The Heterotrimeric Protein G_o Is Required for the Formation of Heart Epithelium in *Drosophila*

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Abstract. The gene encoding the α subunit of the *Drosophila* G_o protein is expressed early in embryogenesis in the precursor cells of the heart tube, of the visceral muscles, and of the nervous system. This early expression coincides with the onset of the mesenchymal-epithelial transition to which are subjected the cardial cells and the precursor cells of the visceral musculature. This gene constitutes an appropriate marker to follow this transition. In addition, a detailed analysis of its expression suggests that the cardioblasts originate from two subpopulations of cells in each parasegment of the dorsal mesoderm that might depend on the *wingless* and *hedgehog* signaling pathways for both their determination and specification.

In the nervous system, the expression of $G_o\alpha$ shortly precedes the beginning of axonogenesis.

Mutants produced in the $G_o\alpha$ gene harbor abnormalities in the three tissues in which the gene is expressed. In particular, the heart does not form properly and interruptions in the heart epithelium are repeatedly observed, henceforth the *brokenheart* (*bkh*) name. Furthermore, in the *bkh* mutant embryos, the epithelial polarity of cardial cells was not acquired (or maintained) in various places of the cardiac tube. We predict that *bkh* might be involved in vesicular traffic of membrane proteins that is responsible for the acquisition of polarity.

Key words: heterotrimeric protein G_o • *Drosophila* • heart epithelium • cell polarity • signal transduction

In *Drosophila*, the larval heart (or dorsal vessel) is a simple linear tube composed of inner myoendothelial cells (cardial cells) and outer unpolarized and nonmyogenic cells (pericardial cells) (Rizki, 1978; Rugendorff et al., 1994; Bodmer, 1995; Bodmer et al., 1997). It extends from the T2 thoracic segment to the A7 abdominal segment. The dorsal vessel is surrounded by a network of extracellular matrix components (Rugendorff et al., 1994), among which some are localized in specialized areas of its surface (Zaffran et al., 1995). The heart behaves as a pump to insure a continuous flow of hemolymph in the organism.

The heart precursor cells arise from a group of ventral blastoderm cells in the presumptive mesoderm anlagen which invaginates at gastrulation (Bate, 1993). Later, the mesoderm spreads and forms a monolayer of cells in close apposition to the ectoderm in the direction of the dorsal region of the embryo. Meanwhile, the cells divide three to four times during the process. A proper dorsal-lateral migration is controlled by the activity of *heartless*, a gene encoding a fibroblast growth factor receptor (Beiman et al., 1996; Gisselbrecht et al., 1996). Once the mesodermal cells have reached their ultimate location, they require inductive signals from the overlying ectoderm to accomplish both their determination and differentiation. The progenitors of the cardial cells derive from a particular region in the mesoderm that receives simultaneously the ectodermal signals of the Decapentaplegic (Dpp) and the Wingless (Wg) secreted proteins and that constitutes the cardiogenic region (Staehling-Hampton et al., 1994; Frasch, 1995; Lawrence et al., 1995; Wu et al., 1995; Bodmer et al., 1997).

In stage 11 embryos, the precursor cells of the cardiac lineage are metamerically organized in groups of mesenchymal cells that do not, however, fully mirror the structure of the mature heart (see for example Dunin-

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Borkowski et al., 1995; Zaffran et al., 1995; Riechmann et al., 1997). From stage 11 onwards and at the beginning of germ-band retraction, the heart cell progenitors acquire their specification by expressing a combinatorial set of marker genes specific for each cell subpopulation.

We are interested in the genetic control of cardiogenesis in *Drosophila* which is viewed as a simple model to study organogenesis. The cardial founder cells become specified in two subpopulations from a unique mesodermal functional domain and further differentiate into cells exerting markedly different functions that are the myoendothelial cells (the cardial cells) and the pericardial cells. Along this determination/differentiation process, the cardial cells acquire a polarity that is the result of a typical mesenchyme-epithelium transition. To better understand these transformations at the molecular level, we have focused our attention on genes that are specifically expressed in distinct subpopulations of heart cells such as the gene encoding the α subunit of the G_o protein. Indeed, an expression of this gene early in embryogenesis in the precursors of the cardial cells has been described (Zaffran et al., 1995) in addition to its expression in the embryonic nervous system (Guillèn et al., 1991; Wolfgang et al., 1991).

The G proteins, which are heterotrimeric GTPases, behave as signal transduction proteins and they are widespread proteins that have been conserved from yeast to humans. Their three subunits α, β, and γ form a complex that relay external signals inside the cell via a receptor (serpentine receptor) composed of seven transmembrane segments, activable by a variety of ligands, including hormones or neuropeptides. In the inactive complex, GDP is bound to the α subunit and activation of the receptor results in an exchange between GDP and GTP and in the dissociation of the heterotrimer in a monomer (α subunit) and a dimer (β and γ subunits). Both entities can be partners in the regulation of the activity of various enzymes and of ionic channels that in turn control the level of intracellular second messengers (see for review Clapham and Neer, 1993; Neer, 1995).

In *Drosophila*, genes coding for proteins that belong to the three major families of α subunits (α_s, α_i, and α_o) have been cloned and characterized. Two $G_s\alpha$ proteins differing by four amino acids are produced by alternative splicing of a unique gene (Quan and Forte, 1990). The unique gene for $G_i\alpha$ produces two transcripts which encode the same protein (Provost et al., 1988). Finally, two $G_o\alpha$ proteins have been deduced from cDNA sequence analyses and are issued from a unique gene transcribed in three different transcripts (de Sousa et al., 1989; Schmidt et al., 1989; Thambi et al., 1989; Yoon et al., 1989). Two other α subunits have been identified as homologues of vertebrate $G_f\alpha$ (Quan et al., 1993) and $G_q\alpha$-3 (Talluri et al., 1995), respectively. All these proteins are expressed throughout embryogenesis in various tissues in a dynamic pattern (Wolfgang et al., 1991) and, consequently, may play major roles in development although no precise developmental function has been ascribed to any of them. In contrast, it is known that the activity of *concertina* which encodes the α subunit of a G_{12} or G_{13} protein (Wilkie and Yokoyama, 1994) is necessary for the coordination of cell shape changes in the ventral furrow at gastrulation (Parks and Wieschaus, 1991). In addition, it is a likely candidate to act downstream of a putative secreted protein, Fog, whose receptor is as yet unidentified (Costa et al., 1994).

Among the different families of G proteins, the G_o protein is perhaps the less well characterized in terms of the nature of its associated signal transduction pathway. On the plasma membrane, G_o proteins may regulate Ca^{2+} and K^+ channels (Van Dongen et al., 1988; Warnick et al., 1988; Kleuss et al., 1991; Llédo et al., 1992), distinct types of phospholipase C (Moriarty et al., 1990), and the mitogen-activated protein kinase cascade (Van Biesen et al., 1996). In addition to interacting with heptahelical receptors such as opiate, α_2-adrenergic, D_2 dopaminergic, and somatostatin receptors, the G_o protein has been described recently as a regulator of vesicular traffic (Lagriffoul et al., 1996; Gasman et al., 1998).

In *Drosophila*, the amount of G_o protein is affected in memory mutants (Guillèn et al., 1990) and is expressed in the nervous system during development at the onset of axonal growth (Guillèn et al., 1991; Wolfgang et al., 1991). We show here that the function of G_o is required for the formation of the heart, the visceral musculature, and the nervous system. Based on the phenotype observed in mutants for the gene encoding the α subunit of the G_o protein, we have chosen *brokenheart* (*bkh*) as its name.

Materials and Methods

Generation and Analysis of Mutations

The *Df(2R)47A* deficiency in the *bkh* gene region was issued from a cross between AQ65 (a P-element (P[w^+, *lac*Z]) enhancer trap line in 47A) homozygous males which were γ-irradiated (4,000 rad) and (*w; Sp/Cyo*) females. Males and females from the F1 generation which were *Cyo*, but not *Sp*, were crossed together to obtain the strain. Among the 5,000 chromosomes which were screened, only one line affected the *bkh* mRNA expression: *Df(2R)47A*.

Three P-lines P[*w, lac*Z] (Török et al., 1993) l(2)k06915, l(2)k11003, and l(2)k07810, inserted on the second chromosome, had been located in the 47A region and were homozygous lethal. PCR analysis of the P-lines' genomic DNA was used for mapping these insertions. The primers were the 31-bp terminal sequence of the P-element (5′-CGACGGGACCACCTTATGTTATTTCATCATG-3′) and *bkh* specific primers: (bkh_6: 5′-CAGCCCATTGTGGTTGGG-3′; bkh_3: 5′-AAGTCCTCAGCAGTGAAGCCGCTCTCG-3′). P07810 was localized 1.5 kb upstream of the class II cDNA ATG and the 1.5-kb PCR fragment obtained was sequenced by Genome Express. P06915 and P11003 were localized 800 bp upstream of the class I cDNA ATG. All the mutant stocks were balanced with *Cyo* (*wg, lac*Z) to identify the homozygous mutant embryos.

A genomic region in the 5′ end of the *bkh* gene was identified with the aid of the DS01583 P1 phage obtained from the *Drosophila* Genome Project. The 5′ end of the DS01583 P1 phage was located 4.5 kb downstream of the class II cDNA ATG. Amplifications of genomic sequences from this region were performed with the pair of specific primers *bkh*6 and *bkh*16 (bkh16 5′-CTGAACTTCGAGCGTCGTC-3′). Sequences were made by Genome Express and analyzed with Gene Finder to identify transcription units.

The bkh^{007} mutation was generated by P-element mobilization in the P07810 line and a cross with flies bearing Δ(2-3) as a source of transposase and of *w; Sp/Cyo; Δ(2-3),Sb/TM6B* genotype.

P-Element–mediated Transformation

Germline transformation was performed using standard procedures as described by Rubin and Spradling (1982). The *bkh* cDNA (class II) was subcloned in the pUAST vector (Brand and Perrimon, 1993) and injected at a concentration of 300 ng/μl with the Δ(2-3) helper plasmid (100 ng/μl) (Robertson et al., 1988). Several independent transformants were obtained and mapped, and lines of interest were made homozygous with the help of a double balancer stock *w; Sp/CyO; MKRS/TM2*.

Rescue with the GAL4 System

To rescue the *Df(2R)47A* phenotype at the embryonic stage, we used the yeast GAL4-directed transcription system (Brand and Perrimon, 1993). Females homozygous for a UAS-*8.31bkh* class II cDNA on the X chromosome (UAS-*bkh*) and bearing the *Df(2R)47A* deficiency were crossed to homozygous males bearing the homozygous 24B-GAL4 driver inserted on the third chromosome and the *Df(2R)47A* deficiency on the second chromosome. Homozygous animals were sorted out with the aid of a labeled *CyO* balancer and scored by observation of their phenotypes.

Immunohistochemistry and In Situ Hybridization

Digoxigenin (DIG)[1]-labeled antisense or sense RNA probes were generated from DNA with T3 or T7 RNA polymerase (Promega) and DIG-UTP (Boehringer). They were used for whole mount in situ hybridization of fixed staged embryos prepared as described in Zaffran et al. (1997). The DIG-labeled RNA probes were detected with the aid of a preadsorbed anti-DIG antibody coupled to alkaline phosphatase (Boehringer) and NBT/BCIP as substrate. The embryos were mounted in Geltol medium (Immunotech) for further observation. A specific probe for class I *bkh* cDNA was obtained by PCR amplification of genomic DNA with the oligonucleotides bkh_4 and bkh_7. A probe for class II cDNA was prepared by amplification of the cDNA inserted in pBlueScript with the T7 primer and the *bkh6* oligonucleotide (bkh_4: 5′-GCCCATGGTGCGTATTGCCTCGACGC-3′; bkh_7: 5′-CCGCTAGCGGTCAAATCGGGTATCCT-3′; bkh_6, see above).

For immunohistochemistry experiments, embryos were fixed and stained with antibodies according to the protocol described by Ashburner (1989). β-Galactosidase in embryos was detected by using a mouse anti–β-galactosidase (Promega) antibody diluted 1,000-fold. The following primary antibodies were used: anti-BKH (1:100 dilution; Guillèn et al., 1991); anti-DMEF2 (1:2,000 dilution; Bour et al., 1995); anti-EC11 (1:2 dilution; Zaffran et al., 1995); anti–Fas II, anti–Fas III, anti-Eve and anti-En (1:10, 1:2, 1:500, and 1:10 dilutions, respectively; Developmental Studies Hybridoma Bank); anti–α-spectrin (1:500 dilution; Lee et al., 1993); anti-Nrt (1:500 dilution; Piovant and Lena, 1988) and anti-Tin (1:800 dilution; Azpiazu and Frasch, 1993). Affinity-purified secondary antibodies (Jackson ImmunoResearch Laboratories) were either coupled to alkaline phosphatase or Biotin and used at a 1:1,000 dilution or conjugated to TRITC or FITC and used at a 1:100 dilution. The stained embryos were mounted in Geltol medium (Immunotech) for further observation under an Axiophot Zeiss microscope or, when fluorescent, in Permafluor (Immunotech) for observation under an Axiophot or a LSM 410 Zeiss confocal microscope.

In double labeling experiments, the conditions were the same as above. In situ hybridizations were performed before staining with antibodies. After dehydration of the embryos labeled with antisense RNA probes, they were rehydrated in PBS containing 0.3% Triton X-100 for at least 1 h and saturated in the same buffer containing 10% FCS before incubation with the primary antibodies.

Results

The Dynamic Expression of bkh during Embryogenesis Suggests a Developmental Function

The organization of the gene *brokenheart* (*bkh*) encoding the α subunit of G_o, as described by Yoon et al. (1989), is schematized in Fig. 1. Two classes of cDNAs (class I and class II) have been characterized (de Sousa et al., 1989; Schmidt et al., 1989; Thambi et al., 1989; Yoon et al., 1989). They arise from an alternative splicing in a unique gene and differ only in the ATG-containing first exon. Three developmentally regulated transcripts (3.4 kb of maternal origin, 4.2 and 6 kb) were revealed (Wolfgang et al., 1991) and by using specific probes, we have shown that the class I cDNA corresponded to the 6-kb mRNA appearing after 12 h of development whereas the class II cDNA corresponded to the 4.2-kb mRNA more abundant in earlier stages (data not shown).

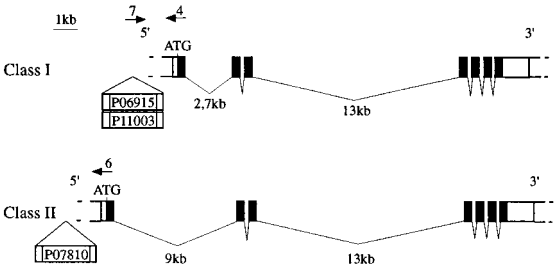

Figure 1. Organization of the *brokenheart* (*bkh*) transcription unit. The structure of the class I and class II cDNAs resulting from an alternative splicing is depicted (from Yoon et al., 1989). Filled boxes indicate coding sequences and open boxes untranslated sequences. The location of the three enhancer trap elements P06915, P11003, and P07810 (Török et al., 1993) is shown in 5′ of the two cDNAs. Numbered horizontal arrows refer to the position and sense of specific oligonucleotides described in Materials and Methods.

Both cDNAs encoded proteins of the same size (354 amino acids) that diverged only by 7 amino acids among their 21 NH_2-terminal residues and for which it is not known whether they exert different functions.

By using the class II cDNA as probe, a strong expression of *bkh* was observed in preblastoderm embryos due to the presence of the 3.4-kb maternal transcript (not shown). In early stage 11 embryos, the zygotic transcript could be detected for the first time in clusters of cells within 11 segments (Fig. 2, a and b). The cells appear to be cardial and visceral muscle progenitor cells since, based on *bkh* staining patterns in later stage 12 embryos, the cells became integrated into the conspicuous monocellular layer of cardial and visceral mesoderm cells on each side of the embryo (Fig. 2, c–e). This assumption was further supported by *bkh* expression in embryonic tissues which were unambiguously constituted of such cells (Fig. 2 e) and, also, in *tinman* (*tin*) loss of function mutants. In *tin* mutants, neither heart nor visceral muscles are formed (Azpiazu and Frasch, 1993; Bodmer, 1993) and, correlatively, *bkh* expression was completely abolished in the territories from which the precursor cells for these two tissues originated (Fig. 2 g). From the middle of stage 11 onwards, neuroblasts of the central nervous system (CNS) became labeled (Fig. 2 c) and *bkh* expression persisted in the neurons of the CNS in later stages of embryogenesis (Fig. 2, d–f). In a similar way, all the neurons of the peripheral nervous system (PNS) expressed the *bkh* mRNA from stage 12 onwards, slightly before the onset of axonogenesis (Fig. 2, d and f). Probes for either cDNA gave identical spatial patterns of expression although class I transcripts were quantitatively less abundant and were expressed later than the class II transcripts (not shown).

Antibodies directed against a COOH-terminal peptide whose sequence was conserved in the α subunit of all G_o proteins showed that the pattern of expression of the pro-

1. *Abbreviations used in this paper:* CNS, central nervous system; DIG, digoxigenin; PNS, peripheral nervous system.

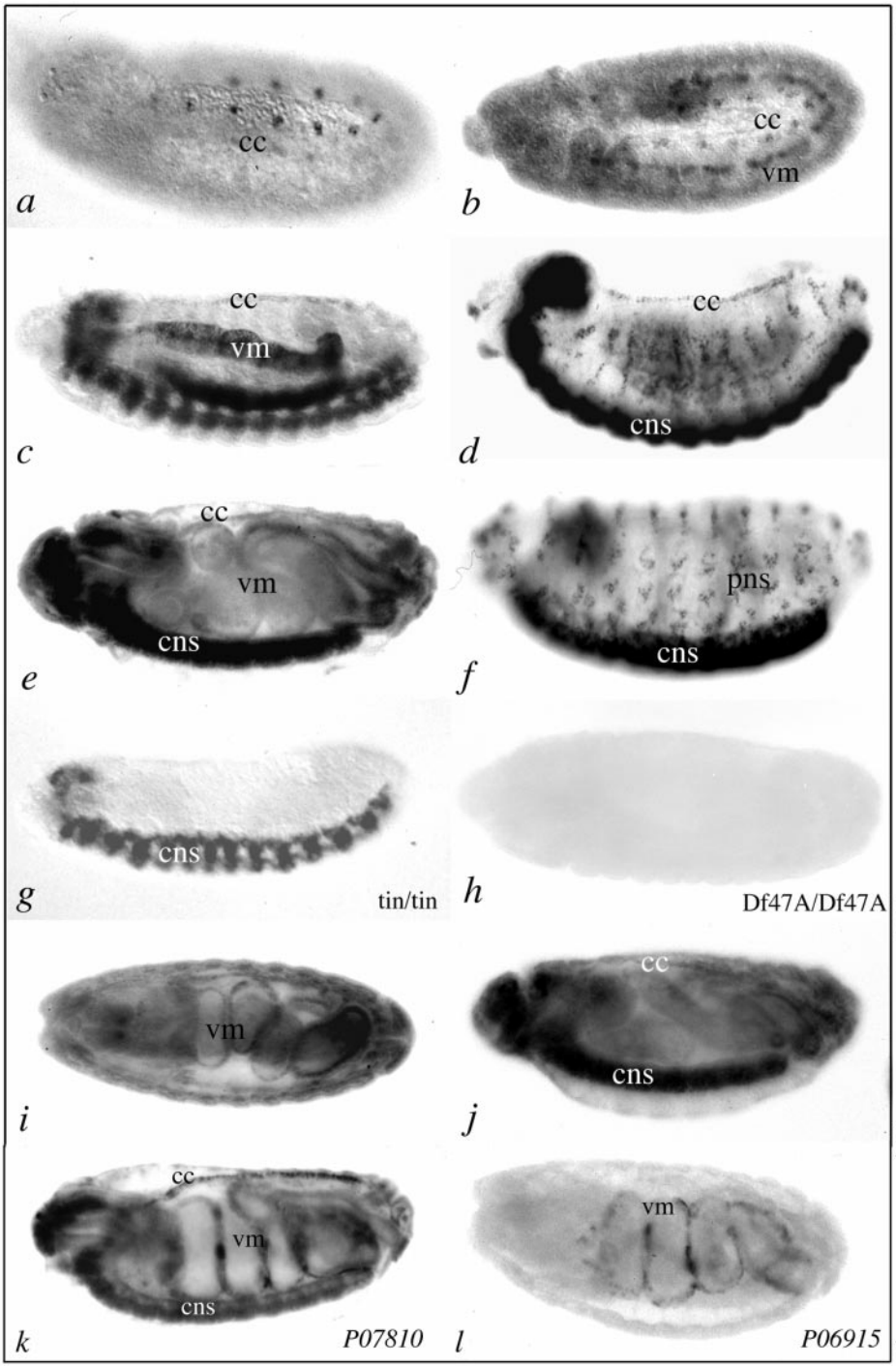

Figure 2. Expression of the *bkh* mRNA, of the protein, and of P-lacZ lines during embryogenesis. (a–h) In situ hybridization of whole mount wild-type (a–f) or mutant (g and h) embryos with an antisense *bkh* mRNA probe (class II cDNA). (a and b) The probe reveals the zygotic mRNA in stage 11 embryos as 11 clusters (one per segment) of (a) cardial and (b) visceral muscles cells. In late stage 11 to stage 12 embryos (c and d), the cells become integrated in the monocellular layer of cardial and visceral mesoderm cells and *bkh* begins to be expressed in the CNS and in the PNS. In stage 15–16 embryos (e and f), the *bkh* expression persists in these tissues. In a *tinman* mutant (g), the heart and the visceral muscles are not formed and the *bkh* expression is abolished in both tissues. No *bkh* expression is revealed in embryos homozygous for the Df(2R)47A deficiency (h). (i and j) Whole mount embryos stained with an antibody directed against the Bkh protein. The pattern of expression is superimposable to that of the mRNA including the dorsal vessel. The general high background probably results from the expression of the maternal protein. (k and l) Whole mount P07810 and P06915 embryos stained for β-galactosidase. The reporter gene displays the same pattern of expression as the *bkh* mRNA in P07810 (k) and labels only the visceral mesoderm in P06915 (l). cc, cardial cells; vm, visceral mesoderm. In all the views, anterior is left and dorsal is up.

tein was superimposable to that of the mRNA during embryogenesis (Fig. 2, i and j). However, probably due to the presence of the protein of maternal origin, these antibodies were poorly efficient in detecting a significant signal in the cardial cells as early as did mRNA probes.

bkh Is an Early Marker of the Mesenchymal-Epithelial Transition Undergone by the Cardioblasts

In stage 11 embryos, the zygotic *bkh* mRNA was detected at first in the precursors of the cardioblasts in a repeated pattern of 11 clusters in the dorsal mesoderm (Fig. 3, a and b). These clusters, constituted initially of two to four cells, were located in the anterior compartment of each mesodermal parasegment in a position anterior to the domain of En ectodermal stripes (Fig. 4) and closely neighboring the Wg-expressing cells in parasegments 2–12 of the overlying ectoderm. These cardioblast precursors belonged to the functional domain of Ladybird (Lbd) and of Even-skipped (Eve) expressing cells in the mesoderm which are the precursors of a fraction of the pericardial cells and of a dorsal muscle (see for example, Lawrence et al., 1995; Az-

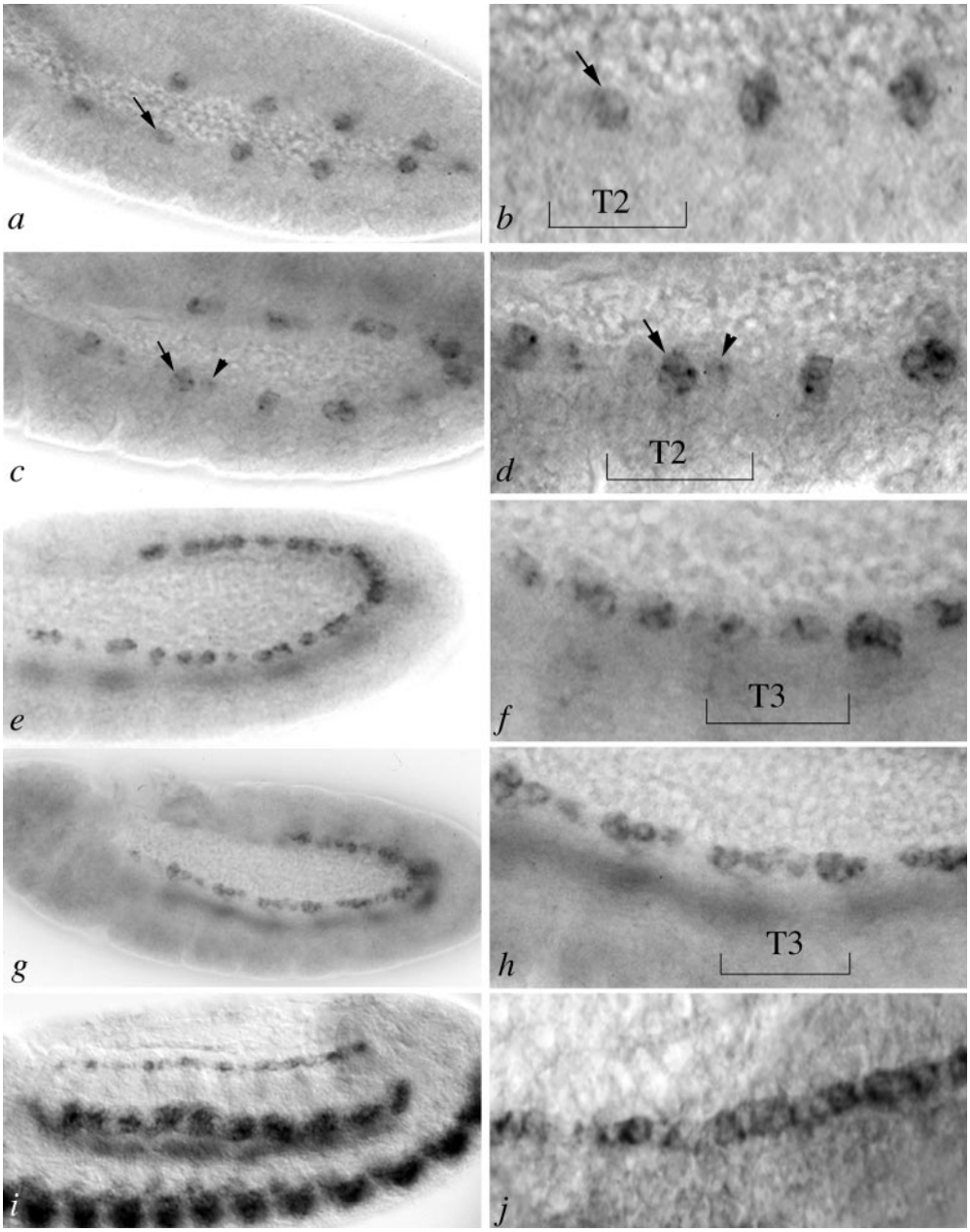

Figure 3. ***bkh*** expression during the mesenchymal-epithelial transition of the cardioblasts. (a, c, e, g, and i) In situ hybridization of whole mount embryos with an antisense ***bkh*** mRNA probe. (b, d, f, h, and j) Enlargements of these same images. In a and b at early stage 11, the ***bkh*** mRNA is first detected in 11 clusters formed of two to four cells (A-cardioblasts) within the dorsal mesoderm located in the anterior compartment of mesodermal parasegments underneath the ectodermal parasegments 2–12 (arrow in b). In c and d during stage 11, a second cluster (arrowhead) of two ***bkh*** positive cells (P-cardioblasts) appears posteriorly and not in continuity with the previous one (arrows). The relative apparent importance of the A- and P-clusters depends on the focus which, in that case, is centered on the T1 and T2 segments. In addition, when they are at first observed, and depending on the segment, there is no synchrony neither in their timing of appearance nor in the numbers of cells that constitute them. (e and f) During late stage 11, the A- and P-cardioblasts are detected in all the segments. Concomitantly and especially in the central region of the embryo, A- and P-cardioblasts have already joined. (g and h) During stage 12, the cells adopt a linear organization composed of six cardioblasts in each segment. (i and j) At stage 13, the cardioblasts form a continuous line of epithelial cells, each cell being in contact with two cells, one on each side. The positions of segments T2 and T3 in b, d, f, and h are indicated by black brackets. In all views, anterior is left and dorsal is up.

piazu et al., 1996; Jagla et al., 1997). Within the cluster, the expression of the *bkh* mRNA was initially very intense in only two of the cells and then it became detectable in the four cells with an almost uniform intensity. Due to their position relative to the parasegment boundary, we call these cells A (for anterior) cardioblasts. Soon after the onset of *bkh* expression in the first cluster, a second cluster of stained cells appeared posterior to the first one and not in continuity with it (Fig. 3, c and d). This group of cells called P (for posterior) cardioblasts was located in the posterior domain of the next parasegment and it never contained more than two *bkh*-expressing cells. Their position coincided with that of an area posterior to En stripes in the ectoderm (Fig. 4).

During germ-band retraction (late stage 11 and stage 12), the cardioblast progenitors that are mesenchymal in nature at that stage began to establish contacts between themselves, to extend filopodes (Rugendorff et al., 1994; Dunin-Borkowski et al., 1995), and to reorganize their shape to form a continuous layer on each side of the dorsal opening (Fig. 3, e–j). The origin of this process was the result of the encounter of the A and P groups of *bkh*-expressing cells in each parasegmental domain (Fig. 3, g and h). Later in development and due to the shortening of

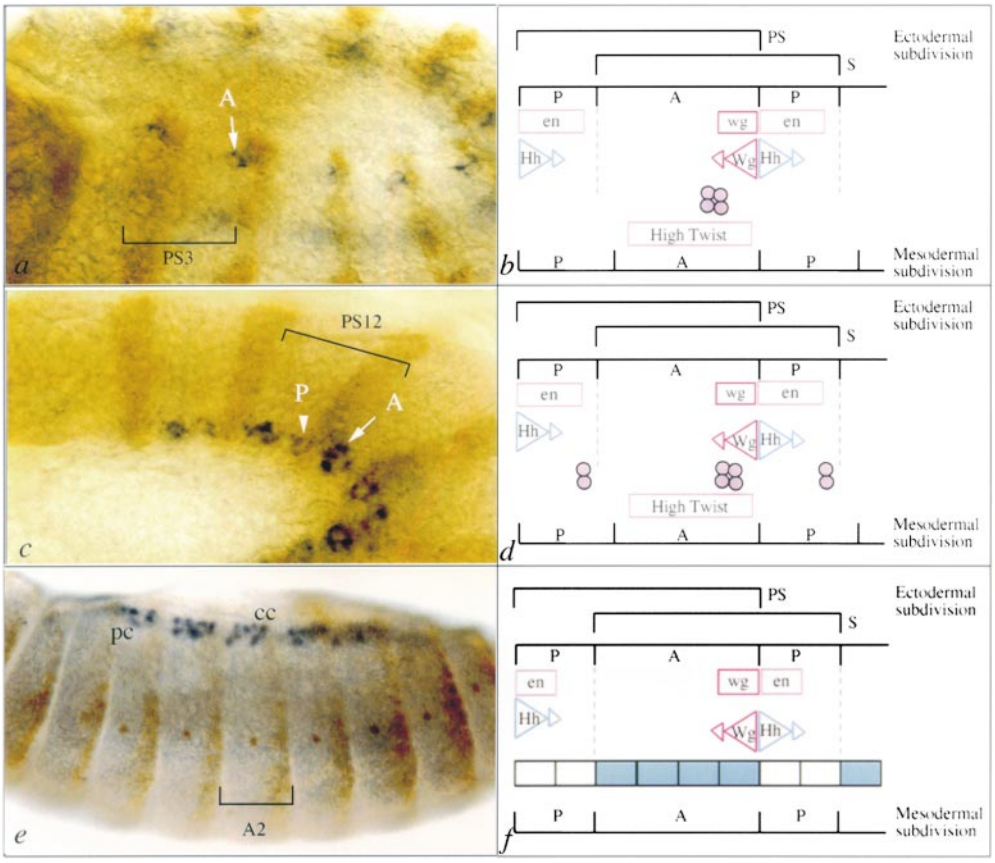

Figure 4. Parasegmental localization of *bkh*-expressing cells. (a and c) *bkh* (in blue) expression revealed with an antisense *bkh* mRNA was positioned with respect to the ectodermal Engrailed stripes. En (in brown) was revealed with an anti-En antibody. In a, in an early stage 11 embryo, *bkh* is expressed in the clusters of A-cardioblasts (white arrow) anteriorly to the En positive cells. In c, during stage 11, A- and P-cardioblasts clusters appear on each side of the En stripe (white arrow and arrowhead, respectively). (e) Stage 13 embryos were double labeled with an anti-Tin antibody (blue) and an anti-En antibody (brown). The four cardial and pericardial cells, stained by the anti-Tin antibody, lie anteriorly to the En stripe. The two Tin-negative cardial cells are therefore situated at this stage below the cells of the ectodermal posterior compartment. The positions of the ectodermal segment A2 and parasegments PS3 and PS12 are indicated by black brackets. cc, cardial cells; pc, pericardial cells. b, d, and f are schematic drawings of the pictures in a, c, and e. Precursors of the cardial cells are in blue. The vertical dotted lines mimic the segment boundaries.

the segment during germ-band retraction, the segmentally repeated six cell clusters spread along the anterior-posterior axis to finally join as a monolayer of polarized epithelial cells (Fig. 3, g–j). Still further in the process, the two rows of cardial cells, together with the pericardial cells that are attached to their basal membrane, fused at the dorsal midline to form the heart tube. Our observations led to the conclusion that a mature heart at the end of embryogenesis was constituted of 52 cardial cells per hemiembryo partitioned in six cells in every segment from T3 to A7 and in only four cells in the T2 segment. The results described above suggest the existence in each segment of two distinct populations of cells composed of four and two cardial cells that derived from mesodermal cells and that were located in domains corresponding, respectively, to the anterior and posterior compartment of each parasegment (Fig. 4). None of the cardioblasts present in parasegments 2 and 3 and only a part of those found in PS4 participated in the mature dorsal vessel, but it was, however, difficult to follow their fate since the expression of *bkh* vanished rapidly from these cells during development.

Similarly, by using the EC11 antibody specific for the pericardial cells (Zaffran et al., 1995), we counted 36 such cells distributed in 4 cells per segment from T2 to A7. Eve-expressing cells from the labial segment and the segment T1 did not contribute to the pool of pericardial cells and they probably gave rise to other structures that we were unable to recognize.

The cardial cells, which are also muscular cells, were determined as epithelial (Rugendorff et al., 1994; Tepass, 1997), based on the expression of several markers for polarity (see below). For example, antibodies directed against α-spectrin specifically labeled the basal-lateral membrane of the epithelium (Lee et al., 1993) (see Fig. 8 a) and the staining by anti-Neurotactin antibodies (Nrt) (Piovant and Lena, 1988) was restricted to the lateral and apical domains of the cell surface (see Fig. 8 e). In contrast, phosphotyrosyl proteins (Woods et al., 1997) and Armadillo (Müller and Wieschaus, 1996) were weakly expressed in the heart cell membranes and had no polarized localization (not shown), in good agreement with the lack of authentic *Zonulae adherens* in *Drosophila* secondary epithelia (Tepass and Knust, 1993; Tepass and Hartenstein, 1994). Finally, the basal membrane of the epithelium was visualized with the mAb EC11 (Zaffran et al., 1995). This antibody recognized an antigen probably secreted by the pericardial cells which was detected on the basal membrane of the cardial cells and around the pericardial cells (see Fig. 8 a).

In the visceral mesoderm that also undergoes a mesenchymal-epithelial transition before its differentiation into the visceral musculature, *bkh* was expressed in a repeated

pattern of clusters of cells in the same 11 parasegments as the precursors of the cardial cells and anterior to them (data not shown; Azpiazu et al., 1996; Riechmann et al., 1997). These precursor cells in parasegments 4–12 were ultimately transformed in a polarized epithelial ribbon of one cell in width (Bate, 1993), whose polarity was assessed by the localization of Fasciclin III (Patel et al., 1987) to cell-cell contacts (see Fig. 8 i).

bkh Is Required for the Formation of the Cardial and of the Visceral Mesoderm Epithelia

The embryonic expression of *bkh* suggests a function of the protein G_o in the formation of the cardial and visceral mesoderm epithelia as well as in axonal growth. A deficiency in the 47A region in which is localized the *bkh* gene was obtained by using as a starting tool the homozygous viable AQ65 line whose lac Z reporter gene was specifically expressed in the entire nervous system. Among the 5,000 chromosomes mutagenized by x-ray irradiation and screened for the loss of the phenotypic *white* marker, a lethal deficiency Df(2R)47A was obtained. It was shown by cytology that it had lost half of each of the A and B bands in 47 (not shown). Quantitative analyses of Western blots revealed that in animals heterozygous for that deficiency, half the amount of the BKH protein was produced as compared with its production in the initial AQ65 line (not shown). Moreover, no mRNA could be detected in homozygous mutant embryos (Fig. 2 h).

Three P-insertion lines, issued from the systematic screen of lethal P-insertions on the second chromosome (Török et al., 1993), have been located within the cytologi-

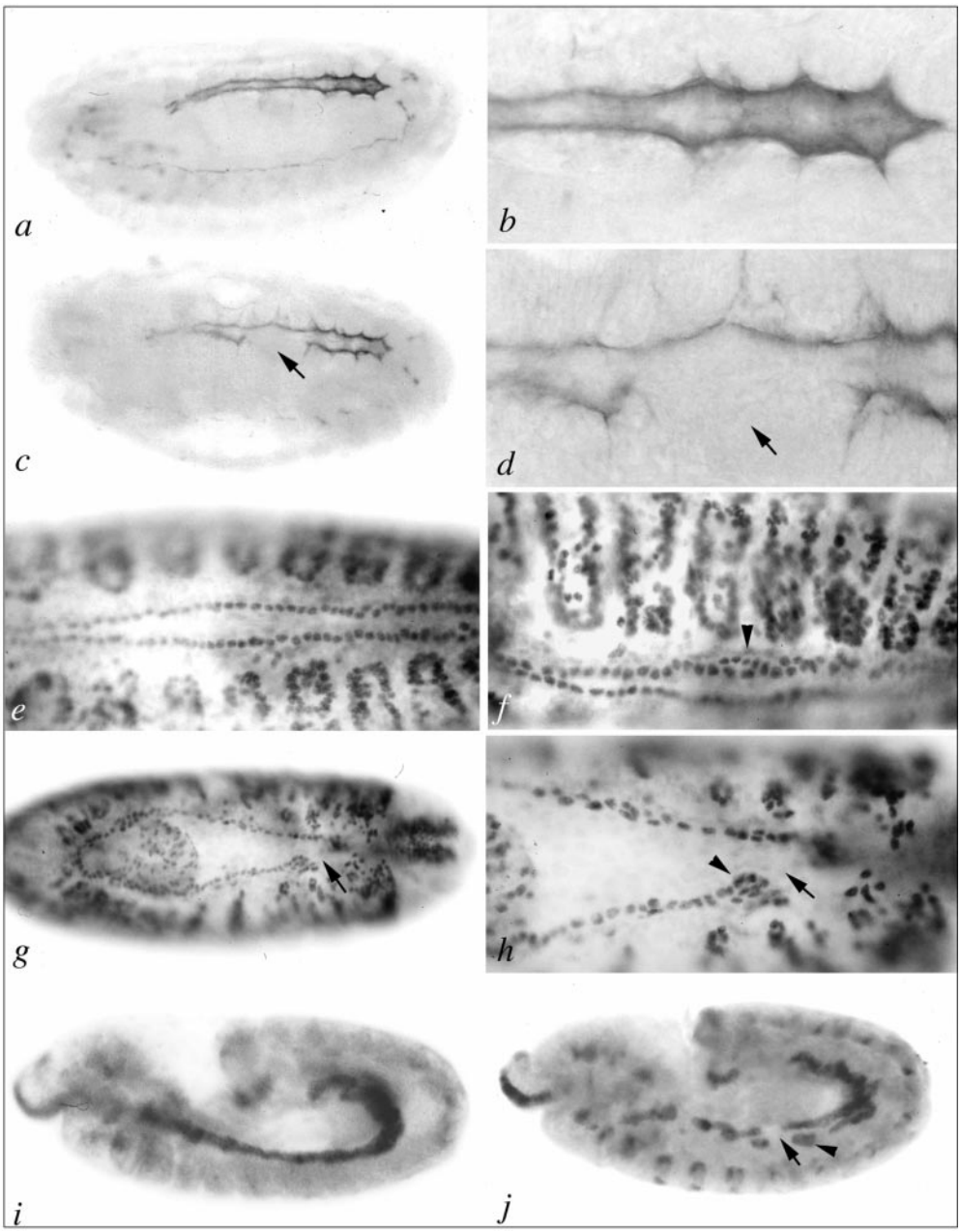

Figure 5. Heart and visceral musculature phenotypes in *bkh*-deficient embryos. The phenotype of homozygous Df(2R)47A embryos was analyzed in the heart by using anti-EC11 (c and d) and anti-DMef 2 (f–h) antibodies as probes. The phenotype in the visceral mesoderm was assessed with an anti–Fas III antibody (j). Figures in b, d, f, and h are enlargements of those in the left panel. (a, b, e, and i) Wild-type embryos. A frequently observed heart phenotype is interruption of the dorsal vessel (c, d, g, and h; black arrows). In f and h, the cardial cells are present in dorsal position but they are organized as clusters of cells (arrowheads) no longer forming a continuous line. The same types of defects are seen in the visceral mesoderm (j; arrowhead and black arrow). In all views, anterior is left, dorsal is up.

cal region of the *bkh* gene. In the P07810 insertion, the lac Z reporter gene expression was superimposable to that of the *bkh* mRNA (Fig. 2 k), whereas, in the P06915 and P11003 insertions, the β-galactosidase activity was restricted to the visceral mesoderm (Fig. 2 l). The different insertions were mapped by PCR in the 5′ region of the class II and class I cDNAs, respectively (see Fig. 1 and Materials and Methods). The lethality in these lines was not consecutive to the insertion of the transposon but was rather due to a secondary mutation since the homozygous lethal lines were perfectly viable in trans of Df(2R)47A and of other deficiencies uncovering the same region [Df(2R)E3363, Df(2R)X1, Df(2R)Stan2, Df(2R)12].

In contrast, in the P07810 line, mobilization of the P-element has resulted in the obtention of several viable revertants and of one lethal bkh^{007} $white^{-}$ revertant in trans of Df(2R)47A. Homozygous bkh^{007} embryos had the same heart and visceral muscle phenotype as that displayed by the homozygous Df(2R)47A-deficient animals (see below).

To analyze the molecular lesions resulting from the imprecise excision of the P07810 transposon, the 5′ region of the gene in which P07810 lies has been further characterized. A P1 phage (DS01583) covering the whole length of the *bkh* gene contained 4.5 kb of genomic sequence upstream of the class II cDNA ATG. This fragment was cloned, fully sequenced, and analyzed for a more detailed definition of the class II transcription unit. Three canonical potential TATA boxes were located within a 510-bp sequence downstream of the P07810 insertion site. The analysis of the bkh^{007} allele showed that a segment of 839 bp was deleted that contained the three potential TATA boxes together with 311 bp of the 5′ end of the transcription unit. As a consequence, no class II mRNA was produced in the homozygous bkh^{007} embryos.

Homozygous Df(2R)47A embryos (recognized by the lack of a labeled *CyO* balancer) harbored defects in the three tissues in which *bkh* was expressed with a total penetrance meaning that all homozygous Df(2R)47A embryos had defects in the heart, the visceral mesoderm, and the nervous system. When probed with anti-EC11 antibodies (Zaffran et al., 1995), the mutant embryos showed interruptions in their dorsal vessel (Fig. 5, c and d) that were also visible when DMef 2 expression was investigated in all the muscle cells including the cardial cells (Fig. 5, f–h) (Bour et al., 1995). The cardial cells were unambiguously present and they had migrated properly in dorsal position, but, in some places, they were no longer arranged as a continuous layer but rather as unorganized clusters of cells (Fig. 5, f–h). Fas III expression revealed the same types of defects in the visceral mesoderm (Fig. 5 j). Similar alterations were detected in bkh^{007} mutants with the same total penetrance (not shown).

In the nervous system, longitudinal axons were often missing (Fig. 6 b) and important modifications were observed in the guidance and axonal growth of motoneurons (not shown). It should be recalled here that the *lola* gene was also deleted in the Df(2R)47A deficiency and that mutations in this gene result in missing longitudinal axons in the CNS (Fig. 6 c; Seeger et al., 1993; Giniger et al., 1994). Since the Df(2R)47A phenotype was slightly stronger (in terms of penetrance of the phenotype) than that provoked by a loss of function of *lola*, this suggests a function per se for G_o in axonal growth or guidance. This was confirmed by the analysis of the bkh^{007} mutation which fully complemented mutations in the *lola* gene but continued to display a neuronal phenotype. In homozygous bkh^{007} mutants, axons of the motoneurons were clearly misrouted (not shown). However, in contrast to the Df(2R)47A mutation, the longitudinal axons in the CNS were missing with a lower frequency but were often pinched (Fig. 6 d).

The heart and visceral mesoderm phenotypes could be rescued at least partially by expressing the class II cDNA under the control of 24B-GAL4 (Brand and Perrimon,

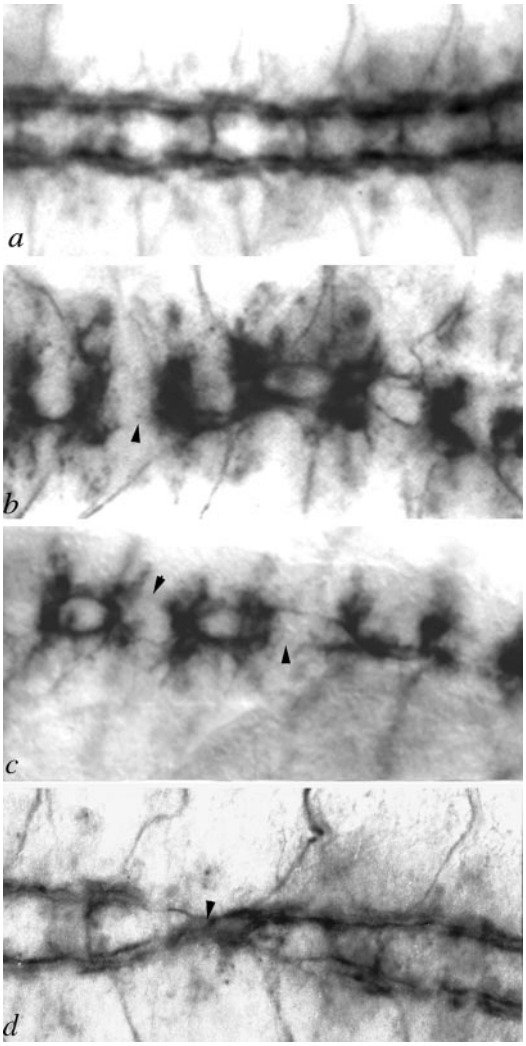

Figure 6. Mutants in the *bkh* gene display a disrupted CNS. Ventral view of a part of the CNS of whole mount embryos stained with an anti–Fas II antibody (Garcia-Alonso et al., 1995) which decorates longitudinal fascicles in the CNS and motoneurons axons. (a) Wild-type embryo; (b) homozygous Df(2R)47A embryo; (c) homozygous $lola^{B97}$ embryo; (d) homozygous bkh^{007} embryo. In b, longitudinal axons were missing (arrowhead). (c) In *lola* mutant embryos, the same phenotype was observed. (d) In *bkh* mutants, longitudinal axons were sometimes missing and the two longitudinal fascicles met at the dorsal midline (arrowhead).

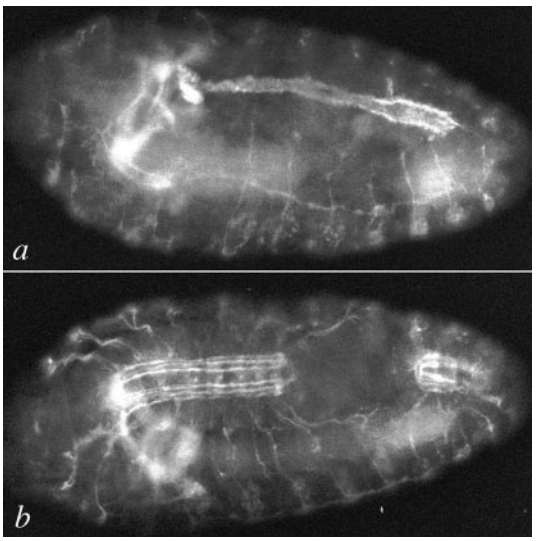

Figure 7. Expression of *bkh* in the whole mesoderm rescues the heart phenotype. a and b represent two different plans of focus on the same embryo, homozygous for the Df(2R)47A deficiency probed with the anti-EC11 antibody (a) and the anti–Fas II antibody (b). The class II *bkh* cDNA under the control of 24B-GAL4 is expressed in the whole mesoderm. The heart appears similar to that in wild-type embryos (a) while the nervous system remains disrupted (b). In both views, anterior is left and dorsal is up.

1993; Zaffran et al., 1997) that drives the expression of UAS-cDNAs in the myogenic lineage including the cardioblasts and the visceral mesoderm. In >65% (n = 250 observed embryos) of homozygous Df(2R)47A embryos, a normal development of these tissues had been restored (Fig. 7 a). In contrast, the phenotype in the nervous system was not abolished (Fig. 7 b) as expected, first because the deficiency uncovers the *lola* gene, and second because of the restricted specificity of expression of the 24B-GAL4 line. The bkh^{007} mutation that affects only the *bkh* gene could also be rescued by a *bkh* transgene (>80% of homozygous bkh^{007} embryos were rescued over a total of 147 embryos examined).

bkh Is Required for the Acquisition of Epithelial Polarity of the Cardial and Visceral Mesoderm Cells

The loss of *bkh* function produced heart and visceral mesoderm phenotypes that suggest a participation of the G_o protein in epithelia formation. During the mesenchymal-epithelial transition, subsets of membrane and cytoskeletal proteins localize to distinct regions of the cell surface to create the apical and basal-lateral membrane domains (Drubin and Nelson, 1996) that confer to the cells their epithelial polarity. The *bkh* mutant phenotype was more accurately described with the aid of markers localized specifically to these different domains. We focused our analysis on regions of the mutant heart in which the cells formed a continuous uninterrupted layer but which displayed abnormal marker expression.

As shown in Fig. 8, b and d, the localization of two polarity markers was affected in several places in the heart epithelium of *bkh* mutant embryos. α-Spectrin, specific for the basal-lateral membrane of epithelial cells, was now expressed on the entire surface of the mutant cells, however with a somewhat lower intensity than in wild-type cells. In addition, these mutant cells remained round, had no signs of shape remodeling, and failed to express the EC11 antigen in their basal membrane. The pericardial cells which are normally attached to the basal membrane of the cardioblasts were absent from these same regions and were rather often associated in clusters in the domains of high EC11 antigen expression (not shown). Similarly, Nrt which was localized to the apical and lateral membranes of wild-type cardial cells (Fig. 8 e) was, in *bkh* embryos, scattered in several locations on the entire surface of those cells which were round and in which the epithelial array was disorganized (Fig. 8, f and h). Finally, Fas III expression also revealed phenotypes in the visceral mesoderm characterized in some regions by a uniform and lower Fas III staining on the surface of still round cells with a partially destroyed epithelial structure (Fig. 8, j and l).

Discussion

The brokenheart Gene Function Is Required for the Morphogenesis of the Heart and Visceral Musculature

In a deficiency, Df(2R)47A, which completely deleted the *brokenheart* gene encoding the α subunit of the heterotrimeric G_o protein, the morphogenesis of the heart and of the visceral mesoderm was impaired. The defects associated to the nervous system have not been studied in detail, essentially because this deficiency also uncovered the *longitudinal absent* gene (*lola*) whose requirement for axonogenesis of longitudinal fascicles is known (Seeger et al., 1993; Giniger et al., 1994). However, several arguments strongly suggest that the phenotypes associated to the heart morphogenesis and to the visceral musculature were a consequence of a loss of the function of *bkh*. First, in deficient animals, alterations were restricted to the tissues that expressed *bkh* and they appeared in synchrony with the temporal expression of *bkh*. Second, P-element mobilization in enhancer trap lines located within the *bkh* gene and imprecise excision of the P-transposon led to the isolation of mutants with phenotypes identical to those observed in homozygous Df(2R)47A embryos. Finally, we were able to rescue the heart and visceral mesoderm embryonic phenotypes in homozygous *bkh* mutants by expressing the cDNA encoding *bkh* in transgenic flies. The failure to totally rescue the phenotype probably resulted from the fact that 24B-GAL4 did not drive the expression of the UAS-*bkh* cDNA in mesodermal cells early enough to be totally efficient (Michelson, 1994).

Although the heart was perturbed in the totality of the mutant embryos (100% penetrance), defects were observed only in some of the cells that constituted the dorsal vessel. This result does not support well the contention that *bkh* is an essential partner in the acquisition of polarity for the epithelial cardial cells since, if this were the case, all the cells would be equally affected. The maternal protein which is abundant and stable until mid-embryogenesis could have partially compensated the loss of zygotic transcript. Production of germline clones lacking *bkh* activity could help to elucidate this point. Moreover, an-

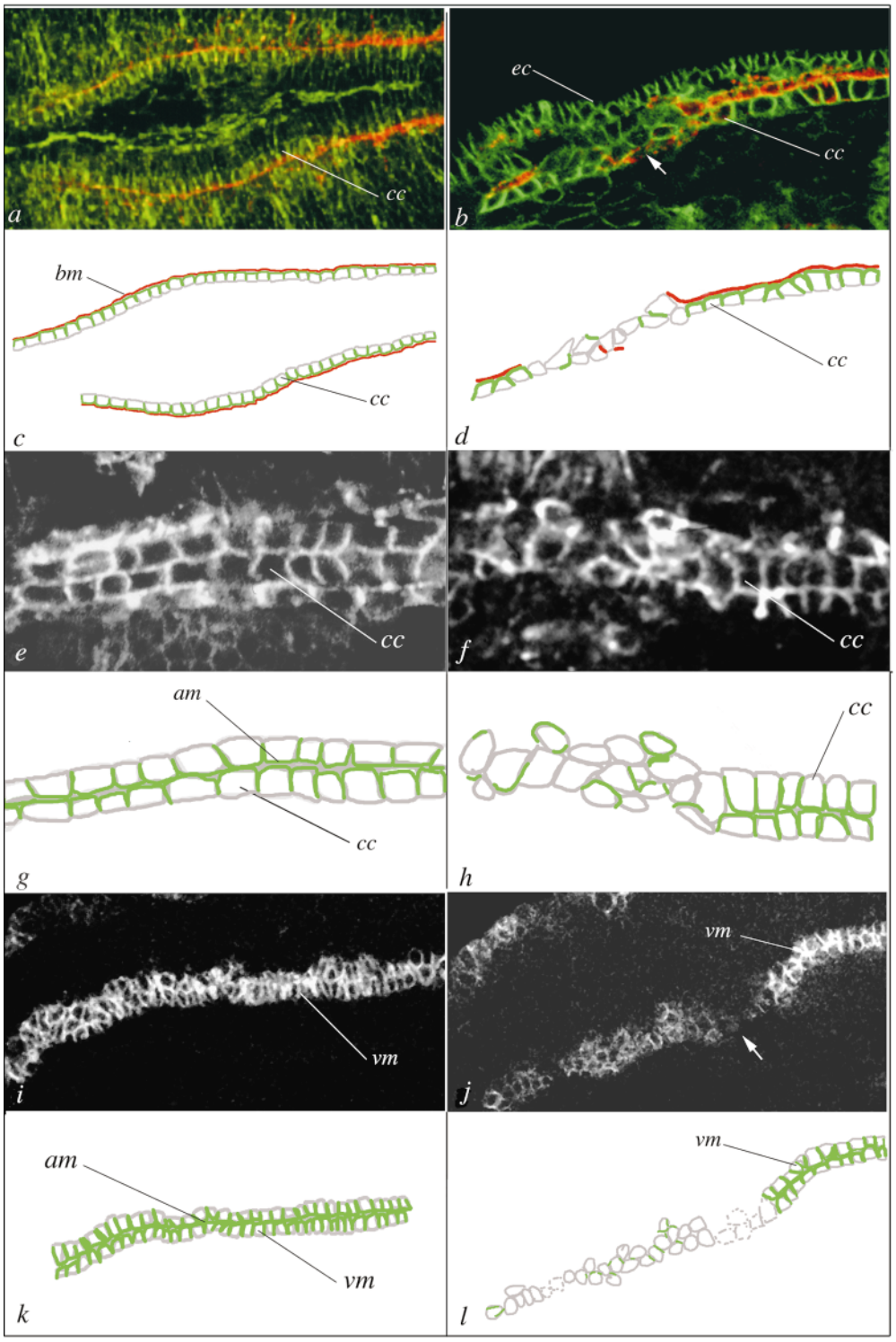

Figure 8. The heart and visceral mesoderm epithelia as observed by confocal microscopy are not properly polarized in *bkh* mutants. (a, e, and i) Wild-type embryos; (b, f, and j) homozygous Df(2R)47A embryos. The images of double labeling experiments (a and b) with anti–α-spectrin (green) and anti-EC11 (red) antibodies or of labeling with anti-Nrt antibody (e and f) and anti–Fas III antibody (i and j) have been obtained with the aid of a confocal microscope. c, d, g, h, k, and l are drawings of the epithelial heart (c, d, g, and h) or of the visceral mesoderm (k and l) seen in the corresponding photographs. In these drawings, only the epithelia of interest are shown to avoid a confusing impression due to other layers that are also labeled by the antibodies. Antibody staining is in green or in red. Membranes of cells that are not labeled or of mutant cells that are poorly labeled are outlined by gray lines or gray dotted lines. (a and c) The polarized nature of the heart epithelial cells is revealed by the anti–α-spectrin antibody (green) which stains the basal-lateral but not the apical face of the cells and by the anti-EC11 antibody (red) which labels the basal membrane. The two rows of epithelial cells have not yet fused at the dorsal midline to form the heart tube. (d) In the mutant, the polarity of the cardial cells is impaired in some places as visualized by the lower level of expression of α-spectrin and its uniform localization on the surface of the cardial cells. In addition, the EC11 labeling is no longer detected on the basal face (white arrow in b). Only one row of epithelial cardioblasts is shown in the optical section below the ectodermal layer. (e and g) Nrt is mainly localized to cell-cell contacts in the cardial epithelium in a stage 16 embryo, in which the two rows of cells have joined at the midline. (f and h) Nrt is hardly detectable in some places and the cells appear round without polarity. (i and k) Fas III is localized to the lateral membrane at cell-cell contacts. At the stage shown, the visceral mesoderm is constituted of two rows of cells. (j and l) In the mutant, Fas III is expressed to a lower extent and in a nonpolarized manner (arrow). ec, ectoderm; cc, cardial cells; bm, basal membrane; am, apical membrane; vm, visceral mesoderm.

other heterotrimeric G_i protein is expressed in the cardial cells (Wolfgang et al., 1991) and functional redundancy might have been at work.

The bkh Gene Function Is Required for the Acquisition of Cell Polarity in the Morphogenesis of the Heart and of the Visceral Musculature

The myoendothelial heart tube is considered as a secondary epithelium that forms by a mesenchymal-epithelial transition (Rugendorff et al., 1994; Tepass and Hartenstein, 1994; Zaffran et al., 1995; Tepass, 1997). The mesenchymal cardial precursor cells, after their migration from the ventral site of gastrulation in the direction of their final dorsal position, reorganize their plasma membrane to acquire their cellular polarity and establish cell junctions to build up the cardial epithelium. A signal must be received by the mesenchymal cells to create the first asymmetry on their surface (for review see Drubin and Nelson, 1996). Interactions with localized extracellular matrix components are believed to be largely responsible for this first event (Drubin and Nelson, 1996; Tepass, 1997) and *Drosophila* mutants, for example, in Laminin A (Yarnitzky and Volk, 1995; Tepass, 1997) or in an integrin subunit (Stark et al., 1997), present severe disruptions in their dorsal vessel. The initiating signal is probably emitted in response to inductive interactions between the mesenchymal cells and the overlying dorsal ectoderm. The newly created asymmetry could then trigger a reorganization of the cytoskeleton. Finally, the different membrane domains (apical and basal-lateral) will be established by the acquisition of different combinatorials of membrane proteins that have been specifically routed towards them; this sorting-out step relies on an absolute specificity of the vesicular traffic (for review see Rodriguez-Boulan and Powell, 1992). In the absence of *bkh* function, the mesenchymal cardial cells did not remodel their shape and, in the case of the most extreme phenotype, they failed to form a continuous row of cells. In addition, whenever such a layer was eventually formed, in several places some but not all cells neither acquired nor maintained a proper polarity.

Based on the timetable of *bkh* expression, its participation in the first step creating the asymmetry of the cardial cells is not very likely. It might rather be required for the subsequent steps that concern cell shape change and polarization by addressing membrane proteins to their respective domains.

It has been suggested that heterotrimeric G proteins could contribute to the vesicular protein traffic by regulating early steps in the secretory pathway (for review see Nuoffer and Balch, 1994). This hypothesis stems from the observation that $AlF4^-$, an activator of heterotrimeric but not of monomeric G proteins, inhibits ER to Golgi and intra-Golgi transport as well as vesicle budding from the trans-Golgi network. In particular, G_o proteins have been implicated in granule exocytosis from chromaffin cells (Vitale et al., 1993; Gasman et al., 1998), insulin secretion (Lang et al., 1995), and transcytosis (Bomsel and Mostov, 1992). It has been shown that the secretion of the protease Nexin-1 by glioma cells was under the control of G_o1 (Lagriffoul et al., 1996). The $G_o\alpha1$ protein was detected on the membrane of small intracellular vesicles and the secretion of Nexin-1 was stimulated by $G_o\alpha1$ overexpression and by activators of G_o proteins such as mastoparan. It was further suggested that the GTPase activity of the $G_o\alpha1$ protein could be stimulated in the absence of a classical serpentine receptor (Lagriffoul et al., 1996).

Thus, we are tempted to predict that the *Drosophila* G_o protein has a function in a particular type of vesicular traffic responsible for the acquisition or maintenance of cell polarity in the cardial and visceral mesoderm cells. Preliminary observations on the subcellular localization of Bkh in embryonic cells were consistent with this prediction. In the totality of the cells examined, Bkh was located to the cytoplasm rather than associated to the cell membrane and the staining pattern revealed a typical granular appearance (data not shown).

Since the exportation and the localization to the plasma membrane of the protein markers we have used were all equally affected in *bkh* mutants, we conclude that *bkh* is involved in a general aspect of vesicular traffic rather than in the specific process of the sorting out of membrane proteins.

bkh might also be required for the reorganization of the cytoskeleton. The protein G encoded by the *concertina* gene participates in cell shape changes taking place at gastrulation (Parks and Wieschaus, 1991), probably via a modulation of the invaginating blastoderm cell cytoskeleton resulting from the activation of RhoA (Hall, 1998).

The early role of the G_o protein in the formation of the heart epithelium does not exclude a function in later events leading to the formation of the heart or to the acquisition of its function. It has been shown recently that knocking-out the $G_o\alpha$ gene in the mouse resulted in heart dysfunction. G_o-deficient mice had lost the muscarinic inhibition of isoproterenol-stimulated cardiac L-type Ca^{2+} currents (Valenzuela et al., 1997; Jiang et al., 1998). It will be interesting to investigate whether the *Drosophila* G_o protein could also be involved in such a process.

The Lineage of the Cardial Cells

Before the determination of cardiac precursors in the mesoderm, the overlying ectoderm is subdivided in segmentally repeated units partitioned into an anterior compartment (A-compartment) and a posterior compartment (P-compartment). Analysis of the expression of genes involved in the specification of mesodermal derivatives and other observations lend support to the idea that, after gastrulation, the mesoderm also becomes subdivided into segmentally repeated units, each of which consists of two separate domains (Dunin-Borkowski et al., 1995; Azpiazu et al., 1996; Riechmann et al., 1997). The domains that are located below the ectodermal P-compartments are subject to influences from the striped regulators *eve* and *hh* and have been termed "P-domains" or "*eve*-domains." By contrast, the development of the metameric domains that are located below the A-compartments depends largely on the striped regulators *wg* and *slp* (*sloppy-paired*) and these domains have been termed "A-domains" or "*slp*-domains."

In that scheme, three basic groups of genes are at work to pattern the mesoderm either along the dorso-ventral axis (*dpp* and *tinman*) to specify the dorsal mesoderm, or along the anterior-posterior axis (*wg* and *slp*) to subdivide

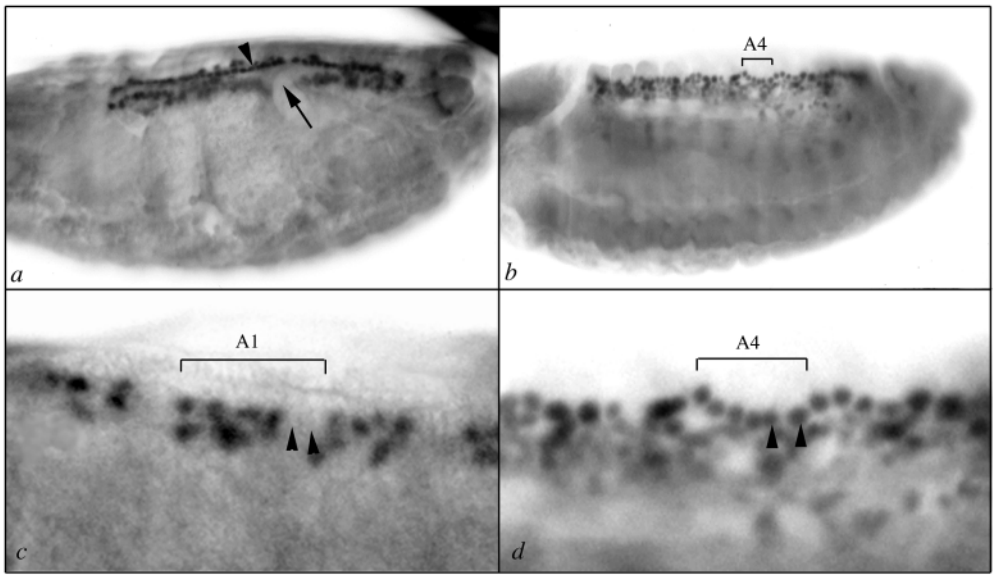

Figure 9. The domain of Tin expression in the cardial cells is expanded in *hedgehog* (*hh*) mutant embryos. The stage of development after egg laying 4h30–5h30 (early stage 10) old hh^{ts2} (Ma et al., 1996) embryos laid at 18° were shifted at 29° for 6 h and stained with an antibody directed against Tin (a, b, and d). (a and b) In homozygous hh^{ts2} embryos, either the absence of Tin-positive cells (arrows) in some areas of the heart as already described in Park et al. (1996) (this is probably due to a loss of function of *wg* consecutive to the loss of the early function of *hh*) or expansion of this expression to the six cardial cells per hemisegment (arrowhead in a) is observed. (c) Tin expression in a wild-type embryo is detected in the four cardial cells and the four pericardial cells. The two cardioblasts that do not express Tin are pointed with arrow heads. (d) Enlargement of b showing that two additional cardial cells (arrowheads) are labeled per hemisegment in hh^{ts2} embryos. Brackets represent the segmental unit.

it into segmental units, or at defined positions to control tissue specification. For example, recent evidence suggests that *wg*, whose expression is restricted to striped domains in each of the A-compartments and which is required for a variety of inductive signaling events during embryonic development, is directly involved in heart formation, in that it is necessary for further subdividing of the dorsal mesoderm and for specifying cardial cell fates. Elimination of the *wg* function shortly after gastrulation, at a time when *tin* becomes restricted to the dorsal mesoderm, results in the selective loss of heart progenitor cells with little effect on segmental patterning of the cuticle or other mesodermal derivatives (Baylies et al., 1995; Lawrence et al., 1995; Wu et al., 1995; Azpiazu et al., 1996; Park et al., 1996). From these and other observations, a picture has emerged in which specification of precardiac and dorsal somatic muscle precursors requires intersections of the dorsal domains of *dpp* expression with the transverse stripes of the dorsal expression of *wg* (Bodmer and Frasch, 1998).

However, the results presented herein are merely consistent with some precursors of the cardial cells originating from the P-domains and subjected to the influence of *hh* signaling rather than to that of *wg*. This hypothesis is somewhat difficult to verify because *wg* expression after gastrulation requires *hh* and vice versa (Klingensmith and Nusse, 1994), but, later in embryogenesis, the two signals become independent. Therefore, we have investigated the expression of Tin in temperature-sensitive hh^{ts2} mutant embryos submitted to a temperature shift ~5 h after egg laying (stage 10) (Fig. 9). In these embryos, the wild-type expression of Tin in four cardial and four pericardial cells was expanded to the two cardial cells located in the P-domains of each segment. We predict that these two *tin*-expressing cells are the same as the two *bkh*-expressing cells in this same domain.

The observation that the cardial progenitor cells can be divided into two cell subpopulations is consistent with the situation in the mature heart tube in which two genetically distinct populations of cardial cells have been described (Bodmer et al., 1997). For example, *tin* (see for example Bodmer, 1993; Jagla et al., 1997) as well as β3-tubulin (Leiss et al., 1988) and several P-lacZ reporter genes from enhancer trap lines (Hartenstein et al., 1992) are expressed in only four cell pairs per segment among the six pairs present. In the same line, a *D-mef2* enhancer element directed lacZ transcription in four cardial cell pairs per segment consistent with a direct regulation by *tin* which is expressed in these same cells (Gajewski et al., 1997). Interestingly, these two subpopulations of cardial cells, respectively, reside below the anterior and the posterior ectodermal parasegmental domains (Fig. 4).

These different observations could mean that the two P-cardioblasts were specified by the inductive instruction of *hh* rather than by that of *wg*. However, it is not known whether the *hh* pathway provides a direct late cardiogenic signal or exerts its effect via suppressing the *wg* function in the posterior domain. This hypothesis is unlikely in that, at that stage, reducing the function of *hh* in the epidermis does not lead to any visible effect on the *wg* signaling pathway (Heemskerk and Di Nardo, 1994). Indeed, *wg* expression in the dorsal epidermis was not expanded in a *hh* mutant (not shown). We predict then that *hh* might behave as a repressive signal for *tin* expression in the two P-cardioblasts. Cell heterogeneity in terms of gene expression could then be achieved along the anterior-posterior axis by an efficient cooperativity of *wg* and *hh* signals in the specification of the cardial cells.

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