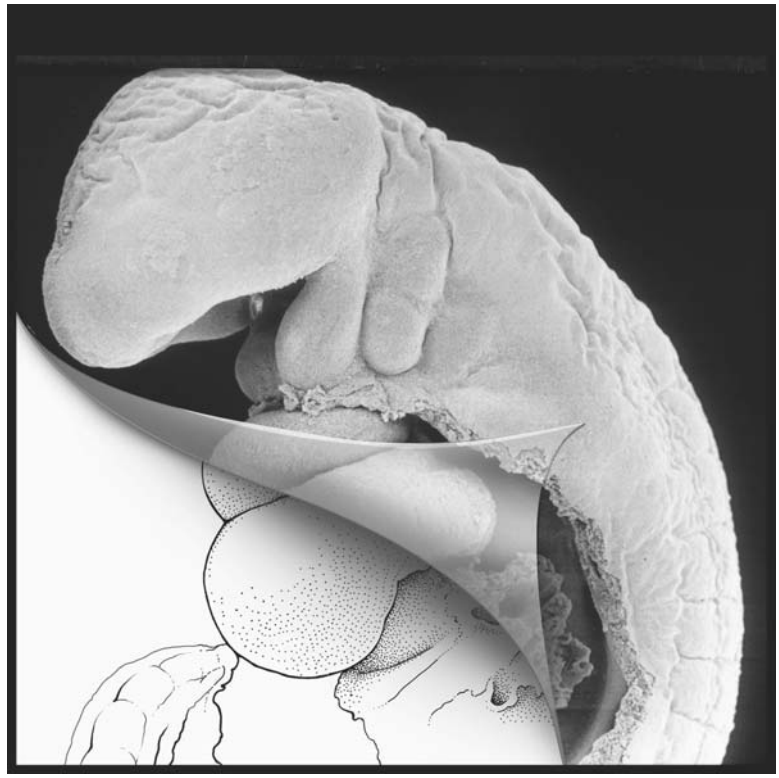

Steding's and Virágh's Scanning Electron Microscopy Atlas of the Developing Human Heart

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Springer

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Preface and acknowledgments

Morphogenesis of the embryonic heart in humans and other higher vertebrates is a complex process which involves an intricate program of tissue remodeling. In particular, the process of septation, as a result of which right and left cardiac chambers become separated, continues to fascinate scientists for over a century now. Thanks to the recent advances in molecular biology, especially in immunohistochemistry and (whole mount) in situ hybridization, it has become increasingly possible to investigate the biological processes that effectuate cardiac morphogenesis. As a consequence, molecular and biochemical scientists urgently require an updated morphological reference of cardiac embryology for topographic correlation of the results of their experiments, which, in the end, must lead to a better understanding of normal and of abnormal heart development.

This atlas contains about 180 scanning electron microscopy (SEM) pictures, together with several explanatory figures, showing the essence of human cardiac development. Apart from serving a unique overview on cardiac development in the human embryo, this atlas enables the projection of experimental results in animals to the human situation. The material for this atlas is largely based on the collection of Prof. dr Gerd Steding, with additional material from the late Prof. dr Szabolcs Virágh. The differences in approaches and specific interests guarantee the diversity in images which is necessary to give a complete and extensive exposure of all spatial and temporal aspects of human cardiac development, as presented in this atlas.

Embryos were staged according to the Carnegie classification. However, it should be taken into consideration that the cardiac developmental state varies individually even in embryos of the same stage. A table is appended that summarizes the hallmarks in human cardiac morphogenesis during the various developmental stages. The development of the heart in the human embryo is known only from material collected through spontaneous miscarriages and legally terminated pregnancies. Thus, obtaining material with a gestational age of less than three weeks that is suitable for scanning electron microscopy is nowadays virtually impossible. Therefore, although heart development commences at stage 9 (20–21 days p.c.), the youngest embryos we dispose of are from stage 11 (23–25 days p.c.).

The first chapter comprises an illustrated textual overview of the early developmental stages in the vertebrate embryo and the formation of the primitive heart tube up to the stage of compartment formation. The subsequent chapters comprise the SEM pictures, which start off at Carnegie stage 11, i.e., when looping has completed. Each chapter begins with a concise description of the relevant developmental events followed by the SEM pictures. The text and the pictures are intended to be separately comprehendible, i.e., the text, which mostly represents the current opinions, should merely give a "backbone" to the reader, whereas the SEM pictures together with their legends speak for themselves. All photographs are

accompanied by line drawings that carry the legends, thus leaving the pictures themselves untouched. A list of terms we used to describe the structures we encountered is appended. We found these terms to be the most appropriately usable, although we realize that some of them may be controversial. We refrained from using adjectives such as "embryonic" and "primitive," which we consider to be superfluous when describing developmental morphology, although we are well aware of the fact that several mature cardiac structures are only partially represented by their embryonic synonyms. Following the initial orientation of the heart tube in human and animal embryos, we used the terms "anterior" and "posterior" with reference to the outflow and inflows parts respectively. The terms we used to describe the various angles, section planes, and topographic relations are summarized in the figures below (Figures 0 A, B).

With this atlas we hope to have succeeded in our intentions to make a readily accessible reference aid for scientists working in the fields of molecular, biochemical, genetic, and morphological investigation of cardiac development. Additionally, we believe that this atlas may serve as a helpful tool in the cardiological education of medical students, clinicians, pathologists, and geneticists.

We wish to express our gratitude to all who contributed to the completion of this atlas, in paticular, the following people:

At the Department of Embryology, Georg-August-Universität, Göttingen, Germany:

Mr. Hans-Georg Sydow for his untiring and conscientious efforts in collecting and preparing the specimens for scanning electron microscopy, his inimitable skill in the complicated setting-up of the microinstruments for dissection and for his valuable support in the solution of laborious technical problems with the scanning electron microscope.

Mrs. Kirsten Falk-Stietenroth for the photographic work she carried out in the darkroom with unsurpassable care, great dedication and her own very special aesthetic aptitude.

Mrs. Anja Aue for archiving the specimens and photographs, which she carried out with great care, thus playing an important role in the maintenance of order and in keeping the records straight.

Dr. Jörg Männer, who, during his time as acting head of the Department of Embryology, generously provided the opportunities to allow the authors to complete this book in familiar surroundings.

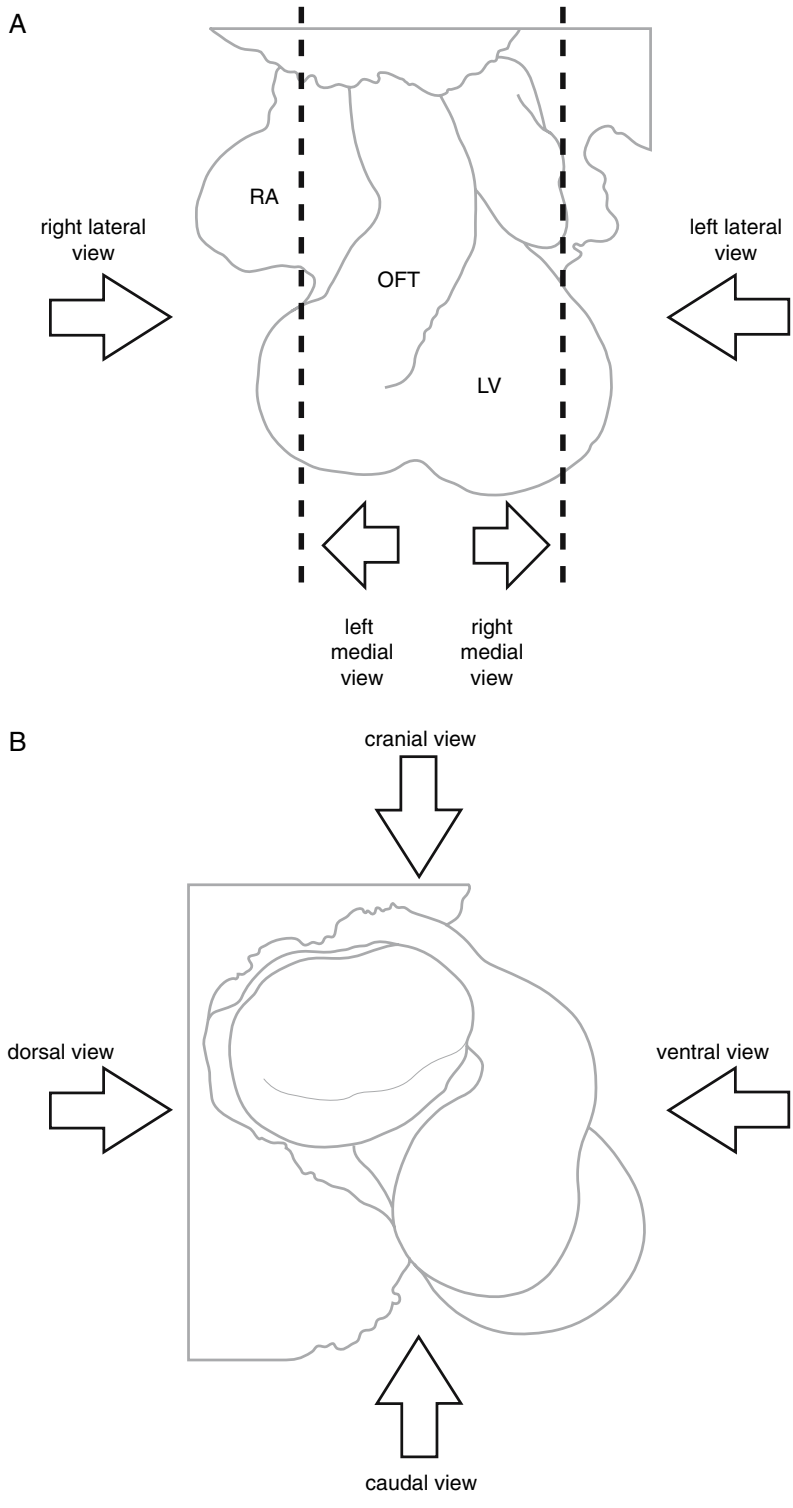
At the Department of Pathology, National Health Centre, Budapest, Hungary:

Mr. Gyula Szabó, photographer; Mr. József Farsang EM-emgineer; Ms. Margit Stark, technician; and the late Mrs. Ilona Kennedy, technician, all of whom assisted Prof. Dr. Virágh in making the EM specimens and photographs.

Dr. Gabriella Arató, former collegue of Prof. Dr. Virágh, who generously assisted in collecting the materials and took care of his heritage.

At the Department of Anatomy and Embryology, Academic Medical Centre, University of Amsterdam, The Netherlands:

Mrs. Lara Laghetto (Visualmedics) for making the drawings of the depicted specimens with meticulous precision and for the additional illustrations, which beautifully clarify the intricate processes of cardiac septation.



Figures 0 A, B Terminology used to describe the angles, section planes and topographic relations.

Mr. Cees Hersbach and Mr. Cars Gravemeijer for scanning and archiving the numerous photographs that were used in making this atlas.
Last but certainly not least we wish to thank Drs. R.H. Anderson, A.E. Becker, B Christ, V.M. Christoffels, F. de Jong and M.B.J. van den Hoff for their helpful discussions.
Ths making of this atlas was financially supported by the Netherlands Organization of Scientific Research (NWO) and the Hungarian Scientific Research fund (OTKA).

In Memoriam

In March 2001, Prof. Dr. Szabolcs Virágh passed away. Dr. Virágh, pathologist at the former Imre Haynal University (presently the National Health Centre) in Budapest, Hungary, was one of the instigators and driving forces in the making of this atlas. His enormous collection of scanning electron micrographs concerning the developing human heart in various embryonic stages, dating back from the early 1980s onward, initiated the idea of making an atlas out of it. Realizing that his material did not cover all aspects of human cardiac development, he readily agreed with Prof. Dr. Steding to join in. Dr. Virágh's expertise, enthusiasm, dedication, and, not in the least, his social skills inspired the co-workers in this project to continue despite several drawbacks. Sadly, he will never to see the results of his efforts. We dedicate this atlas to his memory.

1

Introduction

THE EARLIEST STAGES OF CARDIAC DEVELOPMENT

The morphogenesis of the four-chambered heart is one of the most intricate processes in higher vertebrate embryology, which involves a program of gene expression, differential growth, spatial organization, and cell movement. Since diffusion of nutrients from the surrounding tissues, which nourishes the rapidly growing embryo in its earliest stages, soon becomes insufficient, the embryonic cardiovascular system is the first functioning organ to appear. Thus, a primitive but functioning circulation with a beating heart is already accomplished during the beginning of the fourth week of development. During development, the primitive single-circuited tubular heart must evolve into a four-chambered double-circuited structure, while it is already committed to its lifelong task, i.e. maintaining circulation. This requires profound and complex remodeling, which is difficult to comprehend and makes great demands on one's spatial insight. With the advent of molecular technology a new era in cardiac embryonic research has begun and the mechanisms involved in the sequential processes of cardiac development are now starting to become unraveled.

During the third week of development (stage 7, 16 days p.c.), ingressing epiblast cells spread in various directions over the embryonic disc between the former epiblast and the hypoblast, thus giving rise to the third embryonic layer: the intraembryonic mesoderm. During stage 8 (17–19 days p.c.), two areas of mesoderm in each half of the embryonic disc meet each other anterior to the stomatopharyngeal membrane and form an arch-shaped structure. The anterior part of this structure is dubbed the cardiogenic crescent (heart forming region). This is the area where the future heart will start to develop. In this arch-shaped band of mesoderm, small cavities appear. The adjacent mesodermal cells will be transformed into epithelial cells. These cavities coalesce and form the first intra-embryonic coelom, i.e. the pericardial cavity. This lumen separates the part of the lateral plate mesoderm that faces the ectoderm, the parietal (or somatic) mesoderm, from that apposed to the endoderm, the visceral (or splanchnic) mesoderm. When the first vacuoles coalesce, the cranial lumen, which becomes the pericardial cavity, expands in caudal direction, forming the pleuro-peritoneal ducts (coelomic ducts) that connect to the peritoneal cavity. The cranial most rim of mesoderm in the embryonic disk will later contribute to the septum transversum. The visceral mesoderm that forms the floor of the pericardial cavity gives rise to the myocardium and endocardium.

At various sites in the extraembryonic mesoderm, and later also the intraembryonic mesoderm, mesenchymal cells appear that form vacuolated masses and cords. These cells known as angioblasts give rise to both blood cells and to a layer of endothelial cells that encloses the blood islands, a process known as vasculogenesis [Poole and Coffin, 1989]. These endothelial cavities and strings fuse to form

primitive vessels. Thus, in the intraembryonic visceral and parietal mesoderm, the outlines of the major systemic arteries and veins appear on each side of the embryonic midline. Close to the notochord the two dorsal aortae are formed, whereas the venous plexuses are situated more laterally in the body wall. In the same manner, angioblasts in the area of the cardiogenic cresent, formed by the cardiogenic plate, give rise to the primitive endocardial plexus [Viragh et al., 1989]. These angioblastic islands coalesce and form a plexus in the midline of the myocardial mantle. The branches of this plexus coalesce and form the unpaired endocardial tube which acts as anastomoses between the rostral ends of the dorsal aortae and the venous plexuses (Figures 1 A–E). Gene expression studies in chicken and mouse embryos have shown that this coalescence takes place at the site that, at a later stage, will contribute to the formation of the left ventricle. The parts of the heart tube that will later on develop into the right ventricle and outflow tract are added later at the arterial end of the heart tube by the so-called secondary or anterior heart-forming region [Kelly et al., 2001; Mjaatvedt et al.; 2001; Waldo et al., 2001]. In between the endocardial tube and the myocardial mantle a glycosamin rich extracellular layer is present which is generated by the cardiogenic plate. This material is known as cardiac jelly, which at this stage is acellular (see Chapter 5). Shortly after the primitive heart tube has been formed, the first cardiac contractions can be seen in the region where the future ventricles will develop, even before the circulation is completely closed.

LOOPING OF THE HEART TUBE

Following the formation of the heart tube, two more or less simultaneously occurring processes accompany the formation of the primitive heart tube. Firstly, during neurulation, which commences at stage 9 (19–21 days p.c.), the embryonic disk flexes and folds along both its transverse and longitudinal axes, resulting in head and tail folds and lateral folds. As a results of the head fold, the most anteriorly positioned structures, being the stomatopharyngeal membrane, the septum transversum, and the primitive heart, curve ventrally at an angle of more than 180° and acquire their definite position in front of the foregut. Secondly, the heart tube starts to loop at the beginning of stage 10 (21–23 days p.c.), a process that in man, as well as in most vertebrate species, already commences before coalescence of the endothelial plexus has formed the unpaired cardiac lumen. Seen from its eventual position ventral to the foregut, the first loop to appear in the primitive heart tube is found in the region where the ventricles develop and causes the tube to curve in dextroventral position; it is dubbed the D(extro)-loop or ventricular loop. The second loop occurs in the region of the future atria and the atrioventricular connection and proceeds in the opposite direction, thus causing a levodorsal curving of the heart tube; it can be dubbed the atrial loop the two loops result in an S-shaped configuration of the heart, both in its ventral and lateral aspects. Subsequently, the ventricular loop bulges over the atrial loop, and twists to the right. As a result, the initial left and right sides of the ventricular loop will become the ventral and dorsal sides respectively (Figures 1 F–L).

It has been debated to what extent extrinsic and intrinsic factors are responsible for the initiation, proceeding and completion of normal cardiac looping. Manasek and Monroe [1972] investigated the morphogenesis in asystolic hearts of live chicken embryos. In contrast to the current opinion at the time, they found that “pre-looped” hearts of HH stage 10 embryos undergo normal looping during 5 hours of potas-

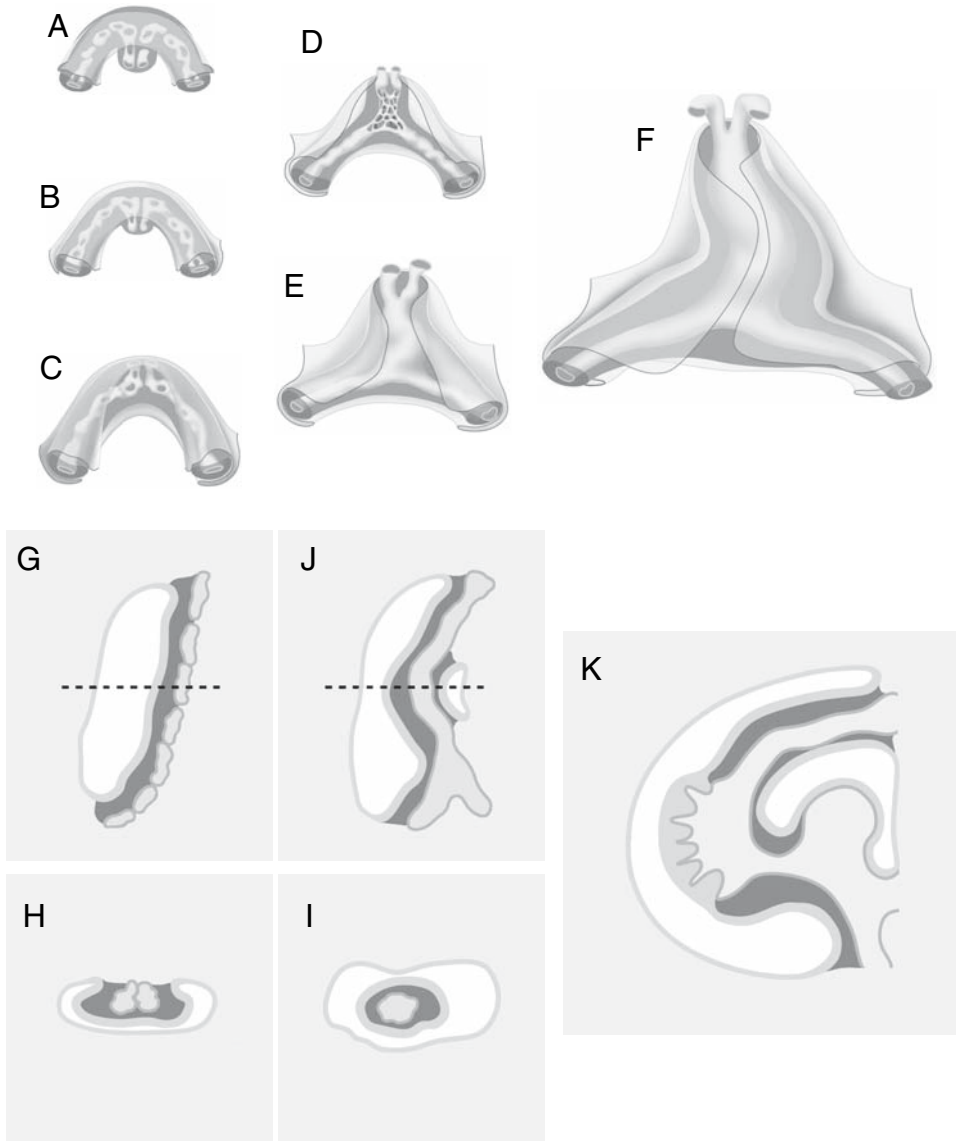


FIGURE 1 **A–F** Formation of the heart tube in the heart forming region during folding of the embryonic disc (dorsal views), involving the endocardial plexus and tubes (light grey), cardiac jelly (dark grey) and the myocardial layer which is continuous with the pericardial lining (transparent sheet). **G–K** Formation and looping of the heart tube (cranial and left lateral views) and its protrusion in the pericardial cavity (white), involving the endocardial plexus and tubes (light grey), cardiac jelly (dark grey) and the myocardial layer which is continuous with the pericardial lining (medium gray).

sium induced cardioplegia and they therefore concluded that normal looping of the heart does not require either cardiac perfusion or contraction. Manning and McLachlan [1990] examined explanted hearts of HH stage 8–10 chicken embryos and found that normal looping occurred in almost all cases, thus supporting the assumption that cardiac looping is an intrinsic process. On the other hand, Hogers

et al. [1997, 1999] recently studied the effects of ligation of vitellinous veins on cardiac morphogenesis in HH stage 17 chicken embryos and found that long term ligation, irrespective of which vitellinous vein was clipped, resulted in a spectrum of cardiovascular malformations, which they, at least partially attributed to impaired looping of the heart tube. Resuming the results of all forementioned studies, it seems plausible to assume that cardiac looping is in essence intrinsically controlled i.e. at a molecular level but that intracardiac blood flow patterns may have a profound effect on the eventual morphological outcome, especially during later stages of cardiac looping. Besides this, it has been suggested that longitudinal flexion of the embryonic disk, at the site of the head or the neck is essential for a proper looping of the tubular heart. Männer et al. [1995] observed that experimental prevention of cranial flexion in chicken embryos did not interfere with a normal looping pattern, whereas different studies show that interference with cervical flexion may lead to cardiac anomalies that are based on defective looping [Männer et al., 1993; Kosaki et al., 1996]. It must be noted however, that cranial and cervical flexion in chick embryos are temporally separated processes, whereas in mammals the two occur much more simultaneously.

During the process of looping the heart tube projects into the pericardial cavity but remains connected, initially over its entire length, with the midline of the dorsal body wall by means of the dorsal mesocardium. Subsequently, this mesocardium becomes very thin, perforates and disappears almost completely, except for the atrial part, which continues to be connected to the embryonic body wall by a pedicle formed by the epithelial bridge between pericardium and the heart that contains scarce mesenchyme: the persisting dorsal mesocardium or heart stalk [Webb, 1998]. The thus arisen dorsal communication, between the left and right sides of the pericardial cavity and known as the transverse pericardial sinus, can still be recognized in the mature heart. It should be noted that in chicken embryos the cardiogenic plate, including the intraembryonic coelom that gives rise to the pericardial cavity, remains separated in two lateral parts until folding of the embryonic disc has completed, hence a ventral mesocardium is temporarily present.

2

Outlines of external development

BLOOD FLOW IN THE TUBULAR HEART

At stage 11, the heart tube has looped completely and despite the fact that it has lost its left-right symmetry, it can still be considered a homogeneous structure from both morphological and functional points of view. The embryonic cardiomyocytes, derived from the cardiogenic plate mesoderm, form a layer, which is dubbed primary myocardium. Impulse propagation and the subsequent contraction waves, which initially have a peristaltoid form, run from the inflow to the outflow end of the heart tube, i.e. in postero-anterior direction. The presence of cardiac jelly guarantees adequate propulsion of blood. From the outset on, pacemaker activity is dominant at the inflow end of the heart tube, although coupling of excitation and contraction is first achieved in the future ventricular area [Van Mierop 1967]. Which mechanisms are responsible for the pacemaker dominance of the inflow end is as yet unknown but it may well be related to the anteroposterior differentiation of the heart tube as a whole, in which retinoic acid signaling plays a quintessential role [Xavier-Neto et al. 2001]. Moreover, many molecular factors involved in early cardiac development [reviewed by e.g. Franco et al. 1998 and Xavier-Neto et al. 2001] exhibit an antero-posterior expression gradient and thus a gradual change of electrochemical properties along the heart tube.

Different blood flows can already be observed soon after looping of the heart tube has completed, i.e. long before physical septation of the embryonic heart is initiated. It has been hypothesized that these flow patterns not only mold the developing heart tube but play a crucial role in the septation processes as well. Many studies have been performed in the past in chicken embryos to elucidate the stream patterns in the early looped heart [e.g. Bremer 1932, Jaffee 1965, Rychter and Lemez 1965, Leyhanne 1969]. Yoshida et al. [1983] performed microangiography with methylene blue injections in live chick embryos and found two consistent blood stream patterns through the heart from Hamilton-Hamburger (HH) stage 14 on. They observed that one of the streams, which runs a ventral intracardiac course, goes to the left branchial arteries, whereas the other one, which runs dorsally, drains onto the right branchial arteries. Steding and Seidl [1990] noticed in chicken embryos that the circular lumen of the tubular heart achieves a halter shape during contraction, thus dynamically bring about two separated blood streams. However, although the flow patterns described by these author groups differ considerably, they both indicated that the patterns they observed cannot explain the morphological characteristics in the development of the atrioventricular and outflow tract septa. They therefore concluded that intracardiac blood flow patterns are not primarily involved in the initiation of cardiac septation. Hogers et al. [1995]

performed labeling studies in stage HH 12–17 chicken embryos and observed flow patterns which again differed from those published by others, but they agreed with Yoshida et al. and Steding and Seidl on the absence of a hemodynamic explanation for the morphology of the outflow tract septum. It seems presently impossible to be conclusive about the correlation between early embryonic blood stream patterns and the subsequent septation of the heart, firstly because almost all hemodynamic studies disagree profoundly with respect to the precise routes of the intracardiac blood streams and their venous and arterial connections, and secondly because both the stream patterns themselves as well as their peripheral vascular connections, according to most of these studies, may change completely within the course of a single developmental stage.

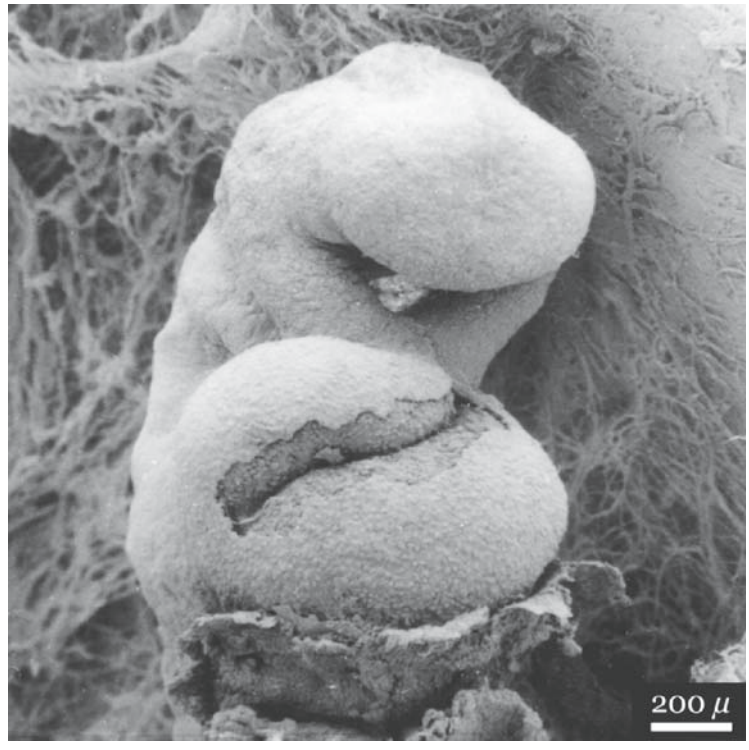
BALLOONING OF THE CARDIAC COMPARTMENTS AND SUBSEQUENT DEVELOPMENT

In the looped heart, an inner (lesser) and outer (greater) curvature can be distinguished, which are important landmarks in the view of subsequent developmental events. The looping process itself is accompanied by a certain degree of torsion since fate map studies have indicated that the outer curvature is initially at the ventral side of the straight heart tube [De la Cruz 1999]. From stage 11 onward, four progressively enlarging balloon-shaped distensions appear at the outer curvature of the atrial and ventricular loops, which will become the cardiac compartments. These compartments expand from the heart tube and should therefore not be considered as segments of the heart tube itself. As a consequence, the inner curvature part is not involved in this process of chamber formation [Moorman & Christoffels, 2003]. It should be noted that the atrial compartments appear as parallel structures on either side of the outer curvature whereas the ventricular compartments are formed successively as serial distensions of the outer curvature. Because of the newly formed cardiac compartments, different parts of the original heart tube can now be identified at the outer curvature. These are the outflow tract at the anterior end, the inflow tract at the posterior end, and the atrioventricular canal in between the developing atrial and ventricular compartments. These parts remain connected with each other at the side of the inner curvature.

The externally visible features of subsequent cardiac development are mostly the results of intricate internal processes. Since they are defined to specific parts of the heart, they will be depicted and described in detail in the according chapters. Between stages 11 and 13 (28 days p.c.) the posterior most part of the inflow tract gradually shifts to the right, resulting in a sinus venosus that drains exclusively to the right atrium. During later development the myocardium that surrounds the sinus venosus and the dorsal mesocardium increasingly contributes to the formation of the dorsal parts of the right and left atria respectively [Soufan et al., 2004], whereas the embryonic atria themselves gradually loose their function in blood propulsion and become transformed into auricles. This process begins around stage 14 (32 days p.c.) but the different shapes of these auricles cannot be clearly appreciated until stage 17 (41 days p.c.). In the early post-looping stages, the atrioventricular canal is located predominantly on the left side of the heart, despite the fact that separate blood flows are already present (see above). During later stages the atrioventricular canal gradually shifts towards the right and becomes largely incorporated in the atria [Wessels et al., 1996]] until its position is merely indicated by the atrio-ventricular sulcus.

The ballooning ventricles, separated by an initially deep interventricular groove, differ in size from their first appearance during stage 11 onward but although the left ventricle is larger than the right one, it is not until stage 17 that the apex of the left ventricle projects beyond the apex of the right ventricle. From that stage onward, the primarily spherical shape of the ventricles changes, the right ventricle will become tetrahedral, the left one cylindric. The interventricular groove becomes less pronounced and by the time embryonic development has completed, the apical parts of the ventricles have formed a single contour. Externally visible development of the coronary arteries can be noted during stage 19 (47–48 days p.c.) by the appearance of a fine reticulum that covers the surface of the ventricles. Soon afterwards, the main vessels and their branches can be distinguished.

The outflow tract, which connects the ventricles with the aortic sac, initially forms a tube-like structure with a single lumen. The knick in this tube roughly separates the proximal part of the outflow tract (conus) from the distal part (truncus). Whether or not this knick represents a fixed anatomical landmark is not known. During stage 15 the regular convexity of the contour is gradually lost with the appearance of a spiralized longitudinal flattening. This flattening corresponds with the formation of endocardial ridges, which, especially in the distal part of the outflow tract, already start to fuse, thus creating two physically separated lumens. From stage 15 onward the myocardial mantle of the distal part of the outflow tract becomes patchy and starts to disappear, leaving a well recognizable and steadily retreating boundary between the proximal and distal parts by the end of stage 17. At this level, the semilunar valves will develop. The proximal part will form the aortic and pulmonary infundibulums and becomes gradually incorporated in the bases of the ventricles. By the end of stage 18, the distal part of the outflow tract has been transformed into the intrapericardial parts of the aorta and pulmonary trunk. The eventual mature contours of the heart as a whole are achieved during stages 19 (47–48 days p.c.) till the end of the embryonic period.



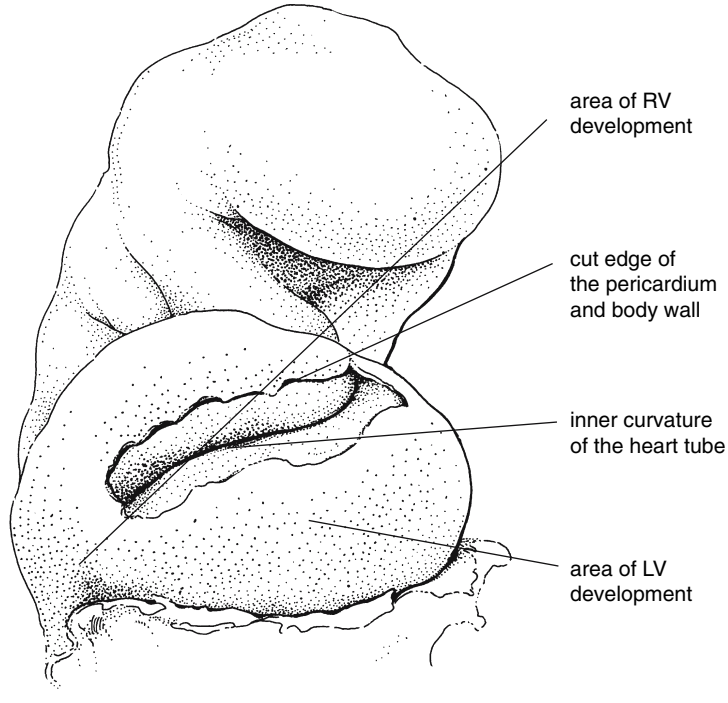
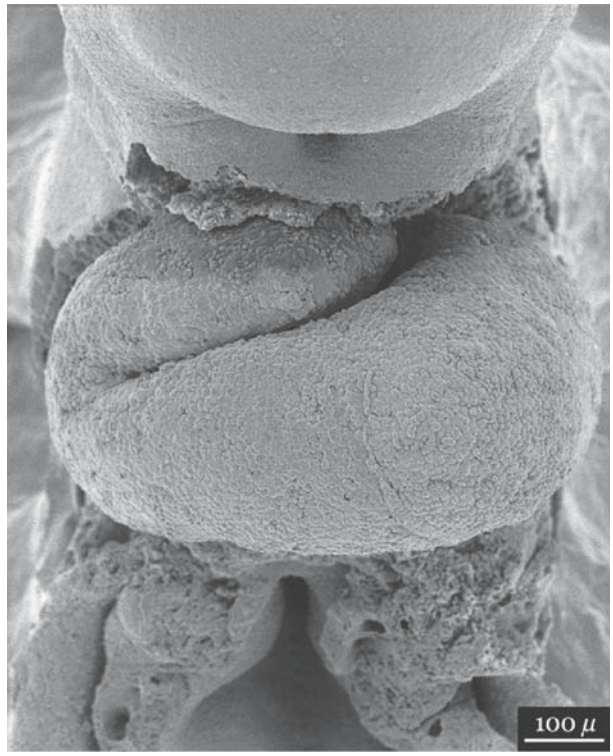
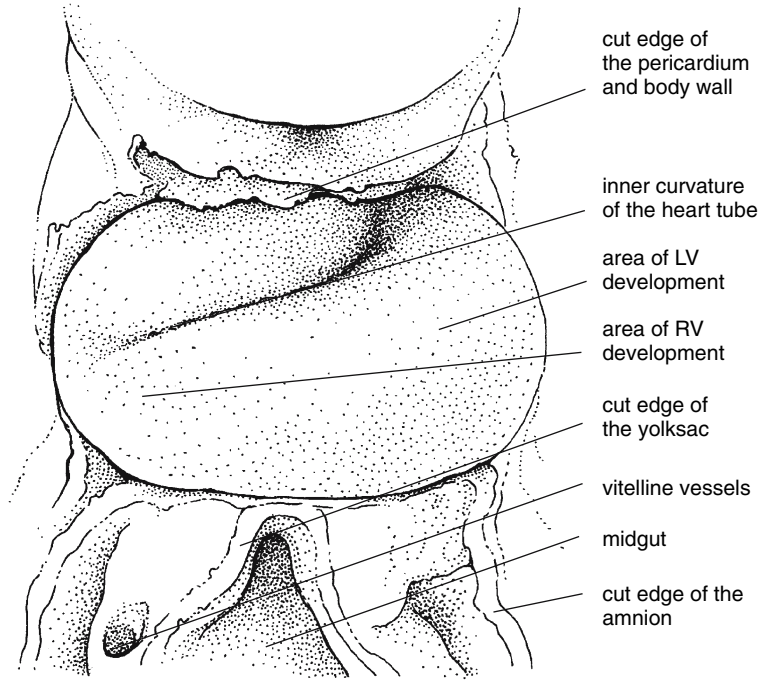
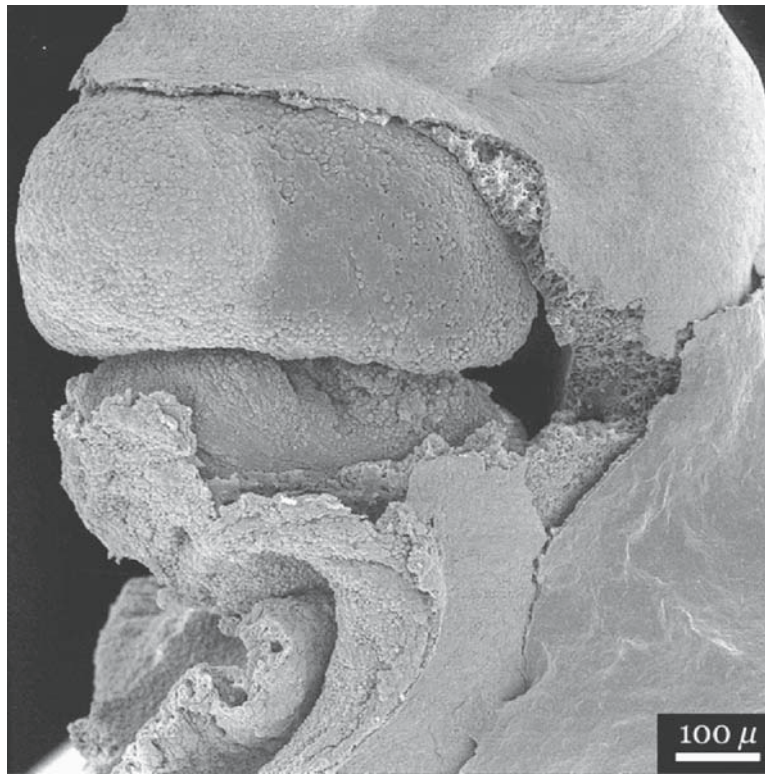


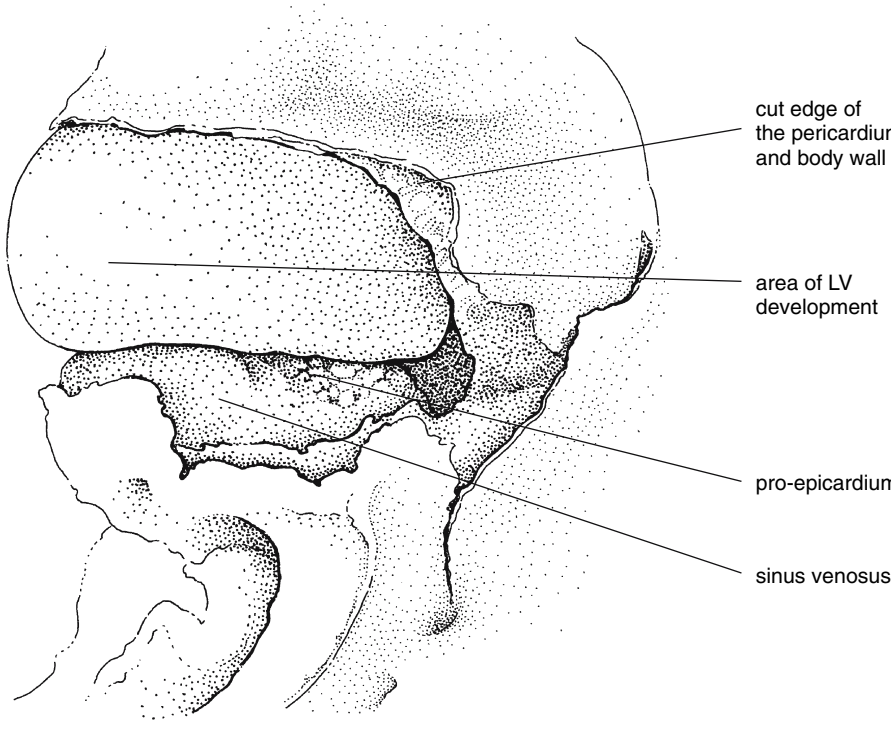
FIGURE 2.1 Complete embryo in situ (stage 11), ventral view. The yolk sac and the amnion are removed. The ventral body wall and the pericardium are opened.



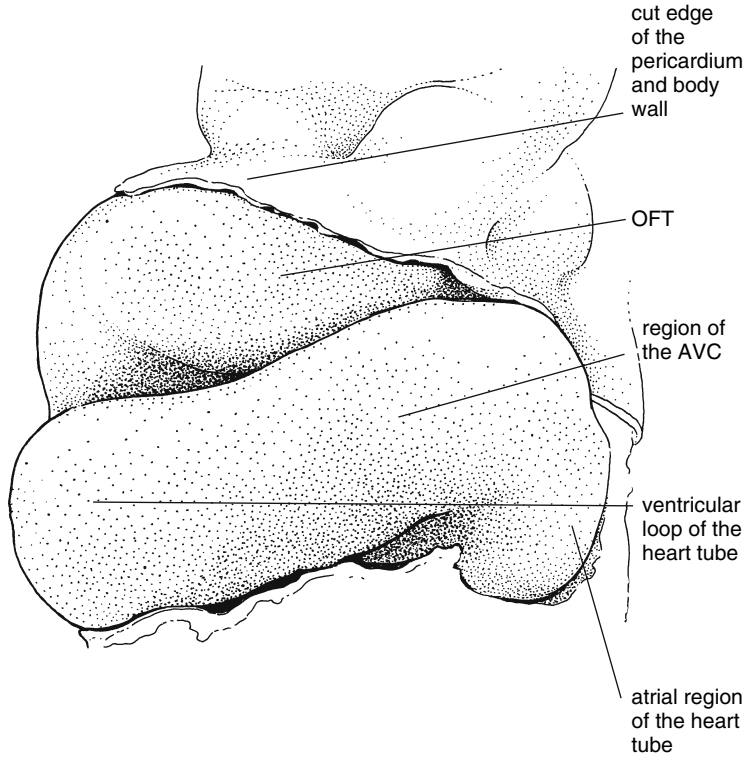


FIGURES 2.2 and 2.3 Complete embryo in situ (stage 11), ventral and left lateral views. The yolk sac, the amnion, the ventral body wall and the pericardium are removed.

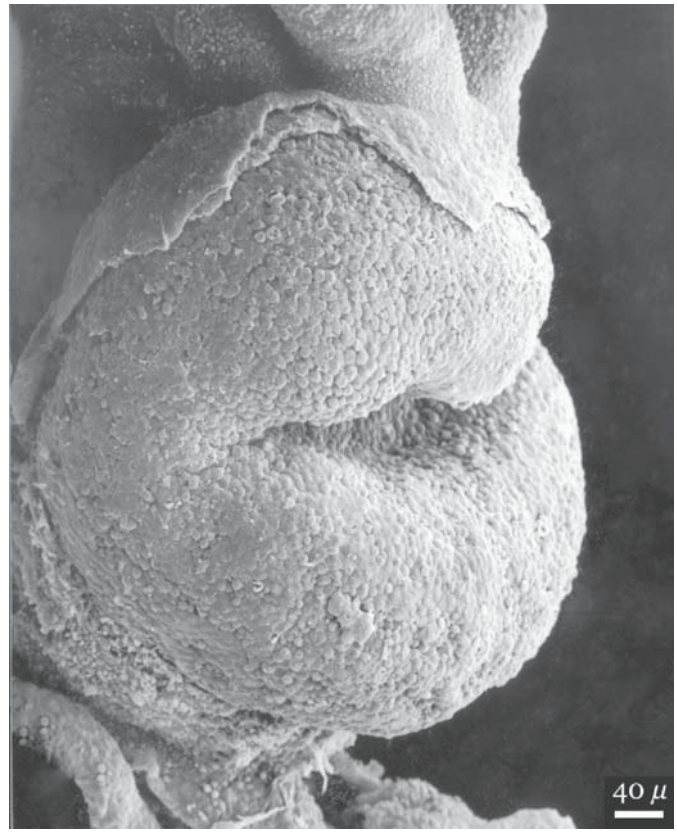


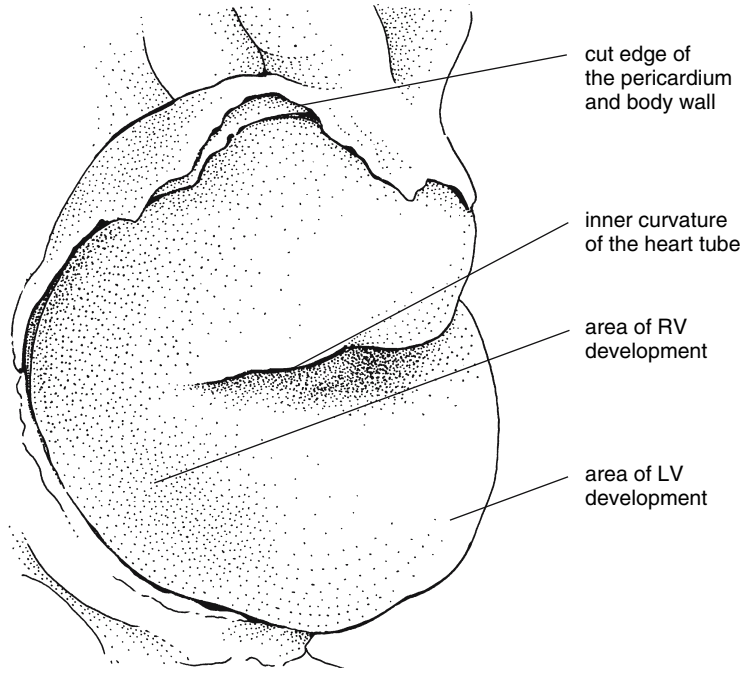


FIGURES 2.2 and 2.3 *Continued*

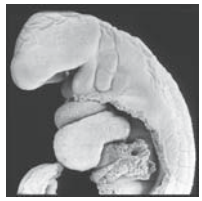


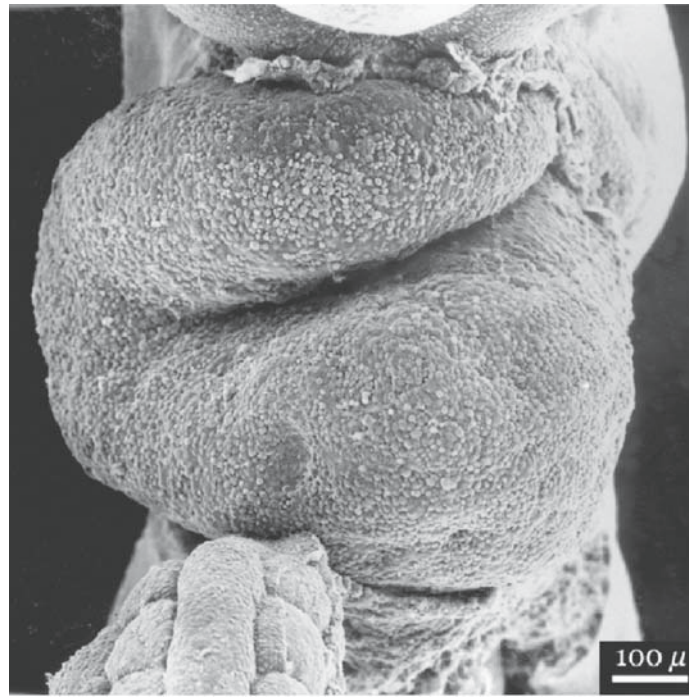
FIGURES 2.4 and 2.5 Complete embryo in situ (stage 11), ventral, left and right lateral views. The yolk sac, the amnion, the ventral body wall and the pericardium are removed. The heart tube has looped completely and the ventricular loop covers the atrial region. There are no externally visible signs of any compartments yet.

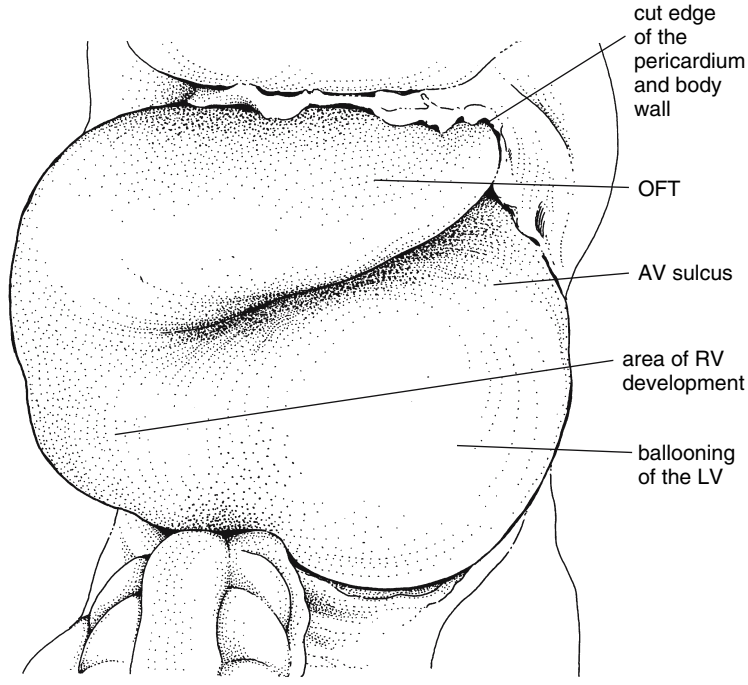




FIGURES 2.4 and 2.5 *Continued*







FIGURES 2.6–2.8 Complete heart in situ (stage 12), ventral, right and left lateral views. The ventral body wall and the pericardium are removed. The heart tube shows a dilatation at the posterior part of the left limb of the D-loop. This is the site where the LV will develop. Other compartments are still only very faintly recognisable externally.