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Developmental Expression of *Hox11* and Specification of Splenic Cell Fate

Charles W. M. Roberts,* Anais M. Sonder,* Andrew Lumsden,† and Stanley J. Korsmeyer*

From the Howard Hughes Medical Institute and the Division of Molecular Oncology, Departments of Medicine and Pathology, Washington University School of Medicine, St. Louis, Missouri; and MRC Brain Development Programme, Division of Anatomy and Cell Biology, UMDS, Guy's Hospital,† London, United Kingdom*

Hox11 *is the first member of a novel class of orphan homeobox genes. We report that* Hox11 *is expressed in a discrete temporal and spatially segmented pattern during embryonic development and appears critical for the specification of splenic cell fate. Expression is first observed in the developing muscle plates of branchial arches 1, 2, 3, and 4/6, and subsequently within motor neurons of cranial nerves V, VII, IX, and X, which innervate these muscles.* Hox11 *serves as a molecular marker distinguishing branchial from somatic motor nuclei. Additionally,* Hox11 *is expressed in the surface ectoderm of the first branchial arch in the region destined to become the tongue and teeth and then in ganglia innervating this area. However,* Hox11*-deficient mice have no apparent morphological or functional defects within these structures.[1] Notably the closely related homeobox genes,* Hox11L1 *and* Hox11L2*, were not expressed in a redundant pattern. Neither* Hox11L1 *nor* Hox11L2 *was expressed in the branchial arches or their motor nuclei within wild-type or* $Hox11^{-/-}$ *mice. Beginning at E11.5,* Hox11 *is normally expressed at a single site in the abdomen within splanchnic mesoderm destined to form the spleen, and* $Hox11^{-/-}$ *mice have no spleen.[1] We noted no increase in cell death within the dorsal mesogastrium of* Hox11*-deficient mice. Instead the dorsal mesogastrium fails to separate from the stomach.* $Hox11^{-/-}$ *mice display a larger stomach and possibly pancreas, suggesting that these mesodermal cells now contribute to other organs. (Am J Pathol 1995, 146:1089–1101)*

Homeobox genes are evolutionarily conserved transcription factors that act as developmental master switches to control the implementation of segmental or regional identity as well as cell lineage fate choices.[2] In vertebrates, tandemly linked *Hox* genes in four clusters are expressed in overlapping domains along the anteroposterior axis of the embryo.[3] Experimental data suggest that the clustered Hox genes encode a combinatorial system of positional specification along the anteroposterior axis. They appear to be critical for implementing overall pattern formation within the hindbrain and branchial arches.[4–7] However, other homeobox-containing genes are located outside of the Hox clusters, and the function of the majority of these orphan genes has yet to be identified.

A common theme is emerging for the genes identified at interchromosomal translocation breakpoints in T-cell leukemias. These genes belong to classic families of transcription factors, and their predominant expression is normally in lineages other than T cells.[8–13] Upon chromosomal translocation into a T cell receptor locus the expression of these transcription factors is redirected to T cells. *Hox11* was originally isolated from the recurrent t(10;14)(q24;q11) breakpoint found in human T-cell acute lymphoblastic leukemia (T-ALL).[14–17] This translocation results in high-level expression of the normal Hox11 product within thymocytes.

We report that *Hox11* is expressed in a segmentally restricted pattern in the branchial arches, hindbrain, and spleen of the developing murine embryo. *Hox11* is first expressed within the muscle plates and select ectoderm sites of the branchial arches and subsequently within the branchial motor nuclei and sensory ganglia that innervate these targets. Because of the lack of phenotype within the branchial arches of $Hox11^{-/-}$ mice we examined the expression pattern of two closely related homologs. Given that $Hox11^{-/-}$

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Address reprint requests to Dr. Stanley J. Korsmeyer, Howard Hughes Medical Institute, Washington University School of Medicine, Box 8022, 660 South Euclid, St. Louis, MO 63110.

mice are asplenic, we also examined the fate of the cells that would normally form the spleen.

Materials and Methods

Embryos

The morning on which vaginal plugs were observed was considered to be E0.5. Staging of embryos was done according to the criteria of Theiler.[18]

In Situ *Hybridization*

Murine embryos were harvested and placed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4 C for 12 to 24 hours followed by 0.5 mol/L sucrose in PBS for 24 hours and then frozen in ornithine carbamoyltransferase on dry ice. Five-μ sections were cut and mounted on untreated SuperFrost Plus microscope slides (Fisher). The remainder of the *in situ* hybridization was carried out as previously described[19] except that the slides were counterstained with hematoxylin and eosin without use of ammonia water. Hox11 was detected using a 200-bp antisense probe from the 3′ open reading frame and untranslated region amplified using the 5′ sense primer (5′AGCTGCAGCAGGAAGCCTTCC3′) and the 3′ antisense primer (5′TGGTTCTCCGGAAATCCTC3′). This probe was labeled with ^{35}S-UTP or [^{33}P]UTP and used for hybridization.

Whole-Mount in Situ *Hybridization*

Whole-mount *in situ* hybridizations were carried out as previously described.[20] *Hox11* was detected using a 0.45-kb probe beginning 3′ of the homeobox and continuing into the 3′ UT defined by the upstream sequence 5′AGAGGAACGTGAGGCCGAGA3′ and downstream sequence 5′GGATCCCAGAAGCCTTCCGG3′. *Hox11L1* was detected using a 0.5-kb probe amplified using the upstream primer 5′TGCTGCACCTGCAGCAAGA3′ and the downstream primer 5′TCCCTGCTTCATCCACAAT3′. *Hox11L2* was detected using a 0.6-kb probe amplified using the upstream primer 5′TGGGAGGAGGACAGTTCCAA3′ and the downstream primer 5′TTATTAAATAAAACGACTTA3′.

TUNEL

Cells undergoing programmed cell death were labeled *in situ* by using terminal deoxynucleotidyl transferase to nick end-label DNA fragments with biotinylated poly-du. Staining was performed using avidin-conjugated peroxidase and diaminobenzidine as previously described.[21]

Results

Temporal Course of Hox11 *Expression*

An initial survey of *Hox11* expression revealed no sites of expression in adult tissues except for a minority of liver samples. Because most homeobox genes are expressed during embryogenesis, we searched for expression of *Hox11* in embryos. Murine *Hox11* was isolated and found to be 96% identical overall to human *Hox11* at the amino acid level and 100% identical within the homeobox and COOH-terminus. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed to define the temporal course of expression. Message is present beginning at E8.5 and continues to be expressed through E17.5.

Expression of Hox11 *in Branchial Muscle Plates*

To spatially localize *Hox11* expression within the developing embryo, RNA *in situ* hybridization was performed on both tissue sections and whole-mount embryos. *Hox11* expression was first detected at E8.5 in the newly forming branchial arches (Figure 1A–C). At this time, only the first two arches have developed significantly, and *Hox11* is expressed within a portion of both. No expression of *Hox11* is detected in the segmented rhombomeres or migrating neural crest where expression of the 3′ clustered Hox genes has been detected (Figure 1D).[22,23] By E9.0, in addition to the first two arches, the third, fourth, and sixth have also begun to form. The fifth arch does not significantly develop within mammals. *Hox11* is expressed within a small portion of each arch (Figure 2, A and B). At E9.5, whole-mount embryos demonstrated that *Hox11* is expressed discretely in the center of each of the arches with expression being most intense in the second arch (Figure 2C). A ventral view of an E9.5 embryo reveals a band-shaped pattern of hybridization in the center of the first arch (Figure 2D). At E10.5, expression is beginning to be down-regulated within the center of the branchial arches. *Hox11* is no longer expressed within the center of the first arch but expression is still detectable in the other arches (Figure 3, A and B). By E11.5, *Hox11* is no longer expressed in the first three arches (not shown).

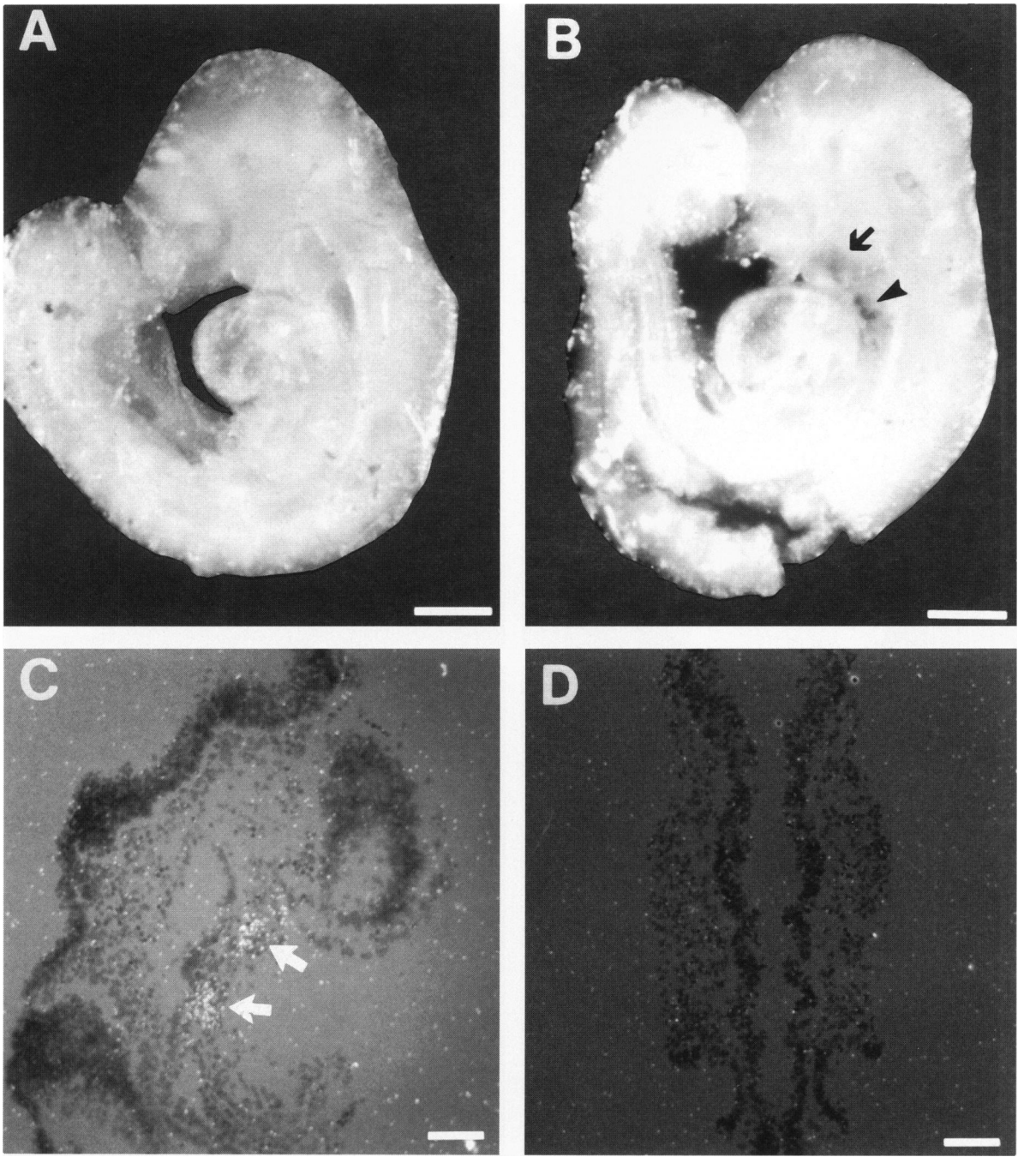

Figure 1. Hox11 *RNA* in situ *hybridization at E8.5.* (**A**) *E8.5 negative control embryo hybridized with* Hox11 *sense probe.* (**B**) *E8.5 embryo hybridized with antisense* Hox11 *probe. Expression is detected within branchial arches 1 (arrow) and 2 (arrowhead).* (**C**) *Sagittal section of E8.5 embryo demonstrating expression in the newly forming branchial arches 1 and 2 (arrows).* (**D**) *Coronal section at E8.5 revealing no expression in the rhombomeres.*

The band of expression in the center of each branchial arch suggests that *Hox11* is expressed in the developing muscle plates. The muscle plate gives rise to all of the voluntary muscles arising from each arch and is derived from paraxial mesoderm, which migrates into the core of each arch.[24] The muscle plate of the first arch gives rise to the muscles of mastication or chewing, the second arch to the muscles of facial expression, the third arch gives rise only to the stylopharyngeus muscle, and the fourth and sixth arches together give rise to the intrinsic muscles of the pharynx. In contrast, the vast majority of other

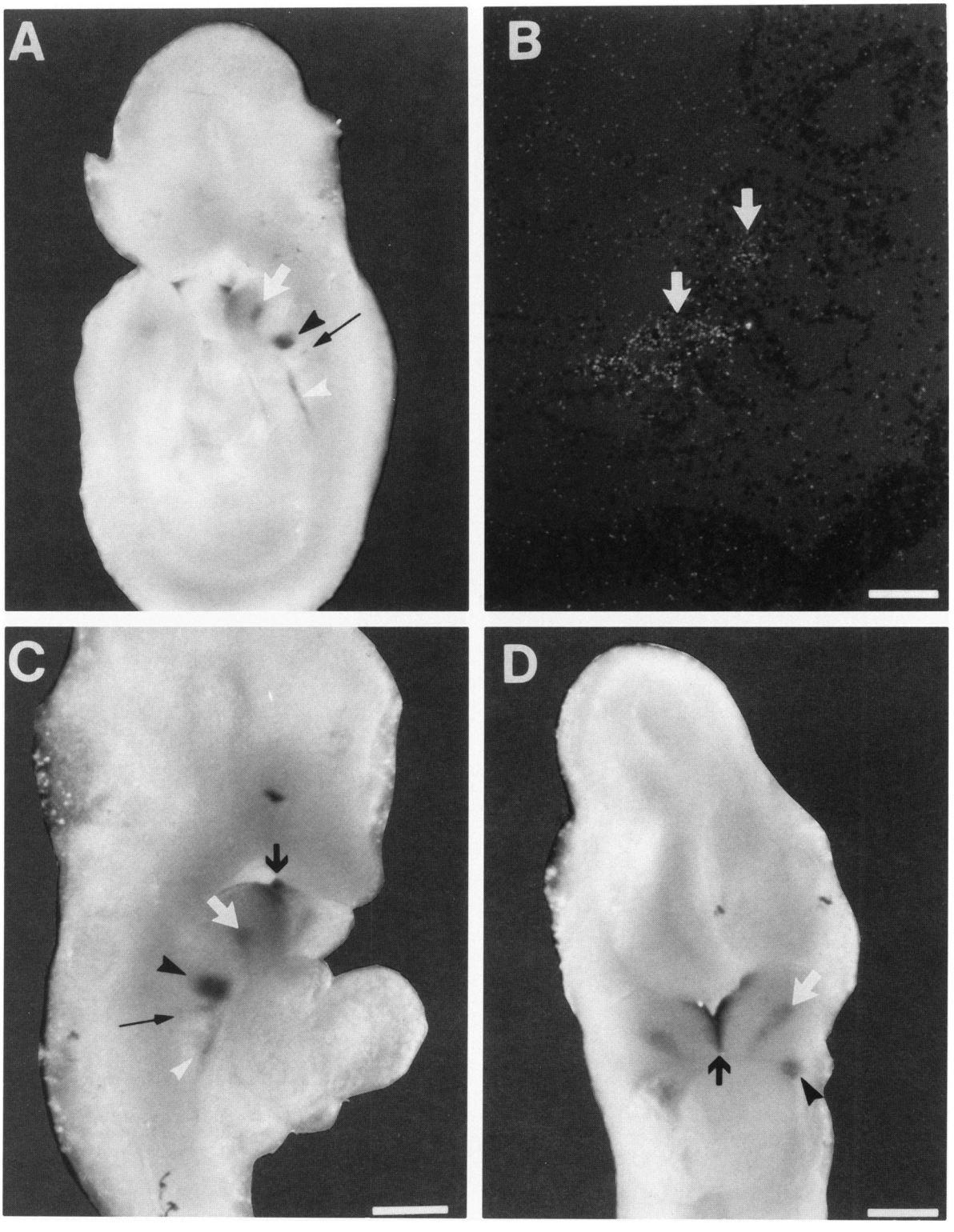

Figure 2. (A) *E9.0 embryo hybridized with antisense* Hox11 *probe. Expression is detected within branchial arches 1 (white arrow), 2 (black arrowhead), 3 (black arrow), and 4 and 6 (white arrowhead).* (B) *Parasagittal section at E9.0 showing expression in the central portion of the first arch and less-developed second through fourth arches. Ventral is up and caudal is to the left.* (C) *E9.5 embryo hybridized with antisense* Hox11 *probe. Expression is detected in the center of branchial arches 1 (white arrow), 2 (black arrowhead) 3 (thin black arrow), and 4 and 6 (white arrowhead). The surface ectoderm of the mandibular portion of the first arch (thick black arrow) is positive.* (D) *Ventral view of E9.5 embryo demonstrating band-shaped pattern of expression in branchial arches 1 (white arrow) and 2 (black arrowhead) and in the surface ectoderm of the mandibular portion of the first arch (black arrow).*

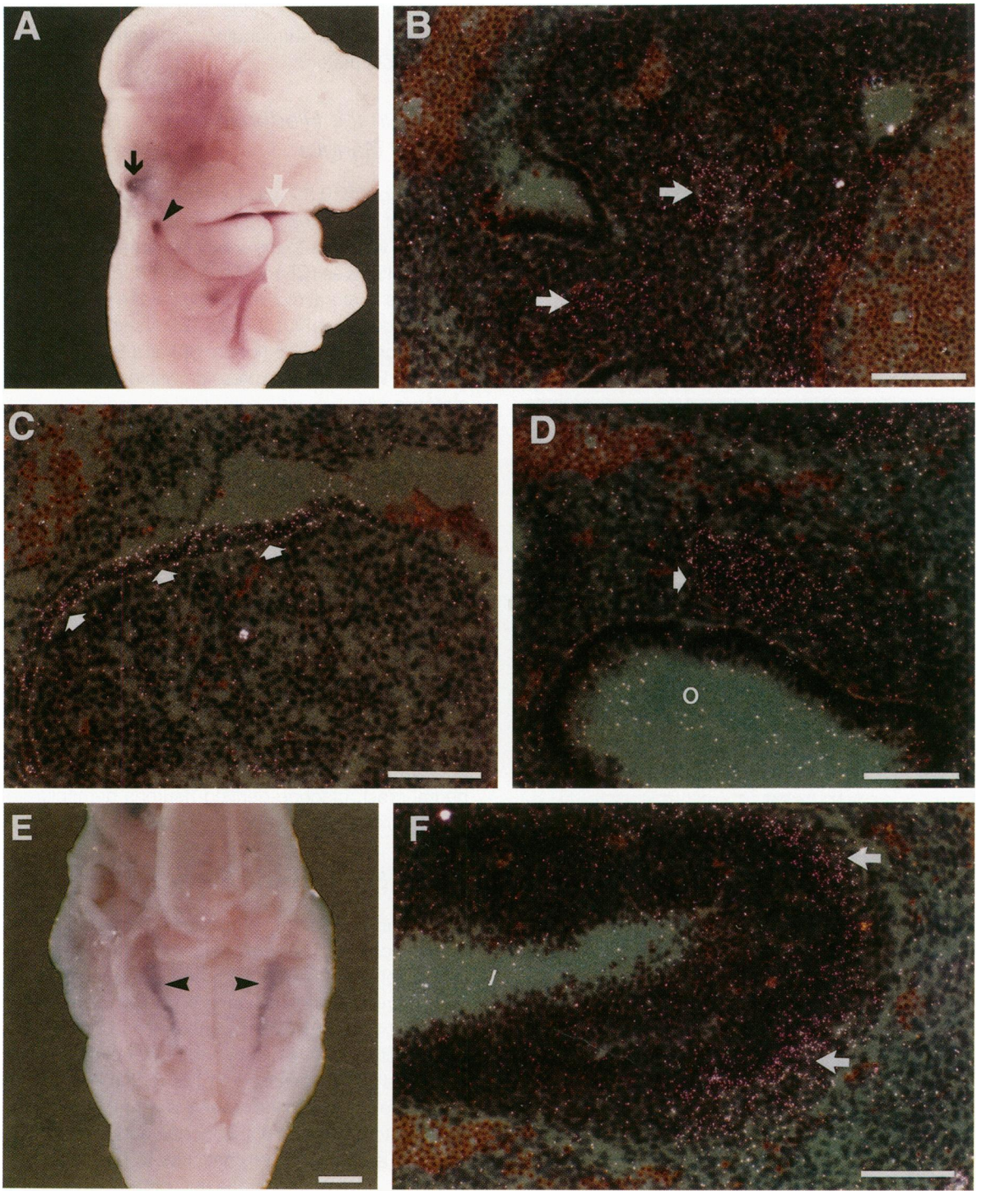

Figure 3. Hox11 *at E10.5.* (**A**) *In the E10.75 embryo, the developing cranial nerve motor nuclei (black arrow) and vestibuloacoustic ganglion (black arrowhead) express* Hox11. *Expression is no longer present in the first branchial arch, but is still weakly present in the second and third arches. The surface ectoderm of the first arch is positive (white arrow).* (**B**) *Parasagittal section at E10.5 showing expression of* Hox11 *in the center of branchial arches 2 and 3 (arrows).* (**C**) *Parasagittal section at E10.5 through the mandibular portion of the first branchial arch demonstrating expression in the portion of surface ectoderm destined to become the floor of the oropharynx.* (**D**) *Parasagittal section of E10.5 embryo.* Hox11 *is localized to the dorsal portion of the facial-acoustic ganglion complex in the vestibuloacoustic ganglion. O: otic vesicle.* (**E**) *Dorsal view of the hindbrain of an E10.75 embryo. The roof of the hindbrain has been removed to reveal expression within the branchial motor columns (arrowheads).* (**F**) *Parasagittal section at E10.5 ventral to the IVth ventricle demonstrating expression in the newly developing branchial motor nuclei. Scale bars represent 0.2 mmol/L.*

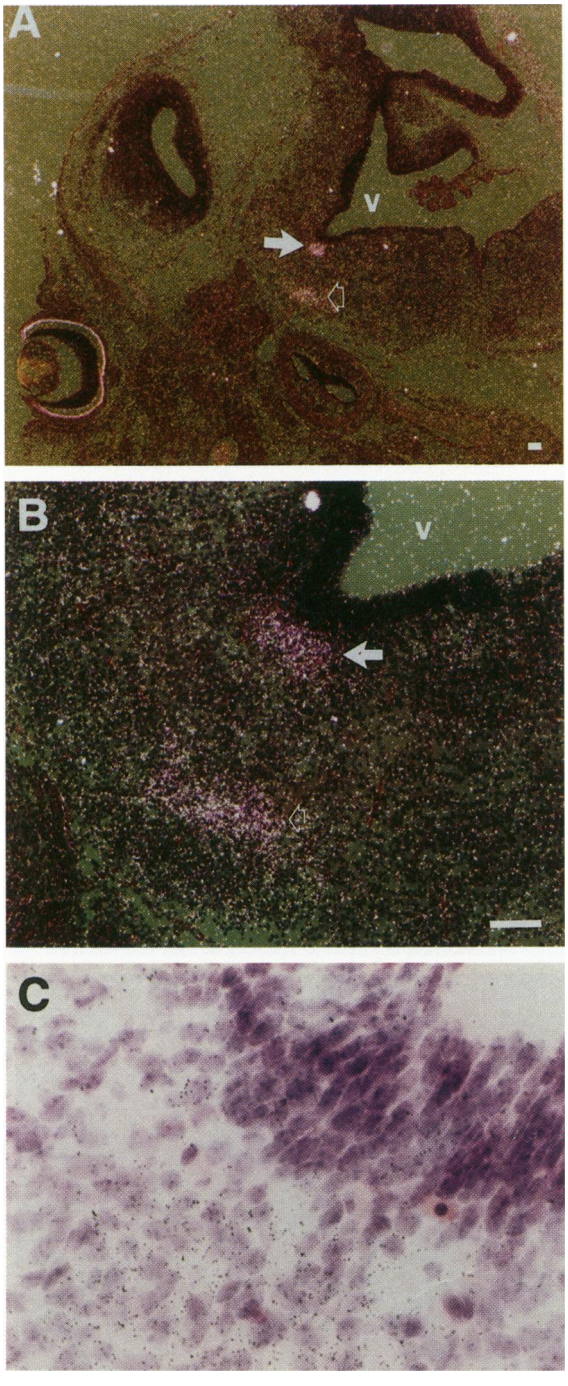

Figure 4. (**A**) *Parasagittal section at E13.5 showing IV ventricle (V), midbrain and hindbrain.* Hox11 *is discretely confined to the motor nuclei of cranial nerves V and VII.* (**B**) *Higher power E13.5 section of the V motor nucleus (closed arrow) and VII motor nucleus (open arrow).* (**C**) *Parasagittal section of E13.5 embryo showing silver grains over the neural precursors of the motor nucleus of CN V. Scale bars represent 0.1 mmol/L.*

structures within the arches are derived from neural crest including all bone, tendon, cartilage, dermis, and stroma.[24] Neural crest originates from the lateral margin of the neural folds and migrates into the branchial arches surrounding the central mesodermal muscle plate.[25]

Hox11 *in the Hindbrain and Cranial Ganglia*

Beginning at E10.5, *Hox11* is expressed in the ventral portion of the developing hindbrain (Figure 3A). Expression localizes to the newly forming motor nuclei of cranial nerves (CN) V, VII, IX, and X, and is visible within the bilateral motor columns of the hindbrain in whole-mount embryos (Figure 3E). A section through the hindbrain at E10.5 (Figure 3F) and E13.5 (Figure 4, A and B) confirms expression within the branchial motor nuclei. *In situ* hybridization indicates that *Hox11* is expressed in the neural populations and not in the supporting cells (Figure 4C). Expression in these motor nuclei continues through E14.5, but was not detected thereafter.

Hox11 is also expressed in select sensory cranial ganglia, specifically the vestibuloacoustic ganglion of CN VIII (Figure 3, A and D), the geniculate ganglion of CN VII, and weakly in a portion of the trigeminal ganglion of CN V. Expression in these ganglia is first detected at E10.5 and continues through E13.5. Studies in the chick model indicate that neurons that constitute these sensory ganglia are predominantly derived from the neurogenic ectodermal placodes and not neural crest. Compared with neurons derived from neural crest, placode-derived neurons demonstrate different responses to several neurotrophic factors.[26]

Hox11 *Expression in Surface Ectoderm*

The surface ectoderm of the mandibular portion of the first branchial arch also expresses *Hox11*. Expression is first detected there at E9.5 and is restricted to the ectoderm covering the central portion of the arch (Figure 2, C and D). By E10.75 the left and right halves of the first arch have fused, and *Hox11* expression is observed to be localized to the region that corresponds to the floor of the oropharynx that will give rise to the anterior portion of the tongue and teeth[27] (Figure 3, A and C). *Hox11* continues to be expressed within the developing tongue until birth.[28] Of note, the tongue and teeth are innervated by the trigeminal and geniculate ganglia, which also express *Hox11*.

Fate of Dorsal Mesogastrium Cells in Hox11-*Deficient Mice*

Beginning at embryonic day 11.5, *Hox11* is expressed at a single site within the abdomen localized

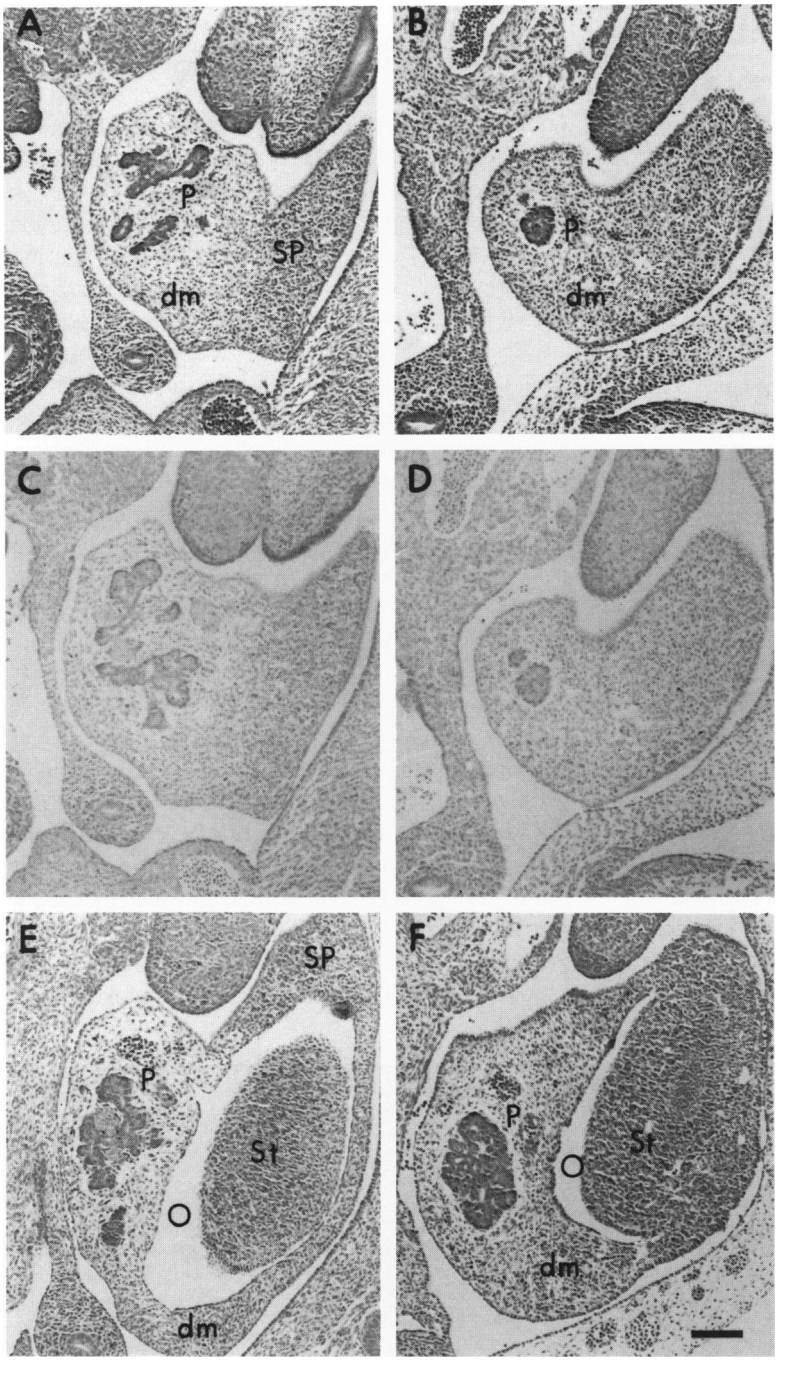

Figure 5. *Dorsal mesogastrium in E12.0 normal and* Hox11$^{-/-}$ *embryos.* (**A**) *Transverse section through dorsal mesogastrium* (*dm*) *of E12.0 normal embryo caudal to the stomach.* (**B**) *Transverse section through same level of a* Hox11$^{-/-}$ *embryo. Note lack of organization and condensation in region where spleen would normally develop.* (**C**) *TUNEL of adjacent section in normal embryo revealing minimal cell death in dorsal mesogastrium.* (**D**) *TUNEL of* Hox11$^{-/-}$ *embryo showing no increase in cell death.* (**E**) *Transverse section through slightly more rostral portion of normal embryo demonstrating the separated dorsal mesogastrium encircling the stomach* (*St*). (**F**) *Transverse section through* Hox11$^{-/-}$ *embryo showing dorsal mesogastrium fused to entire lateral wall of stomach. Pancreas* (*P*), *spleen* (*SP*), *dorsal mesogastrium* (*dm*), *stomach* (*St*), *sac of the omentum* (*O*).

to the dorsal mesogastrium where mesodermal cells destined to form the spleen are beginning to condense.[1] *Hox11* continues to be expressed in the splenic mesenchymal cells as they migrate and proliferate to form the definitive spleen. *Hox11*-deficient mice have the remarkable phenotype of being completely asplenic but are otherwise apparently normal.[1] Because the spleen never forms within *Hox11*$^{-/-}$ embryos, we sought to determine what happens to the cells of the dorsal mesogastrium that would normally form the spleen. One possibility is that these cells undergo programmed cell death in the absence of *Hox11*. Apoptotic cells can be detected *in situ* by the use of the TUNEL technique.[21] Serial sections at multiple time points from *Hox11*$^{-/-}$ embryos revealed no increase in cell death within the dorsal mesogastrium at the site where the spleen would normally develop (Fig. 5, A–D).

An alternative possibility is that cells within the dorsal mesogastrium would adopt a new differentiative fate in

Table 1. *Stomach and Pancreas of Normal and* Hox11$^{-/-}$ Mice

Characteristics	Normal mice	*Hox11*$^{-/-}$ mice	*P*
n	14	17	
Sex (*% female*)	43	47	
Weight (*g*)			
Total body	27.2 ± 8.2	28.4 ± 6.7	
Stomach	0.26 ± 0.12	0.36 ± 0.08	8.8×10^{-3}
% Stomach/ total body	0.90 ± 0.17	1.32 ± 0.38	5.8×10^{-4}
Pancreas	0.15 ± 0.04	0.18 ± 0.04	0.12
% Pancreas/ total body	0.58 ± 0.06	0.64 ± 0.07	0.024

Stomach and pancreas was harvested from age-matched normal and *Hox11*$^{-/-}$ mice. Weights are listed for total body, stomach, and pancreas. The percent of total body weight due to stomach and pancreas are also listed and serve as controls for size differences between individual mice.

the absence of the genetic program for spleen development. At E12.0 in normal embryos the spleen is continuous with the pancreas, whereas the dorsal mesogastrium has completely separated from the stomach at points (Figure 5E). However, in *Hox11*$^{-/-}$ mice the region within the dorsal mesogastrium where the spleen would normally develop remains fully fused with the stomach throughout all serial sections (Figure 5F). To assess whether the mesodermal cells destined to form the spleen could have contributed to either stomach or pancreas, these organs were harvested from age-matched normal and *Hox11*-deficient mice (Table 1). The weights of organs indicate that *Hox11*$^{-/-}$ mice have significantly larger stomachs than controls. There was also a trend for the pancreas to be larger.

Hox11L1 *and* Hox11L2 *Expression in Normal and* Hox11*-Deficient Embryos*

An extensive series of dissections revealed that the muscles derived from the branchial arches are anatomically normal in *Hox11*$^{-/-}$ mice.[1] Whole-mount *in situ* hybridization performed on *Hox11*$^{-/-}$ embryos using a *Hox11* antisense probe confirmed that the transcript was lacking at all sites of normal expression. (Figure 6) Immunostaining was performed with the 2H3 monoclonal antibody, which recognizes a 155-kd intermediate neurofilament protein. This revealed that the sensory ganglia that normally express *Hox11* have normal morphology and position in *Hox11*-deficient embryos (not shown). Given that disruption of *Hox11* caused the absence the spleen but resulted in no apparent defects within the branchial arches, we assessed the expression of the closely related genes *Hox11L1* and *Hox11L2* to determine whether either of these genes could functionally substitute for *Hox11*.

To determine whether *Hox11L1* or *Hox11L2* expression overlapped with *Hox11* within the branchial region, normal embryos were harvested and used in whole-mount *in situ* hybridization with *Hox11L1* and *Hox11L2* antisense probes. Hybridization to E9.5 embryos revealed that both *Hox11L1* (Figure 7A) and *Hox11L2* (Figure 7B) are expressed within the newly forming trigeminal (CN V), facioacoustic (CN VII, VIII), glossopharyngeal (CN IX), and vagus (CN X) cranial ganglia. At this time, *Hox11L1* is also expressed

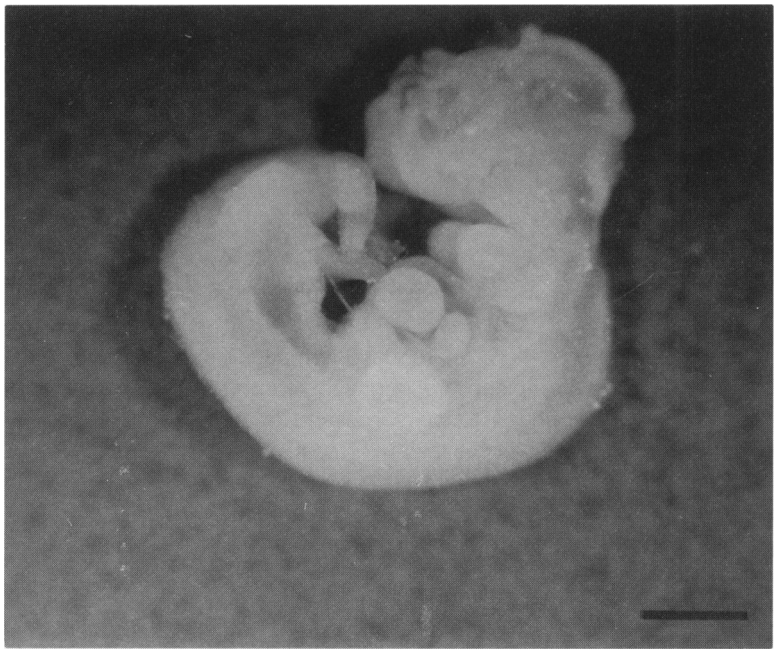

Figure 6. In situ *hybridization of* Hox11$^{-/-}$ *embryo. E9.5* Hox11$^{-/-}$ *embryo hybridized with* Hox11 *antisense probe. Absence of expression confirms disruption of* Hox11 *message.*

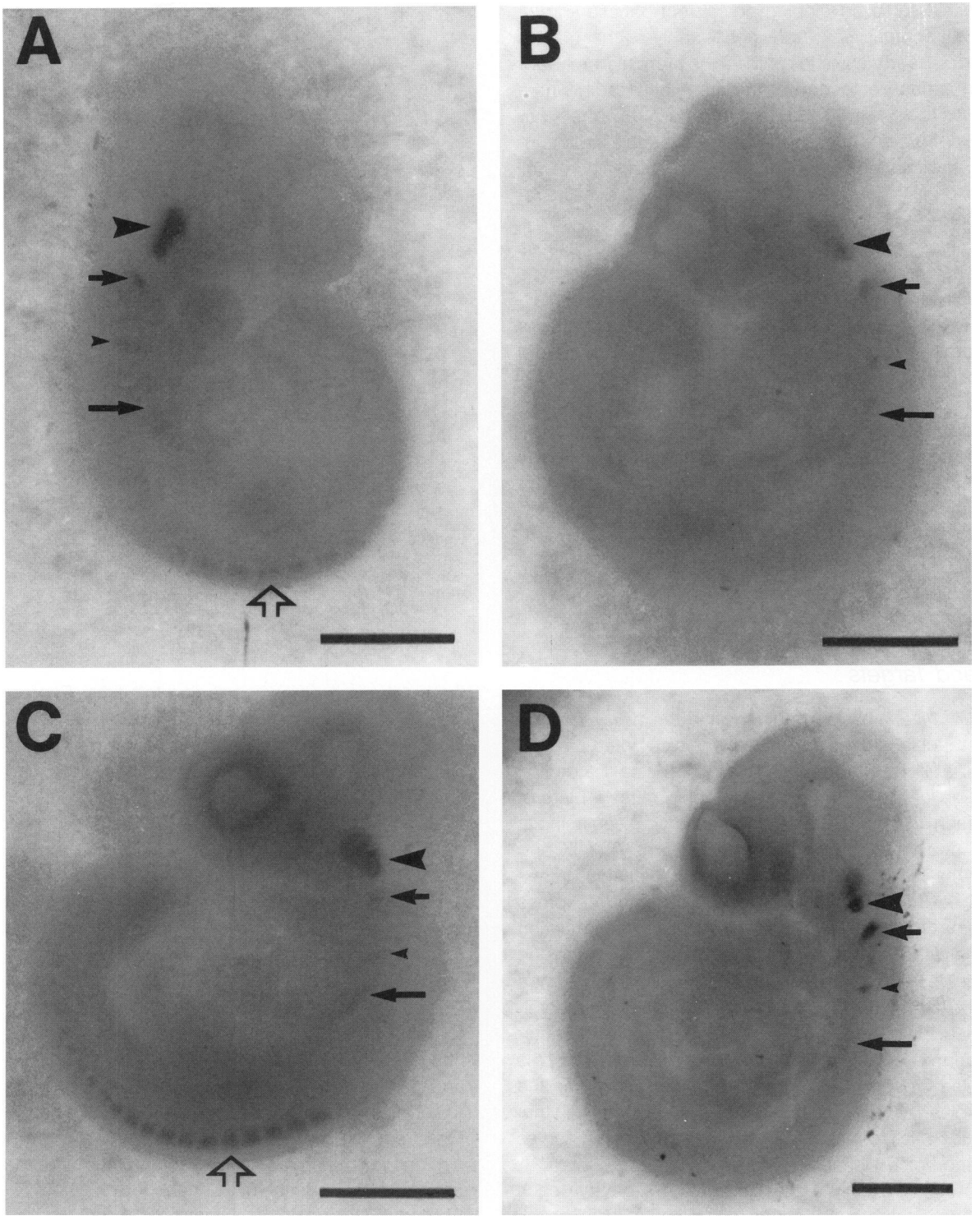

Figure 7. *Expression of* Hox11L1 *and* Hox11L2 *does not overlap with* Hox11 *in branchial arches of normal or* Hox11$^{-/-}$ *embryos.* (**A**) *E9.5 normal embryo hybridized with* Hox11L1 *antisense probe.* (**B**) *E9.5 normal embryo hybridized with* Hox11L2 *antisense probe.* (**C**) *E9.5* Hox11$^{-/-}$ *embryo hybridized with* Hox11L1 *antisense probe.* (**D**) *E9.5* Hox11$^{-/-}$ *embryo hybridized with* Hox11L2 *antisense probe. Trigeminal ganglion (large arrowhead), facioacoustic ganglion complex (short arrow), glossopharyngeal ganglion (small arrowhead), and vagus ganglion (long arrow).*

within some developing dorsal root ganglia (Figure 7A), whereas *Hox11L2* is not. Notably, neither gene is expressed within the center of the branchial arches or in surface ectoderm of the mandibular arch where *Hox11* is expressed. Also, neither *Hox11L1* nor *Hox11L2* is expressed within the branchial motor nuclei within the hindbrain at any time (not shown). Full expression data on *Hox11L1* and *Hox11L2* will be reported elsewhere.

Hox11$^{-/-}$ embryos were examined to assess whether either *Hox11L1* or *Hox11L2* expression was activated in new sites in the absence of *Hox11*. *Hox11*$^{-/-}$ embryos showed hybridization patterns identical to normal embryos (Figure 7, C and D). Specifically, neither *Hox11L1* nor *Hox11L2* was expressed within the branchial arch muscle plates, surface ectoderm, or hindbrain in *Hox11*-deficient embryos. Consequently, the two genes most closely related to *Hox11* do not perform a backup function for *Hox11* in these structures.

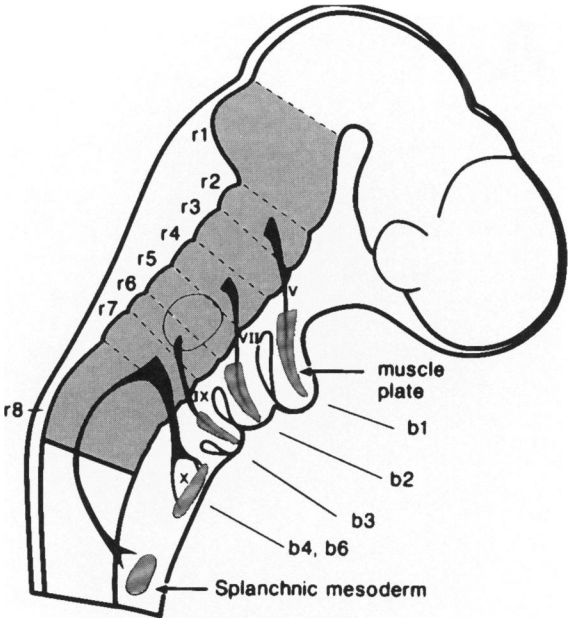

Figure 8. *Relationship between branchial motor nerves and the muscles of the branchial arches in the developing embryo. Branchial motor nerves (V, VII, and IX) and branchial arches (b1–b3) are indicated. The hindbrain is divided into rhombomeres (r1–r8, stippled). The motor nuclei of CN V, VII, IX, and X innervate the muscle plates of branchial arches 1, 2, 3, and 4/6 respectively. CN X also innervates the splanchnic mesoderm that becomes the spleen.*

Discussion

Expression in Branchial Neurons and Targets

Hox11 expression begins within the newly forming branchial arches at E8.5 as was also noted by Raju et al.[28] By E9.5 expression localizes to the central core of the arch in the developing muscle plate of each arch. In addition, we observed that beginning at E10.5, the motor nuclei of cranial nerves V, VII, IX, and X that innervate the muscle plates of branchial arches 1, 2, 3 and 4/6, respectively, also begin to express *Hox11* (Fig. 8). Consequently, there is a correlation between expression in the muscle plates and the nerves that innervate them. Similarly, we confirmed the expression of *Hox11* in the geniculate and trigeminal sensory ganglia, which send fibers to the ectoderm on the floor of the oropharynx where *Hox11* is also present.[28] *Hox11* is also expressed within the spleen, which is innervated by CN X. It is striking that *Hox11* is expressed both in these developing nerves and in their innervation targets.

Hox11 is only expressed for a few days in each location and corresponds to the time of initial development and innervation. Development of the branchial arches proceeds in a ventral-to-dorsal direction.[25] Migrating neural crest first fills the ventral portion of the arch at E8.5, surrounding the mesoderm destined to become the muscle plate. Next, the dorsal portion of the arches is filled, and the cranial sensory ganglia are formed. Patterning and differentiation of the hindbrain itself occurs during this time. Expression of *Hox11* closely parallels the temporal course of these developments. It is first expressed in the muscle plates at E8.5, then in the cranial ganglia, and finally in the motor nuclei in the hindbrain.

Branchial Versus Somatic Motor Nuclei

The hindbrain contains two major classes of efferent neurons distinguished by their pattern of exit. The branchial motor neurons exit the hindbrain dorsolaterally and innervate the muscle plates of the branchial arches. The muscle plates ultimately develop into the voluntary muscles derived from each arch. The motor neurons of CN V arise from rhombomeres 2 and 3 (r2 and r3), the VII motor neurons from r4 and r5, the IX motor neurons from r6 and r7, and the X motor neurons from r7 and r8 (Figure 8). The somatic motor neurons primarily exit the ventral region of the hindbrain and innervate the eye and tongue muscles. CN IV, VI, and XII contain the somatic motor neurons that originate within the hindbrain. These motor neurons arise in r1, r5 and r6, and r8, respectively. The fact that *Hox11* is expressed in branchial motor nuclei but not in any somatic motor nuclei, including those originating from the same region of the hindbrain, is noteworthy. Previously, no known genetic differences distinguished these two types of motor nerves. *Hox11*

provides a molecular marker to begin to dissect the development of these two populations.

Co-Localization with Ttg-1 (Rhombotin)

Ttg-1 (RBTN1) is another gene that was originally identified in T-ALL, being deregulated by the t(11;14)-(p15;q11).[8,11] *Ttg-1* expression is restricted to limited regions of the central nervous system including the motor nuclei of cranial nerves V and VII.[29] It is intriguing that two genes deregulated in T-cell leukemia both localize to these nuclei at the same time in development. *Ttg-1* contains two highly conserved cysteine-rich motifs that may serve as metal binding Zn fingers dubbed the LIM domain. LIM domains are present in *lin-11* (required for asymmetrical division of vulval precursor cell types in *C. elegans*), *Isl-1* (rat insulin gene enhancer binding protein), and *mec-3* (required for differentiation of touch neurons in *C. elegans*).[30–32] It has previously been suggested that *Ttg-1* may be a downstream gene regulated by the clustered Hox genes.[33] Perhaps a gene program implemented in the branchial motor nuclei is particularly oncogenic when redirected to T cells.

Could Hox11 *Be Regulated by the Clustered Hox Genes?*

The 3′ members of the clustered Hox genes have discrete anterior limits of expression that correspond to the segmental boundaries of the rhombomeres. The anterior limit of expression of these genes occurs at two segment intervals such that each gene is expressed two rhombomeres anterior to the limit of its 5′ neighboring gene. Consequently, pairs of rhombomeres express a unique combination of the genes from the four vertebrate Hox clusters. Notably, these pairs are out of phase with the pairs of rhombomeres that give rise to the neurons that constitute each of the branchial motor nuclei. This argues that the clustered Hox genes do not directly specify development of the branchial motor nuclei. Indeed, mice that possess disrupted Hox genes as a result of gene targeting display phenotypes that support this argument.[4–7] Rather than directly controlling specification of structures, it has been proposed that expression of the clustered Hox genes constitutes a combinatorial code that activates appropriate downstream genes. This combinatorial pattern of Hox genes that is present within the hindbrain is also transferred to the neural crest.[34]

In addition to contributing neurons to several sensory ganglia, neural crest gives rise to most structures within the branchial arches. Heterotopic neural crest transplantation experiments revealed that the crest plays a critical role in directing the three-dimensional development of the branchial arches. Replacement of presumptive second (hyoid) arch crest with first (mandibular) arch crest resulted in the development of first arch structures within the second arch.[35] Not only were the structures directly derived from the crest appropriate for the first arch, but muscles in the second arch developed with first-arch alignments and attachments. These experiments clearly demonstrate that the crest is capable of organizing development within arches through inductive interactions with non-neural crest-derived tissues. *Hox11* is expressed within the branchial arch mesoderm only after neural crest has begun to arrive. Consequently, *Hox11* may prove to be downstream of the clustered Hox genes.

Novel Family of Homeobox Genes

Hox11 is the first reported member of a novel class of homeobox genes. The most closely related gene previously reported, *BarHI* of drosophila, shares only 33 of 60 amino acids within the homeodomain with *Hox11*.[36] *Hox11L2* shares 56 of 60 amino acids with *Hox11*.[37] In addition, *Hox11L1* shares 52 of 60 amino acids with human *Hox11*.[17] The homeodomains of these three genes, together with *BarHI* and the recently reported genes *Nkch4* and *Hex*, constitute the only known homeodomains that contain threonine in place of the more common isoleucine at position 47 within helix 3. Further, threonine at this position causes *Hox11* to favor recognition of the unique DNA motif TAAC rather than the antennapedia class DNA recognition motif TAAT.[37] Perhaps other members of this novel family of orphan homeobox genes may pattern specific structures.

Hox11L1 *and* Hox11L2 *and the Concept of Functional Redundancy*

All homeobox genes that have been disrupted to date have had areas of expression that are apparently unaffected in the homozygous null animal. It has been proposed that areas lacking a phenotype represent sites where a closely related gene substitutes for the missing gene. In the case of the clustered Hox genes, functional redundancy has been difficult to test because the number of genes clustered within four groups that could potentially compensate for a disrupted member is quite large. However, *Hox11* constitutes an orphan homeobox gene with a novel DNA binding motif. Moreover, three independent research

groups have only been able to identify the same two closely related genes, *Hox11L1* and *Hox11L2*. All other homeobox genes show marked divergence from *Hox11* within the homeodomain. Consequently, we investigated whether either of these two genes may provide functional redundancy for *Hox11*. Neither gene overlaps with *Hox11* within the branchial arch mesenchyme or hindbrain in normal or *Hox11*$^{-/-}$ embryos. These results suggest that functional redundancy among these closely related genes does not explain the lack of phenotypical effect in the branchial arches of *Hox11*-deficient mice. It is possible that subtle defects exist but remain undetected within these areas. Alternatively, although a gene is expressed within a given tissue, it may not serve a unique function there. Certain sites of expression might reflect the evolutionary heritage of a gene rather than a locale of influence.

Hox11 *Specification of Differentiative Fate*

Expression of *Hox11* within mesodermal cells destined to form the spleen begins at embryonic day 11.5 and continues to be expressed within the splenic anlage through E14.5. It was previously reported that this expression was within the pancreas, which also develops within the dorsal mesogastrium.[28] The spleen and pancreas are closely juxtaposed during development. Although it is possible that there may be a small amount of expression within a portion of the developing pancreas, our evidence indicates that the vast majority of expression occurs within the developing spleen.[1] This area of expression is particularly important for the function of *Hox11*, given that *Hox11*$^{-/-}$ mice have congenital asplenia. The pancreas of the mice is fully formed and has a normal histological appearance.

In an attempt to ascertain the fate of the mesodermal cells normally destined to form the spleen, we searched for increased cell death within the dorsal mesogastrium of *Hox11*$^{-/-}$ embryos. The lack of increased cell death combined with the increased size of the stomach and perhaps pancreas in *Hox11*$^{-/-}$ mice suggests that cells normally destined to form the spleen may adopt the fate of surrounding cells in the absence of *Hox11*. The inactivation of homeobox genes can lead to homeotic transformations resulting in one segment or part of the body developing in the likeness of another.[2] However, transformations involving individual organs are much less well documented. In *Drosophila*, the homeobox-containing *cut* gene specifies the identity of external sensory organs.[38] In the absence of *cut*, chordotonal sensory organs develop instead.[39] Within vertebrates, however, *Hox11* is the first example of a gene that controls the specification of differentiative cell fate that results in a single organ.

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