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Heart tube patterning in *Drosophila* requires integration of axial and segmental information provided by the *Bithorax Complex* genes and *hedgehog* signaling

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SUMMARY

The *Drosophila* larval cardiac tube is composed of 104 cardiomyocytes that exhibit genetic and functional diversity. The tube is divided into the aorta and the heart proper that encompass the anterior and posterior parts of the tube, respectively. Differentiation into aorta and heart cardiomyocytes takes place during embryogenesis. We have observed living embryos to correlate morphological changes occurring during the late phases of cardiogenesis with the acquisition of organ function, including functional inlets, or ostiae.

Cardiac cells diversity originates in response to two types of spatial information such that cells differentiate according to their position, both within a segment and along the anteroposterior axis. Axial patterning is controlled by homeotic genes of the *Bithorax Complex* (*BXC*) which are regionally expressed within the cardiac tube in non-overlapping domains. *Ultrabithorax* (*Ubx*) is expressed in the aorta whereas *abdominal A* (*abd-A*) is expressed in the heart, with the exception of the four most posterior cardiac cells which express *Abdominal B* (*Abd-B*). *Ubx* and *abd-A* functions are required to confer an aorta or a heart identity on cardiomyocytes, respectively. The anterior limit of the expression domain of *Ubx*, *abd-A* and *Abd-B* is independent of the function of the other genes. In contrast, *abd-A* represses *Ubx* expression in the heart and ectopic overexpression of *abd-A* transforms aorta cells into heart cardiomyocytes. Taken together, these results support the idea that *BXC* homeotic genes in the cardiac tube conform to the posterior prevalence rule.

The cardiac tube is also segmentally patterned and each metamere contains six pairs of cardioblasts that are genetically diverse. We show that the transcription of *seven up* (*svp*), which is expressed in the two most posterior pairs of cardioblasts in each segment, is dependent on *hedgehog* (*hh*) signaling from the dorsal ectoderm. In combination with the axial information furnished by *abd-A*, the segmental *hh*-dependent information leads to the differentiation of the six pairs of *svp*-expressing cells into functional ostiae.

Movies available on-line

Key words: *Drosophila melanogaster*, Heart, Cardiogenesis, *hedgehog*, *abdominal A*, *Ultrabithorax*, Patterning, Heart ostiae, Aorta

INTRODUCTION

The *Drosophila* heart is a pulsatile organ, composed of a simple linear cardiac tube subdivided into an ‘aorta’ in the anterior region and a ‘heart’ in the posterior region (Rizki, 1978). The heart beat ensures the flow of hemolymph – insect blood – in an open circulatory system. In the adult, hemolymph enters the cardiac cavity through four pairs of valve-like ostiae situated in the heart and is propelled in a caudal to rostral direction by the contractions of the heart muscle (Rizki, 1978; Curtis et al., 1999; Molina and Cripps, 2001).

The general organization and structure of the larval cardiac tube are similar to that of the adult but with some differences (Curtis et al., 1999; Molina and Cripps, 2001). In particular, there are three pairs of ostiae, which are genuine openings, as opposed to the four differentiated adult valve-like ostiae. In addition, the alary muscles, which support the heart, are less developed in the adult than in the larva and an additional layer of longitudinal muscle cells has been added in the ventral region of the adult cardiac tube (Rizki, 1978; Molina and Cripps, 2001).

The *Drosophila* cardiac tube is composed of two cell types: cardiac cells, arising from cardioblast precursors, and pericardiac cells. These two cell types differentiate from

segmentally arranged clusters of progenitor cells which are located in the dorsal-most strip of mesoderm. The mesenchymal cardiac progenitor cells undergo a mesenchymal-epithelial transition, during germband shortening, to generate two bilateral rows of epithelial cardioblasts that are flanked by pericardiac cells (Dunin-Borkowski et al., 1995; Frémion et al., 1999). Through the process of dorsal closure, the two rows join at the dorsal midline to form the lumen of the dorsal vessel, which is surrounded by irregularly arranged pericardiac cells (Ruggendorff et al., 1994; Zaffran et al., 1995). From that stage onwards, the cardiac tube is composed of repeated units, or segments, each containing six pairs of cardiomyocytes, with the exception of the most posterior segment that contains only two pairs of cells (Bodmer and Frasch, 1999). The segments of the cardiac tube are in register with the segments of the epidermis and are accordingly annotated T3, A1-A8. Each segment boundary is defined by the position of the alary muscles that are attached, on one side, to the segment borders and, on the other side, to the two most posterior cardiomyocytes that express *svp* (Gajewski et al., 2000; Lo and Frasch, 2001; Molina and Cripps, 2001).

The expression of several genes is metamerically repeated in cardioblasts suggesting an intrasegmental specification of cell identity and potential functional diversification. Within each segment, the four anterior-most pairs of cardioblasts express *tinman* (*tin*), β3-tubulin and *D-sulfonyl-urea receptor (D-sur)* (Bodmer and Frasch, 1999; Kremser et al., 1999; Nasonkin et al., 1999) whereas the two posterior-most pairs express *seven up* (*svp*) and *Tb66F2* (also known as *Dorsocrss1*) *D-sur* (Gajewski et al., 2000; Lo and Frasch, 2001). The two anterior-most pairs of *tin*-expressing cardioblasts also express *ladybird* (Jagla et al., 1997). All cardioblasts undergo myogenic differentiation and express D-Mef2 and myosin heavy chain. Based on this combination of molecular markers, the cardioblasts may be subdivided into at least three different subpopulations that could respond to specific developmental programs and differentiate into cells with specific physiological functions. Such a concept is illustrated by the differentiation of ostiae from *svp*-expressing cardioblasts (Molina and Cripps, 2001).

Inductive extrinsic and intrinsic signals dictate and co-ordinate myocardial patterning. It is generally acknowledged that, in the segmented mesoderm, mesodermal precursor cells differentiate into specific tissues depending on their position within the segment (Azpiazu and Frasch, 1993; Riechmann et al., 1997). According to this view, the anterior region of the dorsal mesoderm in each segment (A domain) gives rise to the cardiogenic region within which cardioblasts respond to two inductive signals secreted by the dorsal ectoderm, namely Decapentaplegic (Dpp) and Wingless (Wg) (Frasch, 1999). The cardioblasts that express *svp*, which are integrated into the cardiac tube, have been suggested, however, to originate from the posterior domain (P domain) of each segment (Frémion et al., 1999; Gajewski et al., 2000). The information provided by these extrinsic signals is complemented by intrinsic effectors, including the transcription factors *sloppy paired*, *even skipped* and *muscle specific homeobox gene* (*msh/Dr; Drop*) (d'Alessio and Frasch, 1996; Halfon et al., 2000; Lee and Frasch, 2000). Concomitantly, a further level of diversity is achieved, at least in part, by asymmetric cell divisions (Park et al., 1998; Gajewski et al., 2000; Halfon et al., 2000; Ward and Skeath, 2000).

Finally, more general positional information along the anteroposterior axis is superimposed on this segmental information to complete patterning and differentiation of the cardiac tube. The mature larval cardiac tube broadens in its posterior region to generate the heart with a large cavity or lumen. The heart beat originates from a pacemaker activity in the most posterior region of the tube (Rizki, 1978). The heart is the only part of the larval tube where ostiae differentiate (Molina and Cripps, 2001). A cardiovascular valve develops at the junction between the aorta and heart, in segment A5, the role of which is probably to prevent a backflow of hemolymph (Rizki, 1978; Molina and Cripps, 2001).

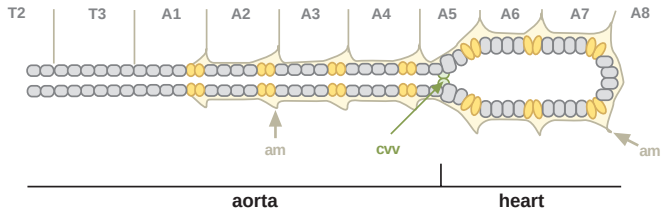

Fig. 1. Schematic representation of the larval cardiac tube. Only cardiac cells are shown in this scheme. The cardiac tube is formed by two rows of cardiac cells which are both epithelial and muscular cells. There are six pairs of cardiac cells per segment from T3 to A7 and only two pairs in T2 and A8. The boundary between aorta and heart is situated within segment A5. The cells shaded grey express *tin*, *β-3 Tub* and *D-sur*. The cells colored yellow express *svp* and *Tb66F*. It has been proposed by Molina and Cripps (Molina and Cripps, 2001) that the *svp*-expressing cells in the heart form the larval ostiae. This scheme has been drawn according to the actual knowledge on cardiac tube differentiation. cvv, cardiovascular valve; am, alary muscles.

Fig. 1 is a schematic synthesis of our current knowledge of the morphology of a fully mature larval cardiac tube.

In the absence of well-characterized molecular markers with which to investigate the anteroposterior subdivision of the cardiac tube, the aim of this work has been to develop tools and methods to study the differentiation and acquisition of cardioblast diversity. Such knowledge is essential for an understanding of the genetic programs underlying functional organogenesis. We show here that the homeotic genes of the *Bithorax Complex* (*BXC*), *Ubx* and *abd-A*, are responsible for the aorta and heart cardioblast identities, respectively, thus providing instructional genetic information along the anteroposterior axis. In addition, part of the segmental information results from secretion of the Hedgehog morphogen, which induces *svp* expression in the two most posterior pairs of cardioblasts in each segment. The superimposition of such segmental and axial information is likely to trigger the differentiation of ostiae from the *svp*-expressing cells exclusively in the heart region.

MATERIALS AND METHODS

Drosophila strains

The *Drosophila* mutant lines used in this study were: hh^{9K}

(temperature-sensitive), hh^{AC} (Heemskerk and DiNardo, 1994), svp^{e22}, svp^{AE127} (Kerber et al., 1998), $abd\text{-}A^{m4}$ (Karch et al., 1990), $Ubx^{9.22}$ (Kerridge and Morata, 1982). $Df(3)Ubx^{109}/Dp(3;3)P5,Sb(1)$ (*Ubx*, *abd-A* double mutant) was obtained from the Bloomington *Drosophila* Stock Center. Homozygous mutant embryos were distinguished from heterozygous embryos in different genotype backgrounds either by the lack of Act-GFP or Dfd-lacZ markers carried by the TM3 balancer, or by the lack of 2nd and (or) 3rd midgut constrictions, or by the absence of oenocytes labeled by anti-Prc.

The following UAS and GAL4 lines have been used: UAS-Ci^{rep} (generous gift from Dr T. Kornberg), UAS-Hh (generous gift from Dr P. Ingham), UAS-HhGPI (Burke et al., 1999), UAS-abd-A (second chromosome), UAS-Armadillo S10 (Pai et al., 1997), UAS-Ubx and UAS-mCD8GFP (second chromosome) (Bloomington *Drosophila* Stock Center), *twist*-GAL4 (Greig and Akam, 1993), 24B-GAL4 (Brand and Perrimon, 1993), *en*-GAL4 (Bloomington *Drosophila* Stock Center).

The strains w;wg^{cx4}UAS-$Armadillo^{S10}$/*Cyo*, hh^{AC},UAS-Hh/TM2 *Ubx* and hh^{AC},UAS-HhGPI/TM3, *Sb*,*Ser* have been constructed by recombination and verified. Several recombinants were obtained in each class and behave in the same manner.

In situ hybridization and antibody staining of whole-mount embryos

In situ hybridization was performed as described previously (Frémion et al., 1999), using digoxigenin (DIG)-labeled antisense RNA probes specific for *svp*I cDNA (Mlodzik et al., 1990). Fixed, staged embryos were stained as described by Frémion et al. (Frémion et al., 1999) with the following primary antibodies: mouse or preadsorbed rabbit anti-β-galactosidase (Promega and Cappel, respectively) 1:1000; rabbit anti-Tinman (Azpiazu and Frasch, 1993) 1:800, preadsorbed; mouse anti-Pericardin (EC11) (Zaffran et al., 1995) 1:2; rabbit anti-D-Mef2 (Nguyen et al., 1994) 1:1000, preadsorbed; rabbit anti-α-Spectrin (Lee et al., 1993) 1:500, preadsorbed; mouse anti-En (4D9, Developmental Studies Hybridoma Bank) 1:4; mouse anti-Abd-A (Karch et al., 1990) 1:1000; mouse anti-Ubx (White and Wilcox, 1985) undiluted; mouse anti-Abd-B (Celniker et al., 1989) 1:100. Affinity purified secondary antibodies were either coupled to alkaline phosphatase or to biotin (Jackson Immuno Research Laboratories) and used at a 1:1000 dilution or were either Alexa-488 or Alexa-594 conjugated (Molecular Probes, Inc.) and used at a 1:500 dilution. In some cases, the signal was amplified with the aid of a Tyramide Signal Amplification kit (NEN Life Sciences). The stained embryos were mounted in Geltol Medium (Immunotech, France) or, when fluorescent, in Vectashield (Vector Laboratories) for further observation under an Axiophot Zeiss microscope or a LSM 410 Zeiss confocal microscope.

Larval histology

Wandering third instar larvae were removed from the culture vessel and initially pinned through the head to a Petri dish containing Sylgard 184 (Dow Corning Corp., Midland, MI, USA) with the dorsal side down. A longitudinal incision was made along the ventral midline using fine scissors, and the body wall was stretched and pinned out to generate a flat fillet. Viscera were removed, taking care not to dislodge the heart in its location at the dorsal midline. The nervous system and other anterior structures including the imaginal discs were left intact because the anterior end of the heart is not attached to the dorsal body wall and the heart would otherwise move too freely.

All dissections were carried out in phosphate-buffered saline (PBS). Samples were fixed in 3.7% formaldehyde in PBS for 20 minutes on ice, then washed twice for 5 minutes in PBS and further processed for antibody labeling as indicated above.

Movie capture

The embryos were dechorionated, glued onto glass slides with sticky tape, covered with a drop of oil (Voltalef 10S) and observed under an inverted Olympus CK40 microscope, with a ×60 objective. Films were recorded, using Adobe Premiere, every 15 minutes between 15 and 23 hours of embryonic development at 25°C.

RESULTS

Differentiation of the cardiac tube during embryogenesis

Whereas the morphology and physiology of the mature larval heart have been extensively studied, little is known about functional cardiogenesis that takes place during embryogenesis. We have developed suitable tools to observe the morphology of cardiomyocytes along the anteroposterior axis and describe their features with precision. We have chosen morphological criteria that are more appropriate than markers of expression to investigate the organogenesis and differentiation of the cardiac tube. Whole-mount embryos labeled with antibodies were observed under a confocal microscope. The antibodies used include anti-α-Spectrin (Lee et al., 1993) that marks the basal-lateral membrane of epithelial cardiomyocytes (Zaffran et al., 1995; Frémion et al., 1999), anti-Pericardin (Chartier et al., 2002) that labels the basal membranes of the cardiomyocytes, anti-β-galactosidase that detects a *lacZ* reporter gene product inserted in the *svp* gene, which reflects *svp* expression (Gajewski et al., 2000; Lo and Frasch, 2001) and anti-DMef2 (Bour et al., 1995) that labels the nuclei of all myogenic cells including cardioblasts.

Cardioblasts undergo a mesenchymal-epithelial transition during embryonic stages 12 and 13, which results in the formation of two bilateral monolayers of cardioblasts situated beneath the most dorsal ectodermal cells, on either side of the dorsal aperture (Zaffran et al., 1995; Frémion et al., 1999). This and subsequent morphological steps occur without further cell division. At these stages, cardioblasts display no evident signs of heterogeneity with respect to their size or morphology along the whole length of the cardiac tube. At stage 14, during dorsal closure, when the two rows of cardioblasts migrate towards the dorsal midline in a movement coordinated with that of the ectoderm (Chartier et al., 2002), clear morphological differences become apparent (Fig. 2). Cells in the presumptive heart region increase in size, elongate along the transverse axis and adopt a columnar shape, while the cells in the aorta region remain smaller and more cuboidal (Fig. 2D,E). Likewise, the round nuclei of the cardioblasts become larger in the heart than in the aorta (Fig. 2K,L). Moreover, differences among the cardioblasts within the heart region begin to emerge. Three pairs of metamerically repeated cells in both rows of cardioblasts develop more even cell contours than the other cardioblasts and become thinner. Their nuclei elongate, adopting an egg-like shape.

The cardiac tube of stage 16 embryos displays the same general morphology as the larval cardiac tube and presents evidence of differentiation along the anteroposterior axis. The cardiac cells are two to three times larger in the heart than in the aorta and this leads to a broadening of the cardiac tube in the heart region (Fig. 2F,G). At the same time, seven segmentally repeated pairs of cardioblasts per half embryo adopt a morphology that distinguishes them from the other cardioblasts, both in the heart and in the aorta. Their nuclei are more elongated (Fig. 2M,N) and the cells are thinner and have

less sharp edges than the other cells of the cardiac tube. In addition, these cells are unambiguously larger in the heart than in the aorta.

These seven pairs of cardioblasts express *svp* (Fig. 2O,P) and lie just beneath the alary muscles, which can be precisely localized by their labeling with anti-Prc (Fig. 2G,L). The *svp*-expressing cells differentiate in the larva into two distinct structures depending on their position along the anteroposterior axis (Molina and Cripps, 2001). In the aorta, the *svp*-expressing cells form a butterfly-like structure that evenly interrupts the epithelial sheet constituting the cardiac tube (Fig. 3A), without differentiating into functional ostiae since there are no openings between the two cells (Fig. 3C). In contrast, these same cells give rise to larval ostiae in the heart region (segments A5-A7) (Fig. 3B,D) (Molina and Cripps, 2001).

Monitoring late differentiation and function of the cardiac tube in living embryos

Analysis of later differentiation of cardioblasts by immunohistochemistry is considerably hampered by the concomitant differentiation of an insulating cuticle, which restricts the penetration of the antibodies into the embryos. We have therefore studied the late differentiation of cardiac cells by direct observation of the development of the cardiac tube in living embryos. Moreover, in vivo observation offers the additional advantage of being able to monitor the

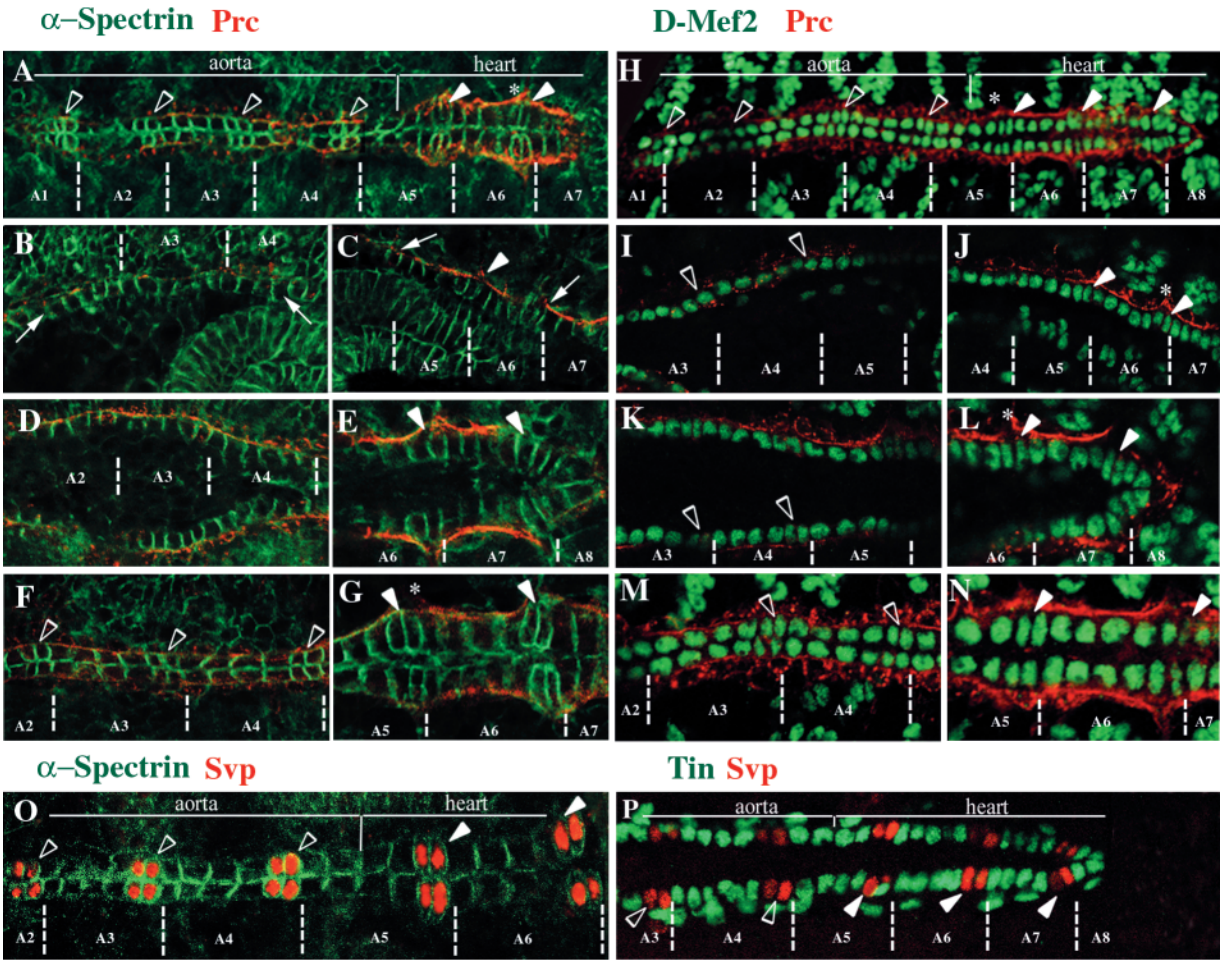

Fig. 2. Differentiation of the cardiac tube during embryogenesis. Dorsal confocal images of the embryonic cardiac tube double labeled with (A-G) anti-Prc (red) and anti-α-Spectrin (green); (H-N) anti-Prc (red) and anti-DMef2 (green); (O) anti-α- Spectrin (green) and anti-β-gal in svp^{AE127}P-line (red); (P) anti-Tin (green) and anti-β-gal in svp^{AE127}P-line (red). (A,H) Whole cardiac tube; (B,D,F,I,K,M) part of the aorta; and (C,E,G,J,L,N) part of the heart. (A,H) At late stage 16, the cardiomyocytes in the cardiac tube display signs of morphological heterogeneity. The cells in the aorta (open arrowheads) and in the heart (arrowheads) with morphological features that distinguish them from the other cardioblasts situated between them are the cells that express *svp* (O,P). The other cardioblasts are the *tin*-expressing cells (P). Their nuclei in (H) have an egg-like shape as compared to the nuclei of the other cardioblasts. These morphological features are more pronounced in the heart than in the aorta. Note the larger size of the heart cardioblasts compared to the aorta cardioblasts. (B,C,I,J) At the onset of dorsal closure (stage 13), all cardioblasts along the whole length of the cardiac tube appear similar (arrows). The *svp*-expressing cardioblasts, however, which will differentiate into the second ostia in the heart, have initiated a slight change in their shape (arrowheads). Only one row of cardioblasts is shown. (D,E,K,L) At late stage 14, the cardiac tube is not yet closed but the cardioblasts in the heart are already larger than in the aorta and the *svp*-expressing cells (arrowheads) have acquired their distinctive morphology. (F,G,M,N) At stage 16, the difference in size between heart and aorta cardiomyocytes is accentuated. The specific morphology of the *svp*-expressing cells is clearly visible in the aorta (open arrowheads) as well as in the heart (arrowheads). The asterisks in A,G,H,J,L indicate the alary muscles. In all views, anterior is to the left.

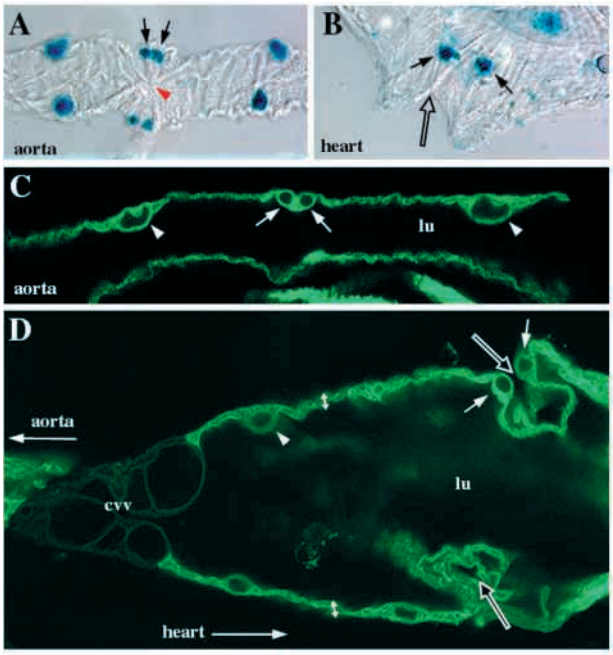

Fig. 3. Differentiation of the larval ostiae. Dissected third instar larval cardiac tube stained with anti-DMef2 (A,B) or with mCD8GFP (C,D) whose expression is driven in the myogenic lineage by 24B-GAL4. (A,B) The large nuclei labeled by anti-DMef2 are *tin*-expressing cells nuclei whereas the small nuclei are *svp*-expressing cell nuclei (Molina and Cripps, 2001). In the aorta, these latter cells (arrows) form a butterfly-like structure in which the striated muscle fibers from each cell converge in a central position (red arrowhead). No sign of opening is apparent in the aorta while, in the heart, the cardiomyocytes with small nuclei form genuine ostiae (large open arrow). (C,D) mCD8GFP labels the membranes of all the cardiomyocytes. The two cells with small nuclei (arrows) in the aorta are flanked on either side by two cells with larger nuclei (arrowheads) and they do not form a functional ostia. Genuine opening is visible in the heart between the first pair of ostiae cells (long open arrow). The aorta is separated from the heart by a non muscular cardiovascular valve (cvv). The double headed arrow points to the very thin cytoplasm in the heart. lu, lumen.

acquisition of cardiac function, namely heart beating and ostiae function.

Fig. 4 shows a series of photographs extracted from a video movie that are representative of the main features of embryonic heart development (movie available at http://dev.biologists.org/supplemental/). Immediately after the tube has closed, the cardiomyocytes became columnar and discernible size differences are already observed between aorta and heart. Rapidly, around 17 hours after fertilization at 25°C, the first signs of muscular activity are observed in the posterior region of the heart. Simultaneously, the lumen in the heart region dramatically increases in size and the cell wall of the cardiac cells become thinner, the cells change from columnar to a more corrugated form that results from the protrusion of the nuclei of *tin*-positive cells into the lumen. In contrast, the aorta does not exhibit many signs of differentiation. A lumen forms but remains small and no autonomous muscular activity is perceptible. Fig. 4E-V depicts the cardiac tube of embryos shortly before hatching.

Observation of living embryos led to the conclusion that ostiae were functional very soon after the first signs of heart muscle activity. They open and close, allowing the entry of rare and large hemocytes that, once in the cardiac tube, are readily propelled towards the aorta (Fig. 4 and movie). The two most posterior pairs of ostiae are the first to form and to become functional. The aperture of the ostiae is regulated by the movement of the two component cells as illustrated in Fig. 4M-V. A filamentous network links together the ostiae cells on either side of the cardiac tube. This filamentous material might serve to isolate the different compartments or chambers of the heart. The hemocytes stay for some time in a chamber, moving backwards and forwards like a ping-pong ball, before eventually being expelled into the aorta.

The recording of heart activity in living embryos has also provided the opportunity to measure another important aspect, namely the sizes of end-diastolic and end-systolic diameters. We calculated these values at eclosion (22 hours at 25°C) to be 22.6 μm and 15.1 μm, respectively.

These results establish morphological and functional criteria that have been used to subsequently characterize the state of differentiation of subpopulations of cardioblasts in various genetic backgrounds.

Expression of the homeotic genes of the *Bithorax Complex* (*BXC*) is regionalized in the cardiac tube

We hypothesized that *BXC* homeotic genes are likely candidates to control patterning of the cardiac tube along the anteroposterior axis, primarily because these genes are responsible for axial patterning of the whole organism. Secondly, *Ubx* and *abd-A* have been reported to be expressed in the cardiac tube in which they can be expected to display a regionalized expression profile (Karch et al., 1990; Bate, 1993). We have analyzed in detail the sites of expression of BXC proteins in the embryonic cardiac tube. The sites of Abd-A and Abd-B expression were analyzed with respect to the *svp*-expressing cells, which were identified by monitoring *lacZ* reporter gene activity in the svp^{AE127} line. To investigate Ubx expression, cardioblasts were double-labeled with anti-Ubx and anti-Tin in wild-type embryos instead of svp^{AE127} embryos to circumvent the fact that the TM3 balancer carries a mutation in *Ubx* that might affect the wild-type level of Ubx expression.

The level of expression of Ubx in the cardiac tube was low compared to that in the ectoderm or gut. Strongest expression was observed in the cardiac and pericardiac cells of the aorta, from segment A2 to the middle of segment A5 (Fig. 5A). Weaker expression was detected in the more anterior segments A1 and T3 and only extremely low expression was observed more posteriorly, in the heart region.

In contrast, Abd-A expression was observed in all the cardioblasts in the heart region, except in the most posterior cardioblasts in segment A8 and in the two pairs of most anterior cardioblasts in segment A5, in which the expression of Abd-A was considerably lower (Fig. 5D). The region of strong Abd-A expression encompasses the two pairs of cells expressing *svp* in segment A5 anteriorly and, the two last pairs of *svp*-expressing cells that belong to segment A7 posteriorly. A subpopulation of pericardiac cells present in segments A5 to A7 also strongly expresses Abd-A.

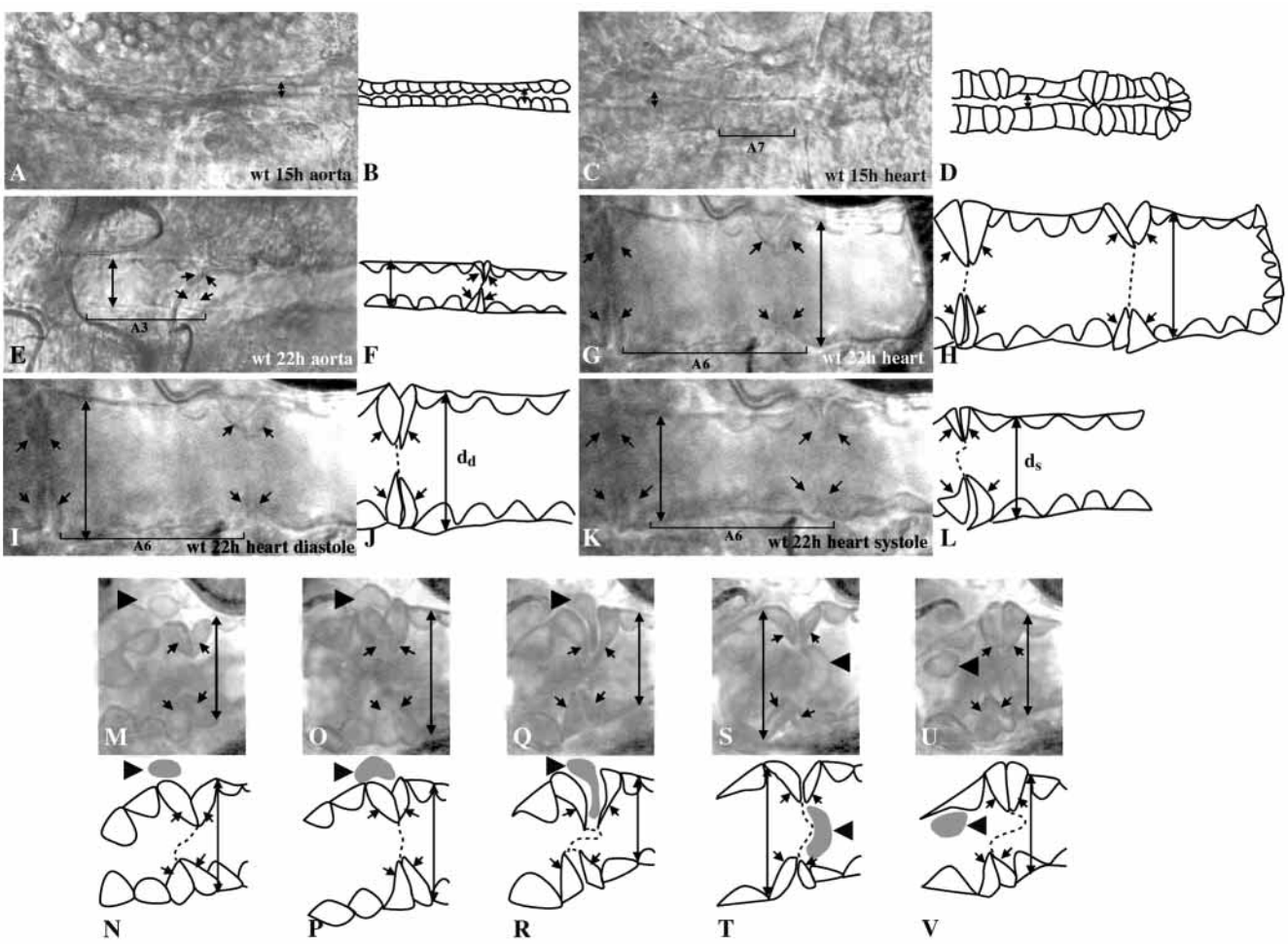

Fig. 4. Observation of the cardiac tube in wild-type embryos. A schematic drawing of the cardiac cells in the cardiac tube is presented for each photograph. Double-headed arrows delineate the internal limit of the cardiac tube, small arrows show the ostiae cells and large arrowheads, the hemocytes. (A-H) Progression of the development of the aorta (A,B,E,F) and of the heart (C,D,G,H). The cardiomyocytes enlarge with the maturation of the cardiac tube. Ostiae are fully functional at 22 hours. (I-L) Recordings of heart beat at 22 hours allow measurement of the end-diastolic (d_d) and end-systolic (d_s) diameters. (M-V) Entry of hemolymph through the ostiae into the cardiac cavity is apparent from the passage of a large hemocyte (large arrowhead). The ostiae cells and the hemocytes adjust their shapes to allow transport of the hemocytes. Note the filamentous material (dotted lines) linking the two pairs of ostiae on either side of the cardiac tube.

Abd-B expression in the cardiac tube is restricted to the 4 most posterior cardioblasts in segment A8 (Fig. 5G). The two pairs of *svp*-expressing cells in segment A7 also express Abd-B, although to a lesser extent.

This specific anteroposterior distribution of the three BXC gene products in the cardiac tube was analyzed in different mutant backgrounds. In embryos homozygous for a null *abd-A* mutation (*abd-A*^{m4}), Ubx expression extended into the heart region (Fig. 5B). In contrast, Abd-A expression, in embryos homozygous for a null mutation in *Ubx* (*Ubx*$^{9.22}$) was similar to that in wild-type embryos (Fig. 5E). Likewise, Abd-B expression was not affected in *abd-A* mutants or in *Ubx,abd-A* double mutants (Fig. 5H,I).

Overexpression of Abd-A in the whole mesoderm, including cardiac cells, using *twist*GAL4 as driver, led to a significant decrease of Ubx expression in the aorta (Fig. 5C). In contrast, ectopic overexpression of Ubx did not result in any significant change in Abd-A expression (Fig. 5F).

Ubx and *abd-A* are required for anteroposterior patterning of the cardiac tube

In homozygous *abd-A*^{m4} mutant embryos, in contrast to the situation encountered in wild-type embryos, the general morphology of the cardioblasts was uniform along the whole length of the cardiac tube even at late stage 16. All the cells had the configuration of aorta cardioblasts, including the *svp*-expressing cells which displayed the features of *svp*-aorta cells (Fig. 6A,B). This observation was further supported by analysis of cardiac tube development in living embryos. In *abd-A* mutants, the heart region never differentiated and the entire cardiac tube remained as an aorta (Fig. 6C,D). Cardiac cells in the heart region did not modify their shape nor did the heart beat. No sign of ostiae function was observed.

In contrast, in *Ubx* loss-of-function mutant embryos, heart cardioblasts were properly differentiated and the function of the heart was similar to that in wild-type embryos (not shown). The most anterior part of the aorta showed, however, some

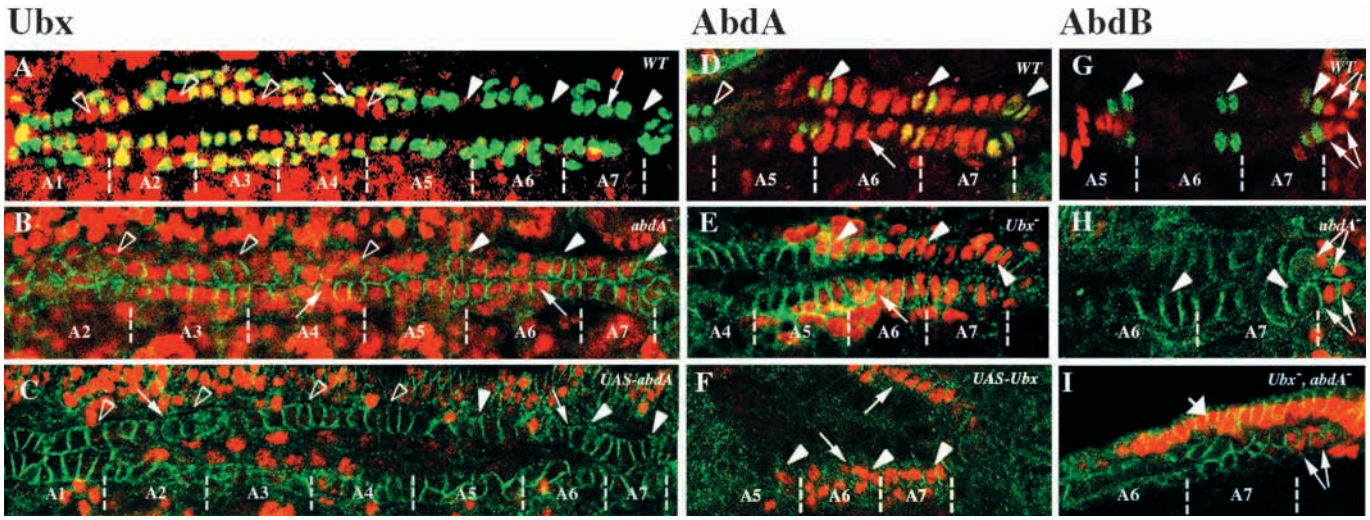

Fig. 5. Expression of Ubx, Abd-A, Abd-B in the cardiac tube. Immunostaining with anti-Ubx (A-C; red), anti-Abd-A (D-F; red), anti-Abd-B (G-I; red) or double labeling with anti-Tin (A, green), anti-α-Spectrin (B,C,E,F,H,I; green) and anti-β-gal (D,G; green). Open arrowheads indicate *svp*-expressing cardioblasts in the aorta; solid arrowheads indicate *svp*-expressing cardioblasts in the heart. (A,D,G) Wild-type embryos. Ubx (in A) is expressed in all the cardioblasts (arrow) in the A2 to A5 segments (yellow) including the *svp*-expressing cells (red) that do not express Tin and a subset of pericardiac cells (asterisks). Abd-A (in D) is expressed in the cardioblasts (arrow) in the A5 to A7 segments, including the *svp*-expressing cells (yellow). Abd-B (in G) is expressed in the four posterior most cardioblasts (arrows) and, to a lesser extent, in the *svp*-expressing cells in segment A7. (B,H) *abd-A*^{m4} mutant embryos. Ubx (in B) is ectopically expressed in cardioblasts in the posterior A5 to A7 segments (arrows), including the *svp*-expressing cells. Abd-B (in H) expression is as in wild-type embryos (arrows). (C) UAS-*abd-A*, *twist*-GAL4 embryos. Ubx expression in the aorta (A1-A5 segments) is considerably less than in wild-type embryos. *tin*-expressing cardioblasts (arrows) and *svp*-expressing cardioblasts. (E) *Ubx*$^{9.22}$ mutant embryos. The expression of Abd-A is as in wild-type embryos (arrows). (F) UAS-*Ubx*, *twist*-GAL4 embryos. Abd-A expression is as in wild-type embryos (arrows). (I) *Ubx, abd-A* double mutant embryos. Abd-B expression is as in wild-type embryos (arrows). The large arrow points to the ectodermal cells expressing Abd-B.

perturbations (compare Fig. 6E and 6F). Polarization and thus differentiation of the cardioblasts are not clearly apparent in segments T3 and A1 in *Ubx* mutant embryos as is the case in wild-type embryos (Fig. 6E,F). These features seem, however, to extend more posteriorly to segment A2 in mutant embryos (Fig. 6E). In addition, pericardiac cells were disorganized in several positions, particularly along these anterior segments.

In *Ubx-abd-A* double mutants, the early stages of cardiac tube morphogenesis proceeded as in wild-type embryos but later differentiation of cardiomyocytes was impaired. The number of cardioblasts in the cardiac tube as well as in each segment was not modified and the segmental expression of *svp* was normal (Fig. 6I,J). No heterogeneity in the morphology of the cardioblasts could be observed along the anteroposterior axis nor did the tube differentiate into heart and aorta (Fig. 6G,H). The cells were not conspicuously polarized along the whole length of the cardiac tube and had a configuration similar to that of the cells in the anterior-most segments (T3 and A1) of wild-type and *Ubx* mutant embryos. Prc-expressing cells were disorganized as in *Ubx* single mutations. Other cells that did not express Prc and that were hypothesized to be lymph gland cells were found in ectopic locations along the tube.

All these observations suggest that in the absence of *Ubx* function, aorta cardiomyocytes could be transformed in cells and structures normally present in segments more anterior than A2 in wild-type embryos.

Overexpression of *abd-A* in the whole cardiac tube resulting in a gain-of-function of *abd-A* in the aorta led to a partial transformation of aorta cardioblasts into heart cardioblasts (Fig. 6K). The most posterior segments of the aorta (A4 and A5) were invariably transformed into heart, including the *svp*-expressing cells that give rise to functional ostiae in these segments (Fig. 6L,M). Although varying from embryo to embryo, the transformation of more anterior segments into a heart phenotype was more or less complete. Also, overexpression of *Ubx* significantly impaired the general morphology of the cardioblasts particularly in the heart region (Fig. 6N). In living embryos, the heart lumen and the timetable for heart beat appeared normal. An important delay was observed in cardioblasts differentiation (Fig. 6O,P) and the development of ostiae was perturbed and they never became functional.

The function of *svp* is required for the differentiation of ostiae

Complete loss-of-function of *svp* (*svp*^{e22} or *svp*^{AE127}) resulted in embryonic lethality. However, the development of the embryos proceeded as in wild-type embryos although muscular movements were impaired at the end of embryogenesis and the embryos never hatched and eventually died. The cardiac tube (Fig. 7A-D) differentiated properly into aorta and heart and cardiac muscular activity was observed, although with some delay. Similarly, the ostiae cells exhibited a delay in their shape remodeling and appeared more like *tin*-positive cardiomyocytes (Fig. 7A-D) in stage 16 embryos. In 22-hour-old *svp* mutant embryos, the ostiae cells regained part of their characteristic morphological features, but were still incompletely differentiated (Fig. 7F,G). They were not functional as evident from a lack of opening and closing and no hemocytes were ever observed entering the apertures.

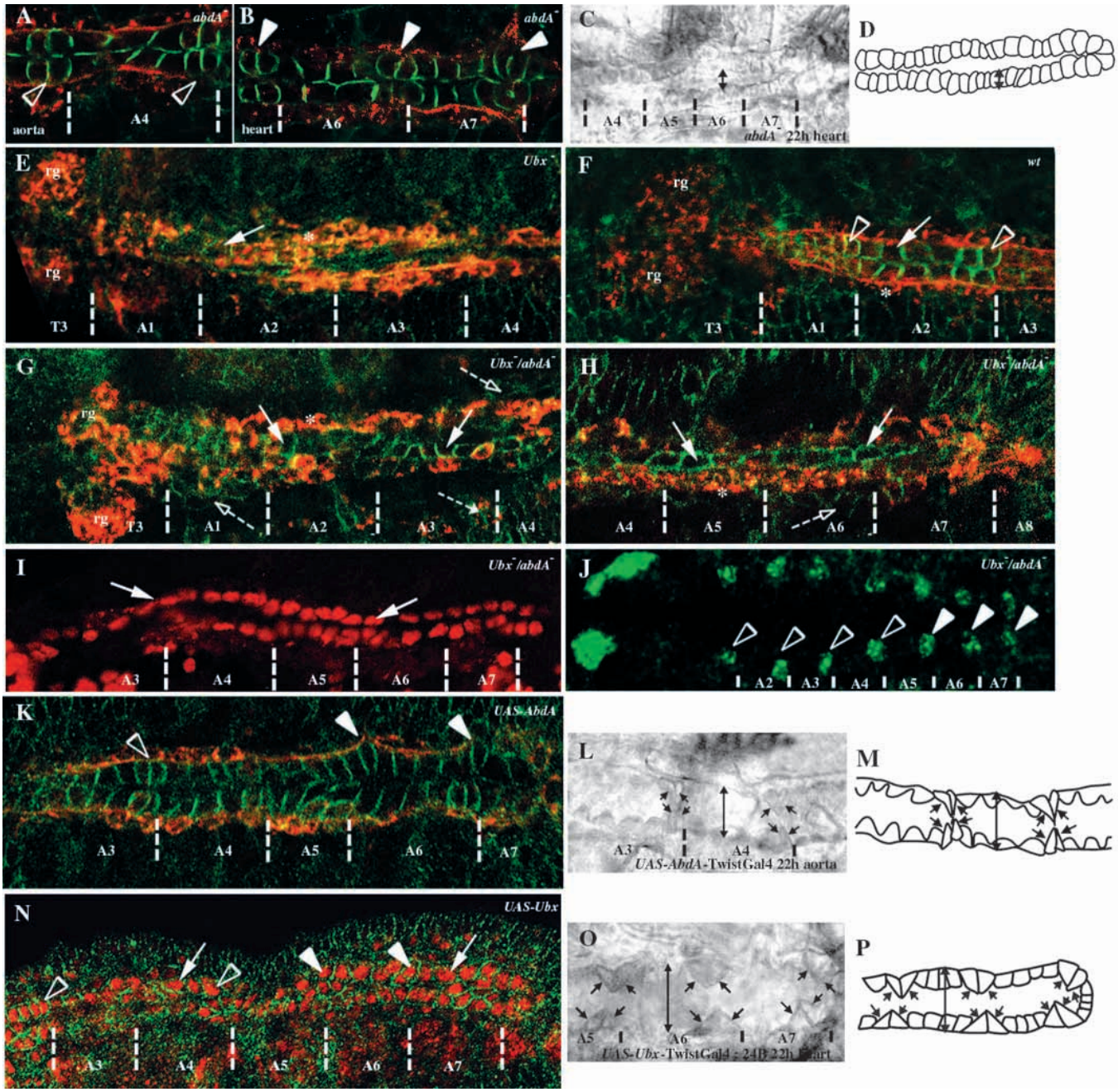

Fig. 6. Function of *abd-A* and *Ubx* in the differentiation and the function of the cardiac tube. Double labeling of cardiac tubes with anti-α-Spectrin (green) and (A,B,E,F,G,H,K) anti-Prc (red) or (N) anti-Ubx (red). (I,J) Staining with anti-D-Mef2 (red, I) and *svp*-RNA (green, J). (C,L,O) In vivo observations (D,M,P) and their schematic representations. In all panels, open arrowheads indicate *svp*-expressing cardioblasts in the aorta and arrowheads indicate *svp*-expressing cardioblasts in the heart. (A-D) In *abd-A* homozygous mutant embryos, the heart cardiomyocytes differentiate as aorta cardiomyocytes along the whole length of the cardiac tube. They are same size along the anteroposterior axis (double-headed arrow). The *svp*-expressing cardioblasts in the aorta and in the heart have the same morphology. As a consequence, the heart does not beat (or only very weakly) and the ostiae are not functional. (E,F) In $Ubx^{9.22}$ mutant embryos (E), the aorta does not differentiate normally, particularly in the anterior region (compare to wild-type anterior aorta in F). (G,H) In *Ubx, abd-A* double mutant embryos the cardiac tube shows no heterogeneity along the anteroposterior axis and the aorta and heart do not differentiate properly. Cardioblasts appear smaller and less polarized than in wild-type embryos (arrow). Prc-expressing cells are disorganized (asterisk) and sometimes located in ectopic positions (dotted arrow). Non epithelial cells, which do not express Prc, form clusters along the cardiac tube (open dotted arrow). (I,J) In double mutant embryos, cardioblasts differentiate into the same number of cells expressing D-Mef2 as in wild-type embryos (arrows). Their size and shape are uniform along the whole length of the tube. (J) *Svp* expression is observed in the most posterior cardioblasts in each segment. (K-M) In UAS-*abd-A, twist*-GAL4 embryos, the aorta is transformed into heart in segments A5, A4 and part of segment A3. *svp*-positive cells differentiate as in a wild-type heart and functional ostiae (arrows in L,M) are visible in segments A4 and A3. (N,O,P) In UAS-*Ubx, twist*-GAL4, 24BGAL4 embryos, Ubx is ectopically expressed in heart region (arrows in N). Cardioblasts in the whole tube do not have a normal morphology (O,P) nor are the ostiae functional. Double-headed arrows delineate the internal limit of the cardiac tube. rg, ring gland.

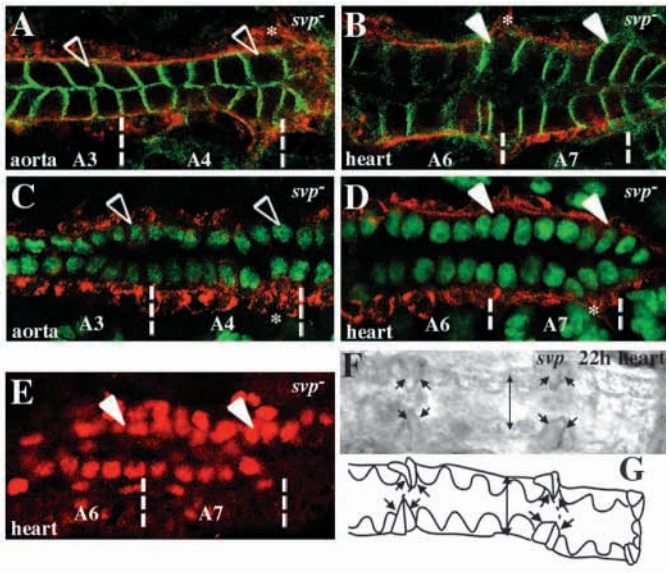

Fig. 7. Differentiation of ostiae in *svp* mutant embryos. (A-D) Confocal sections of late stage 16 embryos double labeled with anti-Prc (red) and either anti-α-Spectrin (green) (A,B) or anti-D-Mef2 (green) (C,D). Differentiation into aorta and heart occurs correctly but the change in morphology (A,B) or in the shape of nuclei (C,D) of the *svp*-expressing cells (arrowhead and open arrowhead) is not apparent, in either the heart or the aorta. The asterisks mark the alary muscles. (E) In *svp* mutant embryos, expression of Abd-A (red) in all cardioblasts in segments A5-A7, including *svp*-positive cells (arrowheads) is similar to that in wild-type embryos. (F,G) In vivo observation of the cardiac tube in *svp* mutant embryos. The heart starts beating normally but almost completely stops after 22 hours of development. The ostiae cells (arrows) differentiate after an initial delay; differentiation, however, remains incomplete and they do not function (or open and close). Double arrows mark the internal limit of the lumen.

Finally, the loss of *svp* function did not affect the limits of the expression domains of the *BXC* genes, including *abd-A* (Fig. 7E). These observations suggest that the function of *svp* is required for functional ostiae to differentiate properly but that *svp* does not participate in the process that leads to differential patterning of the aorta and heart.

The expression of *svp* and the segmental diversity of cardioblasts require the *hedgehog (hh)* signaling pathway

The segmental diversity of the cardioblasts is unlikely to result from the action of the *BXC* genes, since the pattern of segmental expression of *tin* and *svp* were not altered in *Ubx* and *abd-A* mutants (see Fig. 5 and Fig. 6). A more likely hypothesis is that segmental diversity relies on a segmental information, which could be delivered by inductive morphogens secreted by the segmented ectoderm. *svp* expression in the cardiac tube is restricted to the two most posterior pairs of cardioblasts in all segments from A1 to A7 (Gajewski et al., 2000; Lo and Frasch, 2001) (Fig. 8A-C), a position that could be influenced by Hedgehog signaling, which is secreted by cells in the posterior domain of ectoderm parasegments.

In *hh* mutant embryos, the expression of *svp* RNA was dramatically reduced or even abolished (Fig. 8E). A temperature-sensitive mutant allele of *hh*, hh^{9k}, has been used in our experiments and the temperature shift was performed at a time where the activities of the *hh* and *wg* signaling were no longer dependent on each other (Bejsovec and Martinez-Arias, 1991) such that the observed effects are due to specific inhibition of *hh* signaling activity. In parallel, Tin expression was ectopically extended (Fig. 8F) due at least in part, to the repression of *tin* expression by *svp* (Gajewski et al., 2000; Lo and Frasch, 2001). It has been shown that *tin* and *svp* expression in cardioblasts are exclusive (Gajewski et al., 2000; Lo and Frasch, 2001). In a *svp* mutant embryo, *tin* is ectopically expressed in the *svp*-expressing cardioblasts, and reciprocally, ectopic expression of *svp* represses *tin* expression. The expression of Engrailed (En) was taken as a reference for the position of the segments.

The decrease in *svp* expression in *hh* mutants was confirmed by expressing a repressor form of Cubitus interruptus (Ci^{rep}) (Aza-Blanc et al., 1997) in the whole mesoderm, including cardiac precursor cells, using *twist*-GAL4/24B as driver. In this situation, in which Ci^{rep} inhibits *hh* signaling in the cells that receive it, *svp* expression was significantly reduced in the cardioblasts (Fig. 8G), although not completely abolished and the reduction was less effective than that observed in a hh^{9k} mutant embryo. One explanation could be that part of the *hh* signal was transmitted by a Ci-independent pathway (Gallet et al., 2000; Apidianakis et al., 2001) or, more likely, low level expression of Ci^{rep} in the *svp*-expressing cardioblasts was insufficient to fully repress *hh* signaling. Nonetheless, the marked reduction of *svp* expression in repressed cardioblasts suggests that *hh* signaling is required for the activation of *svp* expression in cardioblasts.

In support of this conclusion, the lack of *svp* could be rescued by expressing *hh* in the posterior compartment of the ectodermal segments, using the *en*GAL4/*hh*UAS system in a hh^{AC} null mutant background (Fig. 8H). It should be noted that no rescue of *svp* expression was obtained by using, in the same genetic context, an Hh form that cannot be secreted and that remains bound to the membrane (Burke et al., 1999) (Fig. 8I). These results suggest that *svp* expression in cardioblasts is under the control of Hh produced and secreted by ectodermal cells.

Contribution of Wg signaling to *svp* expression was ruled out by maintaining *hh* expression in a *wg* null mutant background. A constitutively active form of Armadillo (Arm^{S10}) was expressed under the control of En in wg^{cx4} embryos, which can be identified by the widened En positive stripes (Pai et al., 1997). In such embryos, *svp* expression was similar to that in wild-type embryos (Fig. 8J), confirming that Hh secretion by the ectoderm is required for *svp* expression in the mesoderm.

In conclusion these results indicate that the segmental diversity of the cardioblasts is provided in part by the secreted morphogen Hh.

DISCUSSION

Generation of cell diversity within an organ is critical to its physiological function. In *Drosophila*, the functional cardiac tube contains only 104 cardiomyocytes that diversify to

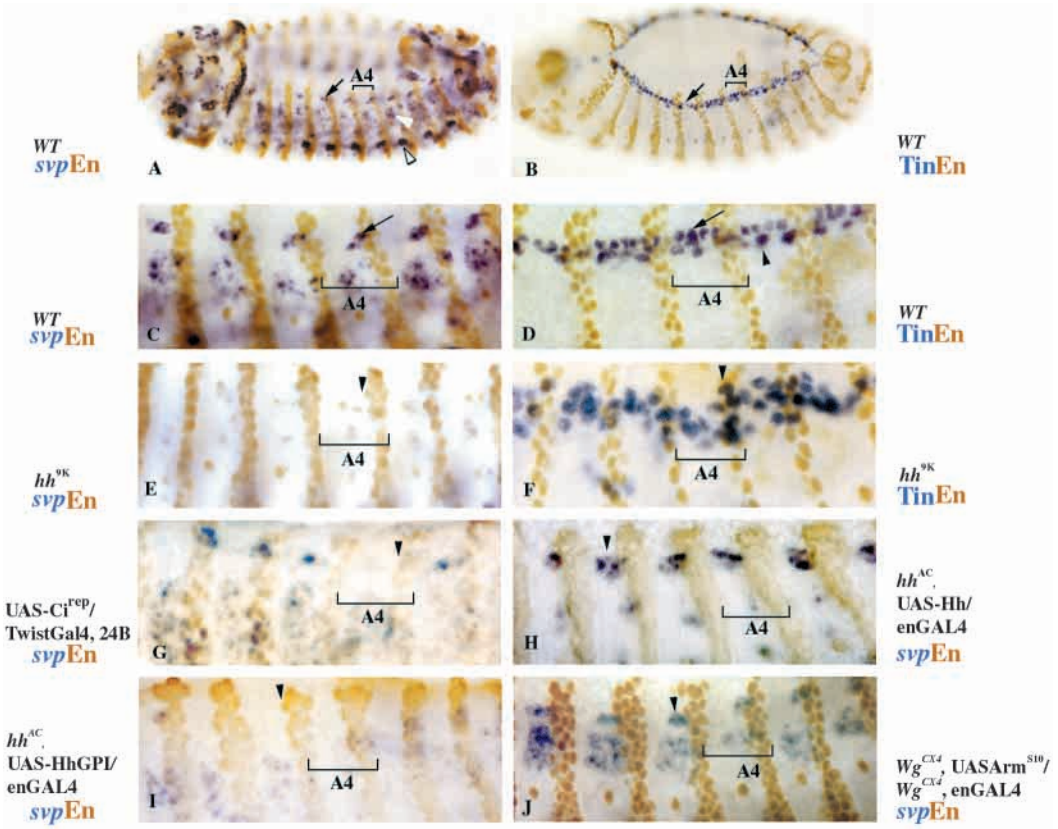

Fig. 8. The activity of the *hh* signaling pathway is required for the transcriptional activation of *svp*. Stage 13 embryos double stained with an anti-Engrailed (En, brown) antibody to position the segmental boundaries and either a DIG-labeled *svp* riboprobe (blue, A,C,E,G-J) or anti-Tin (blue, B,D,F). Anterior is to the left and dorsal is up. (A,B) Whole embryos; (C-J) enlargements showing the central abdominal region of the embryo. (A-D) Wild-type embryos. *svp* is expressed in seven pairs of cardioblasts per hemi-embryo in the segments A1-A7 (arrow), in the chordotonal organs (white arrowhead) and in the oenocytes (open arrowhead). Tin is expressed in four cardioblasts (arrow) per hemi-embryo in the segments T3 to A7 and in a subset of pericardiac cells (arrowhead). Tin and *svp* are expressed in different subsets of cardioblasts (see Fig. 2P). (E,F) In hh^{9K}, expression of *svp* is abolished in *svp* cardioblasts (arrowhead) as well as in the chordotonal organs. More cells express Tin (arrowhead), including cardioblasts that express *svp* in the wild type. (G) The expression of Ci^{rep} driven by *twist*-GAL4/24B represses *svp* expression in the cardioblasts (arrowhead), although not completely (compare with E). (H) Expression of Hh in ectodermal En-expressing cells rescues the expression of *svp* (arrowhead). (I) Expression of a GPI membrane-bound form of Hh does not rescue *svp* expression (arrowhead). (J) Hh expression is maintained in the En-expressing cells in an otherwise *wg* mutant background. The function of *wg* is not required for the expression of *svp* (arrowhead).

generate distinct subtypes of cells with specific developmental and cellular properties. Owing to its relative simplicity, *Drosophila* cardiogenesis constitutes a unique model with which to investigate the genetic programs that provide cell diversity and specific cell differentiation within an organ.

Two types of seemingly independent positional information, axial information along the anteroposterior axis and segmental information, are required to pattern the cardiac tube. In regard to the first type of information, the results presented herein indicate that the homeotic genes, *Ubx* and *abd-A*, are responsible for the identity of aorta and heart cardiomyocytes, respectively. Concerning segmental information, the cardiac tube is indeed segmented and the *hh* signaling pathway induces (by turning on *svp* expression) the diversification of a subpopulation of cardiomyocytes within each segment in the tube. It is the intersection of these two types of positional information that triggers the differentiation of heart cardiomyocytes expressing both *abd-A* and *svp*, into three pairs of ostiae. The scheme in Fig. 9 summarizes these observations (Fig. 9).

The observation that the segmental diversity of cardiomyocytes was not influenced by the function of homeotic genes suggests that the two programs operate independently, in parallel pathways. The segmental restriction of *svp* expression to two cardioblasts per segment was unchanged in a *Ubx,abd-A* double mutant cardiac tube (Fig. 6). Similarly, the pattern of expression of Abd-A was maintained (Fig. 7). How information from these two independent pathways is interpreted by specific transcriptional targets will be a major goal for future studies. A likely target in the heart cells forming the ostiae could be *wingless*, which is expressed exclusively in the mature cardiac tube in *svp*-positive cardiomyocytes of the heart (our own observations) (Gajewski et al., 2001). It can also be anticipated that *tin*-positive cells in the heart will express specific genes responsible for the distinct physiological activity of the heart versus the aorta cardiomyocytes. One can expect that the transcription of these candidate genes will be activated by *tin* and *abd-A* but not by *tin* and *Ubx*.

Axial patterning of the cardiac tube

The morphological and functional criteria that we have defined in this study have allowed us to subdivide cardiomyocytes into two distinct populations that acquire different identities and differentiate according to their positions along the anteroposterior axis. *Ubx* is expressed in almost all cardiomyocytes of the aorta whereas *abd-A* is expressed in almost all cardiomyocytes of the heart. The lack (or a very low level) of *Ubx* expression in the T3 and A1 segments of the aorta suggests that cardiomyocytes in these segments may be exposed to a distinct mode of differentiation. In support of this

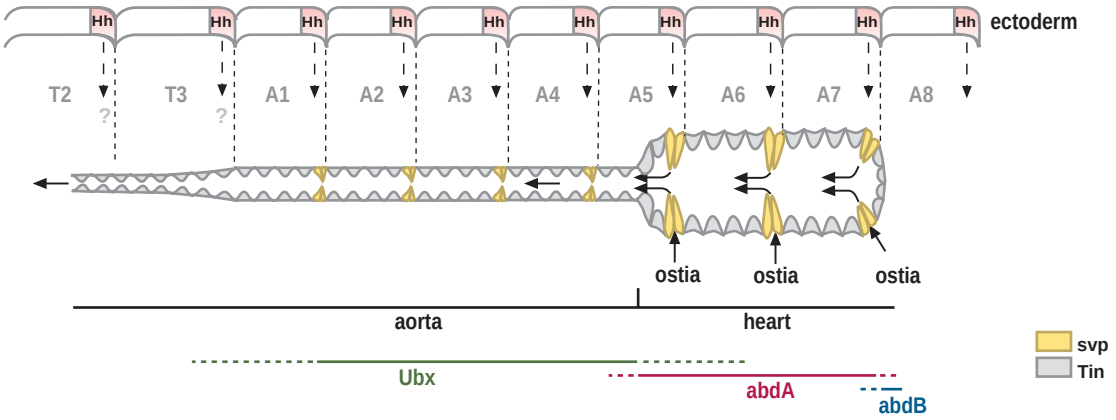

Fig. 9. Schematic representation of the cardiac tube patterning. The combined action of the regionalized expressions of Hox genes and of Hh provided by the ectoderm leads to segmental and axial cell diversities.

hypothesis, our morphological analysis has revealed distinct features in the most anterior region (segments T3,A1) of aorta. These particular traits were nonetheless difficult to unmask owing to a hindering of the aorta inside the embryo and to the presence of the ring and lymph glands surrounding the cardiac tube. Similarly, the lack of *abd-A* expression (and the strong *Abd-B* expression) in the four most posterior cardioblasts of the heart implies that these cells respond to specific genetic and differentiation programs that do not operate in more anterior heart cardiomyocytes. While no obvious morphological features permit these most posterior cardioblasts to be distinguished, their position in the caudal most region of the cardiac tube suggests that they are likely candidates to form the pacemaker center of the organ (Rizki, 1978).

In contrast to the situation in the ectoderm (Akam, 1998), the domains of expression of the *BXC* homeotic genes in the cardiac tube do not overlap, are contiguous and mutually exclusive. The same type of regionalized expression is also encountered in the visceral mesoderm in which, for example, *Ubx* is expressed in PS7 while *abd-A* expression encompasses the segments PS8 to PS12 (Bienz, 1994). Nevertheless, whatever the tissue, ectoderm or visceral mesoderm, the more posteriorly expressed gene represses (or dominates over) more anteriorly expressed genes, conforming to the phenotypic suppression (Gonzalez-Reyes and Morata, 1990) or posterior prevalence (Duboule, 1995) rules. Accordingly, loss-of-function of *abd-A* leads to a posteriorization of *Ubx* expression and a concomitant transformation of the heart into aorta. Similarly, the anterior boundary of the expression domains of *abd-A* and *Abd-B* were not modified in *Ubx* and *abd-A* mutants, respectively. Reciprocally, overexpression of *abd-A* in the whole cardiac tube repressed *Ubx* expression and transformed the most posterior aorta cardiomyocytes into heart cardiomyocytes. However, ectopic expression of *Ubx* also impaired the differentiation of cardioblasts, although to a lesser extent than when *abd-A* was overexpressed and it did not significantly repress Abd-A expression. This latter result suggests that Ubx and Abd-A may be in competition for common downstream targets.

In *Ubx* embryos, or in *Ubx*, *abd-A* double mutants, differentiation of the most anterior region of the aorta was affected. This observation suggests that, as in the visceral mesoderm, *Antennapedia (Antp)* might be expressed in the anterior domain (segments T3, A1) of the aorta, in which the lymph glands and the ring gland are located and that *Antp* transcription is repressed by *Ubx* in segment A2 and more posterior segments. In the absence of *Ubx* function, the *Antp* expression domain could be extended posteriorly and lead to the formation of ectopic lymph and/or ring gland cells. Finally, the fact that an additional effect on cardioblast differentiation was observed in double mutant embryos when compared with each single mutation, suggests that *Ubx* and *abd-A* participate in cardiomyocyte differentiation independently of their role in axial patterning.

The homeotic genes *abd-A* and *Ubx* are transcription factors which probably induce differential activation of particular gene networks which, in turn, could confer specific physiological function on distinct subsets of cardiomyocytes. For example, studies performed on the cardiac tube of another insect, *Samia caecropia* (McCann, 1970), provide good evidence that the electrophysiological properties of the cardiomyocytes are different in the aorta and in the heart. *abd-A* function may be necessary to activate genes responsible for heart activity or genes that participate in cardiomyocyte growth. Aorta and heart cardiomyocytes respond to a differential control of cell growth since, at the end of embryogenesis, the heart cardiomyocytes are at least two to three times larger than the aorta cardiomyocytes. Alternatively, *Ubx* could repress the growth of the aorta cardiomyocytes analogous to its role in haltere cells (Roch and Akam, 2000). Growth control of cardiomyocytes is probably not the unique function exerted by *Ubx* and *abd-A* in the cardiac tube, since in the absence of both gene activities the cells did not differentiate properly.

Hedgehog signaling in cardiogenesis

The segmentally repeated expression of *svp* is regulated by a positive inductive effect of Hh secreted by cells from the overlying ectoderm. A role for *hh* signaling in *Drosophila* cardiogenesis has not previously been acknowledged. It has been observed that in *hh* mutant embryos heart progenitors were lacking (Park et al., 1996), however this has been interpreted to be an indirect influence of *hh* upon *wg* signaling. The results reported herein strongly favour the idea that *hh* has a direct and positive effect on the determination and specification of the sub-population of cardioblasts that expresses *svp*. It has been proposed that each segment of the trunk is sub-divided into two domains, A and P (Azpiazu et al., 1996; Riechmann et al., 1997). The cells from the anterior domain (A domain) of the dorsal mesoderm would be directed towards a cardiogenic fate while cells from the posterior

domain (P domain) would adopt a visceral mesoderm fate. The *wg* and *hh* signals released, respectively, from the anterior and posterior compartments of the ectodermal parasegments have been proposed to be the determinants in specification of the two domains. Our observations, however, provide strong evidence that a subtype of cardiac cells could originate from the mesodermal P domain. The P domain origin of some cardioblast progenitors had previously been suggested by Frémion et al. (Frémion et al., 1999) who reported the presence at stage 11-12, within the P domains, of *bkh*-expressing cells, which will contribute to the cardiac epithelium later in development. It seems, therefore, that there is not a perfect superimposition between A domains within mesodermal segments and the capacity of the cardiac cells to be integrated into the cardiac tube.

The *hh* signal secreted by cells belonging to the posterior compartments of the segmented ectoderm is sufficient to promote *svp* expression, as illustrated in Fig. 8. The Hh morphogen needs to be secreted and to freely diffuse from the ectoderm to the underlying mesoderm, as judged from the loss of *svp* expression in the cardioblasts when a membrane-bound form of Hh is expressed in the same genetic background in place of endogenous Hh. The existence of a specific mechanism to constrain diffusion of the secreted morphogen to the cardioblasts of the P-domain can thus be postulated. Further investigation of this mechanism will provide insight into how specificity of morphogen signaling is achieved across embryonic germ layers.

Based on gene expression patterns, Hh signaling is likely to be instrumental in the specification of *tin*- and *svp*-cardioblasts by inducing the expression of *svp* in cardioblasts which, in turn, leads to the repression of *tin*. Such a repressive action of *svp* has already been reported (Gajewski et al., 2000; Lo and Frasch, 2001), although a direct interaction between *tin* regulatory sequences and *svp* has not been demonstrated (Lo and Frasch, 2001).

Similar relationships between homologs of *hh*, *svp* and *tin* have been described in vertebrate cardiogenesis. An homolog of *svp*, COUP-TFII is expressed in the posterior region of the mouse primitive heart tube where it is required for heart development (Pereira et al., 1999); furthermore the expression of COUP-TFII is induced by Sonic hedgehog (Krishnan et al., 1997). Shh (and Indian hedgehog) participates in mouse cardiac morphogenesis but, in contrast to the situation in *Drosophila*, induces rather than represses the expression of the *tin* homolog, *NKx2.5* (Zhang et al., 2001). It must be concluded, from these remarks, that the genetic networks can be differently interpreted and utilized in invertebrates and vertebrates. Further studies should give better insights into the conservation of the genetic programs at work in heart development.

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