyploidy. In contrast, imaginal cells remain committed to a future program of mitotic growth, the timing of which is tightly regulated and varies between the different imaginal primordia. For example, the onset of division in the eye disc begins 14 hours into the first larval instar (Madhavan and Schneiderman, 1977), whereas his-
toblast cells remain quiescent throughout larval development, but upon pupariation actively proliferate and replace the histolyzing larval epidermis by 40 hours postpupariation (Madhavan and Schneiderman, 1977; Roseland and Schneiderman, 1979).

What are the mechanisms that maintain the proliferative capacity of quiescent imaginal cells and what regulates the timing of their re-entry into the mitotic cell cycle? Recent progress on the escargot gene has demonstrated that diploidy (and possibly other imaginal tissues) is maintained by the suppression of endoreplication (Hayashi et al., 1993), most likely through transcriptional repression of subordinate genes (Fuse et al., 1994). Studies of the anachronism gene (ana), mutations in which cause premature activation of imaginal neuroblast proliferation in the larval brain, have demonstrated that ana acts non-autonomously to suppress proliferation of imaginal neuroblasts by an unknown mechanism (Ebens et al., 1993). This suppression of neuroblast proliferation is overcome by the activity of the trol gene (‘terribly reduced optic lobes’: Datta and Kankel, 1992), which acts downstream of ana possibly by inactivating the ana repressor or bypassing its requirement (Datta, 1995).

Strict control of imaginal cell proliferation is necessary for synchronizing tissue growth with the hormonal signals initiating metamorphosis. This link between imaginal proliferation and the endocrine system is best illustrated in tumor suppressor gene mutations that result in imaginal disc overgrowth (Bryant and Schmidt, 1990). In general, these mutants exhibit delayed puiariation and an associated reduction in ecdysteroid titer (Sehnal and Bryant, 1993). A similar developmental delay is observed when imaginal disc tissue regeneration is induced in situ by heat-sensitive cell-lethal mutations (Simpson et al., 1980). These and many other observations have led to the model in which proliferating imaginal disc cells control the timing of differentiation by interfering with ecdysone release (Poodry and Woods, 1990); however, the molecular mechanisms remain unclear.

Imaginal development is a powerful model system in which to study the fundamental relationships between primordial cell growth regulation and morphogenesis during organogenesis. Our interests are focused on the problems of how imaginal and larval cell growth are differentially regulated, and how this is linked with the timing of adult organ differentiation. Here we report the molecular genetic characterization of a gene called headcase (hdc), which is expressed in all proliferating imaginal lineages. This specificity has allowed us to identify the embryonic origin of different imaginal primordia and follow their lineage throughout embryonic and larval stages. hdc expression prefigures imaginal cell re-entry into the mitotic cell cycle and continues until the end of primordia growth. Null alleles have been generated and found to cause a pupal lethal phenotype associated with the developmental arrest of many adult organs, all aspects of which can be rescued by expressing Hdc protein from a transgene. Interestingly, the morphology of mutant discs appear normal, suggesting that imaginal growth regulation is unaffected; instead, the defect appears to be in the ability of the imaginal cells to undergo proper differentiation during metamorphosis.

MATERIALS AND METHODS

Stocks
The wild-type stock used was Oregon-R, obtained from John Roote. TMZ is a third chromosome ‘blue balancer’ harbouring an enhancer trap that expresses lacZ uniformly in the epidermis following germline band retraction (called C40.1S3 in Bellen et al., 1989), obtained from Cahir O’Kane. The P[lacZ, ry+] transformatant line, B5, was originally selected by Alex Gould from a collection of lacZ enhancer traps, generated by John Merriam using the strategy of O’Kane and Gehring (1987), Marker mutations are as described in FlyBase (FlyBase, 1994).

Dissections
Embryo fillets were performed as described in White and Wilcox (1984). Embryonic stages of development were scored according to Campos-Ortega and Hartenstein (1985). Larvae were collected as newly hatched first instars and then aged on yeasted apple juice plates, and appropriate larval stages were scored by morphology of mouth hooks as described in Ashburner (1989). Internal tissues were dissected from bisected and inverted half-larvae. For epidermis preparations larvae were heat killed 5 seconds at 55°C before dissection in order to relax the body wall musculature. Pupae were collected as white puparia (stage P1, stages described in Baimbridge and Bownes, 1981), then aged 4-5 days at 25°C on water-saturated Whatman paper kept in a Petri dish. Pherate adults (stage P14/15) were dissected from pupal cases pinned out on a Sylgard-filled Petri dish.

X-gal staining
Embryo whole-mount and flat preparations were X-gal stained as described in O’Kane and Gehring (1987). Devitellinization was performed using ethanol rather than methanol to avoid inhibition of β-gal activity. Larval tissues were stained as described in Prokop and Technau (1991).

Cloning and sequencing the hdc gene
A 450 bp junction fragment (JF, Fig. 3A) flanking one end of the P[lacZ, ry+] element was isolated from a B5 genomic library, made in lambda Zap (Stratagene), and then used to initiate a 65 kb chromosome walk surrounding the insertion site (EMBL3 genomic library kindly provided by John Tamkun). Overlapping DNA fragments of the walk were used for mapping of the hdc transcript by in situ hybridization (see below). Three probes (grey boxes, Fig. 3A) detected a transcript with an identical distribution pattern to B5-lacZ. Probe G’ (Fig. 3A) was used to isolate two overlapping hdc cDNAs, hdc.NB40 and hdc.K, from two different 12-24 hour embryonic libraries (plasmid library kindly provided by N. Brown, described in Brown and Kafatos (1988); gt10 library kindly provided by L. Kauvar). Double-stranded sequence analysis was compiled using Staden and GCG software. Searches for nucleotide and protein similarities between the full-length hdc sequence and known genes in the GenEMBL, OWL and Prosite databases were performed using FASTA, FLASH, BLAST, BLOCKS and MotifFinder programs.

In situ hybridization
Digoxigenin-labelled DNA probes (Boehringer Mannheim) were made from gel-purified restriction fragments (varying in length from 2-8 kb) spanning the chromosome walk. Digoxigenin-labelled RNA probes (Boehringer Mannheim) were transcribed in vitro from phd.NB40 using T7 (anti-sense probe) and SP6 (sense probe) polymerases. Hybridizations were performed as described in Gould and White (1992), with DNA probes at 0.5 µg/ml and RNA probes at 0.1 µg/ml (with respect to starting template).

Generating hdc alleles and heat-shock transformants
All hdc deficiencies were derived from an isogenic B5 line by imprecise excision of the P-element. This was achieved by crossing in a source of transposase (Robertson et al. 1988) and resulting dysgenic male progeny were individually crossed to ry[106]TM3 Sb ry females. 150 individual ry progeny were back-crossed to tester females in order to isogenize each excision chromosome. 8 lethals
were obtained and complementation tests revealed that all 8 lethals fell into the same complementation group. Breakpoints in hdc alleles 43, 50, 97 and 117 were mapped by genomic Southern blot hybridization as described in Daniels et al. (1985) using radioactive probes made from the chromosome walk. The HS-hdc vector was constructed by subcloning the hdc.NB40 cDNA downstream of the heat-inducible hsp70 promoter in the P-element vector pCaSpeR-hs (kindly provided by Carl Thummel) which has a mini-white+ selectable marker. Transformants were made using P-element-mediated germline transformation of y w host embryos as described in Spradling (1986). Six independent white+ transformant lines were isolated, three of which (lines 38.5, 77.1 and 87.1) mapped to the second chromosome and were crossed into a hdc43 ry/TM3 Sb ry background. One of these lines, 87.1, completely rescued the pupal lethality, even without heat shock, as measured by the presence of ry Sb+ progeny from the cross:
y w; P[w+m]; HS-hdc43; hdc43 ry/TM3 Sb ry males × y w; P[w+m]; HS-hdc43; hdc43 ry/TM3 Sb ry females

Analysis of mutants
Pupal lethality
Overnight egg lays were collected in yeasted agar vials, allowed to pupate, and then scored for the number of eclosed or dead pupae once no more emerging flies were observed. Alternatively, individual cohorts of newly pupariated animals from large bottle cultures were transferred to Petri dishes as above and then scored for pupal lethality after 1 week.

Histology
Dissected pharate adult heads were wax embedded and 8 μm serial histological sections were cut with a Reichert manual microtome. Sections were stained with Harris’ haematoxylin and eosin.

Scanning EM
Dissected pharate adults were fixed overnight at 4°C in 0.1 M Pipes (pH 7.4)/2% glutaraldehyde/2% formaldehyde. Fixed specimens were treated with 1% osmium tetroxide, dehydrated in a graded series of alcohols and critical point dried. Specimens were then coated with 20 nm of gold and viewed in a JEOL JSM 35 microscope.

Monoclonal antibody preparation and antibody staining
A 1.25 kb fragment from phdc.NB40, encoding 417 amino acids of Hdc protein (underlined residues in Fig. 3B), was subcloned into pRSETC (Invitrogen) and pGEX-3X (Pharmacia) bacterial expression vectors in order to generate unique fusion proteins of relative Hdc protein (underlined residues in Fig. 3B), was subcloned into pRSETC (Invitrogen) and pGEX-3X (Pharmacia) bacterial expression vectors in order to generate unique fusion proteins of relative

RESULTS
The B5 enhancer trap is a marker of imaginal tissues of the developing Drosophila larva
In an enhancer trap screen for genes potentially regulated by homeotic genes of the bithorax complex (Gould, 1990), we discovered one transformant line, B5, where lacZ is specifically expressed in imaginal cell populations of the mature larva (Fig. 1B-N). At this stage, the imaginal cells can be distinguished from their larval counterparts by a characteristic small cell size, tissue morphology and positioning within the larval body plan (see schematic, Fig. 1A; Bodenstein, 1950; Bryant, 1978). In addition to the classic imaginal ‘discs’, the B5-lacZ pattern also highlights the imaginal precursors of gut, genitalia, CNS, respiratory system and epidermis. We consider each aspect of this expression pattern in detail below.

The B5-lacZ pattern in the ventral nerve cord corresponds to the distribution of imaginal neuroblasts (Truman and Bate, 1988), as well as their postembryonic lineages, with many more expressing cells in the thoracic than in the abdominal neuromeres (in, Fig. 1N). The posterior tip of the ventral nerve cord also has many more lacZ-expressing cells than in abdominal neuromeres. Interestingly, it is this region where sexually dimorphic neuroblasts are proliferating (Truman and Bate, 1988) and most likely represent the precursors of neurons destined to innervate the genitalia. The larval pattern diminishes during the early stages of pupal development, and is replaced by strong expression in the developing central brain and optic centers (data not shown).

In the larval respiratory system, B5-lacZ expression is observed in imaginal tracheoblasts (Whitten, 1980; Manning and Krasnow, 1993), which are easily identified by their small size compared to neighboring larval tracheal cells and close association with the larval tracheal network. Two types of clusters are seen, one round and one elongate. Round clusters are found in thoracic segments only, and are situated next to the dorsal longitudinal trunk (Fig. 1D). Elongate clusters are stretched along the visceral and spiracular branches, which emanate from the transverse connectives (Fig. 1F) and are segmentally repeated down the length of the larval trunk. The other respiratory-associated cell types represented in the B5-lacZ pattern include the dorsal prothoracic discs associated with the anterior spiracles (Fig. 1B) and the spiracular histoblast nest found in the epidermis of abdominal segments A1-A7 (Fig. 1I).

Larval glands that express B5-lacZ include the imaginal ring of the salivary gland (Fig. 1C), the ring gland (Fig. 1N), the lymph gland and pericardial cells flanking the length of the aorta (both not shown). Alimentary expression includes the foregut and hindgut imaginal rings, and midgut imaginal islands (Fig. 1J,M). B5-lacZ is also expressed in the larval gonads. In the male, B5-lacZ is found in apically associated cells of the testis (Fig. 1G). These cells correspond to the mitotically dividing gonial cells (Cooper, 1950; Fuller, 1993) and remain positive in the adult male, where expression is concentrated at the anterior tip of the testis (data not shown). The terminal cells (Fig. 1H) at the posterior tip are of unknown identity; however, they continue expressing B5-lacZ during pupation as this group of cells grow out towards the developing genital disc, presumably forming the seminal duct epithelium (Cooper, 1950). In the female, B5-lacZ is expressed uniformly throughout the ovary (Fig. 1L).

The embryonic development of imaginal primordia can be followed using the B5 marker
Since the lacZ expression pattern of the B5 line is a unique marker for larval imaginal cells, it should allow us to identify the embryonic origins and morphogenesis of these tissues. We focus here on the development of imaginal structures in
Fig. 1. Imaginal tissue specificity of the enhancer transformant line B5. Wandering third instar larvae from the transformant line B5 were stained with X-gal in order to detect the nuclei (blue) of lacZ-expressing cells (B-N). (A) Schematic illustration of a third instar larva dissected along the ventral midline and laid flat on the right, with tracheal trunk pulled towards the right hand edge. Imaginal tissues are highlighted blue. (B) Dorsal prothoracic imaginal disc surrounded by the anterior spiracle. (C) Salivary imaginal ring at the junction between the salivary duct and salivary gland. (D) Clusters of imaginal tracheoblasts clinging to branch points along the larval tracheal tree in thoracic hemisegments. (E) Imaginal discs: w, wing; l1, metathoracic leg and h, haltere. (F) Imaginal tracheoblasts, as in D, illustrating the segmentally repeated nature of the elongated clusters. (G) Gonial cells and (H) terminal cells of the testis. (I) Histoblast nests: v, ventral nest; da, dorsal anterior nest and dp, dorsal posterior nests; sp, spiracular nest. (J) Hindgut imaginal ring. (K) Genital imaginal disc. (L) Ovary. (M) Foregut imaginal ring, fr, located anteriorly along the inner surface of the proventriculus, and surrounding the oesophagus. The midgut imaginal islands, mi, are interspersed as in J, but not in the gastric caeca, gc. (N) Imaginal tissue associated with the larval CNS and head skeleton: e, a, eye-antennal disc; l1, prothoracic leg disc; l2, mesothoracic leg discs; lb, labial disc and cl, clypeolabral discs; in, imaginal neuroblasts; r, ring gland.

Fig. 2. B5-lacZ expression pattern reveals the embryonic origin of imaginal primordia. Homozygous B5 embryos were stained with anti-β-gal antibodies as whole-mount (A-F) or flat preparations (G,H). (A,B) Ventral and lateral aspects of stage 13 embryos highlighting the earliest detectable pattern. Prominent features include: a cluster of neural cells in each thoracic hemisegment of the CNS, white arrow, these persist throughout early development and form the imaginal neuroblast population; a stripe of epidermal cells adjacent to the posterior edge of the T1 segment, open arrow, which gives rise to the prothoracic disc; three leg disc primordia, arrowheads; a pair of epidermal cells, asterisk, of unknown identity repeated from A1-A7. (C,D) Ventral and lateral aspects of stage 15 embryos illustrating the dynamic change in expression pattern, especially in the CNS where the number and intensity of staining cells increases. The three leg disc primordia have increased in staining intensity and are shifting anteriorly as a consequence of head involution, this is much more apparent in F. The genital disc primordia, gd, is detected posterior to the ventral nerve cord, slightly beneath this focal plane. The prothoracic disc primordia has invaginated along with the anterior spiracle (also see F,G). Cells from the T2 and T3 leg primordia have begun migrating dorsally, these cells will populate the wing and haltere disc primordia (arrowheads in D, also see F,G). Intense staining in the brain lobes, oesophageal ganglia, clypeolabral, labial and eye-antennal primordia can be discerned in deeper focal planes (data not shown). (E,F) Ventral and lateral aspects of stage 17 embryos displaying the mature embryonic pattern of imaginal primordia. The leg discs have migrated further anteriorly, forming tear-drop-like pockets stretching dorsally towards the wing, w, and haltere, h, disc primordia. Each abdominal segment exhibits two additional groups of cells: a line of imaginal tracheoblasts, tr, and a second pair of cells of unknown identity, asterisk, just internal to the epidermal cells seen in B and D. Staining is also seen in pericardial cells, lymph gland, and testis (data not shown). (G) Stage 17 embryo with CNS removed to reveal all thoracic imaginal disc primordia: prothoracic, p; wing, w; haltere, h; prothoracic leg, l1; mesothoracic leg, l2; metathoracic leg, l3. (H) Internal organization of abdominal segments in a stage 17 embryo. As in F, each segment contains a string of approximately 8 imaginal tracheoblasts, tr, attached to the visceral branch of the dorsal longitudinal trunk, and two sets of 2 cells, each of unknown identity, one set sitting on the ventral internal oblique muscles, asterisk, and the other pair located in the epidermis underneath this focal plane.
Fig. 3. Molecular analysis of the \textit{headcase} gene. (A) Schematic summarizing how the \textit{headcase} gene was identified and mutated. The junction fragment, JF, 65 kb chromosome walk, and B5 enhancer trap element \textit{P\{lacZ, \textit{p}3.7\} \textit{+}} are included on a thick black line measured out in 1 kb units (below) and selected restriction sites (above). Boxes F, G, G', p3.7 and \textit{H} are representative DNA probes used for mapping the \textit{headcase} transcription unit; those filled grey gave positive signals. Black boxes represent \textit{hdc} cDNAs. Thin black lines underneath genomic walk represent the breakpoint mapping of four independent \textit{hdc} deletion mutants (break in line), allele names are indicated to the right. (B) Nucleotide and theoretical translation of cDNA sequences, with \textit{hdc.K} beginning at nucleotide 14 and ending at 1371, and \textit{hdc.NB40} beginning at nucleotide 356 and ending at 4627. Two large ORFs are separated by a single amber stop codon (boxed) beginning at nucleotide 2980 (sequences encoding the downstream single amber stop codon (boxed) beginning at nucleotide 14 and ending at 1371, indicated to the right. (B) Nucleotide and theoretical translation of cDNA sequences, with \textit{hdc.K} beginning at nucleotide 14 and ending at 1371, indicated to the right. (B) Nucleotide and theoretical translation of cDNA sequences, with \textit{hdc.K} beginning at nucleotide 14 and ending at 1371, indicated to the right. (B) Nucleotide and theoretical translation of cDNA sequences, with \textit{hdc.K} beginning at nucleotide 14 and ending at 1371, indicated to the right. (B) Nucleotide and theoretical translation of cDNA sequences, with \textit{hdc.K} beginning at nucleotide 14 and ending at 1371, indicated to the right.

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Thoracic and abdominal segments, although we have also been able to identify the eye-antennal, clypeolabral and labial disc primordia (data not shown).

The rudiments of the strong staining in the thoracic segments of the CNS are discernible by stage 13 of embryogenesis (Fig. 2A) and mature as the ventral nerve cord condenses (Fig. 2A,C,E). This pattern prefigures the larval pattern of postembryonic imaginal neuroblasts (IN), which is unusual since other IN markers, such as Grainyhead protein (Bray and Kafatos, 1991), do not show segmental modulation until the very latest stages of embryogenesis.

As in the CNS, the pattern of B5-lacZ expression in the epidermis differs significantly between thoracic and abdominal segments. The reason for this is the presence of the imaginal disc primordia in thoracic segments, the relative positions of which are shown in Fig. 2G. The ventral discs (arrowheads) and the dorsal prothoracic disc (open arrow) are the first detected following germ band retraction, early in stage 13 (Fig. 2B). The wing (w) and haltere (h) primordia appear later during head involution and dorsal closure, as cells appear to migrate dorsally from the ventral discs and form small pockets clinging to tracheal branches (Fig. 2F,G). This migration has also been observed for Distalless-lacZ-expressing cells (Cohen et al., 1993). The dorsal prothoracic disc primordia, initially adjacent to the posterior T1 segment boundary, rounds up and invaginates with the anterior spiracle during head involution (Fig. 2B,D,F).

A very different pattern is seen in abdominal segments. Three groups of strong lacZ-staining cells are reiterated in each segment: (1) the imaginal tracheoblasts (tr, Fig. 2F,H), which are situated along the same position in the embryonic tracheal network as the elongate clusters described in the mature larva; (2) two epidermal cells of unknown identity (asterisk, Fig. 2B,D,F) and (3) two cells located on the ventral internal oblique muscles (asterisk, Fig. 2H), also of unknown identity.

We also see B5-lacZ expression in the location of the genital disc primordium (gd, Fig. 2C,E; Hartenstein and Jan, 1992; Whiteley et al., 1992). Expression appears in three cell clusters: two transverse stripes of cells, one on each side of the midline and one, more posterior, median cluster. These clusters subsequently associate together presumably to form the fused genital disc. We do not yet know the relationship of the three cell clusters to the development of the adult analia and genitalia or to the somewhat similar three clusters found in other flies (Dubendorfer, 1971).

Identifying and cloning headcase, the gene responsible for the B5-lacZ expression pattern

The B5 line harbours a single insertion of the P[lacZ,ry+] transposon at cytological position 99F (data not shown). In order to characterize the gene responsible for the B5-lacZ pattern, which we have named headcase, (hdc), we screened neighboring genomic DNA first for hdc transcription units and then for hdc cDNAs (see Methods). The composite sequence of two overlapping cDNAs is shown in Fig. 3B and predicts a message of 4.3 kb. This is consistent with a single 4.3 kb transcript detected by northern blot analysis of embryonic mRNA when probed with the nearly full-length cDNA, phdc.NB40 (data not shown). Importantly, this same probe recognizes the complete B5-lacZ expression pattern in the embryo (compare Fig. 4A and B) and larva (not shown). Southern blot mapping of the cDNA sequences onto the genomic walk (black boxes, Fig. 4A) revealed that the first 2/3 of the transcript maps directly downstream of the insert, whereas the remaining 1/3 is distal to the DNA isolated in the walk. The unmapped 3' domain appears to be a bone fide part of the hdc mRNA, not only because of the correlation with transcript size, but also because in situ probes made from these sequences detect the complete B5-lacZ embryonic expression pattern (data not shown).

Fig. 4. Embryonic headcase gene expression mimics B5-lacZ. Flat preparations of stage 16 embryos. (A) Anti-βgal staining of B5 transformant line. (B) In situ hybridization of wild-type using an antisense RNA probe transcribed from hdcb.NB40. (C) Anti-Hdc staining of wild-type. (D) Double labelling of wild-type imaginal primordia with anti-Hdc and anti-Sna, a marker of the wing and haltere imaginal primordia (Alberga et al., 1991). High-power magnification reveals nuclear staining Snail and cytoplasmically staining Hdc proteins in the same cells of the wing, w, and haltere, h, imaginal disc primordia: Hdc staining alone is present in the two leg disc primordia, l2, l3.
The Headcase protein is a novel cytoplasmic protein present in embryonic imaginal primordia

Theoretical translation of the \( \text{hdc} \) cDNA is shown in Fig. 3B. Surprisingly, there are two large open reading frames (ORF) separated by a single in-frame stop codon. PCR analysis of the genomic sequence verified the existence of the stop codon (data not shown). The upstream ORF predicts a protein with a \( M_r \) of \( 71 \times 10^3 \); whereas translation of the downstream ORF, either by suppressing termination or by internal initiation, predicts proteins of \( M_r \) \( 125 \times 10^3 \) or \( 35 \times 10^3 \), respectively. Mouse monoclonal antibodies were raised against a portion of the upstream ORF (underlined residues in Fig. 3B). Antibody staining with this monoclonal detects a protein expressed in the imaginal cells of the embryo (Fig. 4C) and larva (data not shown).
shown), mirroring the exact patterns observed with B5-lacZ and hdc in situ staining (compare Fig. 4C with A,B).

High-power magnification of antibody-stained tissue revealed that the Hdc protein is located in the cytoplasm. We took advantage of this property in order to establish the percentage of Hdc-expressing cells in embryonic wing and haltere imaginal disc primordia, all of which express the nuclear protein Snail (Sna) (Alberga et al., 1991). Embryos double-antibody labelled for Hdc and Sna proteins reveal that all Sna-positive cells seen adjacent to the discs most likely represent mesodermally derived adepithelial precursors, as these Hdc-positive cells co-express the imaginal mesoderm-specific Twist protein (Fig. 7A).

The headcase null phenotype affects imaginal cell differentiation but not growth

In order to assess the possible role of the hdc gene in imaginal tissue development, deletions removing part or all of the mapped hdc transcription unit (Fig. 3A) were generated by imprecise P-element excision (see Methods). All deletions caused pupal lethality when homozygous and heterallelic combinations show similar mutant phenotypes suggesting that all alleles are functionally null. We confirmed this for the typical allele, hdc43, where neither hdc RNA (Fig. 5) nor protein (data not shown) are detected in embryos or larvae homozygous for the mutation. We can conclude that the pupal lethal phenotype of the hdc mutant chromosomes is caused by the removal of the hdc gene activity, since Hdc function provided from an heat-inducible hdc transformation construct can completely rescue the pupal lethality (see Methods).

Although hdc mutants die as pupae, all larval imaginal discs are present, and their size and shape appear normal. Therefore the loss of hdc does not affect cell growth in any obvious way. The penetrance of pupal lethality is 100%; however, the developmental stage at which the pupae die is variable, ranging from brown pupae with no obvious differentiated tissue, to dead pharate adults. The most prominent hdc defects involve head development (Fig. 6) and can result in complete deletion of the head capsule (Fig. 6C), or duplication of head cuticle or antennae (Fig. 6B,E). Although the majority of pharate adults exhibit normal external head morphology, they possess massive defects in internal head structures, including the CNS, muscular and tracheal tissues (Fig. 6G). Other tissues are also affected (including wings, halteres, legs and epidermis) but vary between mutant individuals. Such pleiotropy is consistent with the broad range of imaginal cell-type specificity of the hdc gene.
gene expression; however, the reason for variable expressivity is unclear.

**hdc expression prefigures imaginal cell proliferation**

One unifying character shared by imaginal cells is their commitment to a strict program of diploid growth. Although hdc is expressed in all imaginal lineages, it initiates in the different imaginal cell groups according to a developmental sequence. Therefore, we were interested in whether hdc expression in a particular imaginal cell type correlated with that tissue’s overall program of proliferation and differentiation.

It is clear from the embryonic expression in imaginal disc primordia (Fig. 2G) that hdc activation in these cells occurs at least 24 hours before their re-entry into the mitotic cell cycle, since mitosis in these cells is not observed until late first/early second instar (Madhavan and Schneiderman, 1977). This observation is also true for the adepithelial cells (Fig. 7A,B). These cells constitute the primordia of adult somatic musculature and are found closely associated with the imaginal discs to which they ultimately attach (Reed et al., 1975; Currie and Bate, 1991); an association that we can trace back into embryonic stages using twist as a marker for adepithelial cells (Bate et al., 1991) and hdc as a marker for the disc primordia (Fig. 7A). Interestingly, at this early stage of adepithelial development, approximately half of the cells express hdc; complete overlap of twist- and hdc-expressing cells is apparent by larval stages (Fig. 7B). Since the muscle precursors are mitotically quiescent until second larval instar (as judged by susceptibility to a DNA synthesis inhibitor (Broadie and Bate, 1991), we can conclude that hdc expression prefigures the activation of imaginal somatic mesoderm cell proliferation. Similarly, for the imaginal histoblast lineage, hdc-positive histoblast cells are not detected in the embryo (Fig. 2), or in 1st, 2nd or early 3rd instar larvae (data not shown), even though histoblast cells are present and can be visualized by several histoblast-specific markers (Alberga et al., 1991; Hartenstein and Jan, 1992; Whiteley et al., 1992; Hayashi et al., 1993). Instead, hdc expression is first detected in wandering third instar (Fig. 11), corresponding to 12-24 hours before the various histoblast nests begin division during pupal stages (Madhavan and Madhavan, 1980).

A correlation also exists between hdc repression and the end of imaginal proliferation and the onset of differentiation (Fig. 7C,D). This is best illustrated for imaginal neuroblast (IN) proliferation which has been studied extensively by bromodeoxyuridine incorporation studies (Truman and Bate, 1988; Truman et al., 1993). Double-labelling experiments using Grainyhead protein as an IN marker (Bray and Kafatos, 1991) demonstrate that during the intense proliferative stages of the second (Fig. 7C) and early third instars (data not shown) hdc is expressed in IN, as well as their progeny. This relationship changes when the larval CNS prepares for pupariation in late third instar. Now the levels of hdc expression are severely reduced in the IN (Fig. 7D), but not their progeny, presumably because these cells are competent to continue cell proliferation. The timing of this change precedes final neuroblast division which, for these neuroblasts, located in the thoracic region of the ventral nerve cord, occurs sometime within the first 12 hours following pupariation (Truman and Bate, 1988). This observation is broadly supported by the pupal expression of hdc during disc morphogenesis. Whereas expression is uniformly strong throughout the proliferative stages, this fades during the first 24 hours of pupal development when cell division is ending. Therefore, the activation of hdc expression correlates with the onset of imaginal proliferation and hdc inactivation correlates with its cessation.

**DISCUSSION**

**Molecular properties of hdc**

The hdc gene encodes two large open reading frames separated by a single in-frame amber stop codon. We were convinced that the first ORF encodes part of, if not all, the Hdc protein for two reasons. Firstly, in vitro translated hdc cRNA produces a product of approximately $70 \times 10^3$ $M_r$, the size predicted for the first ORF (data not shown). Secondly, a monoclonal antibody raised specifically against the upstream ORF recognizes a protein whose tissue distribution coincides with the complete B5-lacZ and hdc mRNA expression patterns. What is the significance of the downstream ORF? Unfortunately, we cannot detect the endogenous Hdc protein by western blotting of embryonic or imaginal disc protein lysates and so the molecular weight of Hdc in vivo is unknown. However, western blot analysis of imaginal disc protein lysates made from heat-shock-treated HS-Hdc transformant line 87.1 revealed two bands: a strongly cross-reacting $71 \times 10^3$ $M_r$ protein corresponding to the upstream ORF, and a weakly cross-reacting protein $>116 \times 10^3$ $M_r$, most probably resulting from inefficient translational readthrough into the downstream ORF (data not shown). This intriguing result suggests that the downstream ORF is potentially translatable, albeit at a reduced efficiency compared to the upstream ORF. Stop codon suppression is not without precedent in *Drosophila*. During the translation of the *kelch* gene mRNA, which encodes a component of ring canals linking nurse cells during oocyte maturation, partial suppression of a stop codon results in synthesis of a large readthrough polyprotein (Xue and Cooley, 1993).

Database searches revealed no sequence similarity or conserved protein motifs within either the upstream or downstream ORF. However, Hdc is cysteine-rich and has a predicted pi of 9.6. Since the Hdc protein is concentrated in the cytoplasm, the extreme basicity may reflect an affinity for protein-protein or protein-RNA interactions.

**The hdc gene is not required for imaginal cell growth, but rather adult differentiation**

To address the role that hdc plays in imaginal morphogenesis, we characterized the developmental consequences caused by small deletions removing the hdc transcription unit. The resulting pupal lethality is associated with defects in the imaginal morphogenesis of many tissue types. The lack of an effect on embryonic and larval development is also consistent with the imaginal specificity of hdc gene expression. Although many late lethals may result from maternal effects (Perrimon et al., 1989), a maternal source of Hdc is not consistent with our in situ hybridizations and anti-Hdc-staining experiments, which fail to detect hdc expression before stage 13 (data not shown). Even so, we cannot completely rule out a maternal contribution until germ line hdc nulls are tested for more severe embryonic or larval phenotypes.
Since Hdc is a pioneer protein, we cannot infer a mechanism for its action based on its amino acid sequence. However, we do have a detailed understanding of its expression pattern. Because hdc expression is strictly limited to imaginal cells, hdc may participate in some process shared by all imaginal cells and distinct from larval cells. We can think of two such cellular behaviors, the first is maintenance of mitotic growth and the second is the response to molting hormones. We are as yet unable to distinguish between these two possibilities. However, our results indicate that hdc expression prefigures the time when imaginal cells re-enter the mitotic cell cycle. Expression continues throughout proliferation, but then ceases just before the final cell divisions, suggesting a role for hdc in the timing of proliferation. However, as hdc mutations exhibit neither a small nor overgrown disc phenotype, it is unlikely that hdc functions in regulating the number of cell divisions or survival of imaginal cells.

**hdc and hormonal signalling?**

The mechanisms coordinating imaginal cell proliferation with differentiation depend on hormonal control. In particular, juvenile hormone (JH) prevents imaginal tissues from undergoing premature metamorphosis in the presence of the molting hormone 20-hydroxyecdysone (ecdysone) (reviewed in Riddiford, 1993). The relative titer of each hormone is critical: abnormally high levels of JH administered before the onset of proliferation in the imaginal discs (Riddiford and Ashburner, 1991) or histoblasts (Madhavan, 1973; Postlethwait, 1974) interferes with their future ability to differentiate without affecting their ability to divide, a phenotype similar to that seen in hdc mutations. Conversely, a reduction in ecdysone titer results in delayed or blocked metamorphosis (Garen et al., 1977; Sehnal and Bryant, 1993). Although several ecdysone-inducible genes have been isolated (reviewed in Fristrom and Fristrom, 1993), little is known about the molecular mechanisms linking hormonal signalling to tissue morphogenesis.

Mutational dissection of the ecdysone-inducible early puff gene E74 demonstrates its role in imaginal tissue morphogenesis during prepupal and pupal development (Fletcher et al., 1995). Most notably, mutations effecting the ‘B’ transcript in E74 fail to undergo head eversion at pupation, a cryptocephalic phenotype reminiscent of that seen in hdc mutants (Fig. 6C). These results raise the possibility that hdc may be involved in the integration or response to hormonal information by imaginal cells.

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