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Zebrafish genetics: **Mutant cornucopia**

Peter D. Currie

The initial characterization of mutations from the large-scale mutagenesis of the zebrafish genome has been reported. What new insights will we gain about vertebrate development from these studies?

Address: Molecular Embryology Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, UK.

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Monumental tasks are manifold in science but saturation mutagenesis of a vertebrate genome must rank high among them. Two laboratories — one at the Max Planck Institute in Tübingen under the direction of Christiane Nüsslein-Volhard, and one at the Massachusetts General Hospital in Boston headed by Wolfgang Driever — have for the past four years been engaged in just such a task, attempting to saturate the zebrafish genome with mutations affecting embryonic development. The fruits of these screens have now been published in a landmark issue of the journal *Development* (December, 1996), devoted in its entirety to papers describing the isolation, classification and preliminary characterization of the many different classes of mutation that have been discovered.

For many years, researchers have been reaping the rich harvest of mutations produced by saturation mutagenesis of the genome of the fruit fly *Drosophila melanogaster* [1]. Analysis of the genes uncovered by these mutagenesis screens has revolutionized our understanding of the signals that control the patterning of early development. Researchers eager to see a similar approach applied to the vertebrate genome have, however, been hampered by the relative intractability at the genetic level of the classical vertebrate model organisms — frogs, chicks and mice. In fact, the elucidation of gene function in vertebrate development has relied heavily on the analysis of homologues of genes first cloned in *Drosophila*, and loss-of-function information has come largely from analysing 'knockouts' of these genes in mice.

Such an *ad hoc* collection of mouse knockouts, together with the numerous spontaneous and insertional mutants that have been generated in mice over the years, is reminiscent of the set of *Drosophila* mutations that were available before the large-scale mutagenesis of the fruitfly genome. Saturation mutagenesis then identified many new genes and, importantly, also produced extensive allelic series of different types of mutation at both known and newly discovered loci. The ability to refer to an allelic series of mutations has been invaluable in uncovering the role of these genes in *Drosophila* development. Random mutagenesis of the zebrafish genome can be seen as a similarly 'forward' genetic approach, providing a complementary methodology to cloning on the basis of sequence similarity and performing gene knockouts in mice. Random mutagenesis makes it possible to survey the vertebrate genome, without bias, for genes that function in particular processes. This approach has the potential to bring about a similar revolution in the understanding of vertebrate development to that achieved for *Drosophila*.

The two large-scale mutagenic screens have used similar general methodologies, employing a classical diploid F_2 screen [2,3]. Mutations are induced by ethylnitrosourea (ENU) into G_0 males; these males are bred with wild-type females, and their offspring (the F_1) are interbred. Mutations are then detected in the diploid offspring of the intercrossed F_2, in which the mutation has been driven to homozygosity. A number of specific attributes of zebrafish have made such screens possible, including the hardiness of adult fish and their ability to produce large numbers of optically clear embryos. Nevertheless, the screens required disciplined fish husbandry, as they involved raising a large number of F_2 'families' (40–100 fish), each representing two mutagenized haploid genomes of the intercrossed F_1. Many tanks containing such families are required for an extensive survey of the genome. The sheer enormity of the task is worth contemplating. Combining the data from both screens, 4 883 F_2 families were raised, representing more than 200 000 adult fish. An average of 4.7 and 6 crosses per family were made in the Tübingen- and Boston-based screens, respectively, and 80 embryos were screened from each pair-mating. This means that the 6 000 or so mutations that were identified resulted from the inspection of around two million embryos. The embryos were systematically scored for specific structures at one, two and five days of age, and in the Tübingen-based screen were also examined for defects in axonal retino-tectal projection in a novel screen devised by Freidrich Bonhoeffer and colleagues [4].

Approximately two thirds of the mutant phenotypes detected fell into four major categories: general necrosis, brain necrosis, general edema or developmental retardation. As mutations within these general classes had similar phenotypes, identifying individual complementation groups would have been extremely time-consuming, so these types of mutant were excluded from further analysis. However, these mutants probably result from perturbation of many interesting genes, and more directed screening attempts

may be required to determine whether they represent defects in a specific process of interest [5].

Despite the mammoth effort, it is clear from an analysis of the number of alleles within each complementation group that saturation has not been reached. Of the approximately 1 800 mutants that were saved for further analysis in the two screens, just over 500 complementation groups have so far been described, with many mutations yet to be assigned to individual complementation groups. Unfortunately, the true number of genes and alleles identified awaits the collation of the complementation data from both groups, an effort that has been largely neglected so far. Taking the data from the larger Tübingen screen alone [6], the allele frequency of identified complementation groups is 2.5, although frequencies vary greatly from 1 to 34 alleles per gene. If a Poisson distribution of frequencies is assumed, the degree of saturation for genes detectable by the methods employed is 90 %. However, if a similar calculation is made using data for only those genes that have one or two identified alleles, the degree of saturation is only 50 %. Given the large difference in mutability of individual loci, making the fit to a Poisson distribution awkward, it is probable that the true frequency lies somewhere between 50% and 90%. Although it must be stressed that this does not represent a genome-wide figure, given that more than half of the mutations have been discarded and the probability that many subtle phenotypes have gone undetected, this calculation can be extrapolated to mean that there are around 700 genes that when mutated produce a specific developmental defect detectable by the morphological screening rationale employed.

It is interesting to compare these figures with the saturation screens that have been performed in *Drosophila*. The approximately 5 000 genes postulated to be lethal when mutated in *Drosophila* are only around one quarter of genes within the genome [1,7]. Of these lethal genes, only around 250 have specific morphologically detectable mutant phenotypes. Given the added complexity of the vertebrate genome, it is clear that the number of developmentally important genes that can be mutated in zebrafish represents a lower proportion of the entire genome. This may reflect the fact that, compared with *Drosophila*, vertebrates have many more genes with redundant or partially overlapping functions within the same developmental process. Unfortunately, there are also few instances of extensive allelic series being generated within individual complementation groups — a function, no doubt, of the degree of saturation obtained. In addition, the lack of mutations that are dominant, antimorphs or hypermorphs, is disappointing, given that mutations of this type have been so instrumental in unravelling the function of *Drosophila* genes.

The phenotypes of the mutants described in the screens are understandably varied. Many groups of mutations, however, affect certain tissues or specific developmental processes (see Table 1). Often, a group of genes share a given phenotype although aspects of the phenotype differ; examples include mutations affecting formation of the pharyngeal arches [8–10], which are derived largely from the cranial neural crest, and those affecting mesodermally derived structures such as the notochord [11,12] and somites [13]. Mutations disrupting the pharyngeal arches affect distinct subsets of arch segments. One class, the ‘anterior group’, of which the mutation *sucker* is an example, disrupts more anterior arch segments; a second class, primarily members of the *flathead* group, disrupt posterior arches (Fig. 1a). These mutations may reflect the way neural crest and associated cell types form, migrate and then interact within the arch environment.

Many mutations have been uncovered that affect notochord development and these have been classified according to whether the gene is required for notochord formation, differentiation or maintenance [11,12]. Invariably, mutants that lack a differentiated notochord have disrupted development of the paraxial mesoderm. Another group of mutations, the *you*-type mutants, have a fully differentiated notochord but lack distinct subsets of somitically derived structures or cell types (Fig. 1b) [13]. Together, these mutants reveal a step-wise cascade of inductive events that

Table 1

Classes of mutation isolated in both screens

Class of phenotype	Maximum number of complementation groups	Mutations not resolved into complementation groups	References
Epiboly/early developmental arrest	15	–	[36]
Gastrulation and axis formation	27	2	[29–31,37]
Notochord	57	18	[11,12]
Somites	8	7	[13,38]
Central nervous system	118	103	[15–18,38–41]
Organs and blood	193	95	[24,26–28,31,41–43]
Pigment	85	75	[25,38,44]
Jaws and gills	53	54	[8–10]
Motility	49	73	[38,45,46]
Adult	22	15	[6]
Retino-tectal	11	14	[23,47]

Mutations are listed under the primary aspect of phenotypes only. Numbers are collated from [6] and [38]. The figures represent the addition of complementation groups identified from both screens; as the complementation groups have not been tested between screens for allelism, the figures are undoubtedly an over-estimate of the number of different genes identified.

occur within the presomitic mesoderm, originating with signals that emanate from notochord. Analysis of these mutants should provide a detailed understanding of how midline signals act to pattern the paraxial mesoderm.

These few examples illustrate how, in a number of instances, the newly isolated mutations define distinct phenotypic classes and presumably identify specific steps in the differentiation of a particular structure. This aspect of the zebrafish screen is particularly satisfying, because in *Drosophila* it is the molecular analysis of individual members of specific phenotypic classes that has led to the detailed understanding of the molecular mechanisms of the processes they control [14]. It is certain that, once the epistatic analysis and molecular cloning of the genes represented by these common mutant classes is achieved, they will do the same for vertebrate development.

Among the most intriguing mutants are members of the large class affecting development of the embryonic brain (Fig. 1c) [15–18]. The ease of inspection of the morphology of the developing zebrafish brain allows the detection of even subtle changes within this complex structure. With detailed characterization of the neuroanatomy of the embryonic brain well under way, it will be possible to pinpoint specific structures that are deleted or altered in these mutants [19–22]. The mutants will then be important tools in unravelling the nature of patterning within the developing zebrafish nervous system, and will help to answer questions about its relationship to the brain of higher vertebrates. For example, the mutations may reveal whether or not there are genetically definable subunits or subdivisions with the developing neural tube that are not apparent using morphological criteria.

On a finer scale, the mutations affecting axon guidance and correct retino-tectal projection will help in addressing

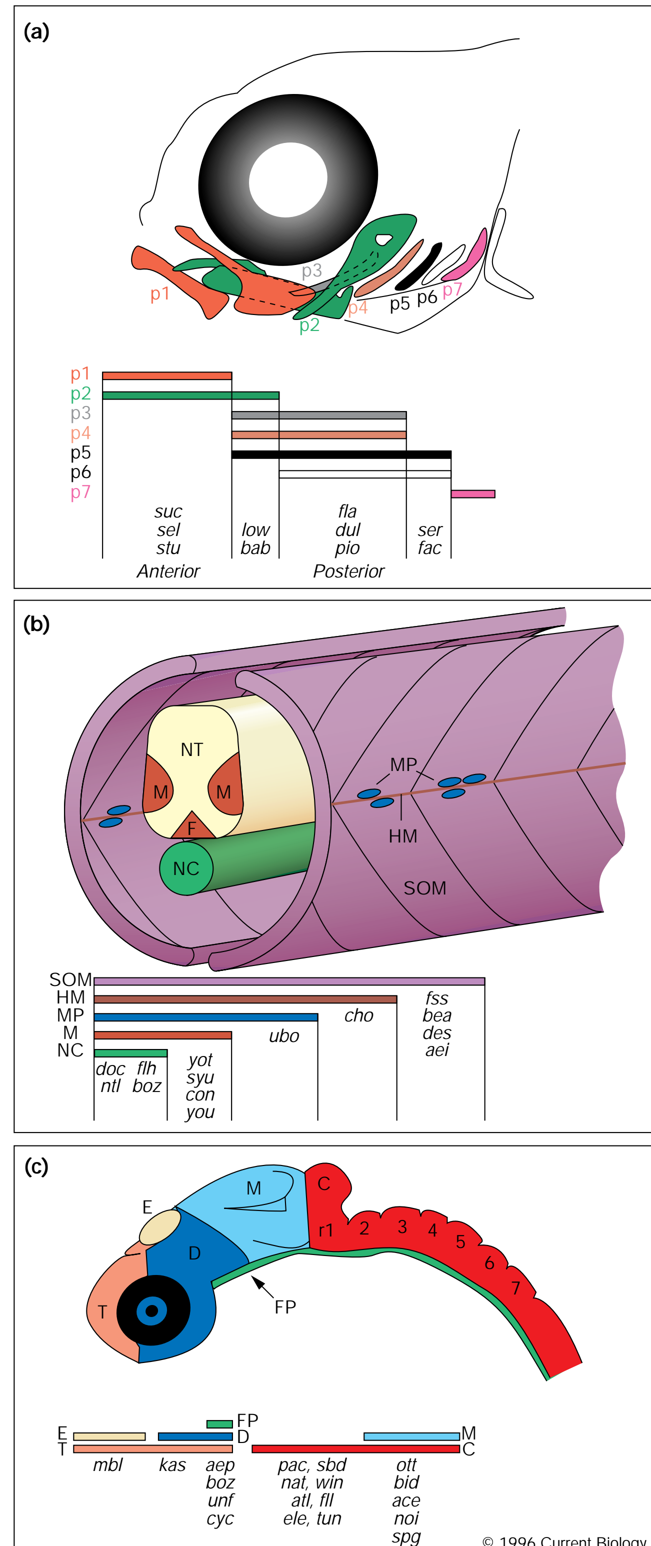

Figure 1

Mutations in specific developmental pathways are listed according to the colour of the structures they affect. Not all mutations affecting the development of the structures shown are listed; many mutations have pleiotropic effects and only the major aspect of the phenotype is highlighted. **(a)** Mutations affecting pharyngeal arches. Seven arches (p1–p7) develop in the head skeleton of the wild-type 5 day larva. A subset of mutants that show regional defects in groups of adjacent arches are shown. A large number of mutants affect all arches and are not shown. Mutant abbreviations: *bab, babyface; dul, duckbill; fac, facelift; fla, flathead; low, lockjaw; pio, pistachio; sel, schmerle; ser, screamer; stu, sturgeon; suc, sucker.* **(b)** Mutations affecting notochord and somite development: F, floorplate; HM, horizontal myoseptum; M, motor neurons; MP, muscle pioneer cells; NC, notochord; NT neural tube; SOM, somites. Many mutations affect the differentiation and maintenance of the notochord and are not shown. Mutants abbreviations: *aei, after eight; bea, beamter; boz, bozozok; cho, choker; con, chameleon; des, deadly seven; flh, floating head; fss, fused somites; mom, momo; ntl, no tail; syu, sonic you; ubo, u-boot; yot, you too; you, u-shaped somites.* **(c)** Mutations affecting brain development: C, cerebellum; D, diencephalon; E, epiphysis; FP, floor plate; M, mesencephalon; R, rhombomeres; T, telencephalon. Many mutations affect the formation of brain ventricles and general brain morphology; these are not shown. Mutant abbreviations: *ace, acerebellar; atl, atlantis; bid, big head; cyc, cyclops; ele, eisspalte; fll, flachland; kas, knollnase; mbl, master blind; nat, natter; noi, no isthmus; oep, one eyed pinhead; ott, otter; pac, parachute; sbd, scabland; spg, spiel ohne grenzen; tun, turned on; unf, uncle freddy; win, wicked brain*

questions about the genes involved in these processes [4,23]. In the retino-tectal projection screen, subsets of retinal ganglion cell axons of single embryos from each F_2 cross were injected with lipophilic dyes to trace the targeting of axons to specific sites in the embryonic tectum. Mutants were isolated in which axons were unable to find their way correctly (pathfinding mutants) as well as mutants in which axons form synapses inappropriately on the tectum (mapping mutants). It is probable that these mutants affect many of the molecular signals that direct axon guidance in the visual system.

These classes of mutations alone would seem to be ample return for the investment, but many more mutants have been uncovered than can be described here. A few, however, are worth a brief description. Haematopoiesis and the development of specific internal organs are altered in a number of classes of mutations. For example, mutants in blood development have been grouped into three different classes [24,25]. Animals homozygous for *vampire* or *moonshine* are defective in blood-cell generation and are the members of the first class of mutants which completely lack blood cells. The second class of mutants are defective in blood cell maintenance/differentiation, and include such mutants as *sticky blood*, *pale and wan* and *clear blood*. The last group includes a number of intriguing mutations that produce photosensitive blood cells; some examples are the colourfully named *dracula* and *desmodius*. When such mutant fish are raised in normal lighting conditions they lack circulating blood cells, but when raised in the dark near normal levels of blood cells develop. This phenotype is reminiscent of known human conditions, in particular the disease porphyria: people with porphyria lack haem biosynthetic enzymes, resulting in the accumulation of haem metabolic intermediates, the porphyrins, which absorb light and release oxidative by-products that lyse blood cells.

The development of the heart and vasculature is affected by mutants in more than 30 genes [26,27]. These can be sub-divided into those affecting heart morphogenesis or those affecting heart function. Examples of mutations affecting morphogenesis are *miles apart* and *bonnie and clyde*, in which fusion of the bilateral heart primordia is perturbed, resulting in cardia bifida. Mutations at the *pandora* locus specifically eliminate the ventricle of the zebrafish heart, and a number of others affect overall heart morphology. Intriguingly, there is also a large class of heart-function mutants that affect contractility of heart cells and the signals that stimulate and propagate the cardiac impulse. The development of the gut and intestinal organs is specifically disrupted in another class of organogenesis mutants [27,28]. The genetic control of organogenesis is largely uncharted, even in invertebrates, and the optical clarity of the zebrafish embryo has provided the first opportunity to screen for mutations that disrupt such processes. The rewards for understanding these events are obvious, given the similarity of organogenesis in zebrafish and higher vertebrates.

It is interesting to consider the kinds of mutation that were not found in either screen. Mutations that cause the transformation of the identity of one segment into another, as exemplified by mutations in the homeotic genes of *Drosophila*, seem to be rare or absent from the zebrafish screens. These types of mutations may, however, be difficult to identify, either because of the similarities between body segments, or because of genetic redundancy. One possible exception is *master blind*, in which the rostral forebrain is apparently transformed into a more caudal diencephalic or mesencephalic identity [15]. Mutants that affect very early events in determining the polarity of the embryo also seem rare, and this may reflect the fact that genes controlling such events are usually active maternally. Thus, the few ‘zygotic mutants’ disrupting axis formation are extremely valuable as starting points for analysis of early patterning events [29–31]. Their scarcity may reflect the fact that yolk, and hence a maternal contribution to development, persists for the entire period of embryogenesis. An exciting but technically daunting task awaits those who wish to undertake a genetic dissection of the maternal component of early zebrafish development.

The immediate task now is to place the available mutations on the genetic map. This will allow the alignment of map positions for cloned genes with those of mutations, a process that has already been successfully completed for two mutations [32,33]. Two separate genetic maps have been compiled, one using randomly amplified polymorphic DNAs (RAPDs) [34] and the other using simple sequence length polymorphisms (SSLPs), and in conjunction with this second mapping endeavour a reference cross has been developed to integrate and facilitate mapping [35]. Positional cloning is the only currently available method — other than the use of cloned candidate genes — for determining the molecular lesion underlying a mutation, and the ease with which this can be performed will be directly proportional to the complexity of the genetic map. A detailed map will certainly help guide us to the real reward these mutations may bring — a molecular description of many aspects of vertebrate embryogenesis. An effort similar in magnitude to that expended in isolating the mutations will now be needed in order to clone the genes they affect.

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