Equivalence in the genetic control of hindbrain segmentation in fish and mouse

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SUMMARY

The vertebrate hindbrain is subdivided into a series of rhombomeres whose segmental organization serves to pattern the architecture and innervation of the developing head. The zebrafish gene valentino is required cell-autonomously in the development of rhombomeres 5 and 6, and valentino mutants lack visible hindbrain segmentation caudal to the r3/4 boundary (Moens, C. B., Yan, Y.-L., Appel, B., Force, A. G., and Kimmel, C. B. (1996) Development 122, 3981-3990). Here we show that valentino is the zebrafish homologue of the mouse segmentation gene kreisler, which encodes a bZip transcription factor. The valentino gene is expressed in a manner consistent with its proposed role in subdividing rhombomeres 5 and 6 from their common precursor ‘proto-segment’ in the presumptive hindbrain, a process that we also demonstrate is reflected in the normal order of appearance of rhombomere boundaries. As well as having similar phenotypes with respect to visible hindbrain segmentation and patterns of marker gene expression, valentino and kreisler mutants have similar pharyngeal arch and inner ear defects, consistent with a conserved role for this gene in hindbrain segmentation and in patterning of the head periphery.

Key words: Hindbrain segmentation, Zebrafish, valentino (val), kreisler, Rhombomere

INTRODUCTION

The rhombomeres of the vertebrate hindbrain correspond to segmental patterns of neuronal differentiation, neural crest migration, and gene expression. These patterns arise with a two-segment periodicity, so that pairs of rhombomeres contribute neural crest and motor innervation to individual pharyngeal arches in the head periphery (Lumsden and Keynes, 1989; Lumsden et al., 1991; reviewed in Guthrie, 1995). The zebrafish segmentation gene valentino (val) is required cell-autonomously in the development of the two-rhombomere unit consisting of rhombomeres (r) 5 and 6 (Moens et al., 1996). Homozygous val- embryos lack r5 and r6, but have a region of one rhombomere’s length that lies between, and fails to form boundaries with, r4 and r7. Genetic mosaic analysis has shown that this region, rX, has a distinct identity from any normal rhombomere, because wild-type cells cannot contribute normally to it. Based on these results, we have proposed that rX is a ‘proto-segment’ that corresponds to the common precursor of r5 and r6 and that val function is required for its subdivision and expansion into the definitive rhombomeres (Moens et al., 1996).

The hindbrain abnormality in val- embryos resembles that of the mouse mutant kreisler (kr), in that both mutants lack visible segmentation caudal to the r3-r4 boundary (Moens et al., 1996; Deol, 1964). In kr mutants this results in a disorganized and cystic inner ear, which in turn causes deafness and the circling behaviour for which the mutant was named (Hertwig, 1942, 1944; Deol, 1964). Based on gene expression patterns and neuroanatomy, the primary defect in kr- embryos has been proposed to be either the respecification of the presumptive r5 and r6 territories towards r4 identity (McKay et al., 1994) or the loss of r5 and part of r6 (Cordes and Barsh, 1994). kr encodes a Maf-related bZip transcription factor, Krm1 expressed in r5 and r6 (Cordes and Barsh, 1994) and, consistent with an early role in the specification of rhombomere identity, kr has been shown directly to regulate the segment-restricted pattern of hoxb3 expression observed in r5 in the mouse (Manzanares et al., 1997).

The similarities between the val- phenotype in the zebrafish and the kr- phenotype in the mouse prompted us to test whether val is the zebrafish homologue of kr. Here we describe the cloning of the zebrafish homologue of kr, and show that it is linked to val on the zebrafish genetic map and is mutated in three val- alleles. Expression of this gene in the zebrafish corresponds temporally and spatially with the requirement for val function in the presumptive r5 and r6 as determined by genetic mosaic analysis. We demonstrate that the similarities between the kr- and val- phenotypes in the mouse and fish, respectively, extend beyond a superficial one in the sculpting...
of the hindbrain, and reflect a highly conserved role for this gene in the early events in hindbrain segmentation and segment specification. Thus val and kr mutants have similar homeotic transformations of pharyngeal arch-derived cartilages and similar inner ear defects which manifest themselves in similar behavioural traits. Differences in the interpretations of the kr− phenotype can be reconciled by proposing that val/kr functions to subdivide the common precursor of r5 and r6 into its constituent rhombomeres. Finally, we provide morphological evidence for the existence of such an r5-6 proto-segment in the zebrafish embryo.

MATERIALS AND METHODS

Cloning

Primers corresponding to nucleotides 745-609 and 1153-1137 of the mouse Krml1 generate a PCR probe which was used to screen 1.5 x10⁶ pfu each from neurula and adult zebrafish libraries. Hybridizations were performed at 37°C; washes were performed at 55°C in 1× SSC, 0.1% SDS. Several partially overlapping val cDNA clones were isolated. 2 from the neurula library, and 4 from the adult library. The GenBank accession number for val is AF006641.

Mapping

To map val, haploid embryos were produced from val⁰³⁷/val⁰ females, which were themselves the progeny of a cross between polymorphic strains *AB (val⁰³⁷/val⁰*) and SJD (val⁰/val⁰*). Genomic DNA was prepared from individual haploid embryos that had been sorted into val⁰ and val− classes following RNA in situ hybridization with krox20 (Moens et al., 1996). We used previously described methods (Postlethwait et al., 1994) to identify a random amplified polymorphic (RAPD) marker, AC6590, linked to val (14 recombinants in 780 haploid individuals tested, or 1.8 cm). We identified snat2 as being a yet more closely linked marker by testing linkage of several other previously mapped markers on the same val mapping cross. A snat2 polymorphism was identified by HhaI digestion of a PCR product generated with the primers 5′-CACTCCG-AGGTGAAGAAGTACC-3′ and 5′-GTGGAA TCAAAACAGGCA-HhaI-3′. To map the zebrafish kr homologue, the primers 5′-GCTGTTGAGAAGTACC-3′ and 5′-GTGGAA TCAAAACAGGCA-3′ were used to amplify a 490 bp fragment of the zebrafish kr 3′UTR from genomic DNA. We identified an RsaI polymorphism in this fragment that was segregating in the linkage map cross that was used to consolidate the zebrafish genetic map (Johnston et al., 1996). Map position was determined by comparing the segregation pattern of this polymorphism in the linkage map cross to the segregation pattern of the other markers that had been mapped using the same cross (Johnston et al., 1996).

The C→T mutation we identified at nucleotide 391 in the zebrafish homologue of kr (see below) produces a PvuII polymorphism (CAGCTG→TAGCTG). To test linkage of this polymorphism with val, diploid embryos from a val⁰³⁷/val⁰ intercross were sorted at the 18-somite stage, when the lack of segmentation in the caudal hindbrain of val− embryos is evident. We used the primers 5′-CCGC-GGACACCTTTCGTCCTAC-3′ and 5′-GTGGAA TCAAAACAGGCA-3′ to amplify the 220 bp surrounding nucleotide 391 from genomic DNA prepared from 600 individual embryos, and digested the PCR products with PvuII.

Molecular analysis of val alleles

To determine whether the zebrafish homologue of kr was affected in either of the two γ-ray induced alleles val⁰³⁰¹ and val⁰⁷⁵⁷, three different pairs of PCR primers were used to amplify regions of the zebrafish kr homologue from 4 pools of genomic DNA each made from 10 diploid wild-type or mutant embryos for each allele. To identify a lesion in val⁰³⁷ we amplified the zebrafish kr homologue from genomic DNA prepared from 10 haploid wild-type or 10 haploid mutant embryos. Wild-type and mutant pools made from resulting PCR products were directly sequenced. The 360 nucleotides surrounding the mutation were sequenced 7 times from mutant pools and 3 times from wild-type pools, and the entire gene was sequenced at least twice each from wild-type and mutant pool.

RNA in situ hybridization

RNA in situ hybridizations using a 490 bp digoxigenin-labelled probe from the 3′UTR of the zebrafish kr homologue were performed essentially as described by Oxtoby and Jowett (1993). Double RNA in situ hybridizations also included a fluorescein-labelled krox20 probe (Oxtoby and Jowett, 1993) and were performed as described (Hauptmann and Gerster, 1994), except that we used X-phosphate/5-bromo-4-chloro-3-indolyl-phosphate (BCIP) alone as a substrate for the detection of the digoxigenin-labelled probe, and 4-nitro blue tetrazolium chloride (NBT) and BCIP together as substrates for the detection of the fluorescein-labelled probe.

Mosaic analysis and single cell transplantations

Genetic mosaics (Fig. 3A-C) were made by transplanting cells between wild-type and val⁰³⁷ embryos at the early gastrula stage as previously described (Moens et al., 1996). After RNA in situ hybridization to detect val expression, mosaic embryos were processed for detection of the biotin lineage tracer in the transplanted cells either using the ABC kit (Fig. 3A; Vector Labs) or using an avidin-Texas red conjugate (Fig. 3B,C; Vector Labs). Mauthner cell transplantations were done by identifying the nascent Mauthner cell in a live embryo mounted in agar using a 40x water immersion lens with Nomarski illumination, and picking it up in an oil-controlled pipette with a 10 μm fire-polished opening. Mauthner cells thus manipulated will survive and differentiate after being placed in heterotopic positions in the hindbrain.

Confocal time-lapse imaging

Embryos were treated from shield stage (6 hours (h) after fertilisation) to bud stage (10 h) with a 200 μM solution of N-(4, 4-difluoro-5, 7-dimethyl-4-bora-3a, 4a-diaza-s-indacene-3-pentanoyl) sphingosine (BODIPY® FL C5-ceramide; Molecular Probes) as described (M. Cooper, personal communication). 4-dimensional images of live embryos mounted in 0.5% agar and maintained at 28°C were obtained using a Zeiss 310 upright confocal microscope. Time-lapse recordings were started by the 3-somite stage (11 h) and were stopped at the 12-somite stage (15 h) at which time rhombomeres could be unambiguously identified by their position relative to the otic vesicle.

RESULTS

The zebrafish homologue of kreasler and valentino map together

We cloned the zebrafish kr homologue by low stringency hybridization using the mouse bZip domain (Fig. 1A). The zebrafish kr homologue exhibits 61% identity with the mouse kr gene, Krml1, at the amino acid level, and 85% identity in the 114 amino acid region that includes the bZip domain and a conserved region adjacent to the basic domain that is required for DNA binding specificity of Maf family proteins (Kerppola and Curran, 1994).

Genetic mapping and molecular analysis of val− alleles demonstrates that val is the zebrafish homologue of kr. We mapped the zebrafish kr homologue by identifying an RsaI
polymerism in its 3'UTR that segregates as alleles in the family of haploid embryos that was used to consolidate the genetic map (Johnston et al., 1996). The segregation pattern of this polymorphism was compared to the segregation patterns of several hundred other markers that have been mapped using this family, and this comparison placed the zebrafish kr homologue on linkage group 23 with the closest linked marker being sna2 (5 recombinants among 86 individuals; data not shown). We mapped several other linkage group 23 markers relative to val, and the resulting genetic map of this region is shown (Fig. 1B). The closest linked marker is sna2, which lies 0.5 cM distal to val because only 4 of the 14 recombination events between val and AC6.590 were also between val and sna2 (Fig. 1D). Thus the zebrafish kr homologue and val both map near sna2 on linkage group 23.

The zebrafish homologue of kreisler is mutated in valentino alleles

Analysis of the molecular nature of the lesions in three independent val alleles further supports the hypothesis that val is the zebrafish homologue of kr. valb361 and valb475 are γ-ray induced alleles of val, one of which (valb475) deletes markers both proximal and distal to val on linkage group 23 and hence is likely to be a deficiency covering the entire region (Fig. 1B). Pairs of primers specific for regions of the zebrafish kr gene amplified the expected fragments from wild-type genomic DNA but did not amplify from genomic DNA prepared from homozygous valb361 or valb475 embryos (Fig. 2A). Thus both
of these alleles are deficiencies that include the cloned zebrafish kr homologue.

We also amplified and sequenced the zebrafish kr homologue from genomic DNA prepared from embryos homozygous for valb337, an ENU-induced putative null allele of val. We observed a single C→T transition at nucleotide 391 in the open reading frame, which changes a glutamine codon (CAG) to a stop codon (TAG) and truncates the protein upstream of its DNA binding and leucine zipper domains (Fig. 2B). No other differences were observed between the wild-type and mutant sequences. This C→T transition constitutes molecular evidence for a null phenotype associated with the valb337 allele, as previously argued from genetic analysis (Moens et al., 1996). Furthermore, the mutation produces a PvuII restriction fragment length polymorphism that co-segregated with val in a valb337/val* intercross. We typed a total of 600 embryos (477 wild-type and 123 mutant, representing a total of 1200 meiotic products), and found no recombinants between the C→T mutation in the zebrafish kr homologue and val (Fig. 2C). Hence the molecular change that accounts for the mutant phenotype maps within 0.17 cM of valb337. We conclude that val is the zebrafish kr homologue.

Expression of valentino

Genetic mosaic analysis has shown that val is required cell-autonomously for the development of r5 and r6 since cells from a val− embryo are excluded from r5 and r6 of a wild-type host (Moens et al., 1996). The domain in which a gene is cell-autonomously required in a genetic mosaic is predicted to correspond to where that gene is expressed, and we combined genetic mosaic analysis with RNA in situ hybridization using a probe generated from the 3′-UTR of the zebrafish kr homologue (henceforth referred to as val) to determine whether such a correspondence exists for val. We observe a tight correlation between the domain of val expression and the domain from which val− cells are excluded in a wild-type host embryo (Fig. 3A).

Mosaic analysis has also suggested that the region of the val− hindbrain that lies between r4 and r7 (named rX) is different from any normal rhombomere because wild-type cells cannot contribute normally to it (Moens et al., 1996). In such a genetic mosaic, we observe that val is expressed specifically in those wild-type cells that form abnormal rounded clumps in rX of a val− host (Fig. 3B,C). This correspondence between expression of the wild-type val gene and abnormal cellular behaviour demonstrates that wild-type cells in a val− host autonomously acquire r5 and r6 identity, and that doing so prevents them from intercalating normally with mutant cells in rX. The results strongly suggest that the primary defect in val− embryos is the failure of the common precursor of r5 and r6 (rX in Fig. 3C) to take on distinct r5 and r6 identities.

val is expressed early during the development of the presumptive hindbrain, with a timing relative to the appearance of rhombomere boundaries that is consistent with its postulated role in hindbrain segmentation (see below). A band of val-expressing cells that spans the width of the neural plate in the presumptive hindbrain is first detected at the end of gastrulation (10 h; Kimmel et al., 1995; Fig. 3D), shortly before the onset of krox20 expression in r5 (Oxtoby and Jowett, 1993). The band of val expression overlaps with, but extends caudal to the r5-specific band of krox20 expression, as shown in double RNA in situ hybridizations at this stage (Fig. 3E). val expression persists homogeneously throughout r5 and r6 until the 20 somite stage (19 h) when it begins to be down-regulated first in r5 and then in r6 (Figs 3F–I, 4A). Although val mRNA

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**Fig. 2.** The zebrafish homologue of kreasler is mutated in three alleles of valentino. (A) The zebrafish kr homologue is deleted in γ-ray-induced alleles of val. Gene-specific primers amplified a 490 bp fragment (arrowhead) from 4 pools of genomic DNA each made from 10 wild-type individuals but not from pools from valb337/valb361 (or valb475/valb475; not shown) individuals. Primers specific for an unlinked gene, nk2.2 (Postlethwait et al., personal communication), amplify a 400 bp fragment (arrow) from all individuals. Dots indicate unlinked gene, (arrow), but not in 10 of their mutant siblings. Embryos typed as homologue from genomic DNA prepared from embryos analzyed in this manner. (B) The zebrafish amplify a 400 bp fragment (arrow) from all individuals. Dots indicate unlinked gene, (arrow), but not in 10 of their mutant siblings. Embryos typed as homologue from genomic DNA prepared from embryos analzyed in this manner. (C) The C fi mutated in 600 and 100 bp size standards. (B) The zebrafish amplify a 400 bp fragment (arrow) from all individuals. Dots indicate unlinked gene, (arrow), but not in 10 of their mutant siblings. Embryos typed as homologue from genomic DNA prepared from embryos analzyed in this manner.
expression is initiated normally in r5 and r6 in the point mutant val\textsuperscript{b337}, mutant embryos can be distinguished on the basis of reduced val expression beginning at the 4 somite stage (11.3 h; data not shown). By the 10 somite stage (14 h) r5- and r6-specific val expression is entirely absent from the presumptive r5-6 region of val\textsuperscript{b337} embryos (Fig. 3F,G). A functional val gene product is therefore required, directly or indirectly, for the maintenance of val expression in r5 and r6. val expression is never detected at any stage in homozygous val\textsuperscript{b361} or val\textsuperscript{b475} embryos (data not shown).

Although val is expressed throughout r5 and r6 well before the onset of cranial neural crest migration, it is not expressed in the earliest migrating neural crest cells that contribute to the third and more caudal pharyngeal arches and which do, in contrast, express krox20 (Fig. 3F,H; Oxtoby and Jowett, 1993; Nieto et al., 1995). However, by the 14 somite stage val expression is upregulated in r5 and r6-derived neural crest cells (16 h; Fig. 3I, arrowhead). This differential regulation of val in the hindbrain versus the neural crest is interesting in that it suggests that positional information is not passively carried from the hindbrain to the periphery but can be independently regulated in migratory neural crest.

In wild-type embryos at the 20 somite stage (19 h) val begins to be down-regulated, first in r5 and then in r6 (Fig. 4A). At the same time, expression is first detected in a number of cell types where we have not previously detected any requirement for val function in our analysis of val mutant alleles. Unlike the situation in r5 and r6 where a functional Val protein is required for maintenance of val expression, in each of these later-expressing cell types val expression is detected both in wild-type embryos and in val\textsuperscript{b337} embryos. We observe Val-independent val expression in a bilateral pair of cells in r4 which we have shown to be the Mauthner cells (Fig. 4A,B; see below), in the lens (Fig. 4D,E), in a cluster of cells in the caudal telencephalon (not shown), in the blood-forming region at the base of the yolk extension (the posterior intermediate cell mass; Detrich et al., 1995; Fig 4F,G); in Rohon-Beard cells in the trunk and tail (Fig. 4F,G), and in a bilateral patch of cells ventral to the 3rd somite which corresponds to the position of the zebrafish primordial germ cells (Yoon et al., 1997; data not shown). At 24 h, val expression in wild-type embryos is extinguished in r5 but persists weakly in r6 (Fig. 4 H), and val expression in r4 has expanded from the single cell to a small cluster of cells, again independent of functional Val protein. In val\textsuperscript{b337} embryos this r4 cluster of val-expressing cells extends caudally into rX (Fig. 4I), consistent with a gradual expansion of r4 identity into rX by this stage (Prince et al., 1998). At 42 hours, the latest stage examined in this study, val is re-expressed in r5 as well as in r4 in a complex pattern whose relationship to earlier val-expressing cells in r4 is unclear (Fig. 4J,K).

We identified the single pair of val-expressing cells in r4 at
19 h as the Mauthner neurons, by single cell transplantation. The Mauthner neuron is a primary reticulospinal interneuron that can be individually identified in live embryos by the 10 somite stage (14 h). In the embryo shown (Fig. 4C), we used a micromanipulator to pick up the single Mauthner cell on the right side of the embryo at 14 h and to move it to a more rostral position. Detection of val expression five hours later demonstrates that the cell that was moved expresses val, and that val expression is absent in the right r4 from which the Mauthner cell was removed. Thus the pair of cells in r4 that normally express val are the Mauthner cells.

**Inner ear and pharyngeal arch defects in valentino mutants**

Zebrafish val− embryos and mouse kr− embryos exhibit striking similarities, consistent with a high degree of functional conservation for this gene across vertebrate species (see Discussion). kr was originally identified because mutant mice exhibit a circling behaviour (Hertwig, 1944) that correlated with an abnormal and cystic inner ear (Deol, 1964), valb337 mutants die as larvae by 9 days of age, before the chambers of the inner ear are fully formed (Haddon and Lewis, 1996), however as for kr− mice, valb337 larvae have variably abnormal inner ears in which the semicircular canals are partially or completely fused (Fig. 5A,B). 21/22 valb337 larvae examined exhibited fusion of the semi-circular canals to varying degrees. Furthermore, 13 of these 22 mutant larvae exhibited a circular swimming behaviour in response to a gentle touch to the head, a behaviour that was detected in only 1/50 of their wild-type siblings.

Abnormalities in the hyoid bone of kr− mice have been interpreted as a partial anterior transformation of the third pharyngeal arch to second arch character (Frohman et al., 1993). In fish, second arch cartilages include the paired dorsal hyosymplectic and ventral ceratohyal which articulate at the posterolateral tip of the ceratohyal via the interhyal cartilage (Fig. 5C; Schilling and Kimmel, 1997). Ventromedially the ceratohylies overlie the unpaired basihyal cartilage. The third and more posterior pharyngeal arches, which are the gill-bearing arches, each have paired ventral hypobranchials, and lateral to these are the ceratobranchial elements which are the segmental homologues of the ceratohyal (Fig. 5C; Schilling and Kimmel, 1997). The gill-bearing arches lack dorsal elements homologous to the hyosymplectic until relatively late in development (Cubbage and Mabee, 1996) and lack segmental homologs of the tiny interhyal cartilage.

In valb337 larvae, arches one and two, and arches four through seven, are normal, but we observe abnormalities in the third arch-derived cartilages which we interpret as anterior transformations. The phenotypes of valb337 third arch cartilages are variable and often bilaterally asymmetric. They include severe truncation and thickening of the first ceratobranchial cartilage in the most strongly affected individuals (7 out of 48 mutants examined; Fig. 5E) and an extension of the first ceratobranchial at its anteromedial end such that it contacts the medial basibranchial element (arrow in Fig. 5F; 7 out of 48 mutants examined). The most common abnormality, which we observed in 29 out of 48 mutant larvae examined, is an ectopic cluster of alcian-blue stained cells either fused to (Fig. 5F, arrowhead) or separate from (Fig. 5G, arrowhead) the posterolateral tip of the first ceratobranchial. This element closely resembles the interhyal cartilage normally only present in the second arch. In each of these respects (thickening, overlap with the medial element, and the presence of an interhyal-like element) the first ceratobranchial more closely resembles its segmental homologue in the second pharyngeal arch.

**The timing of rhombomere boundary formation in the zebrafish**

val is expressed in r5 and r6 and is required for cells to take on r5 and r6 identity. In the absence of val function, the presumptive r5-6 domain persists as a region with a unique identity (rX) in which wild-type cells are unable to intercalate normally. We have proposed that rX is a remnant of the common precursor of r5 and r6, and that val is required for the subdivision of this precursor proto-segment into the definitive rhombomeres (Moens et al., 1996). If this is the case, we would predict that the boundary between r5 and r6 should form only after the onset of val expression, and after the appearance of the proto-segment boundaries (the presumptive r4/5 and r6/7 boundaries). We performed confocal time-lapse analysis of embryos labeled with a vital dye (M. Cooper, personal communication; reviewed by Cooper and Kimmel, 1998) to determine the timing of rhombomere boundary formation in the zebrafish hindbrain. We detected the r3/4 and r4/5 boundaries as early as the 5 somite stage (11.7 h; Fig. 6A) and the r6/7 boundary shortly thereafter, but the r5/6 boundary appears only by the 7-8 somite stage (12.5-13 h; Fig. 6B), about 3 hours after the onset of val expression. The r5/6 boundary is clearly visible by the 10 somite stage (14 h; Fig. 6C). No rhombomere boundaries are visible at bud stage when val expression is first detected (10 h; Fig. 6D). At the 12 somite stage rhombomeres can be unambiguously identified by their position relative to the otic vesicle (15 h; Fig. 6E). In val− embryos at this stage there are no clear boundaries caudal to the r3/4 boundary (Moens et al., 1996) although there is a faint r4/rX boundary visible with the vital dye that lies at approximately the position where we see a narrow strip of krox20 expression by RNA in situ hybridization (Fig. 6F). The timing of rhombomere boundary formation we observe in the zebrafish provides additional support for the hypothesis that r5 and r6 arise from a common precursor proto-segment.

**DISCUSSION**

Hindbrain segmentation is a conserved feature of vertebrate development, and the structures that are patterned and innervated by the hindbrain are among the defining features of the vertebrate body plan (Guthrie, 1995). Given that this is the case, the genetic mechanisms of hindbrain segmentation and segment specification are also expected to be conserved. We have shown that the zebrafish segmentation gene valentino (Moens et al., 1996) is the homologue of the mouse segmentation gene krei{umlaut}sler, which encodes a Maf-related bZip transcription factor (Hertwig, 1942, 1944; Deol, 1964; Cordes and Barsh, 1994). The expression pattern of this gene in the zebrafish confirms its early role in hindbrain segmentation, and the considerable similarities of the val− and kr− phenotypes confirm that this role is highly conserved across vertebrates species. Differences in the interpretations of the kr− phenotype
can be reconciled by proposing that \textit{val}/\textit{kr} functions by subdividing the common precursor of r5 and r6 into its constituent rhombomeres. Finally, we have provided morphological evidence for the existence of such an r5-6 proto-segment in the zebrafish embryo.

\textit{val}b\textsuperscript{337}, \textit{val}b\textsuperscript{361} and \textit{val}b\textsuperscript{475} are null mutations in the zebrafish \textit{kr} homologue

\textit{val} and the zebrafish homologue of \textit{kr} map together on the zebrafish genetic map, and we identified lesions in the zebrafish \textit{kr} homologue in three alleles of \textit{val}. Two of these alleles (\textit{val}b\textsuperscript{361} and \textit{val}b\textsuperscript{475}) are \textit{\gamma}-ray induced deficiencies, and the third, \textit{val}b\textsuperscript{337} is a single base substitution that truncates the encoded protein upstream of its DNA binding and dimerization motifs. This molecular characterization confirms that \textit{val}b\textsuperscript{337} is a null allele, which we argued previously based on the observation that its homozygous phenotype is the same as its phenotype when placed in \textit{trans} to a deficiency (Moens et al., 1996). The original \textit{kr} allele, in contrast, is a hypomorphic regulatory mutation that affects \textit{kr} expression in the hindbrain but not in its other normal domains of expression (Cordes and Barsh, 1994; Eichmann et al., 1997). While the phenotypes of \textit{val}b\textsuperscript{337} and \textit{kr} with respect to the hindbrain and its derivatives are remarkably similar (see below), \textit{val}b\textsuperscript{337} causes lethality in homozygotes during larval stages (8-9 days post fertilization) while the original \textit{kr} allele is homozygous viable. The greater severity of the \textit{val}b\textsuperscript{337} phenotype may be due to differences in the downstream effects of the loss of r5 and r6 in fish and mice, however as for its homologs in the mouse and the chick, \textit{val} is expressed in sites outside r5 and r6 (see below), and loss of essential \textit{val} functions in one or more of these sites may be responsible for the lethality of \textit{val}b\textsuperscript{337} homozygotes.

Expression of \textit{valentino} is consistent with a role in the subdivision of r5 and r6 from their common precursor proto-segment

Genetic mosaic analysis has demonstrated that \textit{val} function is required cell-autonomously in r5 and r6. In the absence of \textit{val} function the presumptive r5-6 domain fails to be subdivided into the definitive rhombomeres and instead persists as a region contributing normally to rX in a \textit{val} \textsuperscript{-} host (Fig. 3B,C), demonstrating that the primary defect in \textit{val} \textsuperscript{-} embryos is the failure of the common precursor of r5 and r6 to be subdivided and expanded into the definitive rhombomeres. Early during hindbrain segmentation \textit{kr}, like \textit{val}, is expressed in the presumptive rhombomeres 5 and 6, consistent with a conserved role in subdividing this region of the hindbrain (see below). However, the expression patterns of \textit{val} and \textit{kr} subsequently diverge, with \textit{kr} being down-regulated in r6 (Cordes and Barsh, 1994) while \textit{val} persists throughout r5 and r6. \textit{kr} has recently been shown to directly regulate \textit{hoxb3} expression in the mouse, and \textit{hoxb3} expression in fish and mice differs in a manner that depends at least partly on these differences in \textit{val} and \textit{kr}, although additional factors are involved (Manzanares et al., 1997; Prince et al., 1998; see below). Since vertebrate \textit{hox} genes specify aspects of segment identity such as the positions of motor neurons (Krumlauf, 1994; Studer et al., 1996; Goddard et al., 1996), differences in \textit{hox} gene regulation between species are expected to contribute to the morphological differences that distinguish them. Indeed, abducens motor neurons differentiate in r5 in mice and in both r5 and r6 in fish (Gillard and Baker, 1993; Chandrasekhar et al., 1997), differences that have been predicted to result from prior differences in \textit{hox} gene expression (Gillard and Baker, 1993). Thus as well as playing a highly conserved role in hindbrain segmentation (see below), \textit{val} and \textit{kr} may differentially regulate \textit{hox} gene expression and thereby contribute to the divergence of vertebrate body plans.

The homogeneous expression of \textit{val} that we observe throughout r5 and r6 during hindbrain segmentation does not imply a mechanism for how \textit{val} functions to specify the difference between these two rhombomeres. This apparent paradox suggests that the \textit{Val} protein interacts with other proteins that are themselves differentially expressed in the presumptive r5-6 territory. There is evidence that \textit{val} homologs interact with other transcription factors to confer regional or cell-type specificity. The chicken homologue of \textit{val}, \textit{MafB}, has been shown to interact directly with the Ets-1 protein in the specification of hematopoietic lineages (Sieweke et al., 1996). In the hindbrain, r5-restricted expression from the mouse \textit{hoxb3} enhancer requires intact binding sites for both the \textit{kr} gene product, \textit{Krm1}, and for an unidentified Ets-1-related protein (Manzanares et al., 1997). It remains to be determined whether \textit{Val} interacts with Ets-1-related partners to distinguish r5 from r6 identity in the zebrafish, and what these partners are.

\textit{val} expression outside r5 and r6 reflects distinct functions and regulatory mechanisms

After its initial phase of expression in the presumptive r5-6 territory, \textit{val} is expressed in other sites where we have not previously detected a requirement for \textit{val} function. Expression outside r5 and r6 has also been noted for \textit{MafB} and \textit{kr}, the chick and mouse homologs of \textit{val} (Eichmann et al., 1997). \textit{val} is expressed in hematopoietic stem cells, which in the zebrafish are found in the posterior region of the intermediate cell mass (Detrich et al., 1995), consistent with the putative role of \textit{MafB} in myelomonocytic differentiation (Sieweke et al., 1996). It remains to be determined whether \textit{val} expression in the posterior intermediate cell mass is associated with any particular hematopoietic lineage, and whether hematopoiesis is affected in \textit{val} \textsuperscript{-} embryos.
val is also expressed in cell types such as the lens, the telencephalon, and the primordial germ cells where expression has not been noted in other vertebrates, and in the Mauthner and Rohon-Beard cells which are primary neurons that have no homologs in terrestrial species. val is not expressed in the roofplate of the hindbrain outside of r5 and r6 as are its homologs in both birds and mice, nor is it expressed in spinal cord motor neurons or interneurons, at least not before 42 h. val is expressed in clusters of cells in r4 and r5 at 42 h, but we have not determined whether these correspond to any of the vestibuloacoustic nuclei that express MafB in the chick (Eichmann et al., 1997). No val expression is observed in the motor nuclei of cranial nerve VII, which by 42 h in the zebrafish lie in r6 and r7, apparently having migrated there from more rostral positions (Chandrasekhar et al., 1997).

val expression in r5 and r6 depends, either directly or indirectly, on the presence of a wild-type Val gene product since expression is lost in valB337 embryos. However in all other cell types where val is normally expressed, the mutant message persists in homozygous valB337 embryos, indicating that val regulatory sequences include an autoregulatory element that is only required for the maintenance of r5-6 expression. Such an element may be disrupted in the original X-ray induced allele of kr, which is a sub-microscopic chromosomal inversion that suppresses kr transcription in the hindbrain but not in its other normal domains of expression (Cordes and Barsh, 1994; Eichmann et al., 1997).

valentino and kreisler play a conserved role in hindbrain segmentation

The many similarities between the val- and kr- phenotypes argue for a conserved role for this gene in hindbrain segmentation. kr was originally named for the circling behaviour of homozygous mutants, a behaviour that results from abnormal development of the inner ear (Deol, 1964). In homozygous kr- mice, the cochlear membrane protrudes through holes in the auditory capsule, the vestibular region fails to be divided into semi-circular canals, and an endolymphatic duct fails to form. Zebrafish do not make an endolymphatic duct or a cochlea, and val- larvae die before the subdivisions of the inner ear – the utricle, saccule and lagena – are fully formed (Haddon and Lewis, 1996). However like kr- mice, valB337 larvae exhibit variable fusions of the semi-circular canals in the vestibular region of the inner ear. And as in kr- mice, this defect manifests itself as a circling behaviour in approximately 50% of the val- larvae tested.

The vestibular defects of val- and kr- mutants are secondary to the hindbrain segmentation defect, which in both mutants is apparent as a lack of visible segmentation caudal to the r3/4 boundary (Deol, 1964; Moens et al., 1996). The segmentation defect in turn results from changes in regional identity in the caudal hindbrain that disrupt the normal alternating odd-even differences required for rhombomere boundary formation (Guthrie and Lumsden, 1991). In both val- and kr- embryos there is a loss of r5 and r6-specific markers, a caudal extension of r4-specific markers, and a rostral extension of markers normally expressed up to the r6/7 boundary (Frohman et al.,

Fig. 4. Other sites of val expression in wild-type and val- embryos.
All embryos are shown in dorsal view with anterior to the left except F,G which are lateral views. (A,B) At the 20 somite stage (19 h), val expression begins to be down-regulated, first in r5 and then in r6, and expression is first detected in a bilateral pair of cells in r4 (arrowheads). In valB337 embryos (B) the r5/6 expression is absent but expression in these two cells occurs normally. (C) In this embryo, the right Mauthner cell was transplanted from r4 to r2 at the 12 somite stage (15 h), and val expression was detected at 19 h. The correlation of val expression with the transplanted cell (arrow) demonstrates that the bilateral pair of val-expressing cells in r4 are the Mauthner cells. (D,E) val expression in the lens in wild-type (D) and valB337 embryos (E) at 19 h. (F,G) val expression in Rohon-Beard cells (arrowheads) and in the blood-forming region at the base of the hind-yolk (arrow) in wild-type (F) and valB337 (G) embryos. (H,I) By 24 h, val expression is gone from r5 in both wild-type (H) and valB337 (I) embryos but persists at low levels in r6 in wild-type embryos. Expression in r4 is no longer restricted to the Mauthner cell but now includes 10-15 cells in a lateral cluster (arrowhead). In valB337 embryos at this stage (I) val expression is similarly upregulated in r4, but the cluster of val-expressing cells extends further caudally than in wild-type embryos (arrowhead). (J,K) val expression in the hindbrain of 42 h wild-type (J) and valB337 (K) embryos. Scale bars = 50 μm.
Fig. 5. Inner ear and pharyngeal arch defects in val larvae. (A,B) Horizontal sections of 5-day wild-type (A) and val -/-(B) larvae showing complete fusion of the developing semi-circular canals in the mutant. Anterior is to the top. This phenotype was observed with varying severity in 21/22 val -/-(B) larvae examined, and 13 of these 22 mutant larvae circled in response to a gentle touch to the side of the head rather than swimming away in a straight line (1/50 wild-type larvae exhibited this behaviour). a and p indicate anterior and posterior semi-circular canals, respectively.

(C-G) Alcian-blue stained pharyngeal arch-derived cartilages of 7-day wild-type (C, D) and val -/-(E-G) larvae. (C) The ventral cartilages of pharyngeal arches 2-7 in ventral view with anterior to the top. The second (hyoid) arch elements include paired ceratohyalals (ch) and interhyals (ih). Medially, ch overlies the unpaired median basihyal (bh). The third through seventh (gill-bearing) arches each include paired ceratobranchials (cb1-5) and hypobranchials (hb1 labelled) which are adjacent to the unpaired median basibranchial (bb). (D) The third arch-derived cartilages of a wild-type embryo, dissected away from the surrounding tissue. Abbreviations are as for C. Note that cb1 is a tapering element, one cell wide through most of its length, and that it is separated from the median bb by hb1.

(E,F) Ectopic interhyal-like elements that are partially fused to (F; arrowhead) or separate from (G; arrowhead) cb1; and medial extensions of cb1 such that cb1 overlies bb (F; arrow). Scale bars in A-C = 100 µm; D-G = 50 µm.

1993; McKay et al., 1994; Cordes and Barsh, 1994; Moens et al., 1996; Prince et al., 1998). Associated with these molecular changes are corresponding neuroanatomical defects, since both val and kr mutants lack r5 and r6-specific neuronal subtypes (the abducens (nVI) motor neurons and, in kr, the superior salivatory nucleus; Moens et al., 1996; Chandrasekhar et al., 1997; McKay et al., 1994, 1997).

These observations on kr -/embryos led McKay et al. (1994) to conclude that in the kr -/hindbrain r5 and r6 are respecified towards an r4 identity, and Cordes and Barsh (1994) and Frohman et al. (1993) to conclude that kr -/embryos lack r5 but retain a region with partial r6 character. Our analysis of val provides a third interpretation. val -/embryos entirely lack r5 and r6, since val -/cells cannot contribute to r5 and r6 of a wild-type host. Like kr -/embryos, val -/embryos exhibit a progressive caudal extension of r4-specific markers, however this is a secondary effect (Prince et al., 1998), and the region of one rhombomere’s length that lies caudal to r4 in val -/embryos (rX) is distinct from r4 or any other rhombomere since wild-type cells cannot contribute normally to it in genetic mosaics. We have proposed that rX is a remnant of the common precursor of r5 and r6, which fails to be subdivided in the absence of val function, and that since rX lacks either odd or even identity, it fails to form visible boundaries either with r4 or with r7 (Moens et al., 1996). Based on the remarkable similarities of the val -/ and kr -/phenotypes, we now propose that the region between r4 and r7 in kr -/embryos has the same unique identity and that the different interpretations of the kr -/phenotype can be reconciled if kr, like val, functions in the subdivision and expansion of a common precursor proto-segment into the definitive r5 and r6.

The order of rhombomere boundary appearance in the zebrafish reflects the subdivision of r5 and r6 from a common precursor

If val is required for the subdivision of the common precursor proto-segment of r5 and r6 into the definitive rhombomeres, we would predict that the boundary between r5 and r6 should form after the onset of val expression. This is indeed the case, since val expression in the presumptive r5 and r6 is first detected before the 1 somite stage (10 h) while the r5/6 boundary is not observed until 3 hours later, at the 7-8 somite stage (Fig. 6). Furthermore, this model predicts that the boundaries of the proto-segment (the presumptive r4/5 and r6/7 boundaries) may form before the r5/6 boundary. This is not necessarily the case, since the proto-segment has neither even nor odd identity, and there is evidence that the apposition of distinct odd and even identities is required for visible rhombomere boundaries to form (Guthrie and Lumsden, 1991). However, we do observe that the r4/5 and 6/7 boundaries are visible approximately 1 hour before the r5/6 boundary in the zebrafish by the use of time-lapse confocal microscopy (Fig. 6). This demonstration of a morphologically visible r5-6 protosegment thus supports our model for val/kr function.

Our observation of a common precursor to r5 and r6 runs contrary to a previous description of rhombomere boundary formation in the chick (Vaage, 1969) which suggested that the r5/6 boundary forms relatively early in development (HH-9), followed by the r4/5 boundary (HH-10) and later still by the r6/7 boundary (HH-12). Vaage suggested that the early-forming r5/6 boundary separates two 'primary' rhombomeres rhB (the precursor of r4 and r5) and rhC (the precursor of r6 and r7). MafB, the avian homologue of val/kr is also expressed in r5 and r6 and just as its function from fish to mice is likely to be conserved, it is also likely to be conserved in birds. Thus a different time-course of rhombomere formation in the chick would reflect a dissociation between the appearance of visible rhombomere boundaries and the underlying genetic events. It should be noted, however, that Vaage made his observations at isolated time-points, without the benefit of the retrospective observations of van/kr mutants.
analysis of time-lapse recordings. We noticed that in the course of our time-lapses, the entire hindbrain moves anteriorly due to convergent extension. As a result, the r5/6 boundary ultimately lies at approximately the same position, relative to the yolk, as did the r4/5 boundary when first detected 3 hours earlier. It is possible that the same movements occur in the chick neuroepithelium, and that the r5-6 proto-segment that \textit{val}/\textit{kr}/MafB serves to subdivide corresponds to Vaage’s rhombomere RhC. A thorough time-lapse analysis of rhombomere boundary formation in the chick is needed to properly resolve this question.

**Conserved homeotic transformations**

Homeosis involves the wholesale change of identity of one differentiated structure to that of another structure, as a result of a simple genetic or epi-genetic event. In the context of segmentation, a homeotic change results in structures in one segment taking on the character of the homologous structures in another segment. Since \textit{hox} genes specify segment identity, homeotic transformations are expected to result from the loss of expression or mis-expression of \textit{hox} genes as has indeed been demonstrated (Rijli et al., 1993; Gendron-Maguire et al., 1993).

Although \textit{val}/\textit{kr} are not \textit{hox} genes, both \textit{val} and \textit{kr} mutants exhibit anterior transformations of the third pharyngeal arch towards a second arch identity. In \textit{kr}− mice, this is apparent as an accessory process on the hyoid bone, which is partially second arch-derived and partially third arch-derived (Frohman et al., 1993). Segmental homologies in the pharyngeal arch-derived structures of the zebrafish (Schilling and Kimmel, 1997) are more apparent than they are in terrestrial vertebrates. In \textit{val}− larvae we observe an anterior transformation in arch phenotype, one that occurs specifically in the homologous pharyngeal arch to that affected in \textit{kr}− mice. In \textit{val}b337 larvae, the third arch-derived ceratobranchial cartilage (cb1) takes on characteristics of its broader segmental homologue in the second arch, the ceratohyal. The mutant cb1 is variably thickened, and in some cases it overlies the unpaired medial basibranchial cartilage element much as the ceratohyal overlies the medial basihyal cartilage in the second arch. Most strikingly, we observe ectopic cartilage elements in the third arch that are similar to a unique second arch element, the interhyal cartilage.

These abnormalities are indicative of an anterior transformation of the third arch. However this transformation is not complete, since the second arch includes large paired dorsal elements, the hyosymplectics, which are never duplicated in the third arch of \textit{val}b337 larvae. The third and more caudal arches of the zebrafish do have putative segmental homologs of the hyosymplectic, the epibranchials, but these do not chondrify until approximately 20 days postfertilization, well after \textit{val}b337 larvae die. It is possible that the ectopic elements in the third arch of \textit{val}b337 larvae are epibranchials, and that the anterior transformation is a heterochronic event resulting in their premature differentiation. However based on their shape, size and position we favour the interpretation that these elements are ectopic interhyal cartilages.

How do \textit{val} and \textit{kr} mutations result in anterior transformations of pharyngeal arch identity? McKay et al. (1994) proposed that third arch neural crest, which normally migrates from the hindbrain at the level of r5 and r6, carries with it an inappropriate identity characteristic of the second arch in \textit{kr}− embryos. Our observations also suggest that the homeotic transformation in \textit{val} can be accounted for by changes in the \textit{hox} code of third arch neural crest. \textit{val}− embryos lack high-level \textit{hoxb3} expression in the neural crest migrating into the third arch, consistent with a direct role for \textit{kr} in the positive regulation of \textit{hoxb3} (Prince et al., 1998; Manzanares et al., 1997). Additionally, rX, the distinct region that replaces r5 and r6 in \textit{val}− embryos, gradually takes on aspects of r4 identity as a secondary effect of the loss of \textit{val} function, and a result, later migrating third-arch neural crest carries with it a \textit{hox} code normally characteristic of the second arch (Prince et

![Fig. 6. Time-lapse analysis of rhombomere boundary formation.](image-url)
REFERENCES


