Of fin and fur: mutational analysis of vertebrate embryonic development

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Vertebrate embryonic development has traditionally been studied in the amphibian and the chick. In these organisms extensive experimental manipulations, and, more recently, molecular characterization have helped to illuminate mechanisms of cell–cell interactions that lead to lineage development and pattern formation. But neither *Xenopus* nor chick is readily amenable to genetic studies. The power of mutational analysis in understanding developmental events in invertebrates has led researchers to consider other vertebrate species in which embryology and genetics might be combined to provide insights into the genetic control of early development. An obvious candidate is the mouse. The early embryology of the mouse is fairly well understood (Rossant and Pedersen 1986), and there is already a storehouse of spontaneous and radiation-induced mutations affecting developmental processes (Green 1989). A number of these mutations have been characterized molecularly by mapping cloned genes to the locus of interest (for review, see Reith and Bernstein 1991), and two, *Brachyury* (Herrmann et al. 1990) and *Sry* (Gubbay et al. 1990), have been cloned by physical means from map position. As the genetic linkage map of cloned genes increases in resolution (Copeland and Jenkins 1991), and as physical mapping initiatives proceed in various regions of the genome, molecular characterization of any mutation of interest should be feasible. However, initiating large-scale screens for new mutations affecting embryonic development in the mouse has been considered to be too slow and expensive an undertaking, mainly owing to small litter sizes, large genome size, the cost of housing large numbers of animals, and the inability to observe and manipulate all stages of the developing embryo easily.

Recently, a rookie player, the zebrafish, a striped 2-inch-long fish from the Ganges River, has been making strong claims to major league status in vertebrate developmental biology because it offers the possibility of combining excellent embryology with genetics. George Streisinger first drew attention to the potential of the zebrafish as a genetic system and was able to establish conditions for γ-ray mutagenesis and screening (Chakrabarti et al. 1983; Walker and Streisinger 1983). The advantages of zebrafish over mouse for classical genetic analysis include its small size and hence, the ability to house a larger number of animals more cheaply, and the large number of embryos produced from one female (usually a few hundred but as many as 1000 eggs a day).

The zebrafish also has a couple of neat genetic tricks up its sleeve (fin!) that aid in genetic screening. Potentially most useful is the ability to prepare haploid embryos by using heavily UV-irradiated, and therefore genetically inactive, sperm to fertilize eggs. The haploid embryos progress almost normally through early development (although they die after several days), allowing the investigator to detect recessive early-acting mutations without breeding them to homozygosity.

Equally important are such features as the accessibility and transparency of the zebrafish embryo and the speed of its early development. In natural matings, eggs are fertilized externally and fall to the bottom of the tank for easy collection. The time from fertilization to gastrulation is only ~5 hr at 28°C; somites form between 10 and 20 hr, and by 24 hr postfertilization, a recognizable animal with rudimentary eyes and brain is wiggling its tail. Thus, the early development of this vertebrate takes only about as long as a phage plaque assay, and it can be viewed under a dissecting microscope. Finally, the zebrafish is a superb embryological model because cell lineages can be traced at all stages in the living embryos (Kimmel 1989; Kimmel et al. 1990).

Despite these attractions, the zebrafish model lacks many of the basic tools that are taken for granted in the mouse. There is no genetic linkage map. The number of cloned genes is still very small, as is the number of accurately described mutant phenotypes. Clearly, embarking on a major search for genes involved in controlling vertebrate embryonic development using the zebrafish as a model would require a substantial investment of time and money in a number of laboratories.

The proponents of the zebrafish usually point to the possibility of applying to a vertebrate the kinds of saturation mutagenesis screens carried out so successfully in *Drosophila* (Nüsslein-Volhard and Wieschaus 1980) as one of the most compelling reasons to invest in the zebrafish. However, in these days of intensive molecular
Rossant and Hopkins

Investigation of developmental processes, the approach of random mutagenesis and delineation of mutant phenotype is not necessarily the only route to a genetic understanding of development. In this review we consider the various mutagenic strategies that can be envisaged in vertebrates. These strategies range from the random to the directed, and all can, in theory, be applied to either zebrafish or mice. Practical limitations favor the use of random approaches in zebrafish and more directed approaches in mice. Which approach will lead faster to the goal of a complete genetic dissection of vertebrate embryonic development remains an open question.

Identifying interesting developmental mutations

Before discussing mutagenic strategies, it is important to consider the criteria to be used to identify those mutations relevant to the control of embryonic lineage development and pattern formation. A simple criterion is embryonic lethality; however, few embryonic lethal mutations actually result from disruption of embryonic patterning genes. In the mouse, embryonic gene activation begins as early as the two-cell stage (Schultz 1986), and reliance on embryonic gene expression is likely to be complete soon after implantation, when the embryo enters a rapid growth phase (Snow 1977). This means that embryonic lethal mutations will include a large number of mutations in genes involved in housekeeping functions. In zebrafish, although embryonic gene transcription begins at the mid-blastula transition, it is probable that there are enough stores of maternal protein for basic metabolic functions to proceed throughout the short period of embryonic development. Thus, a much higher proportion of embryonic lethals will be in the sought-after embryonic-encoded regulatory genes in zebrafish than in mice.

One still requires criteria to distinguish metabolic defects from patterning defects. Phenotypes that cause specific alterations in normal embryonic patterning rather than simple developmental arrest are promising candidates for critical developmental mutations. Not all such patterning gene defects need be embryonic lethals. For example, in mice, the mutations undulated [un] (Grubeberg 1954) and tailkinks [tk] (Grubeberg 1955) both cause skeletal abnormalities in otherwise viable homozygotes. Embryological studies have traced both defects to the critical phases of sclerotome differentiation in the somites, and un has proved to be a mutation in the paired-like gene, pax-1 (Balling et al. 1988; Chalepakis et al. 1991). Clearly, identifying informative phenotypes requires a good understanding of normal developmental processes. This is where the excellent cell lineage map in zebrafish can be a great advantage (Kimmel 1989; Kimmel et al. 1990). However, in mice, knowledge of how the postimplantation embryo is put together is improving rapidly through a combination of classical lineage analysis (Lawson et al. 1991), comparison with experimental studies in other vertebrate species, and identification of developmental domains defined by expression of putative developmental genes. Prior to gastrulation in both species, cell-marking studies have revealed considerable cell mixing and indeterminacy of cell lineage (Rossant 1985; Kimmel et al. 1990), making it less clear whether the phenotypes of early acting mutations will be very informative. Considerable embryological knowledge and a degree of intuition will be required to identify mutations in genes critical for establishing the basic body plan in both mouse and zebrafish.

Mutagenic strategies: random mutagenesis

The classical approach to identification of genes important for pattern formation in development is to undertake an extensive screen, approaching saturation, for mutations whose phenotypes indicate involvement in such processes (Nüsslein-Volhard and Wieschaus 1980; Mayer et al. 1991). Such a screen requires a very efficient mutagen to make it feasible, and, for this reason, chemical mutagenesis has been the usual method of choice. Among vertebrate species, mice have been subjected to a number of different mutagens over the years, of which the most efficient are X-rays (Searle 1974; Russell 1951) and the chemicals ethynitrosourea (ENU) (Russell et al. 1979; Hitotsumachi et al. 1985) and chlorambucil (Russell et al. 1989). The type of mutation produced varies according to mutagen and germ-cell stage targeted, X-rays and chlorambucil induce rather large lesions, usually deletions (Rinchik et al. 1990; Rinchik and Russell 1991), whereas ENU is expected to produce predominantly point mutations. After treatment of male mice with either ENU or chlorambucil, new mutations can be produced at a given locus with a frequency as high as 1/600 to 1/1000 gametes tested, X-rays are ~10-fold less efficient (Rinchik 1991). This frequency is estimated from the specific-locus test in which mutagenized wild-type males are crossed to females homozygous for a number of recessive visible mutations and the offspring are scored for mutant phenotype (Russell 1951). There is much less extensive information on mutagenic frequencies in zebrafish, but they seem likely to lie in similar or possibly higher ranges, based on the frequencies of γ-ray-induced or EMS-induced mutations reported at specific loci (Chakrabarti et al. 1983; W. Dreier, pers. comm.).

What are the logistics of using chemical mutagenesis to screen for mutations affecting embryonic pattern in vertebrates? One approach is to attempt a genome-wide screen, as has been reported recently for Arabidopsis (Mayer et al. 1991). A scheme for such a screen is outlined for zebrafish in Figure 1. This strategy basically involves a three-generation cross in which F1 females, heterozygous for a number of induced mutations, are backcrossed to their father or mated to their brothers to reveal homozygous mutant phenotypes. If we assume that a locus-specific mutation frequency of 1/1000 gametes scored can be achieved in both mice and zebrafish, then according to Poisson expectations one would need to screen at least 3000 mutagenized gametes to approach saturation of the genome. The efficacy of such a screen...
establish mutant pedigrees in the absence of linked markers.

The main advantage of performing a saturation mutagenic screen on a genome-wide basis is that the screen has only to be performed once on one set of 3000 mutagenized gametes. The main disadvantage is that mapping and cloning the mutations generated would be difficult. Chemical mutagenesis always has the drawback that there is no easy route to molecular characterization, but this is exacerbated in situations where no markers linked to the mutations are included in the screening strategy.

Strategies that involve screening the genome a piece at a time provide for inclusion of linked markers and, when combined with physical mapping approaches, can lead more directly to molecular cloning. The main disadvantage of such approaches is that each piece of the genome screened requires the separate analysis of another set of 3000 mutagenized gametes. In Drosophila, saturation mutagenesis was essentially performed one chromosome at a time using an inversion balancer chromosome, which prevents recombination, carries a visible marker, and is lethal when homozygous (Jürgens et al. 1984, Nüsslein-Volhard et al. 1984, Wieschaus et al. 1984). There are only 4 chromosomes in the Drosophila haploid complement, compared with 20 in the mouse and 25 in zebrafish. A number of inversion chromosomes exist in mice, but few carry suitable markers; in zebrafish, no such resources are currently available.

Even in the absence of balancer chromosomes, Haldane (1955) has shown theoretically that animals carrying a number of widely spaced genetic markers can be used to screen a reasonable percentage (~6%) of the genome at a time for embryonic lethal mutations. Application of this technique to the mouse has been rare (Carter 1959) because of the large numbers of animals involved in the three-generation cross required to reveal recessive phenotypes. The largest three-generation screen has been carried out by Bill Dove and his colleagues, who have screened >350 mutagenized gametes and identified a number of new recessive embryonic lethal mutations in the t complex (Shedlovsky et al. 1988; King et al. 1989). This screen, which was aided by the presence of a number of linked markers and an inversion that prevents recombination, still falls far short of saturation levels.

A more generally applicable approach to the idea of analyzing the genome a piece at a time is the use of deletions. In mouse, sets of radiation-induced deletions exist around several known markers (Rinchik and Russell 1990). ENU mutagenesis can be used for fine-structure mutation mapping within a deletion, utilizing a two-generation cross in which mutations can be revealed in the hemizygous state against the deletion (Fig. 2). This provides an enormous reduction in the number of animals required for saturation screening and makes it feasible to approach saturation even in mice. This is exemplified by the work of Rinchik and colleagues, who have screened >3500 ENU-mutagenized sperm for new mutations in a 6- to 11-cM deletion around the albino locus.

In mice, application of this approach on a genome-wide scale has been limited both by the sheer numbers of mice needed and the difficulty in isolating and maintaining a new lethal or detrimental mutation without any linked markers to aid in genotype identification. In zebrafish, setting up a genome-wide saturation screen for embryonic mutations using the standard three-generation cross would be slow, but would be less space- and money-consuming than in mice. Furthermore, identification of embryonic mutant phenotypes would be much simpler because of the short free-living embryonic stage and the large numbers of embryos that can be produced. A further considerable saving in time and space could be achieved if haploid screening turns out to be workable enough for screening purposes. Haploid testing would also ease the burden of the test-breeding needed to establish mutant pedigrees in the absence of linked markers.

Figure 1. Chemical mutagenesis–diploid screen.

Chemical mutagenesis always has the drawback that the number of mutations carried by each mutagenized gamete not be too high, or segregation of the mutation of interest becomes tiresome. The number of actual mutational hits carried per gamete will depend on the number of mutable genes. In mice it has been estimated from ENU mutagenic frequencies that there are 50–100 vital genes in the 15-cM domain of chromosome 17 that encompasses the t complex (Shedlovsky et al. 1988). Extrapolating to the complete genome of ~1600 cM gives an estimate of ~5,000–10,000 genes in which mutations would lead to embryo lethality or a visible phenotype (Dove 1987). We will assume that similar numbers would apply in zebrafish. ENU-treated gametes would thus carry 5–10 such mutations, a number that is not unreasonable for screening purposes.

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Figure 2. A scheme for fine-structure mutational mapping in a deletion region, based on mutagenesis within the *albino* deletion in the mouse (Rinchik 1990). The scheme makes use of easily scored alleles of the tyrosinase gene at the *albino* locus, which cause wild-type coat color (+), *chinchilla* (*c''^h*), or *albino* (*c*). In the case of females carrying putative mutations (*) to males carrying a deletion at the *albino* locus, albino offspring will not be produced when there is a new lethal mutation within the region of the deletion. The mutation can be recovered from heterozygotes that are readily distinguished by their chinchilla coat color.

[Rinchik 1991 and pers. comm.]. The development of high-efficiency deletion mutagens like chlorambucil should aid the generation of more panels of nested deletions in other regions of the mouse genome. A combination of fine-structure mutagenesis and physical mapping utilizing deletion breakpoints should provide molecular genetic access to many interesting areas of the mouse genome (Rinchik and Russell 1990). Similar approaches could be readily applicable to zebrafish, once suitable markers for deletion analysis are in place.

Thus, saturation mutagenic screens are currently feasible in limited regions of the mouse genome, but scaling up to a genome-wide screen is limited by both genetic resources and the numbers of mice needed. In zebrafish, the numbers of fish required need not be so daunting, but the genetic resources that would aid in isolating mutant strains, establishing genetic linkage, and eventually cloning the mutated genes are currently not available. A genome-wide screen [Fig. 1] can proceed in the absence of such resources, and interesting mutant phenotypes will be found. A number of interesting developmental mutants have already been recognized from *γ*-irradiation mutagenesis (Kimmel et al. 1989; Felsenfeld et al. 1990; Hatta et al. 1991). Proceeding from mutant phenotype to molecular clone, although not a small task, need not be an impossible one in these days of genome project initiatives. Microsatellite polymorphic variants (Weber and May 1989; Love et al. 1990), which are highly variable and readily typed by polymerase chain reaction (PCR), have been used to map polygenic traits genetically in both mouse and rat (Hilbert et al. 1991; Jacob et al. 1991; Rise et al. 1991; Todd et al. 1991). The successful application of this approach in the rat, where the linkage map was extremely incomplete, suggests that this kind of approach should be readily transferrable to zebrafish, where the availability of haploid offspring will aid in linkage analysis. A genetic map tagged with molecular markers is a prerequisite for subsequent physical mapping approaches. Both genetic and physical mapping of the zebrafish genome is under way in a number of laboratories (C. Tabin and W. Driever, pers. comm.), and initiatives of this sort would seem to be an important priority for the success of the chemical mutagenesis route in zebrafish.

**DNA-tagged mutagenesis**

Many of the cloning problems associated with chemical mutagenesis could be circumvented if the mutagenic protocol generated molecular tags suitable for following pedigrees, mapping, and molecular cloning. Insertional mutagenesis via introduction of exogenous DNA sequences provides such molecularly tagged mutations. In mice, insertional mutations have been produced by mi-
croinjection of DNA into zygotes, electroporation of DNA into embryonic stem (ES) cells, injection of early embryos or ES cells with exogenous retroviruses, and reinfection of the germ line with endogenous retroviruses (for review, see Gridley et al. 1987; Jaenisch 1988). In zebrafish, only DNA microinjection into eggs has been reported to date. The first published report of germ-line transgenic zebrafish was from Westerfield's laboratory in 1988 [Stuart et al. 1988] and indicated a transgenic frequency of ~4-5% following injection of plasmid DNA into the cytoplasm of the fertilized egg. Nuclear injection, as usually performed in mice, has not been tried because of the difficulty in seeing the pronuclei and the rapidity of early cleavage divisions. More recently, frequencies of germ-line transmission, though not necessarily expression, of injected sequences as high as 20% and even higher have been reported from a number of laboratories (Culp et al. 1991; C. Fulwiler and M. Westerfield, pers. comm.).

DNA microinjection has been widely used in the mouse for a variety of purposes, and its mutagenic potential has been fairly well documented. Approximately 1 in 10 transgenic mice carries a recessive mutation causing embryonic lethality or a visible phenotype [Palmiter and Brinster 1986]. Because each transgenic mouse usually contains only one insertion, DNA microinjection is much less efficient than chemical mutagenesis. One would have to test 300,000 transgensics to be sure of a mutation in each of the estimated 10,000 genes that yields a visible phenotype upon mutation, compared with 3000 gametes for chemical mutagenesis. Generating the transgensics would not be trivial, and the three-generation cross then required to produce homozygotes from the original hemizygous transgenic animal would severely limit the number of insertions that could be studied. Using current PCR-based technologies for tracking transgenic lines, the most efficient laboratory could probably test 200 transgenic lines in a year (R. Woychik, pers. comm.). Use of a visible marker for tracking hemizygous transgensics, such as the tyrosinase minigene that causes variable coat pigmentation on an albino background [Tanaka et al. 1990; Yokoyama et al. 1990], might increase this number twofold, still far short of a saturation level.

By use of haploid screening, obviating the need for a three-generation cross, and by virtue of the ease of identifying embryonic lethals, a proficient zebrafish laboratory could probably test-breed at least 10-fold more transgensics in a year than is possible in the mouse [i.e., 2000], making the numerology not quite so daunting. Because it is easy to generate and inject large numbers of eggs, two people could probably generate the 20,000 potential transgensics per year required to provide 2000 transgenic females for haploid screening [see Fig. 3]. Such a screen would, again, be aided by a dominant visible marker transgene, such as *Escherichia coli lacZ*, which can be detected in the living embryo using the fluorescent substrate fluorescein diothiogalactoside [FDG] [Nolan et al. 1988; C. Fulwiler, pers. comm.; S.C. Lin and N. Hopkins, unpubl.] or in fixed embryos using X-gal. Before vital staining can be used for this purpose, it will be necessary to solve the problem of getting transgenes to express more efficiently after passage through the germ line [Stuart et al. 1990; Culp et al. 1991]. Two thousand transgenic fish should generate 200 visible mutations. At these rates, 150 lab-years would be required to generate the 30,000 mutations needed for saturation. This is still a huge undertaking, and the numbers given are based on the assumption that microinjected DNA will be equally as mutagenic in zebrafish as in mice. This must be tested experimentally, and the answer should be known within the year.

The temptation to proceed with an insertional mutagenesis screen of some sort in zebrafish is great, because the technology is more or less in hand. However, DNA microinjection may not be an ideal means of generating readily clonable insertional mutations. A considerable number of transgenic insertional mutations have been reported in the last few years in the mouse, but it is noteworthy that full molecular characterization of the associated host gene has only been reported so far for one, the *limb deformity* locus [Maas et al. 1990; Woychik et al. 1990]. Studies of several more are close to completion, but the record does not suggest that moving from mutation to gene is necessarily easy. The main reason for this seems to be that transgenic insertions generated by microinjection often cause deletions and rearrangements that can greatly complicate association of a particular transcriptional unit with the phenotype ob-

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**Figure 3.** Protocol for DNA mutagenesis using a haploid screen.
served (Covarrubias et al. 1987; Wilkie and Palmiter 1987).

Retroviruses can also cause insertional mutations, and retroviral insertions are usually simple and cause little or no alteration to the surrounding host DNA. The mutation frequency from retroviral insertion has been estimated at ~5% [Jaenisch 1988]. Jaenisch’s laboratory has cloned host transcripts associated with three different retrovirally induced mutations produced after embryo infection in the mouse [Harbers et al. 1984; Soriano et al. 1987; Gridley et al. 1991]. Clearly, development of retroviruses capable of infecting fish embryos at a reasonable efficiency would be an important advance, especially if the virus could be constructed so as to express a dominant visible marker for ease of screening. What would be even more useful, however, would be a genetic system in which the fish did the work of mutagenesis.

Development of zebrafish stocks in which one could cause a transposable element or retrovirus to hop or re-infect the germ line at high efficiency would take much of the manual labor out of screens for insertional mutations. P-element mutagenesis in Drosophila [Kidwell 1986; Cooley et al. 1988], the Tcl transposon in C. elegans [Herman and Shaw 1987], and, to a lesser extent, SWR-RF hybrid mice [Jenkins and Copeland 1985; Spence et al. 1989], provide examples of such systems.

Promoter- or gene-trapping

DNA-tagged mutagenesis would be more efficient if one could select directly for insertion events that lie within coding sequences. Entrapment vectors, as first described in bacteria [Casadaban and Cohen 1979; Casadaban et al. 1980], would fulfill this goal. In mammals, entrapment vectors can be introduced into pluripotent ES cells in culture and then passed into the germ line via chimeras [Gossler et al. 1989; Skarnes 1990]. Emphasis has been placed on promoter or gene traps [Brenner et al. 1989; Gossler et al. 1989; Kerr et al. 1989; von Melchner et al. 1990; Friedrich and Soriano 1991; Reddy et al. 1991] rather than on the enhancer trap vectors used in Drosophila [O’Kane and Gehring 1987; Bellen et al. 1989; Bier et al. 1989; Wilson et al. 1989]. These vectors contain a reporter gene, usually lacZ, lacking its own promoter and with or without a splice acceptor sequence upstream, and are introduced into ES cells by electroporation or via a retrovirus [Fig. 4]. Activation of the reporter can only occur when the vector is inserted into a unique active host gene and requires generation of a fusion transcript with the host gene. The reporter gene activity then provides the advantages of accessibility to the vectors. This would require isolating ~30,000 cell lines that express the reporter fusion transcripts. What is the optimum strategy from here? One might consider a screen in which all gene-trap integrations that express in ES cells are transmitted through the germ line and examined for mutant phenotype in the resulting mice. To date, ~30 such cell lines have been transmitted through the germ line [Friedrich and Soriano 1991; W.C. Skarnes, A. Auerbach, and A.L. Joyner; H. Ruley, both pers. comm.], and at least 1 in 4 is a recessive embryonic lethal mutation or shows a visible phenotype when homozygous. However, the task of screening 30,000 different mouse lines is still formidable, and the proportion of the mutations that would turn out to be involved in
embryonic patterning is probably quite low. Also muta-
tions in genes that turn on at later stages of development
than those equivalent to ES cells would be missed.

One of the advantages of the gene-trap approach is that
it allows for the possibility of preselecting the insertion
events that are actually passed through the germ line, thus
obviating the need for huge numbers of mice. Sev-
eral such prescreens can be envisaged [Fig. 5]. Pattern
of expression can be an important clue as to the function
of a gene, and use of lacZ gene-trap vectors allows pre-
screening for expression of the host gene. If ES differen-
tiation in vitro can be controlled sufficiently and di-
rected down specific pathways, it may be possible to pre-
screen for expression in particular lineages in vitro and
only take those expressed in the lineage of interest
through chimera formation and germ-line transmission.
This is particularly powerful, as integrations in genes
that are silent in the undifferentiated state but activated
later in development may be detected. Genes with spa-
tially restricted expression indicative of a role in pattern
formation are, however, unlikely to be identified during
morphogenetically disorganized ES differentiation. To
detect such genes, prescreening by expression in ES chi-
meras at the relevant stage of development would be the
method of choice. In a preliminary screen for expression
in 8.5-day chimeras of 80 gene-trap cell lines that ex-
pressed lacZ in vitro, we have found that ~10% show
spatially restricted expression patterns in the embryo [S.-
L. Ang, A. Auerbach, S. Gasca, F. Guillemot, D. Hill, A.
Joyner, J. Rossant, and W. Wurst, unpubl.]. Using this as
the prescreen, one would only follow 30,000 or so lines
though germ-line transmission and phenotypic evalu-
tion. We do not yet know whether this prescreen actu-
ally enriches for mutations with informative embryonic
phenotypes. Finally, one could also use host gene se-
quence as a guide to the importance of the entrapped
gene by taking advantage of the features of the vectors
that allow rapid cloning of the associated host gene.

Can gene/promoter-trap vectors be used in fish in a
broad-based mutagenic screen? In the absence of ES cells,
the question is whether direct injection of vectors into
eggs would be feasible. If one could use the vital fluo-
rescence-generating substrate FDG for lacZ, it might be
possible to screen for patterns of expression directly in
the microinjected progeny and allow only expressors to
proceed to adulthood and germ-line mutational analysis.
Currently used gene-trap vectors are activated in ES cells in ~1% of all integrations (Gossler et al. 1989; Friedrich and Soriano 1991, Reddy et al. 1991). If we assume, for sake of argument, that there are a further 4% of integrations that could express at other stages of development, then as many as 5% of transgenic zebrafish might express the vector. At a transgenic frequency of 20%, 1 in 100 surviving fish would show expression and contain a mutation. One-quarter of these mutations would show a visible phenotype, using the mouse numbers as a guide, giving a visible mutation frequency of just over 1% of transgenics. This is 10-fold less than the mutation frequency hoped for from random integration, but the ability to screen for expression and easily clone the associated gene makes this a potentially attractive proposition. Also, improved vector designs can be envisaged that would increase the efficiency of activation. However, there is frequently mosaic integration and expression in microinjected fish, and it is not yet clear how often a vector integration that results in a pattern of expression in the microinjected progeny will be transmitted through the germ line.

From gene to phenotype: targeted mutagenesis

Whatever the mutagen, any kind of a random mutational screen can proceed at least 10 times faster in zebrafish than in mice. When this factor is combined with the ease of characterizing embryonic lethals, it is clear that a saturation screen for embryonically acting mutations in vertebrates can only be attempted in fish. Any such mutagenic strategy is still, however, a major undertaking, and two questions still remain. First, will all genes important for development be identified by this route? Second, is this the only way to identify the genes vital for the morphogenesis of the vertebrate embryo?

On the first point, it is clear that not every step in the molecular pathways underlying developmental events can necessarily be identified by random mutagenesis. Complete or partial redundancy of gene function may lead to failure to identify a mutant phenotype for a developmentally important gene. The degree of redundancy in the genome can be estimated by comparing the estimates for the number of transcriptional units with the number of genes estimated from visible mutation frequency. In mammals, these estimates differ by a factor of ~10, with the current estimate of transcription units being ~100,000 (Ohno 1986; Brown and Bird 1986), versus 10,000 vital genes estimated by mutation frequency [Dove 1987]. The high degree of redundancy within the genome implied by this difference may be more apparent than real, largely reflecting our inability to recognize subtle phenotypes.

On the second point, one could argue that molecular cloning strategies currently in place and planned for the future will isolate many, if not all, of the genes vital for vertebrate development, without the need to resort to random mutagenesis. Much attention has focused on molecular characterization of genes whose nature, expression pattern, and cross-species conservation indicate possible roles in the critical events in vertebrate embryonic development, such as gastrulation, neural induction, segmentation, and anterior–posterior specification. Though identified by various means, often including homology to Drosophila developmental genes [Kessel and Gruss 1990], such genes have tended to fall into general categories of genes involved in intercellular signaling or genes acting as nuclear transcription factors. The rapid advances in our understanding of the biochemistry of signal transduction and transcriptional regulation and in techniques to identify upstream and downstream genes in such pathways will provide important insights into genetic hierarchies of development. In addition to these directed approaches to identifying developmentally regulated genes, the semidirected gene-trap approach and approaches involving screening embryonic cDNAs for pattern of expression at critical stages will provide an immense storehouse of potentially important developmental genes over the next few years. When put in the context of our rapidly increasing understanding of the morphogenesis of the vertebrate embryo and the delineation of important developmental domains, it should be possible to use all of this information to deduce the general genetic program of vertebrate development.

In this molecule-driven approach to development, mutagenesis would still play a critical role in confirming the function of those genes thought to be most critical for a particular developmental process. Mutagenesis in this case would be targeted to the gene of interest. Dominant gain-of-function mutations can be made by introducing constructs that overexpress or ectopically express the gene of interest. For example, the phenotypes of both frogs [Harvey and Melton 1988, Ruiz i Altaba and Melton 1989] and mice [Kessel et al. 1990] misexpressing members of the Hox gene family have provided strong support for a role for these genes in anterior–posterior patterning. Dominant-negative mutations that inactivate the host gene can also be introduced exogenously. The lack of posterior structures in Xenopus embryos expressing a dominant-negative mutation of the fibroblast growth factor receptor [Amaya et al. 1991] points to the feasibility of using this approach to block specific developmental events.

Technical developments in the mouse in the past 4 years have made it possible also to target recessive mutations to specific genes and transmit such mutations to the germ line (Capecci 1989; Rossant and Joyner 1989; Rossant 1991). Targeted mutagenesis in the mouse is achieved by homologous recombination into the gene of choice in ES cells in vitro. Cells heterozygous for the targeted mutation can then be returned to the environment of the early embryo and will contribute to all tissues of the chimeraic mouse, including the germ line (Gossler et al. 1986; Robertson et al. 1986). A mutation generated in culture can thus be transmitted into the mouse gene pool. Targeted mutations have already been reported in several genes whose products have been postulated by various criteria to be important for patterning in the early embryo. These phenotypes have all been
informative. Already the phenotypes of presumed null mutations in the Wnt-1 [McMahon and Bradley 1990; Thomas and Capocchi 1990] and Hox-1.5 and Hox-1.6 genes [Chisaka and Capocchi 1991; Lufkin et al. 1991] have helped elucidate their roles in development of spatial domains in the early neural tube and neural crest. A mutation in the homeo box of the engrailed-like gene, En-2 [Joyner et al. 1991], led to a defect in postnatal patterning of the cerebellum, rather than a defect in the embryonic development of the engrailed-expressing domain of neural tube. This and the postnatal defects seen following mutation of other embryonically expressed genes indicate that partial redundancy of gene function may be widespread in mammals [Schwartzberg et al. 1991; Soriano et al. 1991; Tybulewicz et al. 1991]. These results simply reinforce the conclusion already drawn from the discrepancy between gene number estimates based on saturation mutagenesis and molecular parameters.

To date, the majority of putative vertebrate developmental control genes have been cloned in mice, and the strategies for targeted mutagenesis, especially by homologous recombination, are most highly developed in this species too. However, it seems extremely likely that most of the genes identified by various means as potential developmental regulators in mouse or Xenopus will also be involved in zebrafish development. In the few instances to date where such homologs have been sought in zebrafish, they have been found, and their expression patterns also show conservation [e.g., En [Eng] (Hatta et al. 1991a), Hox [Molven et al. 1990, Njolstad et al. 1990] and Wnt genes [Molven et al. 1991]]. Patterns of expression of some genes in zebrafish may actually reveal additional information about the evolution of vertebrate developmental compartments, as shown by a recent study of Pax gene expression [Krauss et al. 1991]. There would be enormous value in obtaining mutations in the homologous genes in two different vertebrate species, because conservation of phenotype would strengthen the interpretation of the function of the gene and differences would illuminate evolutionary changes.

Is targeted mutagenesis a reasonable goal in zebrafish? Introduction of dominant-acting constructs by transgenic techniques is certainly possible. Producing recessive mutations by homologous recombination requires some technical advances. There is every reason to suppose that homologous recombination will occur in the fish and so the problem becomes one of how to introduce the targeting vector and recover homologous recombinants at high frequency. Clearly, zebrafish cell lines equivalent to ES cells would be ideal, and several laboratories are trying to generate these and show that they can be reintroduced into the embryo and form chimeras. So far, germ-line chimeras have been obtained by transplanting primary embryonic cells from pigmented to albino embryos [S. Lin, W. Long, J. Chen, and N. Hopkins, unpubl.], but it remains to be seen whether cultured cells will also be able to reenter the germ line. In the absence of ES cells, it may be possible to microinject targeting vectors into eggs directly, as large numbers of eggs can be injected and the targeting frequencies for some genes in mice can be on the order of one in 50–500 of cells that take up DNA. If one could use a positive–negative selection system, such as that devised by Capocchi for mammalian cells [Mansour et al. 1988], directly on the injected embryos, targeting frequencies could be even higher.

The future path!

We have seen that strategies already exist to perform saturation mutagenesis in zebrafish, and several laboratories are embarking on large-scale chemical mutagenic screens in fish. For the reasons we have discussed, this approach has its strong proponents. The current lack of tools to assist in molecular cloning of such mutations is perhaps the strongest argument against the approach but need not be a problem in the long term. The attraction of potentially finding all of the new phenotypes that affect development provides a strong incentive to pursuing this approach. Drosophila genetics has made us dissatisfied with anything short of all of the genes that control developmental processes, and chemical mutagenesis is the fastest route to this goal right now. Insertional mutagenic strategies developed in mice could be applied on a larger scale in fish and could provide a very profitable route to identifying molecularly tagged mutations. Promoter- or gene-trap vectors are particularly attractive in this regard because they allow access directly to the host gene. However, these vectors also allow a number of possible routes for prescreening for integrations relevant to development prior to germ-line analysis. As a result, the number of animals needed for analysis of mutant phenotypes is much reduced, making it applicable to mice as well as fish. Finally, the development of techniques for targeted mutagenesis, combined with the rapid advance in identifying developmentally important genes by molecular cloning, has suggested that the classical approach of pursuing genetic analysis of development from phenotype to gene may be overtaken in vertebrates by the reverse route from gene to phenotype. This route could be pursued in either zebrafish or mice, although techniques are currently more advanced in mice.

It seems likely that mutagenesis in both mice and zebrafish will proceed in various ways over the next few years. Although one can argue the pros and cons of the various strategies, the argument is not about whether zebrafish are better than mice, but rather about how to make the most out of the availability of two systems in which to study the genetics of vertebrate development. There is no real reason to suppose that the fundamental logic of vertebrate development will be revealed more readily by mutations in zebrafish than in mice; there are indications that mutations in homologous genes may have very similar phenotypes in the two species. However, there is no question that the embryology of the fish has distinct advantages for analyzing phenotypes. To be able to follow the development of mutant embryos in real time under the microscope instead of working backwards through fetal stages dissected from the mother is
an immense saving in initial analysis. The elegant cell lineage work of Kimmel and co-workers provides the next level of analysis in which mutant and normal cell lineages can be compared [Kimmel 1989, Warga and Kimmel 1990]. Finally, the ability to perform cell transplantation studies at a variety of stages of development allows the lineage autonomy of a mutant effect to be determined [Hatta et al. 1991b, Ho and Kane 1991]. Similar studies can and have been performed on mouse embryos [Conlon et al. 1991; Rashbass et al. 1991], but the inaccessibility of the postimplantation embryo, except to short-term culture, has limited the mosaic approach to date to preimplantation chimeras.

Developmental biology thrives on comparative studies, where similarities suggest conserved mechanisms and differences illuminate the progress of evolution. Clearly, the task over the next few years will be to integrate the information derived from embryological, molecular, and genetic manipulations in different vertebrate species to define the underlying genetic program of vertebrate development.

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Mutational analysis of vertebrate development

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GENES & DEVELOPMENT 11
Rossant and Hopkins

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