INTRODUCTION

Organogenesis is the process by which cells of different embryonic origins assemble to form discrete structures. As these cells aggregate, a number of organotypic decisions give the organ its final shape and structure. In the case of the heart, the definitive heart tube, once formed, is first patterned in the anteroposterior (A-P) axis to form the different chambers. It then undergoes looping morphogenesis, and specific endocardial cells go through an epithelial to mesenchymal transition to form the prevalvular mesenchyme.

The heart has several advantages for the study of the processes of organ formation. It is a relatively simple structure, consisting of two concentric epithelial tubes, the inner, endocardial and outer, myocardial. It is also the first organ to form and function during vertebrate embryogenesis, and cardiac function can be assessed by simple visual inspection, at least in the optically transparent zebrafish embryo.

Heart development has been well described morphologically in several species including chick (DeHaan, 1965; Viragh et al., 1989), mouse (DeRuiter et al., 1992), and zebrafish (Stainier et al., 1993; Lee et al., 1994). In all vertebrates, the post-gastrulation cardiogenic cells migrate medially as part of the lateral plate mesoderm. They form two primitive myocardial tubes on either side of the midline. These tubes then fuse to enclose the endocardial cells and form the definitive heart tube. Subsequently, this tube is patterned along the A-P axis to form the different chambers, the main ones being the atrium and ventricle, and valves form at chamber boundaries. The heart starts beating at the time when the primitive heart tubes fuse and function.
and once the definitive heart is formed, contraction proceeds in a coordinated and characteristic manner: first the atrium beats, and then the ventricle, with the valves opening and closing to prevent the retrograde flow of blood.

In the zebrafish, cardiogenic progenitors are concentrated in a marginal zone that extends from 90° to 180° longitude (Stainier et al., 1993). Precardiac cells involute during early gastrulation and migrate towards the embryonic axis as part of the lateral plate mesoderm. They form two myocardial tubular primordia on either side of the midline, with a distinct group of cells, the endocardial progenitor cells, sitting medially between them. The myocardial tubes then fuse to enclose the endocardial cells and form the definitive heart tube. By 22 hours post-fertilization (hpf), the heart tube is clearly beating and around 24 hpf, circulation begins. The regionalization of cardiac myosin heavy chain expression distinguishes the cardiac chambers at this stage, although they are not morphologically delineated until 36 hpf. By 36 hpf, the heart tube has looped, is beating at about 140 beats/minute at 28.5°C, and provides a strong circulation to the trunk and head.

The molecular events underlying vertebrate heart formation are poorly understood. A murine homeobox-containing gene, Nkx2.5, has been isolated, based on its homology with the fly gene tinman (Komuro and Izumo, 1993; Lints et al., 1993). In the fly, tinman is expressed in the heart as well as in the visceral mesoderm and tinman mutants do not form a heart (Azpiazu and Frasch, 1993; Bodmer, 1993). In the mouse, Nkx-2.5 transcripts are first detected at the end of gastrulation in myocardial progenitors as well as in a few other tissues. This gene is the earliest known marker for myocardial progenitor cells. The heart of Nkx2.5 mutant mouse embryos does not loop, nor develop endocardial cushions nor trabeculae (Lyons et al., 1995). Other transcription factors expressed in early myocardial cells that may interact with Nkx-2.5 include MEF-2, SP-1, TEF-1 and GATA-4 (Lyons, 1994).

A genetic approach to vertebrate heart formation provides several advantages. Firstly, it makes no preconceived judgement about the role of specific genes in this process. Indeed, recent experiments in the mouse have revealed both unexpected roles for certain genes in heart formation, for example in the case of the retinoid X receptor α gene (Sucov et al., 1994), and phenotypes disappointing in their informativeness about cardiac development, as in the case of TGFβ1 (Shull et al., 1992). Secondly, the mutant phenotypes can point to critical steps of heart formation. Thirdly, the mutations themselves provide relevant entry points into these processes.

In this paper, we categorize and briefly describe the cardiovascular mutations identified in a large-scale mutagenesis screen of the zebrafish genome (Driever et al., 1996). These mutations specifically affect distinct aspects of heart formation and function as well as the integrity of the vasculature.

### MATERIALS AND METHODS

Zebrafish were raised and handled as described by Westerfield (1993). Developmental time at 28.5°C was determined from the morphological features of the embryo. The design of the ENU screen and screening methods are as described in Solnica-Krezel et al. (1994) and Driever et al. (1996). Screening for cardiovascular mutations was at 48 hpf, although effects of some mutations were first noted earlier. Complementation analyses between all members of each group (Tables 1 and 2) were performed by pairwise matings of heterozygous fish bearing different mutations. Different group assignments were made by dint of reproducibly and obviously different visible

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**Table 1. Mutations affecting cardiovascular morphogenesis**

<table>
<thead>
<tr>
<th>Genetic loci</th>
<th>Alleles</th>
<th>Phenotype</th>
<th>Other phenotypes</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I. No endocardium</td>
<td></td>
<td>No endocardium</td>
<td>Blood, vascular</td>
<td></td>
</tr>
<tr>
<td>cloche (clo)</td>
<td>m39,m378</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Group II. Large heart</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>valentine (vtn)</td>
<td>m201</td>
<td>Large, distended heart</td>
<td></td>
<td></td>
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<tr>
<td>heart of glass (heg)</td>
<td>m552</td>
<td>Large, distended heart</td>
<td></td>
<td></td>
</tr>
<tr>
<td>santa (san)</td>
<td>m773</td>
<td>Large, distended heart</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group III. Small heart</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>heart and soul (has)</td>
<td>m129,m567,m781</td>
<td>Small, dense heart</td>
<td>Brain, eye, body</td>
<td>b,c</td>
</tr>
<tr>
<td>Group IV. Ventricle defect</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pandora (pan)</td>
<td>m313</td>
<td>Reduced ventricle</td>
<td>Eye, ear, somite, body shape</td>
<td>c,d</td>
</tr>
<tr>
<td>Group V. Bifid heart</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miles apart (mil)</td>
<td>m93</td>
<td>Cardia bifida</td>
<td>Tail</td>
<td>e</td>
</tr>
<tr>
<td>bonnie and clyde (bon)</td>
<td>m425</td>
<td>Cardia bifida</td>
<td></td>
<td></td>
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<tr>
<td>Group VI. No valve</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>jekyll (jek)</td>
<td>m151,m310</td>
<td>No valve</td>
<td>Branchial arch, jaw reduced</td>
<td>f</td>
</tr>
<tr>
<td>(~)</td>
<td>m27</td>
<td>No valve</td>
<td></td>
<td></td>
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<tr>
<td>Group VII. Vascular</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>integrity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mash for brains (mfb)</td>
<td>m75,m381,m508</td>
<td>Anterior hemorrhage</td>
<td>Degeneration, brain</td>
<td></td>
</tr>
<tr>
<td>bubble head (bbh)</td>
<td>m292</td>
<td>Hemoarraghe (brain)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>leaky heart (leh)</td>
<td>m166</td>
<td>Hemoarraghe (pericardial area)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gridlock (gdl)</td>
<td>m145</td>
<td>Cranial/anterior hemorrhage</td>
<td>Caudal circulation defect</td>
<td></td>
</tr>
<tr>
<td>migraine (mig)</td>
<td>m247</td>
<td>Hemoarraghe (brain)</td>
<td>Degeneration, starting from brain</td>
<td></td>
</tr>
<tr>
<td>(~)</td>
<td>m521</td>
<td>Hemoarraghe (brain)</td>
<td>Degeneration, brain</td>
<td></td>
</tr>
<tr>
<td>(~)</td>
<td>m413</td>
<td>Hemoarraghe (brain)</td>
<td></td>
<td></td>
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</tbody>
</table>

References: (a) sanP253; Tubingen allele; Chen et al. (1996); (b) Schier et al. (1996); (c) Malicki et al. (1996); (d) Abdelilah et al. (1996); (e) milP273; Tubingen allele; Chen et al. (1996); (f) Neuhauss et al. (1996).
Zebrafish cardiovascular mutations

phenotypes. Mutants with incomplete complementation analysis were not given locus names, but rather are referred to by m number alone. Histological analysis was performed as described in Stainier and Fishman (1992). Contractility was assessed visually by examination for both global and regional wall motion abnormalities, with regard to rate and distance of systolic contraction.

Tables 1 and 2 list all the mutations and provide the locus names and abbreviations, and the alleles determined by complementation analysis.

RESULTS

Mutations affecting cardiac form

The wild-type heart is composed of two concentric epithelial tubes, the inner endocardium and outer myocardium (Fig. 1A,B). cloche (clo) deletes the endocardial cells. In this mutant, the myocardial layer forms in the absence of the endocardial cells but is dysmorphic: the ventricle is reduced in size and the walls of the atrium are distended. The clo heart also exhibits a reduced contraction. The original allele, clo m39 (Stainier et al., 1995) was identified in an Indonesian fish background and subsequently an ENU allele, clo m378, was identified with the same phenotype (data not shown). The clo mutation also affects blood cell differentiation, as assessed morphologically and by the expression patterns of GATA-1 and GATA-2, two transcription factor genes expressed very early during differentiation of the hematopoietic lineages (Stainier et al., 1995). clo mutants, like most of the heart mutants described in this study, exhibit progressively more severe edema, first of the pericardial sac and then around the eyes and yolk sac. They die around day 7, as do most other heart mutants.

In the valentine (vtn), heart of glass (heg) and santa (san) mutations, the walls of the heart are grossly distended, yet they have a full complement of endocardium (shown for vtn in Fig. 1C,D). Blood is also present in these mutants. In the heart and soul (has) mutation, the heart is much smaller than normal and no distinctive chambers are evident (Fig. 1E,F). In pandora (pan), there is mainly one chamber, which histologically appears to be atrium (Fig. 2A,B). The ventricle is markedly reduced.

The fusion of the primitive myocardial tubes results in the formation of the definitive heart tube. The cellular and molecular events underlying this process are not understood. Two mutations, miles apart (mil) and bonnie and clyde (bon), disrupt this fusion process, resulting in the differentiation of two hearts, one on either side of the midline, a situation commonly known as cardia bifida. Fig. 3A,B shows a wild-type and mil m93 embryo, respectively, at 36 hpf. Transverse sections in the heart region of mil m93 show two complete heart tubes on either side of the midline (Fig. 3C). In some mutants, the two hearts actually contact each other, yet they do not fuse. The two bilateral hearts have endocardial cells lining their lumen and are composed of two chambers; they also beat independently of each other. There is no blood flow in these mutants, presumably because there is no connection to the dorsal aorta.
Normally, cushions form at the atrioventricular junction and at the outflow (bulboventricular) region of the ventricle at about 48 hpf, and clear valve leaflets are evident in these regions by 96 hpf (Fig. 4A,C). In the valve mutants, \textit{jekyll} (\textit{jek}) and \textit{m27} (Fig. 4B,D), cushions are not evident and valves never form.

Mutations affecting vascular integrity

In several mutants localized hemorrhage becomes evident after initiation of the circulation, in areas without other significant obvious defects, suggesting a problem with assembly or maintenance of the endothelial tube. For example, Fig. 5A shows extravasated blood in ventricles of the brain in \textit{bubble head} (\textit{bbh}), accompanied by ventricular edema. Hemorrhage is restricted to particular regions in other mutants. For example, it is localized to the pericardium.
in leaky heart (leh) and to the forebrain and midbrain region of m413 embryos, (Fig. 5B). In gridlock (gdl), hemorrhage is less penetrant and is associated with a diminished or absent caudal circulation. In several mutants, hemorrhage, usually in the brain, is accompanied by degeneration in the brain and elsewhere. In mush for brains (mfb) the degeneration follows the hemorrhage within a few hours; in migraine (mig) and m521, the degeneration is delayed about 12 hours.

**Mutations affecting cardiac function**

Mutations affecting the function of the heart account for the majority of the cardiovascular mutants identified in the screen. Preliminary characterization has allowed us to arrange separate matrices for complementation analysis of lines with defects in contractility, rhythm or forward flow. As some of the mutants have more than one defect, the first detectable phenotype was used to group them. Cross-matrix complementation analyses have also been done to confirm correct matrix assignment.
Contractility
The contractility mutants include those with chamber-specific abnormalities as well as those with whole-heart disturbances. Hatching, swimming and reflex withdrawal are normal, suggesting that skeletal muscle is not affected. The atrial mutant, weak atrium\(^m^{58}\) (wea), has a silent atrium and a normal ventricle. \(\text{wea}m^{229}\) and \(\text{wea}m^{448}\) are alleles with atria that beat weakly before becoming silent after 48 hpf. In the silent atrium of \(\text{wea}\) mutants, the thickness of the space between the endocardium and myocardium increases as the chamber swells, as shown in Fig. 6A. In the ventricle-specific mutant group the ventricles contract only weakly and, although the atria beat with force, circulation soon comes to a halt. Poor contractile function is often accompanied by poor filling in response to atrial contraction, suggesting that the ventricles are stiff as well. The atria in the ventricle-specific mutants often dilate behind the failing ventricles, as shown in Fig. 6B for \(\text{hal}m^{235}\).

In the group with reduced contractility of both chambers, some circulation is initially established but is absent by 48 hpf. The weak beat is accompanied by dilatation of both chambers in pickwick (pik) mutations, but the other mutations affecting contractility of both chambers develop localized contractures and bulging aneurysms by day 3 (Fig. 6C), suggesting localized weakening of the chamber wall.

The ‘retrograde flow’ group of mutations is marked by regurgitation, or retrograde propulsion of blood from ventricle to atrium and from atrium to yolk sac. Forward flow is diminished even though the valves are present. Normally, the timing of contractions, such that atrial beat precedes ventricular beat, helps to ensure forward flow even before valves become fully functional. The relative timing of contraction may be abnormal in this group of mutants. The valveless mutants, \(\text{jek}\) and \(m^{27}\), do regurgitate, but have been classified as morphology mutants because they lack cushion tissue.

Rhythmicity
The rhythmicity mutants manifest primary defects in the rate, rhythm or conduction of the cardiac impulse. Rate and rhythm heart mutants display their abnormal phenotypes as early as the onset of the initial wave of contraction. The conduction subgroup contains both early- and later- (day 2) presenting phenotypes. Subsequent changes in contractile strength and morphology of the ventricle are also observed in some of these mutants, but are considered secondary.

\(\text{slow mo}\) (\(\text{smo}\)) is the sole member of the heart rate group. This slow rate mutant is also one of the few non-lethal cardiovascular mutants identified. \(\text{smo}\) hearts beat functionally, but the rate and, initially, the conduction along the heart tube is slower than wild type from the onset of regular contractions. As is true for wild type, heart rate in \(\text{smo}\) mutants increases with development, but remains slower than normal. For example, at 72 hpf, 28.5°C, the wild-type rate is 206±5.5 beats per minute and the \(\text{smo}\) rate is 146±6.0 beats per minute (significant, \(P<0.01\)).

The tremblor (tre) locus mutants display chaotic cardiac activity, which we have classified as fibrillation. The atrium is active with uncoordinated contractions that give the impression of a shaking bowl of jelly. The ventricle also fibrillates, although less prominently. Circulation is never established.

Abnormal creation or propagation of the impulse is the evident defect in the remainder of the rhythmicity mutants. In \(\text{island beat}\) (isl) mutant hearts, individual cardiomyocytes contract independently, never triggering contraction of adjacent cells and therefore never generating a functional beat. The ventricle becomes silent on day 2 of development, but single cell contractions continue in the atria through day 5. \(\text{tell tale heart}\) (tel) is similar to the isl mutants, but occasionally several cells beat in unison and with evident directionality to impulse propagation.

Initiation of the beat is aberrant in reggae (reg) mutant hearts. The wave of contraction initiates, as in wild type, in the sinoatrial (S-A) region, but only rarely escapes to cause normal sequential atrial and ventricular contractions, suggesting a partial functional block in the S-A region. In the silent partner (sil) mutant, atrial contraction does not lead to ventricular activity. Because the ventricle is silent from the start, this could be a defect in either ventricular contraction or conduction, although complementation analysis shows it to be a different locus from other members of either group. In the ginger (gin) mutants, propagation across the atrioventricular border is initially normal, but by day 2 atrioventricular block appears, with many atrial beats not triggering ventricular contractions.

DISCUSSION
The zebrafish embryonic cardiovascular system turned out to be propitious for genetic study. The heart is visible on the ventral surface of the embryo, such that individual cells are easily resolved in the living embryo. The sensitivity of the screening is increased by the mutations’ concomitant effects upon contractility and blood flow. The visible phenotype of most of the mutations, especially those affecting cardiac function, is restricted to the heart or vessels. Aside from pericardial edema, the mutant fish are not noticeably affected by the lack of normal blood flow for several days, presumably because embryonic fish are not dependent upon the circulation for oxygen delivery during the first days of life (Burggren and Pinder, 1991).

Mutations of cardiac form and vascular patterning
We have identified 17 loci that are essential for fashioning normal organotypic form. As in the nematode or fruit fly, they serve to point out steps in multicellular assembly, although in this case for vertebrate-specific structures. These loci are active in determining the size of the heart, the demarcation of its chambers, the patterning of the vasculature, and assembly of tubes and distinctive organotypic components, such as valves. One attribute is the apparent non-linearity of certain aspects of the decision-making process, such that deletion of one component, be it endocardium, valves or even an entire chamber, leaves others to assemble relatively normally, at least at the morphological level.

The phenotype of some of these single gene defects resembles consequences of physical or pharmacological manipulations of embryos. For example, hearts with reduced ventricle, like the \(\text{pan}\) heart, can be caused by exposure to retinoic acid at early stages of gastrulation (Stainier and Fishman, 1992). Cardia bifida, as is seen in \(\text{mil}\) and \(\text{bon}\), can be caused by disruption of the midline where the cardiac primordia normally fuse (DeHaan, 1959). These relationships suggest that signals related to axial and midline patterning
could be candidates for the pathways perturbed by these particular mutations.

It is not well understood how vessels assemble from different populations of angioblasts into a seamless set of tubes and develop thereafter to sustain different functions. The mutations that cause localized hemorrhage might perturb signals which guide these processes. Those with accompanying degeneration could be secondary to disruption of the surrounding tissues.

**Mutations of cardiac function**

Unlike most other organ systems, it is easy to assess the function of the cardiovascular system by visual inspection. Certain attributes are necessary for the forward movement of blood under sufficiently high pressure to perfuse the body and to prevent the accumulation of extravascular fluid. In the absence of proper structural development, contractility or electrophysiological coordination, tissues eventually suffer from poor oxygenation, and waste and edema fluid accumulate. We have categorized the functional disturbances by their visible effect, although it should be evident that subtle disorders of cellular form might be noticed first as functional defects, and that both contractility and conduction might be perturbed by the same cellular processes.

Contractility of heart cells is affected by mutations at 12 loci. The heart forms normally in these mutants, and in a majority initiates blood flow. The poor contractility is evident by day 2 and is inevitably followed by deterioration of the affected chamber by day 3. The rate of shortening of the myofibers in adult heart cells, unlike in skeletal muscle, is affected by the degree of stretch, a property known as Starling's law (Starling, 1918). One consequence of defects in contractility of adult hearts is enlargement of the chamber, thereby increasing the force generated to maintain blood flow. Some of the contractility mutants go through a dilated phase prior to deterioration. Components of the myofibril are one potential molecular site of dysfunction, as is the proper regulation of intracellular calcium and its coupling to contraction by the troponin-tropomyosin complex.

The initiation and propagation of the cardiac impulse are disturbed by mutations at 7 loci. The very first contractions of the heart tube are in the form of a peristaltic wave, a mechanism also seen in more primitive hearts, such as that of *Drosophila*. By 36 hpf, the atrium and ventricle contract sequentially, a coordination critical to maintenance of unidirectional flow. This change depends upon development of the specialized conduction system and may depend, in part, upon acquisition of the correct gap junctions and ion channels. The onset of these phenotypes precedes innervation of the heart, so defects in heart-beat initiation (*smo* and *reg*) are possibly due to problems in the region of the sino-atrial pacemaker rather than to defects in neural modulation.

The unidirectionality of blood flow anticipates the generation of cardiac ‘cushions’, which are formed by localized accumulation of extracellular matrix and migration of endocardial cells at chamber boundaries. Cushions develop in these regions by the end of day 2. Regurgitant blood flow thus characterizes mutations which delete the valve specifically (e.g. *jek*). Even before cushions form, however, blood flow is maintained in one direction, which has been ascribed to differences in the rapidity and relative timing of atrial and ventricular contractions (Mooirman and Lamers, 1994), and it may be that mutations such as *png*, *ten* and *yyo* cause retrograde flow by interfering with this coordination.

**Genetics of cardiovascular formation**

How does the zebrafish compare with other ‘genetic organisms’? *Drosophila* is related only distantly to vertebrates in that the heart is a dorsal organ which lacks well-demarcated chambers and valves, and ejects hemolymph by peristalsis through the pores of an open circulation devoid of endothelium (Bodmer, 1995). Several gene-targeting mutations in the mouse affect the heart (Kern et al., 1995), but with the exception of fibronectin (George et al., 1993), appear to affect relatively later steps than those described here, including thickening of the ventricular myocardium, septation of the ventricle and outflow tract (to separate the pulmonary and systemic circulations), and generation of the epicardium. Cardiovascular mutations which act at earlier stages may prove to be difficult to assay in the mouse because the mouse embryo is dependent upon blood flow for survival, unlike the fish. Similarly, human mutations that affect the heart tube stage would be lethal before 3 weeks of gestation. Therefore, these zebrafish mutations provide a window on steps of heart formation and function not easily identified by other means or in other organisms.

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