Vertebrate genome evolution and the zebrafish gene map

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In chordate phylogeny, changes in the nervous system, jaws, and appendages transformed meek filter feeders into fearsome predators [1]. Gene duplication is thought to promote such innovation[2]. Vertebrate ancestors probably had single copies of genes now found in multiple copies in vertebrates[3] and gene maps suggest that this occurred by polyploidization[2–7]. It has been suggested that one genome duplication event occurred before, and one after the divergence of ray-finned and lobe-finned fishes[5]. Holland *et al.*, however, have argued that because various vertebrates have several *HOX* clusters, two rounds of duplication occurred before the origin of jawed fishes[3]. Such gene-number data, however, do not distinguish between tandem duplications and polyploidization events, nor whether independent duplications occurred in different lineages. To investigate these matters, we mapped 144 zebrafish genes and compared the resulting map with mammalian maps. Comparison revealed large conserved chromosome segments. Because duplicated chromosome segments in zebrafish often correspond with specific chromosome segments in mammals, it is likely that two polyploidization events occurred prior to the divergence of fish and mammal lineages. This zebrafish gene map will facilitate molecular identification of mutated zebrafish genes, which can suggest functions for human genes known only by sequence.

We localized genes in zebrafish with respect to anonymous DNA polymorphisms[8–10] (Fig. 1), and then compared the arrangement of zebrafish genes with their mammalian orthologues. (Orthologues are genetic elements in two species that are inherited directly from a single corresponding element in the last common ancestor of the two species. In the absence of gene duplication after species divergence, orthologues share a terminal branch on a phylogenetic tree.) Analysis revealed that a group of genes that is syntenic (on a single chromosome) in humans tend to have orthologues that are syntenic in zebrafish. For example, maps showed that nine genes that reside on zebrafish Linkage Group (LG) 9, have apparent orthologues, and a tenth gene, at least a close homologue on the long arm of *Homo sapiens* chromosome 2 (Hsa2q) and its mouse counterpart (Fig. 2*a*). This set of Hsa2q genes, which is spread over approximately 5% of the human genome, is on two separate chromosomes in mouse. Additional examples of conserved syntenies appear throughout the zebrafish genome (Fig. 2, legend). Our long-range zebrafish mapping results extend earlier results with swordtails[6], and complement sequence analysis of the pufferfish genome, which shows substantial local sharing of neighbouring genes with mammalian genomes[11].

Because fish and mammals share chromosome segments, we can ask whether polyploidization events that are thought to have occurred during vertebrate evolution took place before or after the divergence of ray-finned and lobe-finned fish. If two lineages diverge after disomic chromosome segregation in meiosis has been re-established following a polyploidization event, then a chromosome segment in one lineage would correspond with a single orthologous chromosome segment in the sister lineages (Fig. 3*a*). Alternatively, if two lineages diverge and become chromosomally different before polyploidization events occur independently in both lineages (Fig. 3*b*), then unique orthologous chromosome segments would not be apparent among paralogous chromosomes in the sister lineages—instead, two paralogous chromosome segments in one lineage would be equally related to two paralogous segments in the sister lineage. (Paralogues are genetic elements within a species that are derived from a single ancestral element.)

Portions of the four chromosomes that contain *HOX* clusters in mammals are paralogues presumably derived from chromosome duplication events[3–5,7]. We found that four zebrafish *hox* cluster genes mapped on four different chromosomes (Figs 1,2) and that for at least three of these, the zebrafish chromosome segments are orthologous to individual *HOX*-bearing segments in mammals. Gene orthologies in these cases were inferred from gene trees in which the zebrafish paralogue branches with the mammalian gene of the same name rather than with other zebrafish paralogues (see for references http://zfish.uoregon.edu/). Besides the conserved synteny that includes the *hoxd/HOXD* clusters on LG9 and Hsa2, (Fig. 2A), *hoxc5* and three other genes on LG23 (*pouc*, *rarg*, and *wnt1*) have apparent orthologues on Hsa12 or its mouse counterpart Mmu15 (*HOXC5, Emb, RARG*, and *WNT1*; Fig. 2*b*), and *hoxb5* and *hoxb6* on LG3 are syntenic with four genes including *pyy, rara2b, tra1*, and *hbaa1* with apparent orthologues on Hsa17q or its mouse orthologue Mmu11 (*PYY, RARA, THRA1*, and *Hba*; Fig. 2*c*). The orthology of the fourth *hox*-bearing chromosome segment is less obvious. LG12 contains the locus *hoxa1* assigned as a member of the *hoxa* cluster on the basis of its expression pattern[12], and paralogues of other genes found syntenic with

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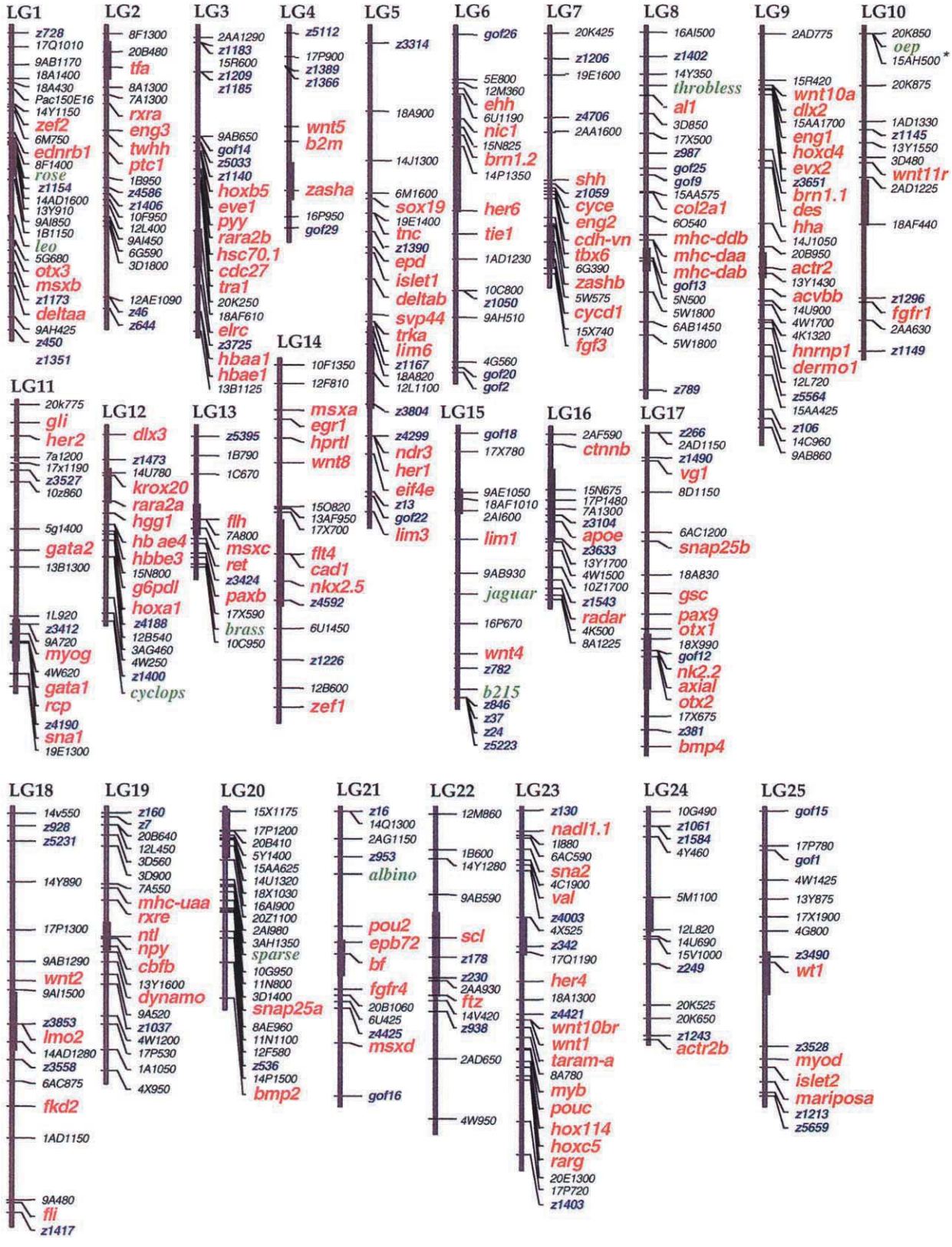

Fig. 1 A gene map for the zebrafish. The locations of 144 cloned genes are represented in a framework of 202 RAPD markers[8,9], 101 microsatellite markers[10], and 15 mutations[8]. Cloned genes are written in large roman script, mutations in italics, microsatellite markers begin with 'z' or 'gof' (ref. 10), and RAPD markers begin with a number and capital letters that refer to the manufacturer's primer name followed by the size of the band in base pairs. Because fewer than 96 individuals were scored for each locus, positions should be viewed as approximate. The mutation b215 is a gamma-ray induced mendelian mutation that displays reduced development of the ventral fin; we localized it to the lower end of LG15 (W.S.T., E.S.E., M. Halpern and C. Walker, unpublished data).

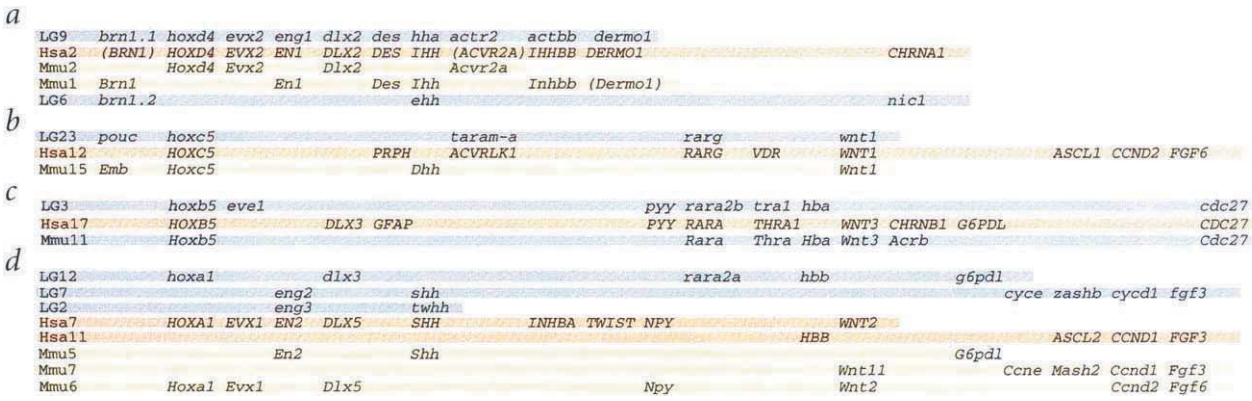

Fig. 2 Conservation of syntenies and paralogies among some zebrafish (LG, blue), human (Hsa, red) and mouse (Mmu, yellow) chromosomes. To ease comparisons, members of multiple gene families are displayed in columns; this necessitates the arbitrary ordering of genes along a chromosome, and so horizontal bars constitute a list of chromosome contents, not map order. The locus order is correct only for the top line. ***a***, Conserved synteny of genes on Hsa2q. The *evx2* gene is localized based on our isolation from a PAC library (L.Z. and C. Amemiya, unpublished data) of a clone containing *hoxd4* which also holds *evx2*. ***b***, Conserved syntenies among *hoxc* containing chromosomes. ***c***, Conserved syntenies among *hoxb* containing chromosomes. ***d***, Conserved syntenies in *hoxa*-containing chromosomes. Orthologues, as judged by sequence comparisons in the literature cited at http://zfish.uoregon.edu, are underlined: *hoxd4/HOXD4; evx2/EVX2; eng1/EN1; dlx2/DLX2; des/DES; hha/IHH; actr2/Acvr2a; actbb/INHBB; dermo1/DERMO1*; both *brn1.1* and *brn1.2* are orthologues of *Brn1* due to a duplication in the zebrafish lineage; *nic1/Chrna1; pouc/Emb; hoxc5/HOXC5; rarg/RARG; wnt1/WNT1; hoxb5/HOXB5; pyy/PYY; rara2a* and *rara2b* both behave as orthologues of *RARA* in a phylogeny due presumably to a duplication in the zebrafish lineage; *tra1/THRA1; hbaa1/Hba; cdc27/CDC27; hoxa1/HOXA1; eng2/EN2; shh* and *twhh* appear to be orthologues of *SHH* due to a duplication in the zebrafish lineage; *cyce/Ccne; cycd1/CCND1; fgf3:FGF3; npy/NPY; hbbe3/Hbb; g6pd/G6pdh*. Additional conserved syntenies follow, with orthologues underlined, as judged by sequence comparisons in the literature cited at http://zfish.uoregon.edu: LG2/Hsa9q (*rxra/RXRA, ptc1/PTCH*); LG5/Hsa9q (*tnc/TNC; trka/NTRK2; lim3/LHX3*); LG6/Mmu4 (*tie1/Tie1, her6/Hes3*); LG8/Hsa6p (*col2a1/COL11A2, mhc-ddb/MHC*); LG11/HsaX (*gata1/GATA1, rcp/RCP*), LG13/Hsa10q (*paxb/PAX2, ret/RET*, and from comparative mapping with mouse *msxc/MSX3*), LG14/Hsa5q (*cad1/CDX1, msxa/MSX2, egr1/EGR1, nkx2.5/CDX, flt4/FLT4*), LG17/Hsa14q (*gsc/GSC, pax9/PAX9, nk22/NKX2A, otx2/OTX2, bmp4/BMP4*), LG18/Hsa11 (*lmo2/LMO2, fli/FLI1*), LG19/Hsa6 (*mhc-uaa/MHC, rxre/RXRB, ntl/T*); LG20/Hsa20 (*snap25a/SNAP25, bmp2/BMP2*); LG21/Hsa6p (*bf/BF, pou2/OCT3*); LG21/Hsa5q (*msxd/MSX2, fgfr4/FGFR4*); and LG25/Hsa11p (*wt1/WT1, myod/MYOD1*).

HOX clusters in mammals (*dlx* and *rar* genes), but LG12 contains *dlx3* and *rara2a*, whose apparent mammalian orthologues *DLX3* (ref. 13) and *RARA* are syntenic with mammalian *HOXB* rather than *HOXA* (Fig. 2*d*). Although it is possible that gene conversion between paralogues or translocations between paralogous chromosomes (homeologues) could have resulted in the linkage of the gene called *hoxa1* to the 'wrong' *dlx* and *rar* genes, that mapping anomaly coupled with the recent discovery that zebrafish has three *hox* genes that do not appear to be closely linked to the four *hox* cluster genes we mapped[14] suggests that the organization of *hox* clusters may differ between zebrafish and mammals. Thus, while more data are necessary to understand the *hoxa* cluster, the chromosome segments that contain *hoxd, hoxc* and *hoxb* cluster genes have clear orthologues in mammalian genomes, consistent with the hypothesis that these chromosomes were duplicated prior to the divergence of ray-finned and lobe-finned fish approximately 420 million years ago[15].

The two other main paralogy segments recognized in mammalian genomes, the 'Hsa5' paralogy group containing parts of Hsa5, Hsa4, and Hsa10 (*5*) and the 'Hsa6p' paralogy group containing parts of Hsa1, 6, 9, and 19 (refs 16,17) also have apparent orthologues in zebrafish (Fig. 2, legend). The identification of

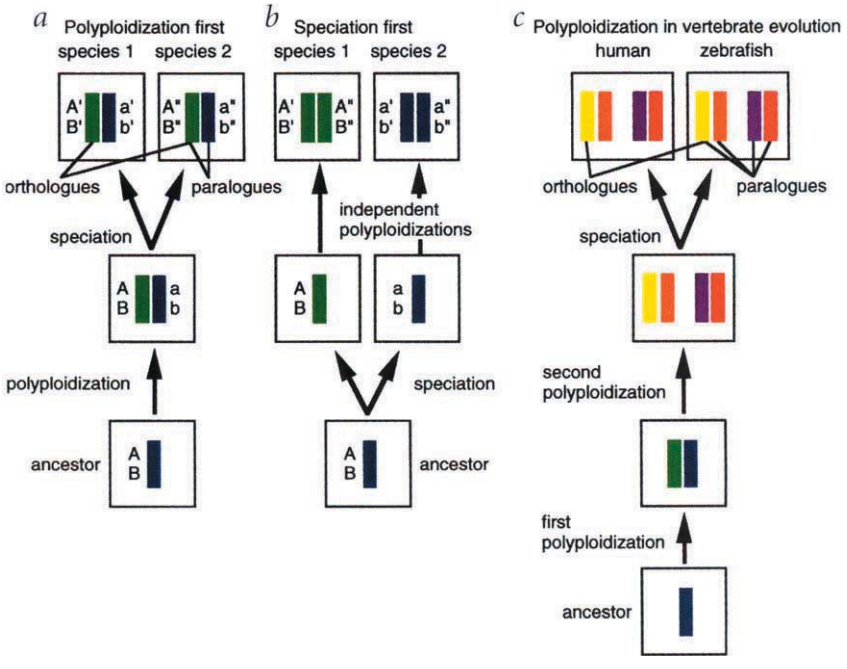

Fig. 3 Models for the evolution of paralogous chromosomes in zebrafish and mammals. ***a***, Polyploidization before speciation. Assume that in an ancestor to modern species, a chromosome containing genes *A* and *B* becomes duplicated in a polyploidization event to make two chromosomes. Subsequent mutations or rearrangements can cause the two duplicate copies to become different from each other, as indicated by the shading. A speciation event will now produce two species in which a chromosome in species 1 is more closely related to its orthologue in species 2 than it is to its paralogue in species 1. ***b***, Speciation before polyploidization. In contrast, if speciation and chromosome differentiation occur before independent polyploidization events in both lineages, then orthologues will not be unique. Instead, two paralogous chromosome segments in species 1 will be more closely related to each other than to either paralogous chromosome in species 2. ***c***, Polyploidization in vertebrate evolution. Comparative mapping analysis reported here supports the model that there were two polyploidization events in a common ancestor of zebrafish and mammals, resulting in four paralogous copies of each chromosome segment in each lineage. Each box represents a portion of a cell nucleus containing one chromosome or its descendants.

orthologous chromosome segments for at least three chromosome paralogy groups in zebrafish and mammals supports the hypothesis that these chromosome segments were duplicated prior to the divergence of zebrafish and mammalian lineages. The presence of more than two copies of each paralogous chromosome segment suggests at least two rounds of duplication (Fig. 3*c*), which would have occurred after the divergence of cephalochordates and craniate chordates, but prior to the divergence of ray-finned and lobe-finned fishes. Thus, these polyploidization events may have occurred early enough to have been associated with the evolution of vertebrate innovations.

Zebrafish often have more members of multigene families than mammals. Assuming that most gene family members have been described in the intensely studied human and mouse genomes, the 'extra' genes in zebrafish could have arisen from either: (i) a greater propensity for tandem gene duplications in the fish lineage; (ii) less loss of paralogous gene copies in fish; or (iii) additional independent chromosome duplication or tetraploidization in fish. Mapping data give no evidence for the clustering of 'extra' genes, thus ruling out tandem duplication as a general mechanism for 'extra' genes in zebrafish. On the other hand, mammals appear to have occasionally lost a paralogue that zebrafish have retained. For example, the sequence of *hox114* (ref. 18) places it in the *hox3* paralogy group and its map location shows that it is near *hoxc5*, suggesting that it is *hoxc3* which has no mammalian orthologue; *HOXC3* may have been present in the common ancestor but lost in the mammalian lineage.

Although some of the 'extra' zebrafish genes can be accounted for if mammals retained fewer paralogues than zebrafish, other 'extra' genes appear to reside on 'extra' chromosome segments. For example, *eng2* and *eng3* are both related in sequence to human *EN2* (ref. 19), and *shh* and *twhh* are about equally related to human *SHH* (ref. 20) suggesting that these genes may have arisen by duplication after the divergence of ray-finned and lobe-finned fishes. Our finding that *eng2* and *shh* are syntenic on LG7 and *eng3* and *twhh* are syntenic on LG2 is consistent with these chromosome segments being duplicated in the fish lineage after the divergence of ray-finned and lobe-finned fish (Fig. 2*d*). In another example, *brn1.1* and *brn1.2* are about equally related to mouse *Brn1* (ref. 21), and they map to two separate chromosomes (LG9 and LG6) that both appear to be partial copies of human chromosome 2q, each with a *brn1* gene and a hedgehog gene and apparent orthologues of other Hsa2q genes (Fig. 2*a*). Finally, portions of LG12 and LG3 containing *rara2a hbae4* and *rara2b hbae1* appear to be duplicates of Mmu11 which contains *Rara* and *Hba*. These examples, and the 'extra' *hox* genes discovered by Prince *et al.*[14], are consistent with the hypothesis that there has been independent chromosome duplication, perhaps even an extra round of tetraploidization, in the zebrafish lineage. Further genomic analysis and mapping of zebrafish *hox* clusters and syntenic loci will determine whether the 'extra' genes are due to a unique evolutionary event as suggested[22], or to tetraploidization in the zebrafish lineage.

Our map (Fig. 1) integrates two maps based on anonymous markers, one utilizing primarily RAPD markers [8,9] and the other based on simple sequence length polymorphisms (SSLPs)[10]. RAPDs provide an inexpensive source of markers for the rapid construction of a genome map and are highly useful for developing a local map around mutations[23]. The utility of RAPDs is diminished, however, because they are highly strain-specific, and because the fully dominant and fully recessive alleles of most RAPD loci work better in haploid than in diploid mapping crosses. On the other hand, SSLPs are expensive to develop, but have the important utility of being informative in most zebrafish mapping crosses[10]. Moreover, because most CA-repeat alleles are co-dominant, they are fully informative in diploid crosses. The map developed in the current work combines the significant advantages of both marker types, and furthermore, adds cloned genes, a functional part of the genome.

The extensive conservation of syntenies between zebrafish and mammals reported here permits the use of comparative gene mapping to provide candidates for genes disrupted by the magnificent recent collections of zebrafish mutations[24–28]. If a zebrafish mutation maps to a conserved chromosome segment, human genes in this segment may become candidates for the zebrafish mutation. Reciprocally, the phenotype of a zebrafish mutation, which can be understood with exquisite cellular precision, can suggest functions for the human gene. In fact, the molecular and phenotypic characterization of genes disrupted by zebrafish mutations may provide an efficient way of assigning functions to essential orthologous human genes which are known only by sequence from the rapidly advancing Human Genome Project. In this way, the investigation of zebrafish molecular and formal genetics can contribute to functional human genomics.

Methods

Sequences of zebrafish genes were obtained from GenBank or other sources, and primers were designed to amplify either 3′ untranslated regions or introns. Polymorphisms were identified in amplified fragments by variation in fragment size, restriction enzyme recognition site, or as single strand conformation polymorphism[29]. Primer sequences, polymorphisms, GenBank accession numbers, and literature citing the genes mapped are displayed at ZFIN, the zebrafish database http://zfish.uoregon.edu/. Primers for microsatellite markers[10] were purchased from Research Genetics. All RAPDs, SSLPs, and 139 of the 144 cloned genes were scored on a haploid mapping panel previously described[9]; the other five genes were mapped on an earlier haploid panel [8] due to the lack of polymorphisms in the primary panel. Maps were constructed using Map Manager (K. Manley and R. Cudmore, http://mcbio.med.buffalo.edu/mapmgr.html) using the 'rearrange' function repeated five times at 99.99% limit, and the consensus map was exported into MapMaker for drawing the maps of each linkage group[30]. The locations of mammalian gene loci were taken from Mouse Genome Database http://www.informatics.jax.org/; Online Mendelian Inheritance in Man http://gdbwww.gdb.org/omim/docs/omimtop.html; the Genome Database http://gdbwww.gdb.org/gdb/; and The Human Transcript Map http://www.ncbi.nlm.nih.gov/SCIENCE96/. We assume that a shared synteny (a case in which a pair of orthologous genes are on a single chromosome in two different species) is evidence that a chromosome segment has been conserved during evolution. Given that a pair of genes are syntenic in humans, the likelihood that the orthologues of those genes would be syntenic in zebrafish purely by chance is about 1 in 25, the number of pairs of zebrafish chromosomes, with the simplifying assumption that all chromosomes are equally long. Thus, most syntenies shared between zebrafish and mammals should be due to conservation through evolutionary history. Exceptions could occur if two genes were on different chromosomes in a common ancestor but become syntenic by chromosome rearrangements occurring independently in fish and mammalian lineages, or if the genes were syntenic in a common ancestor but became unlinked in one or both lineages followed by the re-establishment of synteny by chromosome translocations. These exceptions seem unlikely to account for many of the shared linkages observed. A zebrafish gene is assumed to be orthologous to a mammalian gene if they appear as sister groups on a dendrogram.

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