ERRATUM

Oskar anchoring restricts pole plasm to the posterior of the Drosophila oocyte

The reproduction of Fig. 6 in this article was inaccurate. The correct version of the figure is given below. We apologise to the authors and readers for the mistake.

CORRIGENDUM

A zebrafish sox9 gene required for cartilage morphogenesis

The name of the third author was published incorrectly in the printed version. The correct name is Robert M. Nissen.
INTRODUCTION

Cartilage cushions joints and provides a template for the development of cartilage-replacement bones. Aberrant cartilage development results in craniofacial anomalies and cartilage damage results in diseases such as osteoarthritis (Hamerman, 1989). The genetic pathway leading to chondrocyte differentiation is under active investigation (Cancedda et al., 1995; de Crombrugghe et al., 2001), but less is known about the cellular mechanisms that control the assemblage of chondrocytes into higher order cartilage organs.

As chondrocytes begin to differentiate, they surround themselves with matrix, including collagen encoded by COL2A1. In many cartilages, cells organize themselves into rows called stacks as they begin to form their mature spatial patterns (Kimmel et al., 2001b). Chondrocytes continue directional proliferation, and then hypertrophy. The extracellular matrix mineralizes before the hypertrophic chondrocytes undergo apoptosis and are replaced by their bone-forming cell replacements.

Most cartilage replacement bones fail to develop normally in individuals with campomelic dysplasia (CD), causing...
macrocephaly, small jaw, cleft palate, lowset ears and sometimes lack of olfactory bulbs. Individuals with CD often have underdeveloped sclerotome derivatives, including non-mineralized thoracic pedicles and 11, rather than 12, pairs of ribs; and they have poorly developed limbs, including bowed limb bones, hypoplastic scapula and an insufficiently ossified pelvis (Houston et al., 1983; McKusick, 1990; Mansour et al., 1995). In addition, most XY individuals with CD display a female phenotype. The cartilage and sex-reversal phenotypes of CD are both caused by mutations in the transcription factor gene SOX9 (Foster et al., 1994; Wagner et al., 1994; Hageman et al., 1998; Cameron et al., 1996; Huang et al., 1996; Wunderle et al., 1998). Thus, phenotypic analysis of CD can cause CD or a similar phenotype in mouse (Wagner et al., 1994; Foster et al., 1994; Kwok et al., 1995; Cameron et al., 1996; Wunderle et al., 1998). The SOX9 protein has a C-terminal transcription activation domain (Südbeck et al., 1996; Ng et al., 1997), and bend DNA (Conner et al., 1994; Werner et al., 1995). Sox9 may also participate in transcript splicing (Ohe et al., 2002). The SOX9 protein has a C-terminal transcription activation domain (Südbeck et al., 1996; Ng et al., 1997), suggesting that it acts by regulating expression of other genes.

Consistent with its role in chondrogenesis, Sox9 is expressed in the pharyngeal arches and neurocranium, the sclerotomes and the lateral plate mesoderm (Lefebvre et al., 1997; Chiang et al., 2001). In these domains, the expression of Sox9 slightly precedes and directly regulates the expression of Col2a1, which encodes the major collagen of cartilage (Wright et al., 1995; Bell et al., 1997; Lefebvre et al., 1997; Ng et al., 1997; Zhao et al., 1997; Chiang et al., 2001).

Despite this knowledge of Sox9 activity, we have insufficient understanding of the morphogenetic roles Sox9 plays in chondrogenesis or the pathogenesis of CD. Heterozygous Sox9 mutant mice show phenotypes similar to individuals with CD and die at birth, so permanent lines have not been established (Bi et al., 2001). Delayed or defective pre-cartilaginous condensations are present in heterozygous Sox9 mutant mouse embryos, but the precise morphogenetic steps that require Sox9 function remain obscure. Some bones showed premature mineralization in the heterozygous mouse embryos, suggesting that Sox9 plays a role in regulating the transition to hypertrophic chondrocytes in the growth plates. Sox9 is thought to regulate this transition by mediating the effects of parathyroid hormone related peptide (PTHrP) (Huang et al., 2001).

We have shown that the zebrafish genome contains two duplicate orthologs of the human SOX9 gene, called sox9a and sox9b, and that these map on zebrafish chromosomes that are duplicates of much of human chromosome 17, the location of SOX9 (Chiang et al., 2001). The two zebrafish sox9 genes apparently arose in a whole genome duplication event hypothesized to have taken place!n the base of the teleost radiation (Postlethwait et al., 1998; Amores et al., 1998). The sox9a and sox9b genes are expressed in partially overlapping patterns that together approximate the expression pattern of Sox9 in mouse (Wright et al., 1995; Chiang et al., 2001), as predicted by the duplication, degeneration, complementation hypothesis (Force et al., 1999). Interestingly, however, in zebrafish the testis expresses sox9a but the ovary expresses sox9b (Chiang et al., 2001), whereas in mammals, only the testis expresses Sox9 (Morais da Silva et al., 1996). We reasoned that a mutation in one of the two zebrafish genes might not be a dominant lethal mutation as in mammals, and so we investigated recessive lethal zebrafish mutations with phenotypes similar to individuals with CD. We show that two alleles of jellyfish (jef), one resulting from chemical mutagenesis (jef<sup>nu237</sup>) (Piotrowski et al., 1996; van Eeden et al., 1996) and the other from the insertion of a retrovirus (Amsterdam et al., 1999), disrupt sox9a. Confocal microscopy demonstrated that jef (sox9a) is required not for precartilage condensation formation, but for overt differentiation of cartilage and for three morphogenetic processes: stacking, shaping and individuation. The results suggest that sox9a or its downstream targets, perhaps including extracellular matrix proteins, play morphogenetic roles in chondrogenesis.

**MATERIALS AND METHODS**

**Animals, histology and gene expression**

The jef<sup>nu237</sup> mutation was identified in an ENU screen for abnormal jaw and fin morphology (Piotrowski et al., 1996; van Eeden et al., 1996). The hll34 mutation was isolated in a retroviral insertion screen (Amsterdam et al., 1999). Cartilages were Alcian stained, dissected and flat mounted (Kimmel et al., 1995). Larval bones were visualized with calcein (Molecular Probes, catalog number C-381) (Du et al., 2001) (C. K., unpublished). In situ hybridization was performed as described (Jowett and Yan, 1996) using probes as described (Akimenko et al., 1994; Yan et al., 1995; Chiang et al., 2001). BODIPY-ceramide labeling was performed essentially as described (Cooper et al., 1999). Late epiboly stage embryos were immersed in BODIPY FL C5-ceramide (Molecular Probes, catalog number D-3521) dissolved to 10 mM in DMSO, then diluted to 10 μM in Embryo Medium (EM) (Westerfield, 2001) with 10 mM Hepes. Embryos were placed in 150-250 μL of dye solution in 1.2% agarose dishes and stored in the dark. On day 2, embryos were anesthetized, mounted on bridged coverslips, and one side of the head z-sectioned at 3 μm intervals with a Zeiss 310 upright confocal microscope. Live developing animals were stored in the dark and at subsequent time points, the same side of each animal was re-examined.

**Morpholinos**

Morpholino antisense oligonucleotides (MO) were obtained from Gene Tools (Philomath, OR) with the sequences: intron 1 splice donor junction (i1d), AATGAAATTACCTACCCTCCAAAGTTT; and intron-2 splice donor junction (i2d), CGAGTCGAAGTTAGTGTCACCACCTG. Morpholinos were injected as described (Draper et al., 2001). In the i1d MO, the 14th base from the 5′ end, a C, pairs with the G immediately following the splice junction (the one mutated in jef<sup>nu237</sup>). In MO i2d, the 4th base from the 3′ end, a C, pairs with the conserved G just after the splice junction.
Mapping
To map jef<sup>tw37</sup>, we identified a single strand conformation polymorphism (SSCP) (see Postlethwait et al., 1998) in sox9<sub>a</sub> [(mapping primers were sox9a.<sup>+</sup>-9 (CTTTCGACAGCACCAGCAGA) and sox9a.<sup>-</sup>-190 (CAGTGAAGGGTCAGAGGAGTCTCA)]. Females heterozygous for jef<sup>tw37</sup> were mated to WIK wild-type males, and the F<sub>1</sub> were crossed to make an F<sub>2</sub>, which were scored for recombination with microsatellite markers (Knouf et al., 1998; Shimoda et al., 1999) near sox9<sub>a</sub> and sox9b (Chiang et al., 2001). The 95% confidence interval around the map distance between jef<sup>tw37</sup> and an SSCP in the 5′ untranslated region of sox9<sub>a</sub> was calculated according to Crow (Crow, 1950). To map the insertion allele jef<sup>hi1134</sup>, we used sox9a-RT2 (CTCTTCCACAGGACAGGCTTTTCCA), (2a (GGGACTGAGATGTTGT) and sox9a.r2 (TTTTCGGGGTGGTGGT) primers amplifying a fragment of the sox9a cDNA using the sox9a antisense RNA probe for cloning primer binds in the 5′ untranslated region (UTR, sox9a.+11: TTCGCAGACACCAGCAGAACAACA) and the reverse primer binds near the end of the 3′ UTR (sox9a.-1784: GTCTTTCCATCATCGACTGAACG). These primers amplified a 3.6 kb fragment including most of exon 1, intron 1, exon 2 and intron 2, and nearly all of exon 3. To minimize PCR errors, Platinum Taq DNA polymerase high fidelity (CAT#11304-029 from Gibco BRL) was used in a touch-down PCR protocol. A BAC-containing sox9<sub>a</sub> (clone 174 (I13)) was identified by screening the BAC zebrafish library-5849 from Incyte Genomics with the primers sox9a.+9 (CTTTCGACAGCACCAGCAGA) and sox9a.-691 (CTTATTAGCAGGGTGATCTTTTGGT). We cloned and sequenced DNA fragmenting the pro-viral insert linked to the hi1134 mutant phenotype using inverse PCR as previously described (Amsterdam et al., 1999).

Cloning
Genomic clones of sox9<sub>a</sub> were isolated by amplifying genomic DNA isolated from Oregon AB wild type, Tübingen AB (TU) (the genetic background of jef<sup>tw37</sup>) or homozygous jef<sup>tw37</sup> embryos. The forward cloning primer binds in the 5′ untranslated region (UTR, sox9a.+11: TTCGCAGACACCAGCAGAACAACA) and the reverse primer binds near the end of the 3′ UTR (sox9a.-1784: GTCTTTCCATCATCGACTGAACG). These primers amplified a 3.6 kb fragment including most of exon 1, intron 1, exon 2 and intron 2, and nearly all of exon 3. To minimize PCR errors, Platinum Taq DNA polymerase high fidelity (CAT#11304-029 from Gibco BRL) was used in a touch-down PCR protocol. A BAC-containing sox9<sub>a</sub> (clone 174 (I13)) was identified by screening the BAC zebrafish library-5849 from Incyte Genomics with the primers sox9a.+441 (CCATGCGGTAGGTTGAAACG) and sox9a.-691 (CTTATTAGCAGGGTGATCTTTTGGT). We cloned and sequenced DNA fragmenting the pro-viral insert linked to the hi1134 mutant phenotype using inverse PCR as previously described (Amsterdam et al., 1999). (see Draper et al., 2001).

RT-PCR experiments to amplify the sox9<sub>a</sub> message from jef<sup>hi1134</sup> mutants using the primers sox9a.F (CCATGCGGTAGGTTGAAACG) and sox9a.R (CGTTCGGGCTCGATTG).

RESULTS
Chondrogenesis requires jellyfish activity
Zebrafish homozygous for the recessive lethal ENU-induced jellyfish allele jef<sup>tw37</sup> have severely reduced cartilaginous elements (Piotrowski et al., 1996; van Eeden et al., 1996). In wild-type larvae, jaw elements extend anterior and ventral to the eye (Fig. 1A,AB), but larvae homozygous for jef<sup>tw37</sup> lack these tissues (Fig. 1E,F) (Piotrowski et al., 1996). Likewise, cartilage supports the fin buds of normal larvae (Fig. 1D), but jef<sup>tw37</sup> larvae have mis-shaped pectoral fins and lack the scapulocoracoid cartilage (Fig. 1F,H) (van Eeden et al., 1996). These phenotypes mimic CD (Houston et al., 1983; McKusick, 1990; Mansour et al., 1995).

A screen for lethal mutations induced by retroviral insertion (Amsterdam et al., 1999; Burgess and Hopkins, 2000) identified a mutation (hi1134) giving a phenotype similar to that of jef<sup>tw37</sup> (Fig. I1-L). To determine whether the hi1134 mutation is allelic to jef<sup>tw37</sup>, we mated a male heterozygous for hi1134 to a female heterozygous for jef<sup>tw37</sup> and observed an approximate Mendelian ratio of phenotypically mutant offspring.
Because the phenotype of or phenotype of people with CD, we tried to rule out that jeftw37.

We mapped jeftw37. Small regions of Alcian-positive material remained in both animals heterozygous for the two alleles (Fig. 1C,G,K,O).

Thus ruling out LG12 near sox9a, on the upper arm of z1176 linkage to microsatellite marker (Postlethwait et al., 1994) of an F2 mapping cross revealed 5068 (Accession Number, AY090036) and compared it with genomic DNA cloned from the wild-type stocks AB (Accession Number, AY090034) and TÜ (Accession Number, AY090035). We found differences between the three strains at 49 locations in the 3560 bp consensus sequence. The two wild-type strains differed at 48 positions, and at the remaining position, the two wild-type strains were the same, but the jeftw37 strain was different. All but one of the 48 differences between the two wild strains were in non-translated parts of the gene, the exception being a silent change in codon Ala 406. Eleven of the 49 differences were indels between I and 10 bp long; the others were single nucleotide differences. The TÜ and jeftw37 strains differed at only three locations, all of them in introns. In two of those positions, jeftw37 had the same sequence as the AB wild-type strain, but in the other, the two wild-type strains had a G, but jeftw37 had a T (Fig. 2A). The unique change was in the first base after the splice donor site of intron 1 in codon Arg146, which alters an Hph1 restriction endonuclease site, allowing identification of jeftw37 heterozygotes by PCR.

Because nearly all introns have a G immediately after the splice junction (Mount, 1982; Zhang, 1998), this raised the possibility in sox9a, and uncovered no recombinants between jeftw37 and sox9a among 491 F2 diploid embryos. This represents 982 meioses, and a distance of 0.1±0.2 cM (centiMorgan) with 95% confidence. Thus, if there are on average 600 kb per cM (Postlethwait et al., 1994), this would be a distance of 60±120 kb. We conclude that jeftw37 maps very close to or within the sox9a gene.

To determine whether jeftw37 lesions the sox9a gene, we cloned and sequenced sox9a genomic DNA from homozygous jeftw37 embryos (Accession Number, AY090036) and compared it with genomic DNA cloned from the wild-type stocks AB (Accession Number, AY090034) and TÜ (Accession Number, AY090035). We found differences between the three strains at 49 locations in the 3560 bp consensus sequence. The two wild-type strains differed at 48 positions, and at the remaining position, the two wild-type strains were the same, but the jeftw37 strain was different. All but one of the 48 differences between the two wild strains were in non-translated parts of the gene, the exception being a silent change in codon Ala 406. Eleven of the 49 differences were indels between I and 10 bp long; the others were single nucleotide differences. The TÜ and jeftw37 strains differed at only three locations, all of them in introns. In two of those positions, jeftw37 had the same sequence as the AB wild-type strain, but in the other, the two wild-type strains had a G, but jeftw37 had a T (Fig. 2A). The unique change was in the first base after the splice donor site of intron 1 in codon Arg146, which alters an Hph1 restriction endonuclease site, allowing identification of jeftw37 heterozygotes by PCR.

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**Molecular genetic nature of jeftw37**

The jeftw37 mutation

Because the phenotype of jef mutations is similar to the phenotype of people with CD, we tried to rule out that sox9a or sox9b is disrupted in jeftw37. Bulked segregant analysis (Postlethwait et al., 1994) of an F2 mapping cross revealed linkage to microsatellite marker z1176 on the upper arm of LG12 near sox9a, thus ruling out sox9b on LG3 as a candidate. We mapped jeftw37 at higher resolution relative to a consensus sequence. The two wild-type strains differed at 48 locations in the 3560 bp consensus sequence. The two wild-type strains differed at 48 positions, and at the remaining position, the two wild-type strains were the same, but the jeftw37 strain was different. All but one of the 48 differences between the two wild strains were in non-translated parts of the gene, the exception being a silent change in codon Ala 406. Eleven of the 49 differences were indels between I and 10 bp long; the others were single nucleotide differences. The TÜ and jeftw37 strains differed at only three locations, all of them in introns. In two of those positions, jeftw37 had the same sequence as the AB wild-type strain, but in the other, the two wild-type strains had a G, but jeftw37 had a T (Fig. 2A). The unique change was in the first base after the splice donor site of intron 1 in codon Arg146, which alters an Hph1 restriction endonuclease site, allowing identification of jeftw37 heterozygotes by PCR.

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**Fig. 2. jeftw37 alters a conserved splice site sequence in sox9a and inhibits message splicing.**

(A) The sequence of sox9a in zebrafish wild-type strains AB and TÜ, splice junction (arrow). An in frame stop codon directly followed the G to T change in jeftw37. (B) The primer pair f1/r1 in exon 1 and intron 1 amplified a band of the expected size (544 bp) from wild-type (WT) genomic DNA and from jeftw37 cDNA, showing that intronic sequences of sox9a were present in mutant cDNA. With normal splicing in wild-type cDNA, there was no strong product of this size. (C) RNase protection assays (RPA, upper panel) on RNA extracted from wild-type and homozygous jeftw37 mutants 4 days old. rRNA served as a negative control. odc, the internal control. Probe for RPA is made from a 402 bp of sox9a cDNA amplified using primer pair f2/r2. Quantifying band intensity (lower panel) shows a drastic decrease of message in jeftw37 embryos. (D,E) The jeftw37 allele behaves as a null when heterozygous for the deletion allele b380. bh, basihyal; bsr, branchiostegal rays; cb, ceratobranchials; ch, ceratohyal; ch?, presumed ceratohyal; cl, cleithrum; ed, endoskeletal disk; co, scapulocoracid; hs, hyosymplectic; m, Meckel’s cartilage; m/de? putative Meckel’s cartilage and dentary bone; op, opercle; pq, palatoquadrate. Scale bar: 100 μm.
possibility that the lesion blocks transcript splicing. Because the lesion immediately follows the second nucleotide in codon 146 in the middle of the HMG domain, and an in-frame stop codon follows two codons downstream, a non-spliced transcript should be translated into a truncated protein containing only half of the HMG domain. Such a lesion is likely to lead to an ineffective protein.

The jef\textsuperscript{tw37} mutation inhibits splicing

If the G\textrightarrow{}T transversion in jef\textsuperscript{tw37} causes the jef phenotype, then it should disrupt the splicing of sox9\textalpha{} transcripts. To test this prediction, we made cDNA from homozygous jef\textsuperscript{tw37} embryos and their homozygous wild-type siblings, and amplified various regions of the sox9\textalpha{} gene (Fig. 2A). A forward primer, f1 in exon 1, and a reverse primer, r1 in intron 1, should fail to amplify a product from mature wild-type sox9\textalpha{} mRNA because the mature message lacks intron 1. Unspliced transcripts should give a band of 544 bp. The results showed that genomic DNA from wild-type animals (a positive control) gave a band of the size predicted for a fragment that includes the parts of exon 1 and intron 1, but cDNA from 4-day-old wild-type animals had only a faint band at this location, consistent with normal splicing. By contrast, cDNA extracted from 4-day-old homozygous jef\textsuperscript{tw37} animals behaved like wild-type genomic DNA (Fig. 2B), as expected if homozygous jef\textsuperscript{tw37} embryos accumulated unspliced transcript.

To learn the extent to which sox9\textalpha{} message is reduced in jef\textsuperscript{tw37} homozygotes, we prepared cDNA from 4-day-old mutant and wild-type animals, and then conducted RNase protection assays using as probe a region of sox9\textalpha{} transcripts that included 110 bp of exon 1, all of exon 2 (251 bp), and 40 bp in exon 3 (Fig. 2A). The ubiquitously expressed housekeeping gene, ornithine decarboxylase (odc) provided an internal standard (Draper et al., 2001). The results revealed that jef\textsuperscript{tw37} embryos possessed only 28\% of sox9\textalpha{} transcript compared with wild-type animals. We conclude that the mutation drastically decreases the efficiency of sox9\textalpha{} transcript splicing.

jeftw37 behaves as an amorphic mutation

The molecular genetic analysis of jef\textsuperscript{tw37} did not rule out the possibility that some message may be spliced normally in homozygous mutants. Coupled with the remnant bilateral patches of cartilage (Fig. 1G), the protection assays made us concerned that jef\textsuperscript{tw37} might be a hypomorph rather than a null allele. The classical test for a null allele is the Müller test: for a null allele, the phenotype of a homozygote equals that of a heterozygote for one mutant allele and one deletion allele (Müller, 1932). To perform this test, we crossed females heterozygous for jef\textsuperscript{tw37} to a male heterozygous for the deletion Df(LG12)dlx3\textsuperscript{b380} (Fritz et al., 1996), which removes a region of LG12 containing sox9\textalpha{} (data not shown). Fifteen of 56 offspring examined showed a jellyfish phenotype, and these were confirmed by PCR to be jef\textsuperscript{tw37}\textbackslash{}Df(LG12)dlx3\textsuperscript{b380}. Fig. 2D,E show that homozygous jef\textsuperscript{tw37}/jeftw37 and heterozygous jef\textsuperscript{tw37}\textbackslash{}Df(LG12)dlx3\textsuperscript{b380} had the same severity of skeletal phenotype. We conclude that jef\textsuperscript{tw37} behaves as a null allele in the Müller test.

Molecular genetic nature of the jef\textsuperscript{hi1134} mutation

The jef\textsuperscript{hi1134} mutation

An allele with a molecular lesion that deletes protein function would strengthen interpretation of the mutant phenotype. We identified jef\textsuperscript{hi1134} in an insertion/mutation screen (Amsterdam et al., 1999; Burgess and Hopkins, 2000). The virus inserted into codon Leu147, 2 bp from the 5’ end of exon 2 inside the HMG domain (Fig. 3), which would form a truncated Sox9a protein and probably destroy protein function.

To confirm that the mutant phenotype of jef\textsuperscript{hi1134} homozygotes is due to the viral insertion in sox9\textalpha{}, we mapped the insertion site with respect to the mutant phenotype. We mated a female heterozygous for jef\textsuperscript{hi1134} to the TAB14 wild-type strain and crossed the resulting F1 progeny to obtain an F2 mapping population. We scored 86 F2 individuals for their genotypes by PCR. The mutant and wild-type alleles were distinguished by the forward primer 5’LTR which binds in the insertion, and the forward primer t2a, which binds in intron-1 (Fig. 3). When these are paired with a common reverse primer RT2, which binds in exon-2, the 5’LTR/RT2 primer pair amplified a band of 628 bp with mutant genomic DNA but no band with homozygous wild-type DNA. The t2a/RT2 pair gave a 237 bp fragment with wild-type DNA, and no band with mutant DNA (Fig. 3C). The results showed that of 45 phenotypically mutant F2 individuals tested, all showed only the 628 bp band expected for homozygous insertions. Of 22 phenotypically wild-type segregants tested, 14 showed both bands expected for heterozygotes and eight showed only the
237 bp band expected for wild-type genomic DNA. Thus, among 53 informative individuals, there were no recombinants. This places the insertion within 1.9±11 cM (95% confidence) (Crow, 1950) of the lesion causing the mutant phenotype. Taken with the failure of jefhi1134 to complement jef tw37, we conclude that the viral insertion in sox9a is responsible for the jellyfish phenotype in jefhi1134 homozygotes.

The jefhi1134 mutation inhibits production of mature message

If the insertion in jefhi1134 causes the mutant phenotype, it could block the formation of mature sox9a mRNA by one of at least two mechanisms. First, because it is so close to the splice acceptor site, it might cause the splicing machinery to skip exon 2 entirely. Alternatively, splicing might occur normally, but the message with the insert would be unstable. If the insertion in jefhi1134 causes skipping of the entire 251 bp exon 2, a primer pair in exon 1 and exon 3 (F/R, Fig. 3) would yield a transcript 251 bp shorter than wild-type. To test these possibilities, we prepared cDNA from mutant and wild-type embryos and amplified the cDNAs. The results showed that in wild type, the predominant band is the 627 bp wild-type product. In mutant animals, however, the predominant band is the 376 bp product produced by neatly skipping exon 2. Direct sequencing of wild-type and mutant PCR products from cDNA confirmed that in jefhi1134, exon 1 splices directly to exon 3.

Translation of the resulting transcript should add 15 out-of-frame amino acids derived from exon-3 after Leu147. As a final test of sox9a expression in mutant embryos, we performed in situ hybridization experiments on wild-type and homozygous jefhi1134 embryos. The experiments showed reduced signal in the mutant embryos (see Figs 6, 7). Because the viral insertion alters sox9a mRNA in a way that would produce a truncated protein with a disrupted HMG box, jefhi1134 is highly likely to be a null mutation.

Inhibition of sox9a function with morpholinos

Morpholinos against sox9a produce a phenotype similar to jef

To confirm that reduction in sox9a function results in the jef phenotype, we injected embryos with morpholino antisense oligonucleotides targeted to sox9a. Injecting homozygous wild-type embryos with 5.0 mg/ml of two splice junction MOs (i1d and i2d) greatly reduced cartilage in the pharyngeal arches (Fig. 4E). Treating the offspring of heterozygous jefhi1134 males and females with sox9a MOs gave animals in three phenotypic classes. Some animals had hypoplastic but recognizable cartilages (Fig. 4E); others had rudimentary first and second arch cartilage elements with two rather large blocks of cartilage remaining at about the location of the ceratohyal (Fig. 4H); and some animals had the typical homozygous jefhi1134 phenotype, with two small blocks of Alcian-positive material remaining (Fig. 4K). Genotyping the animals for the HphI site polymorphism in jefhi1134 before Alcian staining showed that among 91 animals, the least severely affected class (20 animals, 22%) were all homozygous wild type; the intermediate class (47 animals, 51%) were all heterozygotes; and the animals with the severe phenotype were all homozygous jefhi1134 (24 animals, 26%). These genotypes were found in the ratio of ~1:2:1 as expected in the progeny of two heterozygotes. We conclude that heterozygous jef animals are more sensitive to sox9a MO than homozygous wild-type animals. The series of phenotypes found in these experiments suggests that the remnant bilateral Alcian-positive regions in jef animals may be portions of the ceratohyal.

The pectoral fin skeletons of MO-injected animals showed a similar pattern. Alcian stained wild-type pectoral fins at 5 dpf (days post-fertilization) showed a flat endoskeletal disc, the basal scapulocoracoid of the pectoral girdle, and the cleithrum, a long straight bone (Fig. 4C) (see Grandel and Schulte-Merker, 1998). The scapulocoracoid cartilage was missing in homozygous and heterozygous jefhi1134 animals treated with MO, but the endoskeletal disk and cleithrum were nearly normal (Fig. 4IL). The scapulocoracoid in the pectoral fins of homozygous wild-type animals injected with the MOs was reduced to two small patches of six to ten cells (Fig. 4F).

To determine the efficacy of splice-
Inhibiting splicing with morpholinos alters the intracellular distribution of sox9a transcript

In normal wild-type animals, sox9a transcript accumulates in the cytoplasm (Fig. 5C). Does the mutation in the splice-donor site or a splice-inhibiting MO hinder the transport of transcript to the cytoplasm? By the two-somite stage (Fig. 5C), the cytoplasm of presumptive cranial placode cells accumulated substantial quantities of sox9a transcript in wild-type embryos. By contrast, the difference in transcript amount between the cytoplasm and the nucleus of the corresponding cells in jefw37 embryos is rather small (Fig. 5C). Wild-type animals treated with the splice junction MOs i1d and i2d showed no apparent sox9a transcript in the cytoplasm, but accumulated substantial quantities of transcript in the nucleus. We conclude that MOs directed against a splice junction can block the transport of transcript from the nucleus to the cytoplasm. The inappropriate localization of transcript can provide an assay for MO efficacy in the absence of an antibody to test for the production of a translated product.

The MO results allow several conclusions. First, the similarity of sox9a MO and jellyfish phenotypes supports the conclusion that jef mutations disrupt the function of sox9a; we therefore call this gene jef (sox9a) according to zebrafish nomenclature guidelines (http://zfin.org/zf_info/nomen.html). Second, the failure of the splice-junction MOs to enhance the phenotype of jefw37 homozygotes is consistent with the interpretation of jefw37 as a null allele. Third, the results suggest that both mutant alleles of jef are more effective at knocking down sox9a activity than are the MOs, perhaps because the morpholinos are less effective at blocking a late phenotype. And fourth, the accumulation of transcript in the nucleus with splice-directed morpholinos can provide an assay for morpholino efficacy independent of phenotype.

An essential role for sox9a in chondrogenesis

These results show that jef (sox9a) is essential for the formation of cartilages in the neurocranium, pharyngeal arches and pectoral appendages, but do not reveal which step in cartilage formation requires the gene. If jef (sox9a) is required for the migration of crest cells, cranial crest, as marked by dlx2 expression (Akimenko et al., 1994), should be aberrant in homozygous jefw37 embryos. In situ hybridization experiments showed that dlx2 and sox9a are expressed in the same groups of cells in 30-somite stage wild-type embryos (Fig. 6 and data not shown). In homozygous jefw37 and jefhi134 embryos, sox9a transcript is weakly detected at this stage (Fig. 6C,E), but the expression pattern of dlx2 is unperturbed (Fig. 6D,F). These results suggest that sox9a is not required for the specification of cranial crest or for the migration of crest cells into the pharyngeal arches.

To determine if postmigratory cranial neural crest is properly specified in jef mutants, we examined two markers of ventral postmigratory crest, dhand and epha3 (Miller et al., 2000). Expression of these genes at 36 hpf showed no distinguishable alterations in jef mutants (data not shown). Thus, at least the ventral postmigratory pharyngeal arch crest in jef mutants is properly specified with respect to these two markers.

The expression of col2a1 marks differentiating chondrocytes in zebrafish (Yan et al., 1995) and Col2a1 is essential for proper chondrogenesis in mammals (Vandenberg et al., 1991). To determine whether jef (sox9a) is essential for col2a1 expression in pharyngeal arches, we compared the expression domains of sox9a and col2a1 in wild-type embryos, and tested whether the expression of col2a1 was altered in jef (sox9a) homozygotes.
Fig. 6. Activity of sox9a is not required for the specification or migration of cranial neural crest. (A,B) Wild type (WT) embryos. (C,D) Homozygous jef<sup>tw37</sup> embryos. (E,F) Homozygous jef<sup>hi1134</sup> embryos. (A,C,E,G,I,K) sox9a expression. (B,D,F) dlx2 expression. All animals were 24 hpf. Dorsal views, anterior towards the left. (B,D,F,H,J,L) Ventral views, vesicle. (A,C,E,G,I,K) Lateral views, anterior scapulocoracoid; ed, endoskeletal disc; ov, otic vesicle. (A,C,E,G,I,K) Lateral views, anterior towards the left. (B,D,F,H,J,L) Ventral views, anterior towards the left. Scale bars: in K, 100 µm for A,C,E,G,I,K; in L, 100 µm for B,D,F,H,J,L.

The results show that the neurocranium, pharyngeal arches and pectoral fins co-express col2a1 and sox9a in wild-type embryos (Fig. 7A,B,G,H), although col2a1 shows additional expression in the presumptive precursors of the cartilage capsule of the ear and eye. In mutant animals, sox9a expression is reduced (Fig. 7C,F), and col2a1 expression appears in only small regions of the pharyngeal arches (Fig. 7I-L). Ventral groups of cells in the first and second arches retain col2a1 expression. The expression in the second arch may correspond to the remaining bilateral Alcian-positive patches found later in mutant animals (see Fig. 1C,G,K,O). These results show that in much of the pharyngeal arch skeleton, the expression of col2a1 depends on sox9a activity.

Prechondrogenic condensations form in jef (sox9a) mutants, but cartilage differentiation and condensation morphogenesis fail to occur

Because migratory and postmigratory cranial neural crest are present in jef (sox9a) mutant embryos (Fig. 6), the severe reduction of differentiated (Alcian-positive) cranial cartilage seen later in jef (sox9a) mutant larvae is due either to the failure of prechondrogenic condensation formation or to the failure of condensation progression into differentiated cartilage. To distinguish between these possibilities, we mated heterozygous jef (sox9a) males and females, and labeled the resulting embryos with the vital fluorescent dye BODIPY-ceramide. This dye fills extracellular spaces, thus labeling cell outlines, and has the powerful advantage of allowing histological identification of nearly every cell type in live preparations (Cooper and Kimmel, 1998). We examined live developing BODIPY-ceramide-stained larvae at multiple time points from 48 hours, when no pharyngeal cartilage differentiation had occurred in wild types (Schilling and Kimmel, 1997), until 76 hours, when the primary scaffold of the larval pharyngeal skeleton had chondrified. At 48 hours, wild type and mutants for both mutant alleles of jef (sox9a) had precartilage condensations in the first two pharyngeal arches (Fig. 8A-B’ and data not shown). Although at this stage no differentiated (Alcian-positive) pharyngeal cartilage was present (Schilling and Kimmel, 1997), the primordia of the major cartilages in the first two arches were readily identifiable in wild type and mutant (Fig. 8A-B’). For example, the hyomandibular foramen was present, as was the rudiment of the symplectic (Fig. 8A-B’). By 54 hours, differentiation had begun in wild type (Schilling and Kimmel, 1997), but had failed to occur in jef (sox9a) mutants (data not shown). Concomitant with differentiation, in wild-type embryos chondrocytes organized into orderly stacks (data not shown) (Kimmel et al., 1998).

By 76 hours, cartilages in the first and second arches of wild-type embryos were well-formed, whereas jef (sox9a) mutants had failed to undergo three major morphogenetic processes. First, jef (sox9a) mutants failed to form stacks of chondrocytes (Fig. 8C-D’ and data not shown), but cells in wild-type precartilage condensations oriented themselves with their long axes parallel to each other (Fig. 8C,C’) (Kimmel et al., 1998). The only pharyngeal cartilages to differentiate in jef (sox9a) mutants, the small bilateral nodules of disorderly cartilage that form in the ventral second arch (see Figs 1, 4) lacked orderly stacks of chondrocytes. Second, the precartilage condensations in jef (sox9a) mutants failed to separate into individualized regions. For example, the prominent dorsal/ventral joint that

Fig. 7. Activity of sox9a is necessary for the expression of col2a1 in most developing cartilage of the neurocranium, pharyngeal and pectoral skeleton. (A-F) Expression of sox9a. (G-L) Expression of col2a1. (A,B,G,H) Wild-type animals. (C,D,I,J) Homozygous mutant jef<sup>tw37</sup> embryos. (E,F,K,L) Homozygous mutant jef<sup>hi1134</sup> embryos. Photographs are a montage with focus on the pharyngeal cartilages, and the pectoral girdle. All animals are 68 hpf. 1 and 2, first and second pharyngeal arches; co, scapulocoracoid; ed, endoskeletal disc; ov, otic vesicle. (A,C,E,G,I,K) Lateral views, anterior towards the left. (B,D,F,H,J,L) Ventral views, anterior towards the left. Scale bars: in K, 100 µm for A,C,E,G,I,K; in L, 100 µm for B,D,F,H,J,L.
Early bone formation is largely unaffected in jef(sox9a) mutants

Mice heterozygous for a Sox9 mutation exhibit expanded...
Fig. 9. Muscle and bone in jef mutants. Expression of titin in wild-type (A) and homozygous jefw37 (B) and jefhi1134 (C) animals at 3 dpf reveals normal patterning of cranial muscles. Compiled stacks of confocal micrographs of wild-type (D) and a jefw37 mutant (E) stained with calcein. Most dermal bones (op, bsr, cl) are relatively unaffected in jef mutants, whereas the dermal dentary (de) and cartilage replacement fifth ceratobranchial bone (cb5) are severely reduced. Teeth (te) are present, as are tiny remnants of bone at the base of the teeth. Bone development occurs on schedule without enlarged ossification centers in jef mutants. am, adductor mandibulae; bsr, branchiostegal ray; cb5, fifth ceratobranchial; cl, cleithrum; de, dentary; hh, hyohyoideus; ih, interhyoideus; ima, intermandibularis anterior; imp, intermandibularis posterior; op, opercle; sh, sternohyoideus; te, teeth; tv1, transversus ventralis. Scale bar: in A, 100 μm for A-C.

DISCUSSION

These experiments identify jef as a mutation in sox9a, one of the two zebrafish orthologs of the human SOX9 gene. Evidence for this conclusion comes from the sequencing of sox9a from animals homozygous for either of two jef alleles, one induced by ENU and the other by retroviral insertion. The ENU-induced mutation changed a conserved G immediately following the splice junction (Mount, 1982; Kreivi and Lamond, 1996). Mutations of this nucleotide at the corresponding site in the second intron of the human SOX9 gene can cause CD (Wagner et al., 1994), and similar mutations disrupt many other genes in humans (Freddi et al., 2000; Sironi et al., 2001; Targovnik et al., 2001) and in zebrafish (Lun and Brand, 1998; Childs et al., 2000). A quantitative assay for sox9a transcript and in situ experiments confirmed that the mutation in jefw37 severely inhibits splicing of the sox9a transcript. The insertion allele results in the skipping of exon 2, and the predicted subsequent translation of a short protein truncated within exon 3.

Two jef (sox9a) alleles behave as null mutations

To infer the role of a gene from its mutant phenotype, it is essential to know whether the alleles investigated lack all function of the gene. This is relevant here because, first, the mammalian mutants retain one normal SOX9 allele and, second, because the zebrafish mutants retain a small patch of Alcian-positive material in the location of the second arch. The zebrafish phenotype could result either from residual activity of sox9a from the mutant allele, or from activity of a different gene. We conclude that the two jef (sox9a) alleles are likely null alleles because: (1) the viral insertion in jefhi1134 causes the skipping of exon 2, which should result in a truncated protein; (2) animals heterozygous for jefw37 over a deletion do not have a more severe phenotype than jefw37 homozygotes; (3) morpholinos that affect wild-type and heterozygous embryos do not make the jefw37 phenotype more severe; and (4) the phenotype of sox9a (Y.-L. Y., unpublished). Although it is well known that MOs can inhibit splicing (Schmajuk et al., 1999; Draper et al., 2001), to our

splice-directed morpholinos provide an independent assay for efficacy

Animals injected with splice-directed morpholinos displayed a weaker phenotype at day 4 than did homozygous jef(sox9a) mutations, presumably because of the rebound of transcript splicing as evidenced by the RNase protection assays. MO-treated animals accumulated sox9a transcript in the nuclei of sox9a-expressing cells, apparently because of a defect in transcript transport. We have also observed this phenomenon for splice-directed morpholinos against sox9b (Y.-L. Y., unpublished).
knowledge this inhibition had not previously been shown to retard the transport of transcript to the cytoplasm. Our novel finding provides an assay for MO efficacy independent of any phenotypic change. This assay or the RNase protection assay, is generally more convenient than an assay for the efficacy of a translation-inhibiting MO because probes to measure nucleic acid quantity are much more readily available than probes to measure the quantity of a specific protein, which often requires a specific antibody. Furthermore, as pointed out by Draper et al. (Draper et al., 2001), splice-directed morpholinos may allow one to distinguish between the effects of different splice variants, and to distinguish between the functions of maternal and zygotic transcript.

**Evaluating jef (sox9a) mutants as a model for campomelic dysplasia**

People affected with CD are heterozygous for a mutation in SOX9, and display a syndrome of clinical features that include bowing of the tibia and femur, hypoplastic scapula, absence of a pair of ribs, cleft palate and a small jaw (Houston et al., 1983; McKusick, 1990; Foster et al., 1994; Wagner et al., 1994; Kwok et al., 1995; Mansour et al., 1995; Cameron et al., 1996; Hageman et al., 1998). Zebrafish homozygous for jef (sox9a) mutations mimic at least two of these phenotypes, but show them in more severe form. In humans and mice (Bi et al., 1999; Bi et al., 2001) heterozygous for SOX9 mutations, the scapulas and jaws form, but they are small and thin. By contrast, the corresponding elements in zebrafish jef (sox9a) mutants, the scapulocoracoid cartilage and the first and second arch derivatives, are almost completely gone.

Why is the zebrafish sox9a mutant phenotype more severe in these aspects than the mammalian SOX9 mutant phenotypes? This question is significant because we need to know whether SOX9 is essential for development of these elements or whether it merely facilitates completion of these cartilages. Many SOX9 mutations in mammals are likely to be null activity alleles rather than dominant negative mutations, judging from their predicted effect on the proteins (Foster et al., 1994; Wagner et al., 1994; Kwok et al., 1995; Mansour et al., 1995; Cameron et al., 1996; Hageman et al., 1998; Bi et al., 1999; Bi et al., 2001). Thus, the heterozygotes probably have about half the normal amount of SOX9 activity in tissues in which the gene is expressed. Homozygous jef (sox9a) animals should completely lack sox9 activity in all cells in which sox9a is expressed in the absence of sox9b.

Before hatching, the time during which the jef (sox9a) phenotype becomes apparent, sox9a is expressed strongly in the first and second arches and in the scapulocoracoid, but sox9b is not expressed in these cells (Chiang et al., 2001). We therefore conclude that SOX9 activity is essential for chondrogenesis of the arches, neurocranium and scapulocoracoid, and that mammals show a weak phenotype because SOX9 activity is reduced, but not completely lost, in the mammalian heterozygous genotypes. The evolution of duplicated zebrafish genes has thus allowed analysis of null-activity embryos not yet available for the ortholog in mammals.

Many individuals with CD have XY sex reversal (Houston et al., 1983; McKusick, 1990; Foster et al., 1994; Wagner et al., 1994; Kwok et al., 1995; Mansour et al., 1995; Cameron et al., 1996; Hageman et al., 1998). Although sox9a is expressed in the zebrafish testis, and sox9b is expressed in the zebrafish ovary (Chiang et al., 2001), consistent with a role in sex determination, the late determination of sex in zebrafish has so far precluded the investigation of sex determination in jef (sox9a) animals. Because both male and female animals heterozygous for jef (sox9a) become sexually mature adults of both sexes, jef (sox9a) appears not to have a fully penetrant dominant effect on sex determination in zebrafish.

**Prechondrogenic condensations form in jef (sox9a) mutants**

The formation of prechondrogenic condensations in jef (sox9a) mutant zebrafish suggests that sox9a is not required for condensation formation in zebrafish. The opposite conclusion was drawn for mouse. Because cells homozygous for a Sox9 mutation failed to contribute to condensations in genetic mosaics, Bi et al. (Bi et al., 1999) concluded that Sox9 was required for formation of condensations. These differences might reflect: (1) species differences in SOX9 function; (2) the presence of duplicated sox9 genes in zebrafish; and/or (3) the difference in experimental paradigms used. Although jef (sox9a) mutant cells form condensations in the context of a totally mutant environment, they might not contribute to condensations when transplanted into a wild-type host, as was found with mouse. Mosaic analyses in zebrafish could test this possibility.

Support for the idea that differences in experimental paradigms could explain the contrasting conclusions on the requirement of SOX9 for condensation formation comes from analysis of the zebrafish valentino mutant. Embryos that lack val (mafb) activity make hindbrain tissue between rhombomeres 4 and 7, yet in genetic mosaics, val (mafb) mutant cells are excluded from this territory in a wild-type host (Moens et al., 1996). Thus, by analogy, homozygous Sox9 mutant mice, like sox9a mutant zebrafish, might form precartilage condensations even though mutant cells are excluded from these domains in a mosaic. A mammalian phenotype analogous to that seen in zebrafish jef (sox9a) mutants might be the L-Sox5; Sox6 double mutant in mouse, where condensations form, but no overt cartilage differentiation occurs (Smits et al., 2001). A conditional allele of mouse Sox9 has been made (Kist et al., 2002), which should facilitate phenotypic analysis of skeletal development in homozygous Sox9 mutant mice.

The simultaneous failure of chondrocyte differentiation and morphogenesis of condensations in jef (sox9a) mutants suggests that jef (sox9a) regulates both morphogenesis and differentiation, and provides another example of specification and morphogenesis going hand-in-hand (see Kimmel et al., 2001a; Kimmel et al., 2001b). These morphogenetic processes are separable from differentiation: the zebrafish pipetail [ppt (wnt5a)] mutation (Piotrowski et al., 1996; Rauch et al., 1997; Hammerschmidt et al., 1996) disrupts chondrocyte stacking but not differentiation. Thus, differentiation does not require stacking. Likewise, individuation of cartilage elements occurs in ppt (wnt5a) mutants (Piotrowski et al., 1996), suggesting that the jef (sox9a)-dependent morphogenetic processes are distinct aspects of morphogenesis under regulation of separate loci.
The failure of differentiation and morphogenesis in *jef* (*sox9a*) mutants correlates in time and space with the *col2a1* expression defect, and raises the possibility that *col2a1* might be involved in one or both of these processes. Because COL2A2 is a major component of differentiated cartilage matrix (Vandenberg et al., 1991), perhaps zebrafish *col2a1* is required for cartilage differentiation, and the failure to activate *col2a1* expression underlies the near complete absence of cartilage in *jef* (*sox9a*) mutants. The lack of Col2a might also underlie the morphogenetic defects in *jef* (*sox9a*) mutants – it is possible that cells require a normal extracellular matrix in order to exhibit stacking cell behaviors. It would be interesting to see if exogenously supplied Col2a could rescue either morphogenesis (e.g. stacking) or differentiation in *jef* (*sox9a*) mutants.

**SOX9** regulates not only COL2A1, but other downstream targets as well. **SOX9** positively regulates expression of **CDH2** (Panda et al., 2001), which may mediate the *jef* (*sox9a*)-dependent stacking, individuation or shaping of pharyngeal mesenchymal cells. Comparing expression of cadherin genes, *col2a1* and orthologs of other **SOX9** downstream targets in *ppt* and *jef* mutants might suggest which genetic pathways underlie stacking behavior and which cartilage differentiation. **SOX9** also regulates cell cycle genes (Panda et al., 2001), providing another potential mechanism for the differentiation and morphogenetic defects in *jef* (*sox9a*) mutants. Mitotic activity is enriched at the second arch dorsal/ventral joint, and was proposed to partially drive the extension of the symplectic cartilage (Kimmel et al., 1998). This region of the second arch condensation never extends in *jef* (*sox9a*) mutants, possibly because mitotic behavior of cells within the precartilage condensation is defective.

**Pharyngeal muscle and bone differentiates in the absence of differentiated cartilage**

Cranial neural crest (CNC) patterns the pharyngeal musculature according to Noden’s experiments transplanting presumptive first arch CNC into the position of presumptive second arch CNC (Noden, 1983). Although recently reinterpreted to be due to the organizing effects of a transplanted isthmus (Trainor et al., 2002), the conclusion remains that the transplant non-autonomously induced host second arch muscles to adopt patterns appropriate to the first arch muscles. Further evidence for an instructive role of CNC comes from the zebrafish mutant *chinless* (*chw*), which lacks both differentiated pharyngeal cartilage and muscles (Schilling et al., 1996). Wild-type CNC cells, when transplanted into homozygous *chw* mutant hosts, induced local differentiation of pharyngeal muscles (Schilling et al., 1996). A third study revealed severe ventral muscle defects in pharyngeal arches of *suc* (*edn1*) mutants, although mutant cells contributed to normal ventral muscles when transplanted into a wild-type host (Miller et al., 2000). These results support the idea that signaling from the CNC patterns the pharyngeal arch mesoderm. The populations of CNC which participate in this signaling, however, remain unidentified. The CNC-derived muscle connective tissue (Kontges and Lumsden, 1996) is a candidate for this activity. The widespread expression of *sox9a* raises the possibility that *jef* (*sox9a*) might function in a CNC-derived connective tissue lineage. The skeletogenic CNC derivatives, some of which are perturbed in *jef* (*sox9a*) mutants, could also signal to the pharyngeal musculature. The presence of a normal pattern of differentiated pharyngeal muscles in *jef* (*sox9a*) mutants shows that signals from CNC to the surrounding mesoderm occur independently of *jef* (*sox9a*) function. Muscles in *jef* (*sox9a*) mutants are shorter and thicker that their wild-type counterparts, suggesting that elongation of the muscles might require stiff, differentiated cartilage.

The precocious ossification and expanded ossification centers in heterozygous **Sox9** mutant mice motivated the characterization of bone formation in *jef* (*sox9a*) mutants. Our calcine labeling confirms observations of bone (Piotrowski et al., 1996), and further demonstrates that cranial and pectoral fin bones form relatively normally in *jef* (*sox9a*) mutants, with the exceptions of the dentary and fifth ceratobranchial bone, a cartilage replacement bone (see Cubbage and Mabee, 1996) that appears to be surrounded by perichondral bone in wild-type 5-day-old zebrafish larvae. The absence of the fifth ceratobranchial bone in *jef* (*sox9a*) mutants could be explained if perichondral ossification requires a cartilage template. The dentary bone, a dermal bone (see Cubbage and Mabee, 1996), is intimately associated with Meckel’s cartilage in wild type. Perhaps the reduction of the dentary in *jef* (*sox9a*) mutants is also secondary to the cartilage defect. Weaker alleles of zebrafish *jef* (*sox9a*) might allow analysis of bone development past the early larval stage of currently available alleles.

Because dermal bones appear without a cartilage template, it might not seem surprising that most dermal bones form normally in *jef* (*sox9a*) mutants. The widespread expression of *sox9a* in postmigratory CNC, however, suggests that CNC osteocyte precursors might express *sox9a*. Expression of *sox9a*, like that of *dlx2* in the zebrafish pharyngeal arches, appears to include the entire CNC population within each arch. The *dlx2*-expressing postmigratory CNC forms a cylinder surrounding the central cores of paraxial mesoderm (Miller et al., 2000; Kimmel et al., 2001a). This population presumably includes precursors of both the cartilage and bone skeleton, based on existing fate maps (Coulby and Le Douarin, 1988; Schilling and Kimmel, 1997; Kontges and Lumsden, 1996). Thus, the absence of a major bone defect suggests that *jef* (*sox9a*) function in the pharyngeal skeleton might actually be required only in a subset of *sox9a*-expressing postmigratory CNC cells, the chondrocyte lineage. Fate-mapping experiments will determine whether chondrocytes and osteocytes share a lineage in the CNC, and whether the chondrocyte lineage is uniquely perturbed in *jef* (*sox9a*) mutants.

**Sox9 and gene duplication**

*Xenopus* embryos treated with a morpholino antisense oligonucleotide that inhibits the production of Sox9 protein lack neural crest progenitors, suggesting that Sox9 is essential for the specification of neural crest in frogs (Spokony et al., 2002). By contrast, neural crest specification is apparently normal in individuals with CD, because they make cranial crest derivatives, but just have hypoplastic skeletons, and in the absence of *sox9a* function in zebrafish, because the expression of *dlx2* is normal, the cranial crest migrates on schedule, and precartilage condensations form. How can we understand these differences? We hypothesize that **SOX9** may play multiple essential roles in cranial crest development, and that these may be revealed by further analysis of the two **SOX9** duplicates in zebrafish. As demonstrated by the work on *Xenopus* (Spokony...
et al., 2002). Sox9 protein plays an early role in crest specification, and as revealed by our experiments on zebrafish and work with mouse, sox9 plays a later role in chondrocyte differentiation that includes the regulation of col2a1 and the morphogenesis that accomplishes stacking. Because the antisense methodology blocked the early step of crest specification in Xenopus, it was not possible to determine whether sox9 is required for the later morphogenetic roles. Zebrafish has two orthologs of SOX9 that have diverged in sequence and expression pattern. The sox9b gene is expressed early in the neural crest of the head and body axis, like the Xenopus Sox9 gene (Chiang et al., 2001; Li, 2002). We predict that sox9b may be necessary for the neural crest specification function revealed in Xenopus (Spokony et al., 2002), and that sox9a is necessary for the later step, as revealed by the jef (sox9a) mutations studied here. In the haploinsufficient mammalian mutants, the level of SOX9 activity is likely to be half the normal level, and this may be sufficient for the early role in neural crest specification, and for much, but not all of the later role in cartilage morphogenesis.

The genome duplication that occurred in the ancestry of zebrafish (Amores et al., 1998; Postlethwait et al., 1998) was followed by nonfunctionalization so that zebrafish retains duplicate orthologs of about 30% of tetrapod genes (Postlethwait et al., 2000). Because subfunctionalization may preserve duplicate genes (Force et al., 1999; Stoltzfus, 1999), ancestral functions may assort to different duplicate copies. The expression patterns of sox9a and sox9b (Chiang et al., 2001) show overlapping subsets of the tetrapod SOX9 expression pattern (Spokony et al., 2002; Wright et al., 1995), consistent with the hypothesis of subfunctionalization. Such subfunctionalization can reveal gene functions that are hidden by analysis of morpholino-injected animals or knock-out mutations in tetrapods because the absence of an early function in a cell lineage may preclude the detection of later functions. As has been the case with Nodal genes, where analysis of zebrafish co-orthologs of Nodal revealed an early function in the induction of mesoderm, and a previously obscured later function in neural plate patterning (Feldman et al., 1998; Sampath et al., 1998; Rebagliati et al., 1998; Blader and Straule, 1998; Nomura and Li, 1998). Such may be the case with sox9a and sox9b as well. In particular, the early expression of sox9b in cranial crest may provide protein that might persist and partially compensate for the loss of sox9a function, even though sox9b is not expressed in post-migratory crest. Further analysis of sox9b can test these possibilities.

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