Cloning zebrafish developmental mutations

Now that so many mutations in developmental loci have been identified working out the corresponding gene sequences has become a major focus of zebrafish research. Geneticists often refer to the process of going from mutation to sequence as 'cloning' a gene. Cloning allows related genes in zebrafish or other species to be identified and the biochemical function of the gene's protein product to be studied. It also makes it possible to look at the gene's expression during development and analyse its regulation and the effects of misexpression using transgenic fish.

The candidate gene approach

It is often possible to clone genes by combining phenotypic analysis of the genes function with information about the approximate genetic map location. This approach to cloning is sometimes referred to as the candidate gene approach to differentiate it from positional cloning, which relies entirely on determining the location of the gene.

Cloning by the candidate gene approach begins with an in depth analysis of phenotype in order to define the gene's biological function (e.g for mutations which result in fish that lack all pigment, the gene is likely to code for an enzyme involved in melanin biosynthesis) .

The next step is to identify zebrafish sequences which could plausible play such a role (e.g. zebrafish homologues of mouse pigment producing enzymes such as tyrosinase or dopachrome tautomerase). Candidate sequences and the mutation are genetically mapped so that candidates, which are not closely linked to the mutation can be eliminated.

Candidate gene sequences which are linked to the original mutation are subjected to various further tests to confirm that they represent the locus which is mutated to give the observed phenotype. One such test involves analyzing the expression of the candidate gene in wild type and in mutant fish. Another is to compare sequences from the candidate gene amplified from mutant fish with the wild type sequence to confirm that mutations which could affect function can be detected for <u>all</u> mutant alleles. This test is especially powerful whn several mutant alleles exist at the locus being cloned. Lastly wild type mRNA or protein can be injected into mutant fish to determine whether it can restore the wild type phenotype (functional complementation).

Examples

<u>Cloning *no tail*</u>
No tail (ntl) was the first zebrafish gene to be cloned (in 1994). Two alleles of *ntl* were isolated in F1 screens of haploid embryos in the early eighties.

On detailed examination these mutants proved to have a defect in mesoderm development, particularly affecting the posterior somites and the notochord.

The phenotype was very similar to that of one of the classical mouse mutants, *T-brachyury*.

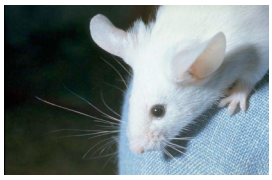
T/T homozygous mice show a failure to make posterior somites and no differentiation of the notochord. It was postulated that zebrafish *ntl* and mouse *T* were homologous.

Mouse T was cloned in 1990 and a zebrafish gene (*Zf-T*) with a 69.7% identical amino acid sequnce and similar expression pattern was isolated in 1992. Linkage analysis showed that Zf-T and *ntl* were closely linked on chromosome 19.

Antibody staining of *ntl160/ntl160* and *ntl195/ntl195* embryos showed no detectable Zf-T protein Sequence analysis of the *ntl*160 allele revealed a frame shift mutation in exon six and a 1544 insertion in exon 2 of *ntl*195. Both mutations would be predicted to lead to a truncated proteins being produced confirming that *ntl* encodes the zebrafish homologue of T.

<u>Cloning nacre</u>
A single *nacre (nac)* mutation was isolated in a screen for neural crest mutants in 1999.

Homozygous *nac* mutants grew to adulthood but lacked pigment cells (melanocytes) in the body although they had the normal amount of dark pigment (melanin) in the eyes.



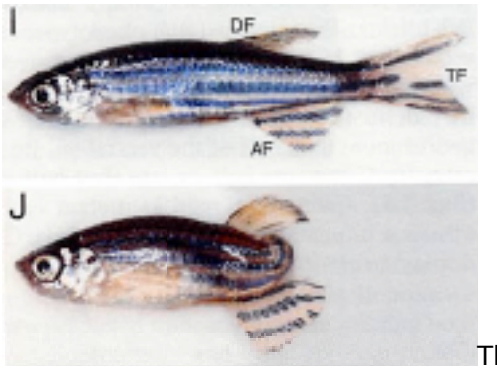
This phenotype is similar to that of several mouse black-eyed white mutant strains. These included the cloned genes *Steel*, *c-kit* and *Mitf*.

Both *c-kit* and *Mitf* were known to act cell autonomously, that is wild type cells injected into mutant embryos could give rise to pigmented melanocytes. Wild type cells injected into *Steel* embryos die because *Steel* encodes a secreted survival factor. Wild type cells injected into *nac/nac* embryos were able to produce melanocytes suggesting that *nac* encoded the zebrfish homologue of a cell autonomous factor like c-kit or Mitf and not a secreted factor like the *Steel* gene product.

The zebrafish homologue of *Mitf* maps close to nac on chromosome 6 making it a good candidate for the *nac* gene. *Nacre* mRNA is expresses in pigment cells in wild type and mutant embryos. This is the type of expression pattern you would expect from the cell autonomous phenotype of the gene. The fact that *nac* mRNA is still expressed in the mutant is consistent with the later finding that the mutant carries a nonsense mutation that would not affect mRNA expression but would lead to a truncated protein being produced. Finally it was shown that injection of wild type nacre DNA into *nac/nac* mutant embryos could rescue the phenotype and result in at least some pigmented melanocytes being produced.

Cloning *minifin*

Adult homozygous *mini fin* fish show partial or full loss of the tail fin.



The developmental origin of this phenotype is a loss of ventral pattern elements from the tail bud in the embryo. Genetic regulation of dorso-ventral patterning is highly conserved (for those of you taking BIOL2010 this applies only to the zygotic genes involved - genes like *Toll* and *Dl* are maternal effect genes and part of an upstream pathway which is not used in vertebrates). In *Drosophila* zygotic mutants with an equivalent D-V patterning phenotype to *mfn* include *dpp* and *tolloid*. Zebrafish homologues of these genes are therefore potential candidates for *mfn*.

Two *dpp* related zebrafish genes have been isolated and mapped to chromosomes 11 and 20. A single *tolloid* related gene has been mapped to chromosome 1. *Mfn* also shows linkage with DNA markers on chromosome 1. This eliminated the *dpp* related genes as candidates.

Tolloid related cDNA clones from all 5 alleles of *mfn* were sequenced and compared with the wild type zebrafish *tolloid* sequence. All five alleles had mutations which were likely to produce non-functional tolloid proteins. Three were nonsense mutations producing premature stop codons and two were misense mutations in highly conserved amino-acid residues. *Mini fin* therefore encodes the zebrafish homologue of the *Drosophila* D-V patterning gene *tolloid*.

References

Linkage maps

Introduction to Genetic Analysis Chapter 4 pp 131-133

http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=iga.section.899

Mapping with Molecular markers

Introduction to Genetic Analysis Chapter 4 pp 146-153

http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=iga.section.919