INTRODUCTION

Craniofacial development involves a complex series of morphogenetic and molecular events in which diverse cell types within the branchial arches give rise to bones, cartilage and nerves of the head and neck (Noden, 1988). Neural crest cells, which originate from the dorsal lip of the neural tube, migrate into the developing branchial arches and execute specialized programs of migration, patterning, proliferation and differentiation in response to extracellular signals and interactions with adjacent epithelial and mesodermal cells (Le Douarin, 1982; Lumsden et al., 1991; Maschhoff and Baldwin, 2000; Trainor et al., 2002). This unique population of cells serves as the source of precursors for the craniofacial skeleton, as well as a subset of peripheral neurons and vascular structures. While it is apparent that neural crest cell diversification is essential for the genesis of these different craniofacial structures, relatively little is known of the transcriptional pathways that subdivide populations of neural crest cells in different territories within the developing branchial arches.

The peptide ligand endothelin-1 (ET-1; also known as Edn1) plays a key role in regulating branchial arch development. Targeted mutations of the genes encoding ET-1, the G protein-coupled endothelin receptor A (ETA, EndrA) and endothelin converting enzyme-1 (ECE-1), show identical phenotypes characterized by abnormalities in branchial arch-derived skeletal elements, arteries and the cardiac outflow tract (Clouthier et al., 1998; Kurihara et al., 1995; Kurihara et al., 1994; Yanagisawa et al., 1998). ET-1 is secreted by the surface epithelium and the paraxial mesodermal core of the branchial arches, and acts on surrounding ectomesenchymal cells that express ETA. Pharmacological interventions with an ETA antagonist in chick embryos showed that ET-1/ETA-mediated signaling is critical for development of the lower beak and other distal branchial arch derivatives during the time period corresponding to colonization of EndrA-positive post-migratory neural crest cells (Kempf et al., 1998). In addition, the gene responsible for the sucker mutation in zebrafish was shown to encode ET-1, and defects observed in sucker mutants, such as severe hypoplasia of the lower jaw and malformations of distal (ventral) branchial arch cartilages can be rescued by injection of ET-1 orthologs or administration of human recombinant ET-1 (Miller et al., 2000). These findings suggest
that a common signaling pathway involving ET-1/ETA is conserved among zebrafish, birds, and mammals, and is essential for development of branchial arch-derived structures.

ET-1 is required for expression of the basic helix-loop-helix (bHLH) transcription factor genes dHAND/Hand2 and eHAND/Hand1 in the menenchyme of the anterior branchial arches (Thomas et al., 1998; Clouthier et al., 2000). We have shown that a 208 bp enhancer upstream of the dHAND gene is sufficient to drive expression of dHAND in the mandibular component of branchial arch 1 and branchial arch 2 (hyoid arch) in mice, and that activity of this enhancer is completely abolished in Ednra null embryos, suggesting that it is a downstream target for ETA signaling (Charité et al., 2001). This enhancer contains a series of conserved A TTA motifs that correspond to the consensus-binding motif for many homeodomain proteins. Mutation of these sites abolishes expression of a linked transgene in branchial arches 1 and 2 of transgenic mouse embryos at E10.5, suggesting that binding of homeodomain transcription factors to these sites is essential for enhancer activity. Consistent with this notion, the distal-homeodomain protein Dlx6 binds these sites and is expressed in a pattern that overlaps that of dHAND in the branchial arches. Expression of Dlx6 is undetectable in the distomedial branchial arches of Ednra null embryos, suggesting that Dlx6 is a key transcription factor involved in ETA-dependent regulation of dHAND in the branchial arch.

Because mice homozygous for a dHAND null allele die from cardiac abnormalities prior to branchial arch development, the specific role of dHAND in development of these structures has been unclear. To address this question, we generated mutant mice in which the ETA-dependent neural crest enhancer of dHAND was deleted by homologous recombination. Mice homozygous for this enhancer deletion fail to express dHAND in the ventral lateral region of the first and second branchial arches and show lethal craniofacial abnormalities that include cleft palate and malformations of the mandible and Meckel’s cartilage. However, expression of dHAND in craniofacial development and reveal unanticipated molecular heterogeneity in the transcriptional pathways that subdivide cells within the branchial arch neural crest.

**MATERIALS AND METHODS**

**Gene targeting**

Three overlapping dHAND phage clones encompassing approximately 18-kb of upstream flanking sequence were isolated from a mouse 129 SV genomic library using the dHAND cDNA as a probe (Srivastava et al., 1997), and cloned into pBlueScript vector (Stratagene, Inc.) for endonuclease restriction mapping. A 754 bp Xhol-BamHI fragment containing a 208 bp branchial arch enhancer SspBam208 described previously (Charité et al., 2001) was replaced by a neo gene cassette driven by the PGK promoter and floxed by two loxP sites (see Fig. 1). A 3 kb fragment 5′ to the unique Xhol site was used as a short arm and a 36 kb BamHII-NotI fragment was used as a long arm; a thymidine kinase gene driven by the MCI promoter was used for negative selection. An Xhol site was introduced adjacent to the 5′ end of an upstream loxP site in the targeted construct.

Linearized targeting vector DNA was electroporated into SM-1 embryonic stem (ES) cells, which were subsequently selected under G418 and FLAU as described previously (Yanagisawa et al., 2000). Genomic DNA was prepared from ES cell clones and digested with SacI for hybridization with a 5′ probe, and with NdeI and XhoI for a 3′ probe. Targeted clones were expanded and injected into blastocysts from C57BL/6 mice and resultant chimeras were bred to C57BL/6 mice to obtain germ line transmission. Heterozygous mutant mice for the dHAND branchial arch enhancer (+neoEdnra) were intercrossed to obtain +neoEdnra homozygous mutants. To obtain heterozygous mutants for the branchial arch enhancer without a neo′ cassette (AneoEdnra), +neoEdnra heterozygous mutants were bred to transgenic mice expressing Cre recombinase under the cytomegalovirus immediate early enhancer-chicken β-actin hybrid promoter (CAG) (Sakai and Miyazaki, 1997). The resulting ΔneoEdnra heterozygous mutants were bred to the +neoEdnra heterozygous mutants to obtain ΔneoEdnra homozygous mutants.

**Genotyping and PCR**

Genotyping was performed by Southern blot analysis using genomic DNA isolated from tail biopsies or yolk sac preparations. We performed PCR amplifications of the neo gene (5′-TTCCACC-ATGATATCGCGACAGG-3′ for an upstream primer and 5′-TATCGGGCTGAGCTGGCAACAG-3′ for a downstream primer), and the Bαenh sequence (5′-TCTGGTCTCTCTACTGGAATTTTCT-3′ for an upstream primer and 5′-ATTCCCGAAGCATHCTGC-3′ for a downstream primer) to identify +neoEdnra, +neoEdnra′ or the AneoEdnra mutant. For detection of the Cre transgene, PCR primers (5′-AGGTTCCGTTACTCATGAAG-3′ for an upstream primer and 5′-TCGACAGGTTATGAACATCCACC-3′ for a downstream primer) were used. For a control, PCR primers (5′-TGGATAATCAAGGATTGAC-3′ for an upstream primer and 5′-ACGCTCTGATGTTATCACTGAT-3′ for a downstream primer) were used. Southern blot analysis was performed to distinguish wild-type mice from the ΔneoEdnra mice.

**Histology and skeletal analysis**

For routine histological analysis, embryos were fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned at 5 μm. Paraffin sections were stained with Hematoxylin and Eosin. For skeletal analysis, postnatal day 1 (P1) embryos were collected, prepared as described previously (Charité et al., 2001; Thomas et al., 1998) with 35S-UTP (Amersham) using the Maxscript In Vitro Translation Kit (Ambion). In situ hybridizations were performed as described previously (Shelton et al., 2000).

**In situ hybridizations**

E10.5 embryos were harvested and fixed in 4% paraformaldehyde overnight at 4°C. Riboprobes for dHAND, eHAND and Dlx6 were prepared as described previously (Charité et al., 2001; Thomas et al., 1998) with 35S-UTP (Amersham) using the Maxscript In Vitro Translation Kit (Ambion). In situ hybridizations were performed as described previously (Shelton et al., 2000).

**Whole-mount in situ hybridization**

Embryos were harvested at E10.5 and fixed in 4% paraformaldehyde overnight at 4°C. Whole-mount in situ hybridizations were performed as described previously (Clouthier et al., 2000) using digoxigenin-labeled riboprobes for dHAND, eHAND, Mlox, Msx1 and Msx2 (Thomas et al., 1998), Gxc, Dlx2 and Dlx3 (Clouthier et al., 2000), Dlx5 [a gift from J. L. R. Rubenstein (Liu et al., 1997)] and Dlx6 [a gift from G. Levi] and Alx3 (ten Berge et al., 1998). At least 3 embryos per genotype were examined per probe. Following whole-mount in situ hybridization, embryos were photographed using an Olympus SZX12 photomicroscope with an attached DP11 digital camera.
RESULTS

Targeted disruption of a dHAND branchial arch enhancer

We have previously identified an evolutionarily conserved 208 bp enhancer that directs dHAND expression in the first and second branchial arches during mouse embryogenesis (Charité et al., 2001). To test whether this enhancer is required for dHAND expression in vivo, and to further clarify the role of dHAND in development of branchial arch-derived structures, we generated a mutant dHAND gene in which a 754 bp XhoI-BamHI fragment containing the branchial arch enhancer was deleted by homologous recombination. The deleted 754 bp region was replaced with a neo cassette driven by the PGK promoter (+neoBAenh). In order to avoid possible interference of the PGK promoter with dHAND expression, we flanked the neo cassette with two loxP sites to permit removal of the neo cassette by Cre-recombinase, yielding a mutant gene referred to as Δ neoBAenh. This strategy has been used in other enhancer deletion studies in mice (Bouvier et al., 1996; Danielian et al., 1997).

The targeting vector was electroporated into ES cells and 480 colonies were screened by Southern blot analysis. Four independent ES clones containing a +neoBAenh mutant allele (data not shown) were injected into blastocysts obtained from C57BL/6 mice, and 6 chimeras were obtained. Three chimeras from two independent ES cell clones transmitted the mutant allele through the germline. The +neoBAenh heterozygous mice were then bred to transgenic mice expressing Cre-recombinase under control of the CAG promoter to establish mice were then bred to transgenic mice expressing Cre-recombinase under control of the CAG promoter to establish mice. The targeting vector was electroporated into ES cells and 480 colonies were screened by Southern blot analysis. Four independent ES clones containing a +neoBAenh mutant allele (data not shown) were injected into blastocysts obtained from C57BL/6 mice, and 6 chimeras were obtained. Three chimeras from two independent ES cell clones transmitted the mutant allele through the germline. The +neoBAenh heterozygous mice were then bred to transgenic mice expressing Cre-recombinase under control of the CAG promoter to establish mice were then bred to transgenic mice expressing Cre-recombinase under control of the CAG promoter to establish mice.

The +neoBAenh+/− mice carrying the Cre transgene were bred to +neoBAenh+/− heterozygous mutant mice, and genotyping of progeny was performed by Southern blot analysis. As shown in Fig. 1B, hybridization of SacI-digested tail DNA with a 5′ probe resulted in a 13.5 kb band for the wild-type and Δ neoBAenh alleles, whereas the +neoBAenh allele gave a 6.5 kb band because of an additional SacI site in the neo cassette. Hybridization of NdeI- and Xbal-digested tail DNA with a 3′ probe yielded bands of 16 kb for the wild-type allele, 12 kb for the +neoBAenh allele and 9.5 kb for the Δ neoBAenh allele. Next, we performed PCR analyses to confirm that the branchial arch enhancer sequence was deleted in +neoBAenh+/− and Δ neoBAenh−/− mice. As shown in Fig. 1C, a 500 bp neo band was absent in the presence of Cre-recombinase and a 300 bp band corresponding to the branchial arch enhancer was absent both in the +neoBAenh+/− and the Δ neoBAenh−/− mutants.

Deletion of the branchial arch enhancer is sufficient to cause craniofacial abnormalities in BAenh−/− embryos

Genotyping of postnatal day 28 mice revealed no viable BAenh−/− mice among more than 100 offspring examined. The BAenh−/− mutants with or without a neo cassette showed an identical phenotype of hypoplastic jaw (Fig. 2A), and all died within 24 hours of birth from failure to suckle. The secondary palate of the mutants failed to fuse along the midline of the oral shelf (Fig. 2B), and the stomach contained no milk. In the homozygous mutants there were no other gross
abnormalities related to branchial arch-derived craniofacial structures, the cardiac outflow tract or the great vessels (data not shown).

Histological examinations of P1 BAenh−/− mutants showed that the mandibular bones were hypoplastic and displaced laterally compared to those of wild-type mice (data not shown). The palate processes were elevated and fused to form the secondary palate in wild-type mice (Fig. 3A). In contrast, the secondary palate was defective in BAenh−/− mice (Fig. 3B, arrows). Consequently, the mutants had a cleft palate. The muscle fibers of the tongue were also less organized and seemed to be oriented randomly in the BAenh−/− mutant compared to wild-type littermates (tg in Fig. 3A,B). The distal symphysis of Meckel’s cartilage was present in both wild-type and BAenh−/− mutant mice (asterisk in Fig. 3C,D). Inner ear structures and middle ear ossicles were all present in the BAenh−/− mutants. To further examine craniofacial structures in BAenh−/− mutants, we compared bone and cartilage staining of homozygous mutants with wild-type littermates at P1. We observed that the mandible of homozygous mutants was much smaller in size and shortened (ma in Fig. 4B,D), compared with the mandible in wild-type mice (ma in Fig. 4A,C). A ventral view of the skull showed deformity of the mandible in the homozygous mutant (Fig. 4D compare with C). The well-developed Meckel’s cartilage was observed extending from the malleus to the middle of the mandible in the wild-type mouse (arrows in Fig. 4C). However in the BAenh−/− mutant mouse, Meckel’s cartilage was disrupted at the proximal end closer to the junction to the malleus (arrow in Fig. 4D). Close examination showed that the angle between the right and left mandibular bones was wider in the BAenh−/− mutant than in the wild-type mouse (Fig. 4K,L). In addition, the angular process was severely reduced, and an ectopic process was observed extending from the ventral surfaces of the mandible (Fig. 4M,N). Defects in the mandible were already apparent in BAenh−/− mutant embryos at E14.5 (Fig. 5B). Cartilage staining at E14.5 revealed the well-developed Meckel’s cartilage in wild-type embryos (Fig. 5A). In contrast, Meckel’s cartilage was obviously truncated in BAenh−/− mutant embryos (Fig. 5B). Interestingly, there was a cartilage primordium in the distal part of the mandible both in the wild-type and the BAenh−/− mutants (Fig. 5C, arrow in D). Meckel’s cartilage normally forms from proximal and distal primordia, and failure of expansion from either primordium could result in a truncated Meckel’s cartilage.

In P1 embryos, the tympanic rings were shortened and deformed in the BAenh−/− mutant mouse (compare arrows in Fig. 4E and F). In the wild-type mouse, bilateral palatine
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processes extended horizontally and fused to form the secondary palate (dotted line in Fig. 4E, arrows in G). In contrast, the palatine processes of the $Baenh^{-/-}$ mouse appeared not to be elevated and thus the secondary palate was not formed (dotted line in Fig. 4F, arrows in H). This causes the underlying presphenoid bone to be visible in ventral view (ps in Fig. 4H). The pterygoid bones were also deformed so that the relative angle to the basisphenoid bone was abnormal in the $Baenh^{-/-}$ mutant mouse (Fig. 4F). Although the remnants of the palatine processes were detectable in the $Baenh^{-/-}$ mouse, they did not fuse along the midline unlike those of the wild-type mouse (arrows in Fig. 4I). The middle ear ossicles seemed to be less affected, however, the projection of the manubrium of the malleus was abnormal (red arrow in Fig. 4P). Skeletal structures affected in the mutant are summarized in Table 1.

![Fig. 4. Craniofacial analysis of wild-type and $Baenh^{-/-}$ mice at P1.](image)

(A,C,E,G,I,K,O) wild-type; (B,D,F,H,J,L,N,P) $Baenh^{-/-}$ mutant mice. (A,B) Lateral view, (C-F) ventral view, (G-P) isolated bones and cartilages. (A-D) The mandible (ma) is hypoplastic and deformed, and Meckel’s cartilage (arrows in C and D) is truncated in the mutant mouse (B,D). (E,F) Tympanic rings (ty) are thickened and deformed in the mutant mouse (F, compare arrows in E and F). Fusion of the bilateral palatine processes observed in the wild-type mouse (E, dashed line) is absent in the mutant mouse (F, dashed lines). Asterisk in F indicates the presphenoid bone. (G,H) In the wild-type mouse, the secondary palate is formed by the fusion of bilateral palatine processes (arrows). In the $Baenh^{-/-}$ mouse, the palatine processes are not formed so that the presphenoid (ps) becomes visible. (I,J) Palatine bones viewed end on. Compare black arrows in I and J, which indicate fused palatine processes and absence of palatine processes, respectively. An asterisk indicates the presphenoid bone. (K,L) Ventral views of the mandibles. Note that the mandible in the $Baenh^{-/-}$ mouse is shorter and deformed. The angle between the left and right mandible is wider than that in the wild-type mouse. (M,N) Lateral views of the mandibles. Note that the gonial bone is hypoplastic and the projection of the manubrium is abnormal in the $Baenh^{-/-}$ mutant. bs, basisphenoid; crp, coronoid process; h, hyoid; i, incus; lo, lamina obturans; m, malleus; mc, Meckel’s cartilage; oc, otic capsule; pa, palatine; pt, pterygoid; tc, thyroid cartilage.

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<th>Arch Structure</th>
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<td>Mandible&lt;br&gt;Meckel’s cartilage&lt;br&gt;Tympanic ring bones&lt;br&gt;Gonial&lt;br&gt;Malleus&lt;br&gt;Pterygoid bones&lt;br&gt;Palatine bones</td>
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<td>Two</td>
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Table 1. Defects in branchial arch-derived structures in P1 $Baenh^{-/-}$ mutants
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dHAND expression in the ventral (distal) region of branchial arches 1 and 2 is unaffected in BAenh null embryos

To examine the effect of deletion of the branchial arch enhancer on dHAND expression, we compared dHAND expression in the +neo BAenh+/− and the ∆neoBAenh–/– mutants and wild-type embryos by in situ hybridization. As shown in Fig. 6, dHAND expression in wild-type embryos was observed in the ventral portions of the first and second branchial arches at E10.5 (Fig. 6A). In contrast, dHAND expression was abolished in all except the most ventral regions of branchial arch 1 in the +neo BAenh+/− (Fig. 6B,E) and the ∆neoBAenh+/− mutants (Fig. 6C). As expected, dHAND expression in the heart and limb was unaffected by deletion of the branchial arch enhancer (Fig. 6E).

We also examined whether eHAND expression was attenuated in the absence of the dHAND branchial arch enhancer. At E10.5, eHAND is expressed in the most ventral portions of branchial arches 1 and 2 of wild-type embryos (Fig. 6F). The eHAND expression pattern appeared identical in the branchial arches of BAenh−/− embryos (Fig. 6G and Fig. 7B), suggesting that a loss of dHAND expression did not induce compensatory up-regulation of eHAND expression. Notably, eHAND was not expressed in the region of the branchial arches where the dHAND neural crest enhancer is active. Since there is evidence for functional redundancy of dHAND and eHAND in some cell types (McFadden et al., 2002), their nonoverlapping expression in this region may account for the craniofacial phenotype in BAenh−/− embryos.

Dlx6 expression is unaffected in BAenh−/− embryos

Our previous studies showed that Dlx6 is an ETA-dependent branchial arch enhancer-binding factor and that the Dlx6 binding sites are essential for activity of this dHAND enhancer (Charité et al., 2001). To test whether Dlx6 is regulated independently of dHAND in the branchial arches, we examined Dlx6 expression in BAenh−/− embryos. At E10.5, Dlx6 expression was observed in the ventral aspect of branchial arches 1 and 2 of wild-type embryos, excluding the most ventral portions (Fig. 6H, Fig. 7G). This expression pattern was maintained in the branchial arches of BAenh−/− embryos (Fig. 6I, Fig. 7G). Most importantly, ventrolateral Dlx6 expression in the first branchial arch of mutant embryos was absent (arrows in B,C,E), whereas heart expression was not affected (h in E). Branchial arches 1 and 2 are indicated.

Fig. 5. Cartilage preparations of wild-type and BAenh−/− mutant embryos at E14.5. (A,C) Wild-type; (B,D) BAenh−/− mutant. In the mutant embryo, Meckel’s cartilage is truncated (compare black arrows in A and B). Close examination shows that the distal primordium of Meckel’s cartilage is formed in the mutant embryo (arrow in D). h, hyoid; mc, Meckel’s cartilage.

Fig. 6. In situ hybridization analysis of dHAND, eHAND and Dlx6 transcripts in wild-type (A,D,H), +neoBAenh+/− (B,E,G,I) and ∆neoBAenh+/− (C) embryos at E10.5. dHAND (A,B,C,D,E), eHAND (F,G) and Dlx6 (H,I) transcripts were detected by in situ hybridization to transverse (A-C,F-H) or sagittal (D,E) sections. Note that ventrolateral dHAND expression in the first branchial arch of mutant embryos was absent (arrows in B,C,E), whereas heart expression was not affected (h in E). Branchial arches 1 and 2 are indicated.
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Effect of the dHAND branchial arch enhancer on expression of other transcription factors in the branchial arches

Several transcription factors, including Gsc, Dlx2 and Dlx3, are down-regulated or absent in ectomesenchymal cells of branchial arches in EdnrA–/– embryos (Clouthier et al., 2000). In addition, activity of the 208 bp dHAND branchial arch enhancer is entirely dependent on ETA-mediated signals (Charité et al., 2001). To determine whether these factors are dependent on dHAND, we examined their expression in BAenh–/– mutants by whole-mount in situ hybridization. We chose panels of transcription factors whose expression patterns in the branchial arches overlapped spatially or temporally with that of dHAND, and/or that depend on ET-1/ETA mediated signals. We first examined dHAND expression in BAenh–/– mutants, and confirmed that dHAND was absent in the ventrolateral portions of the first and second branchial arches, while eHAND expression was not affected in the mutant embryos (Fig. 7A,B).

Msx1 and Msx2 are homeobox transcription factors regarded as general repressors of transcription in developing branchial arches, and are required for normal growth and development of branchial arch-derived structures (Satokata et al., 2000; Satokata and Maas, 1994; Takahashi et al., 2001). Msx1 was previously reported to be down-regulated in branchial arches of dHAND null embryos in an ET-1-independent manner (Thomas et al., 1998). The expression domains of Msx1 and Msx2 are overlapping and are detected in the ventrolateral aspects of the maxillary and mandibular arches of wild-type embryos at E10.5 (Fig. 7C,D). Surprisingly, Msx1 was not down-regulated in the branchial arches of the BAenh–/– mutants. Most likely, the residual expression of dHAND in the ventral portion of branchial arches 1 and 2 is sufficient to induce expression of Msx1 in the anterior branchial arches. Msx2 expression was largely unchanged in branchial arch 1 of BAenh–/– mutants, except that the expression domain appeared to be shifted slightly ventrally (Fig. 7D, ventral view).

As we previously reported, Gsc is expressed in the ventral aspect of the posterior half of the first mandibular arch and the anterior half of the second arch at E10.5, and is severely down-regulated in EdnrA–/– embryos (Clouthier et al., 1998). Inactivation of Gsc in mice results in defects of most of the facial region, suggesting its role in epithelial-mesenchymal interactions (Yamada et al., 1995). As Fig. 7E shows, the Gsc expression domain was maintained in BAenh–/– mutant embryos.

There are six Dlx genes in mice; Dlx1/Dlx2, Dlx7/Dlx3 and Dlx5/Dlx6 are organized as physically linked pairs (Stock et al., 1996). Dlx1 and Dlx2 are involved in development of derivatives of the maxillary primordia (Qiu et al., 1997; Qiu et al., 1995) and Dlx5 and Dlx6 have redundant roles in the development of the mandibular primordia (Acampora et al., 1999; Depew et al., 1999; Robledo et al., 2002), whereas the roles of Dlx3 and Dlx7 in craniofacial development have not been elucidated. Although expression of Dlx2 in the second branchial arch and Dlx3 in the mandibular and second branchial arch are almost undetectable in EdnrA–/– embryos, we did not observe any changes in Dlx2 or Dlx3 expression in BAenh–/– mutants compared with wild-type embryos (data not shown). As Fig. 7F and G show, Dlx5 and Dlx6 are robustly
expressed in the ventral aspects of the mandibular and second branchial arches of wild-type and homozygous BAenh mutants. Alx3, a homeobox gene related to Drosophila aristaless, is expressed in the neural crest-derived ectomesenchyme in the first and second branchial arches (ten Berge et al., 1998). There was no difference in the expression of Alx3 between the wild-type and the BAenh+/– embryos (data not shown).

**DISCUSSION**

**Ventrolateral expression of dHAND in branchial arches 1 and 2 is required for development of distal branchial arch structures**

To investigate the role of dHAND in development of branchial arch-derived structures, and to determine whether the ETA-dependent branchial arch enhancer of dHAND is solely responsible for branchial expression of dHAND in vivo, we deleted this enhancer from the mouse genome by targeted mutagenesis. Mice homozygous for this deletion mutation exhibit lethal craniofacial abnormalities.

Interestingly, the craniofacial defects observed in BAenh+/– mutants are milder than those in ET-1+/+ or EndrA+/– mice. The primordium of Meckel’s cartilage was observed in BAenh+/– mutants at E14.5, and all of the bones and cartilages from the mandibular and hyoid arches were present, though truncated or malformed, indicating that ventral expression of dHAND is not required for specification of the cell lineages that contribute to these structures. Rather, dHAND may be involved in differentiation events in these cell lineages such as regulating the genes required for proper condensation and differentiation of cartilages, namely Bmp2, Bmp4 and Fgf2 (Sarkar et al., 2000). Alternatively, maintenance of dHAND expression may be required for continuous proliferation of mesenchymal cells or maintenance of a local concentration of a survival factor such as FGF8 (Schneider et al., 2000), as suggested in the branchial arches of dHAND null embryos (Thomas et al., 1998).

It is interesting to note that dHAND has also been shown to regulate patterning of zeugopods and digits of the limbs (Charité et al., 2000). Misexpression of dHAND in the anterior region of the limb bud results in preaxial polydactyly and repatterning of posterior skeletal elements. Conversely, forced expression of dHAND mutant proteins that fail to bind DNA or activate transcription results in truncation of the zeugopods (McFadden et al., 2002). The limb patterning activity of dHAND has been attributed to its ability to induce ectopic expression of sonic hedgehog (Charité et al., 2000), a morphogen that establishes anteroposterior polarity in the developing limbs (Lauper et al., 1994). However, the downstream effectors of dHAND activity in zeugopod outgrowth have not been identified. It is not unreasonable to speculate that the same sets of effector genes might mediate the activity of dHAND in the growth of craniofacial and limb skeletal structures.

Msx1 was previously shown to be down-regulated in branchial arch 1 of dHAND null embryos (Thomas et al., 1998), whereas Msx-1 expression was not affected in EndrA+/– null embryos (D. E. C., unpublished observation). In our BAenh+/– homozygous mutants, we did not observe a significant change in Msx1 expression in the area where dHAND expression was abolished. This finding could be explained if the remaining ventral (distal) expression of dHAND induced a soluble factor(s) to maintain expression of Msx1 in adjacent cells within the branchial arches. Loss-of-function and gain-of-function of Msx2 have been reported to cause various craniofacial disorders in humans and mice, suggesting that gene dosage of Msx2 influences chondrogenesis and osteogenesis in vivo (Liu et al., 1995; Satokata et al., 2000; Wilkie et al., 2000). In the developing mandibular process, BMP4 induces expression of Sox9 and Msx2, which function as positive and a negative regulators of chondrogenesis, respectively (Semba et al., 2000). While the possibility that dHAND is involved in BMP4 signaling in the developing branchial arches remains to be investigated, it is interesting to note that BMP4 is sufficient to induce dHAND expression in post-migratory neural crest cells during terminal differentiation to become sympathetic neurons (Howard et al., 2000).

**A Dlx6-dependent enhancer controls dHAND expression in the ventrolateral, but not distal, portions of branchial arches 1 and 2**

Mice homozygous for the deleted enhancer failed to express dHAND in the ventrolateral portion of branchial arches 1 and 2. However, dHAND expression in the ventral (distal) portion was unaffected in the mutants. Since dHAND expression is abolished throughout the branchial arches except in the ventral most tip in ET-1 and EndrA+/– mutant mice, these results strongly suggest the involvement of at least two distinct ETA-dependent enhancers in the control of dHAND expression in developing branchial arches 1 and 2 in vivo.

Mesenchymal cells in the dorsal and ventral (distal) regions of the anterior branchial arches appear to be independently specified (Miller et al., 2000). Mosaic analysis in zebrafish showed that ventral postmigratory neural crest cells adopt a ventral fate when they interact with ventral paraxial mesoderm, which expresses suc/ET-1. These ventral mesenchymal cells were shown to express dHAND, msxE, Dlx3 and EphA3 in a suc/ET-1 dependent manner (Miller et al., 2000). The complexity of dHAND expression revealed in the present study clearly points to the involvement of multiple spatially restricted neural crest enhancers in the control dHAND expression. Once the distal branchial arch enhancer for dHAND is identified, it will be interesting to determine if it shares sequence homology with enhancers that regulate distal arch expression of other genes such as Msx1 and eHAND.

Our results have lead us to define two distinct ET-1/ETA-dependent dHAND sub-domains in the anterior branchial arches. One is a Dlx6-dependent ventrolateral domain controlled by a 208 bp proximal branchial arch enhancer. This dHAND expression domain overlaps with the ventrolateral portion of the Dlx6 expression domain, which depends on ETA-mediated signals (Charité et al., 2001). The other is a ventral domain controlled by a putative distal dHAND branchial arch enhancer(s). A recent finding that the expression of dHAND is abolished in the branchial arches of Dlx5/Dlx6 double mutant embryos, which exhibit homeotic transformation of the lower jaw into an upper jaw, suggests that the ventral domain of dHAND-expressing cells is controlled by a putative enhancer that is potentially regulated by the combination of these two genes (Beverdam, 2002; Depew, 2002). It is plausible that the loss of both Dlx5 and Dlx6 in mandibular arch mesenchyme may affect epithelial expression of soluble factors critical for branchial arch development such as FGF8, a homeobox gene related to H. Yanagisawa and others.
as BMP7 (Depew, 2002) and ET-1. A third sub-domain of dHAND seems to exist in the most ventral region of the branchial arch, which is independent of ET-1/ETA-mediated signals (Fig. 8). Examination of dHAND expression in Dlx6 null embryos may further define dHAND sub-domains required for development of the anterior branchial arches.

**Multiple and parallel signaling pathways involved in craniofacial development**

Craniofacial development is a complex process regulated by a plethora of transcription factors and signaling molecules that comprise multiple independent signaling pathways (Francis-West et al., 1998). One molecule that appears to initiate or maintain one or more of these pathways is ETA, expressed by migratory and post-migratory neural crest cells and their derivatives. We have previously shown that the expression of at least several transcription factors involved in facial patterning is disrupted in EndrA–/– mutant embryos. Among those transcription factors, the interrelationship of ETA signaling and Distalless function appears to be tightly linked. The expression of Dlx3, Dlx5 and Dlx6 are all partially or completely disrupted in the EndrA–/– mutant embryos, while Dlx1 and Dlx2 are largely unaffected. This suggests that ETA signaling is required in a similar manner by linked pairs of Dlx genes. Interestingly, disruption of Dlx5 expression in ETA mutant embryos is only observed in the proximal domains, similar to the pattern observed for Dlx6 (D. E. C., unpublished observation). However, Dlx5 and Dlx6 are likely to be involved in different signaling pathways in facial morphogenesis, as Dlx5 induces Gsc expression, while Dlx6 induces dHAND expression. Mice homozygous for both Dlx5 and Dlx6 develop craniofacial and ear defects, including the failure of Meckel’s cartilage, mandible, and calvaria formation, all of which are more severe than those observed in Dlx5 mutant mice or EndrA–/– mutant mice (Robledo et al., 2002). These findings clearly illustrate the similar but non-redundant roles of Dlx genes in facial morphogenesis.

**dHAND and its branchial arch enhancer as potential targets for mutations in cleft palate syndromes**

Cleft palate has a multifactorial etiology and has been associated with abnormal expression of numerous signaling molecules and transcription factors (Ferguson, 1994). Palate formation involves a complex series of steps that include growth of the palatal shelves, palatal elevation and fusion, and disappearance of the midline epithelial seam (Ferguson, 1988). The role of dHAND in the formation of palatine shelves may not be mediated by a direct effect on palatine bone formation. Rather, dHAND may be required for the elevation of the palatine shelves by sustaining concurrent growth of the mandibular bone, as dHAND-positive cells do not populate the palatine bones as judged from lineage analysis using dHAND-Cre; R26R indicator mice (Clouthier et al., unpublished observation). This hypothesis is supported by the observation that a relationship between growth retardation of Meckel’s cartilage coupled with relative macrognathia and malformation of the secondary palate is a critical determinant in the development of cleft palate in mice homozygous for a semi-dominant Col2α1 mutation (Ricks, 2002).

The T-box gene TBX 22 has been reported to be responsible for CPX (X-linked cleft palate and tongue-tie) syndrome (Braybrook et al., 2001), and a significant linkage-disequilibrium has been found between non-syndromic cleft lip with or without cleft palate (CL/P) and the Msx1 and TGFβ3 genes, and between Cleft Palate Only (CPO) and Msx1 (Lidral et al., 1998). In addition, craniofacial defects often accompany congenital heart defects as seen in the DiGeorge and Holt-Oram syndromes (Murray, 2001). The expression of dHAND during craniofacial as well as cardiovascular development suggests that mutations in the 5′ regulatory region of the dHAND gene could also be associated with cleft palate.

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