DBHR, a Gene with Homology to Dopamine β-Hydroxylase, Is Expressed in the Neural Crest throughout Early Development

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In a screen for genes involved in neural crest development, we identified DBHR (DBH-Related), a putative monooxygenase with low homology to dopamine β-hydroxylase (DBH). Here, we describe novel expression patterns for DBHR in the developing embryo and particularly the neural crest. DBHR is an early marker for prospective neural crest, with earliest expression at the neural plate border where neural crest is induced. Furthermore, DBHR expression persists in migrating neural crest and in many, though not all, crest derivatives. DBHR is also expressed in the myotome, from the earliest stages of its formation, and in distinct regions of the neural tube, including even-numbered rhombomeres of the hindbrain. In order to investigate the signals that regulate its segmented pattern in the hindbrain, we microsurgically rotated the rostrocaudal positions of rhombomeres 3/4. Despite their ectopic position, both rhombomeres continued to express DBHR at the level appropriate for their original location, indicating that DBHR is regulated autonomously within rhombomeres. We conclude that DBHR is a divergent member of a growing family of DBH-related genes; thus, DBHR represents a completely new type of neural crest marker, expressed throughout the development of the neural crest, with possible functions in cell–cell signaling. © 2001 Academic Press

Key Words: DBHR; MOX; monooxygenase; dopamine-β-hydroxylase (DBH); chick development; neural crest; hindbrain.

INTRODUCTION

In vertebrate embryos, the neural crest (Le Douarin and Kalcheim, 1999) is a transient population of cells that undergoes several transitions as development proceeds. Initially, neural crest is induced at the lateral border of the neural plate. Subsequently, neural crest cells migrate extensively throughout the body. Once these cells reach their destinations, they differentiate to form diverse tissues, including neurons and glia of the peripheral nervous system, melanocytes, and bone and cartilage in the head. Thus, neural crest development encompasses three fundamental developmental processes: induction, migration, and differentiation.

Migrating neural crest cells follow distinct pathways that are characteristic of their axial origin. In both the trunk and the head, these cells migrate in segmental patterns: through the rostral half of the somites in the trunk, and lateral to the even-numbered rhombomeres (r) in the hindbrain (Lumsden

et al., 1991). Rhombomere identity is thought to be conveyed by expression of a specific combination of transcription factors, most notably Hox genes, in each rhombomere (Hunt *et al.,* 1991a) and in its neural crest (Hunt *et al.,* 1991b). It was previously believed that rhombomere identity and Hox gene expression were always maintained autonomously, regardless of the environment; however, in transplantation experiments, neural crest cells (Saldivar *et al.,* 1996; Hunt *et al.,* 1998) or rhombomeres transplanted posterior to the otic vesicle (Grapin-Botton *et al.,* 1995; Itasaki *et al.,* 1996) were shown to adopt an identity and expression pattern appropriate for their new environment (reviewed by Trainor and Krumlauf, 2000).

Following migration, neural crest cells begin to differentiate into appropriate derivatives. This differentiation is characterized by the expression of cell-type-specific marker genes. Neuronal markers include neurofilament and the Hu proteins; in the chick, Hu is first expressed in neural crest-derived cells at Hamburger and Hamilton (1951) (HH) stages 17–18, in the forming dorsal root ganglia, and by stage 19 in the sympathetic ganglia (Marusich *et al.,* 1994). Neuronal differentiation is also marked by the expression of

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genes involved in the production of neurotransmitters. A classic marker of sympathetic neurons is dopamine β-hydroxylase (DBH), which converts dopamine into nor-epinephrine; in agreement with the timing of Hu expression, DBH mRNA is first detected in avian embryos at stage 18, the same stage at which tyrosine hydroxylase (TH) mRNA is first detected (Ernsberger *et al.*, 2000). Finally, differentiation is also usually marked by withdrawal from the cell cycle, but this need not be the case; for example, sympathetic neuronal progenitors divide after acquiring adrenergic characteristics (Rothman *et al.*, 1978), including DBH expression (Rothman *et al.*, 1980).

Although neural crest development has been studied for over a century, only recently have researchers begun to discover the molecules that mediate key processes. Using a differential display screen for genes expressed in the early chick neural crest (Martinsen and Bronner-Fraser, 1998), we identified DBHR (DBH-Related), a gene with about 30% similarity to DBH. DBHR is expressed at the border of the neural plate and nonneural ectoderm, where neural crest is induced; this expression persists in the dorsal neural tube, in migrating neural crest cells, and finally in neural crest derivatives. DBHR is also expressed in the myotome and in a dynamic pattern in the hindbrain, where, at one stage, DBHR expression is low or absent in odd-numbered rhombomeres. By microsurgically rotating rhombomeres, we show that DBHR expression is maintained autonomously within rhombomeres. We conclude that DBHR represents a new type of neural crest marker, in terms of its apparent molecular nature as well as its continuous expression throughout the stages of induction, migration, and differentiation.

MATERIALS AND METHODS

Chick Embryos

Fertilized chick eggs were obtained from AA Laboratories (Westminster, CA) and incubated at 37°C in humidified, rocking incubators (Lyon Electric Co., Chula Vista, CA). Embryos were staged according to Hamburger and Hamilton (1951).

Isolation of DBHR

D6U3-1, a 182-bp PCR fragment generated from a differential display screen (Martinsen and Bronner-Fraser, 1998), was cloned into pGEM-T (Promega); the resulting plasmid was used to generate a probe for screening a 2- to 4-day chick embryo library (kindly provided by Sue Bryant) at high stringency, using standard techniques (Sambrook *et al.*, 1989). Four clones were obtained and sequenced, using the ABI Prism DNA Sequencing Kit (Perkin-Elmer Applied Biosystems) and an automated sequencer. These clones were found to represent different lengths of the same gene, which contains the D6U3-1 sequence at its 3′ end [extending into the 3′ untranslated region (UTR) sequence]. However, all clones were incomplete at the 5′ end, lacking an initiation codon or 5′ UTR sequence. To isolate the complete sequence, a probe was generated from an *Nco*I/*Pvu*II restriction fragment at the 5′ end of

the longest clone; this probe was used to screen a HH stage 12–15 chick embryo library (kindly provided by Angela Nieto and David Wilkinson). Two clones were identified and found to overlap each other as well as the previous four clones. Of these, a 2.3-kb clone was found to contain a complete open reading frame, in addition to 175 bp of 5′ UTR and 292 bp of 3′ UTR. This clone was used for subsequent sequence analysis, using DNASTAR Software, the Signal P World Wide Web Prediction Server at Center for Biological Sequence Analysis (www.cbs.dtu.dk/services/SignalP/), and the basic BLAST search facility at National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST/).

In Situ Hybridization

Whole-mount *in situ* hybridization was performed according to the procedure of Wilkinson (1992). For sections of embryos <5 days old, *in situ* hybridization was performed on whole embryos (previously fixed in 4% paraformaldehyde), which were subsequently dehydrated, embedded in paraffin, and sectioned at 20 μm with a Leitz microtome. For sections of 5-day-old embryos, *in situ* hybridization was performed directly on 10-μm paraffin sections, generated from embryos fixed in modified Carnoy's solution [60% ethanol, 30% stock (37%) formaldehyde (11.1% final formaldehyde concentration), 10% acetic acid], using a protocol from Dr. H. Etchevers (Nogent-sur-Marne, France), modified from Strähle *et al.* (1994) and Henrique *et al.* (1995). This detailed protocol is available on request.

Rhombomere Rotations

Eggs were incubated at 37°C for 36–38 h, until approximately stage 10 (9–12 somites). After the removal of 3 ml of albumin from each egg, a window was cut in the shell to allow access to the embryo, which was visualized by injection beneath the blastoderm of 10% India Ink (Pelikan Fount) in Howard Ringer's solution. A segment of hindbrain including rhombomeres 3 and 4 was excised as described in Saldivar *et al.* (1996). Briefly, fine glass needles were used to separate the neural tube (including the surface ectoderm above and the notochord below) from the adjacent mesoderm and the underlying endoderm. Next, perpendicular cuts were made at the r2/3 and r4/5 borders to separate the r3/4 segment. Finally, that segment was rotated 180° and reimplanted in the hindbrain, maintaining the original dorsal/ventral organization, but reversing the rostral/caudal pattern. Eggs were sealed with adhesive tape and incubated for an additional 16 h prior to harvesting.

RESULTS

DBHR/MOX Genes Have Homology to DBH

A short, 3′ fragment of chick DBHR was initially PCR-amplified from cDNAs derived from HH stage 8 midbrain neural folds (including premigratory neural crest), in a differential display screen described previously (Martinsen and Bronner-Fraser, 1998). Preliminary analysis of this fragment by whole-mount *in situ* hybridization demonstrated high expression in neural crest emigrating from the hindbrain (data not shown). By using this fragment to screen chick embryo cDNA libraries, we subsequently obtained a complete clone containing a 1.8-kb open reading

frame, which encodes a predicted 70-kDa protein. Nucleic acid and amino acid sequences of DBHR have both been submitted to GenBank (Accession No. AF327450; see also Fig. 1A).

Analysis of the sequence of this gene showed high homology (73.5% similarity; see Table 1 and Fig. 1A) to an unpublished, unnamed mouse protein (GenBank Accession Nos. NP 067484/BAA95089; mouse DBHR in Fig. 1A), and similar homology (68.8% similarity) to the human gene MOX. MOX was identified by Chambers *et al.* (1998) in a differential display screen for genes that are dysregulated in senescent human fibroblasts. The name MOX, or monooxygenase X (unknown substrate), reflects its similarity to DBH (27.1% similarity), though monooxygenase activity has not been determined.

Although the overall percentage similarity between the DBHR/MOX genes and human DBH (Lamouroux *et al.,* 1987; Kobayashi *et al.,* 1989) is low (29.8% for chick DBHR; see Table 1), there is good conservation of residues that are critical for DBH structure/function. First, DBH genes all contain 14 conserved cysteines (C1–C14, Figs. 1A and 1B). Robertson *et al.* (1994) showed that 12 of these form intramolecular disulfide bonds (Fig. 1B), while the remaining 2 (C11 and C12, red arrowheads in Figs. 1A and 1B) form intermolecular bonds in the dimer. All of these cysteines are conserved in chick and mouse DBHR and human MOX, with the interesting exception of C11 and C12; thus, DBHR/MOX genes may lack intermolecular disulfide bonds.

Also well conserved are residues which are necessary for DBH function. DBH is a copper-containing monooxygenase, with high similarity to the monooxygenase domain of peptidyl-glycine amidating monooxygenase (PAM; Eipper *et al.,* 1992); the mechanisms of both enzymes have been studied extensively, and the structure of the monooxygenase domain of PAM has been solved (Prigge *et al.,* 1997). Both proteins contain two coppers at separate, inequivalent sites, CuA and CuB (Fig. 1B; Blackburn *et al.,* 1990). Six residues have been shown to bind to these copper atoms (Prigge *et al.,* 1997): for CuA, three histidines (H) at positions 235, 236, and 307 (given for the chick DBHR sequence); for CuB, H389, H391, and methionine 464. Additionally, mechanism-based inhibitor studies have shown that two tyrosines, Y203 and Y468, are also required for activity (DeWolf *et al.,* 1988; Farrington *et al.,* 1990). All eight of these crucial residues are conserved in chick and mouse DBHR and human MOX (blue asterisks, Fig. 1A).

Despite their similarities, there are significant differences between the DBHR/MOX genes and DBH, including a potential difference in secretion. Native DBH protein is a secreted protein that exists in both soluble and membrane-bound forms. Like DBH, chick and mouse DBHR genes possess a proposed signal sequence, with a predicted cleavage site in chick DBHR between A20 and A21 (black arrowhead, Fig. 1A). In contrast, human MOX lacks a signal sequence, making it unlikely that human MOX is secreted, at least by the usual mechanism.

While using the BLAST program from NCBI to identify sequence homologues of DBHR, we also noted weak homology (34.1% similarity) with a mouse DBH-like gene (DBHL in Fig. 1A) in the database (GenBank Accession No. AAB69054). Mouse DBHL was identified during the sequencing of the mouse T-cell receptor *β* locus, so no information is available about its function or expression. Like the DBHR/MOX genes, DBHL has low homology (27.2% similarity) to DBH; unlike DBHR, DBHL lacks several conserved residues, including C7, C13, C14, and Y468. Mouse DBHL therefore seems to represent a second, more divergent branch of DBH-related genes.

DBHR Is Expressed Early in Prospective and Migrating Neural Crest

To examine the expression pattern of chick DBHR, we performed whole-mount *in situ* hybridization on chick embryos between stage 6 (head-fold stage) and stage 26 (5 days). Specific expression was first detected weakly at stage 7 (1–2 somites), at the lateral boundary of the neural plate. As the neural tube begins to close, this expression intensifies in the cranial neural folds (arrowhead, Fig. 2A). After the cranial neural tube has closed (8-somite stage), expression is maintained in the dorsal brain, with highest expression in the prospective hindbrain (arrowhead, Fig. 2B). At this stage, expression appears to be uniform throughout the hindbrain. Subsequently, there is a gradual change within the forming rhombomeres, such that expression is maintained at a low level in r3 and r5, and increased in r2, r4, and r6 (Fig. 2C). Neural crest cells that have migrated out from the midbrain also strongly express DBHR (arrowheads, Figs. 2C and 2D).

Expression of DBHR in the dorsal hindbrain and its emigrating neural crest is most evident at the 20-somite stage, with strong staining of the three main streams lateral to r2, r4, and r6 (Figs. 2E and 2F). At this stage, these neural crest cells are beginning to coalesce to form the cranial ganglia (Fig. 2F). There is no staining in or lateral to r3 and increased staining in r5, though this staining is still slightly reduced relative to that of r2 and r4. Expression is maintained in neural crest migrating lateral to the midbrain (black arrowhead, Fig. 2E), though DBHR expression in the dorsal midbrain has largely been lost, with the exception of one region in the rostral midbrain (arrow, Fig. 2E).

Expression of DBHR in migrating neural crest proceeds caudally with the normal rostrocaudal order of development. At stage 16, DBHR expression can be detected weakly in the trunk dorsal neural tube and more strongly in recently emigrated neural crest cells lateral to the tube (arrowhead, Fig. 2I). Thus, DBHR is expressed by both premigratory and migratory neural crest throughout the rostrocaudal extent of the embryo.

A

FIG. 1. Sequences and proposed structure of DBH-related genes. (A) Sequence alignment of chick and mouse DBHR, human MOX, mouse DBH-like (DBHL), and human DBH. Conserved residues are indicated in black; percentage similarities are given in Table 1. The black arrowhead marks the putative cleavage site for the signal sequence of chick DBHR. Conserved cysteines are numbered in red; positions corresponding to interchain cysteines 11 and 12 in DBH (not conserved in DBHR/MOX genes or DBHL) are indicated in (A) and (B) by red arrowheads. Residues required for activity are indicated by a blue asterisk. (B) Diagram of chick DBHR gene, showing the proposed signal sequence (in black), conserved cysteines (numbered in red) and their putative linkages, and conserved active-site residues (in blue) thought to interact with copper atoms at sites CuA and CuB (modified from Oyarce and Eipper, 2000).

TABLE 1

Percentage Similarity between DBH-like Proteins

	Mouse DBHR	Human MOX	Mouse DBHL	Human DBH
Chick DBHR	73.5	68.8	34.1	29.8
Mouse DBHR		74.7	33.2	29.9
Human MOX			30.0	27.1
Mouse DBHL				27.2

DBHR Is Expressed in the Developing Somite and Myotome

Though DBHR is expressed consistently in neural crest and the dorsal neural tube, its expression is not limited to these tissues. In the 20-somite embryo, DBHR expression is clearly visible in the somites, as well as the ventral ectoderm (white arrowheads, Figs. 2E–2H). In rostral, more mature somites, DBHR expression is restricted to the myotome (black arrowhead, Fig. 2G). However, in progres-
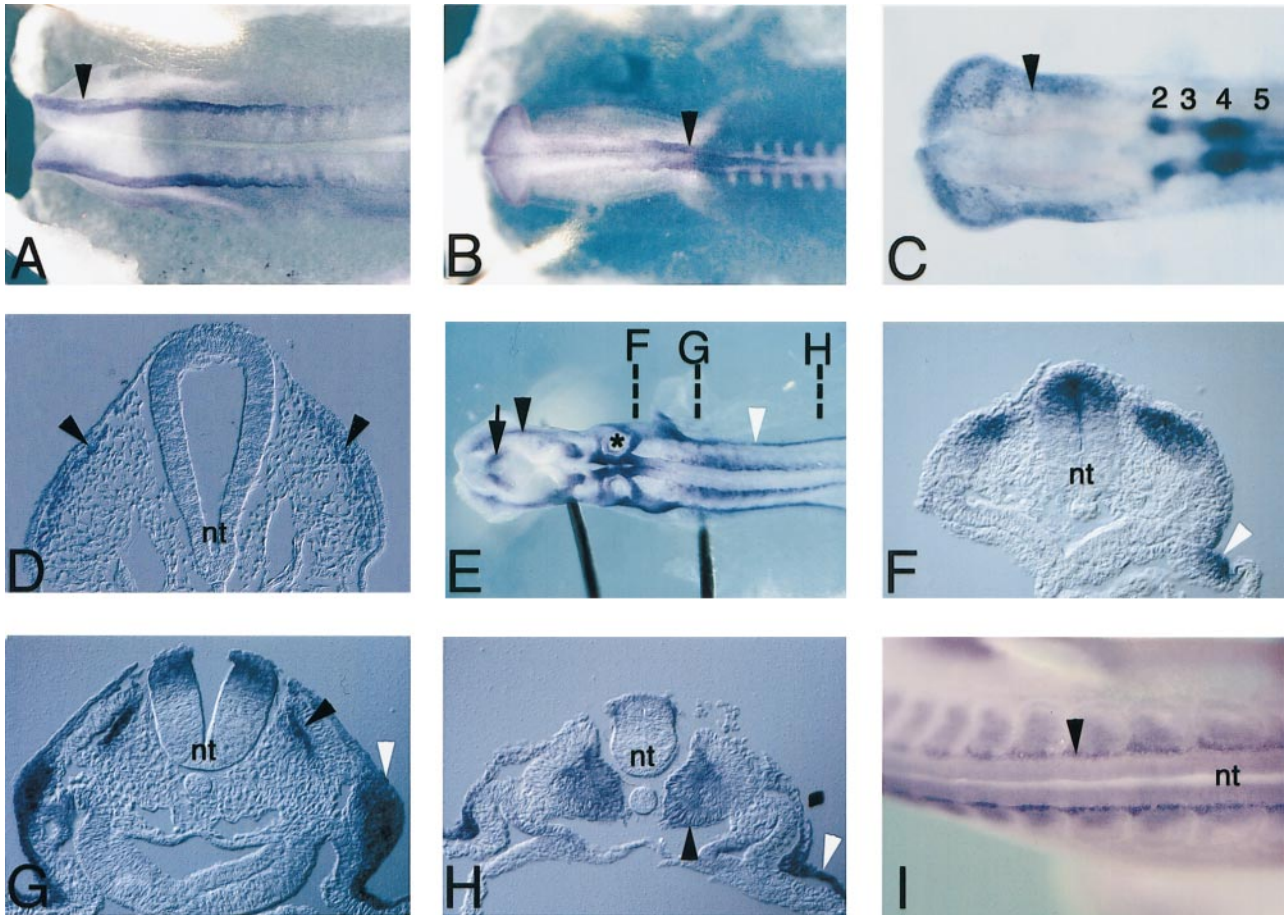

FIG. 2. Early expression of chick DBHR. For all whole mounts, dorsal views are shown, and anterior is to the left. All sections are transverse, with dorsal toward the top. (A) DBHR expression (dark blue) in the neural folds (arrowhead) of a 4-somite embryo (stage 8). (B) An 8-somite embryo (stage 9), showing strong staining in the hindbrain (arrowhead). (C) Expression in a 12-somite embryo (stage 11), in the migrating neural crest (arrowhead), in the midbrain, and in the rhombomeres (numbered) of the hindbrain. Staining is low in r3 and r5, and intense in r2 and r4. (D) Section through the midbrain of an 11-somite embryo, showing staining of neural crest (arrowheads) migrating out from the neural tube (nt). (E) DBHR expression at the 20-somite stage (stage 13), in the dorsal midbrain (arrow), in migrating neural crest in the head (black arrowhead), and in the hindbrain, in rhombomeres 2 and 4–6 as well as neural crest migrating out around the otic vesicle (asterisk). Stripes of expression are also seen in the trunk, medially in the somites, and laterally in the ventral ectoderm (white arrowhead). Dashed lines indicate the level of subsequent sections. (F) Section through the postotic hindbrain, showing staining in the dorsal hindbrain, neural crest, and ventral ectoderm (white arrowhead). (G) Section through a rostral somite, with DBHR expression in the myotome, dorsal spinal cord, and ventral ectoderm (plus underlying mesenchyme; white arrowhead). (H) Section through a more caudal somite, showing weak expression throughout the ventral somite (black arrowhead) and in the dorsal spinal cord, plus strong expression in the ventral ectoderm (white arrowhead). (I) DBHR expression in the trunk of a stage 16 embryo, with strong expression in the emigrating neural crest (arrowhead), and weak expression in the neural tube and myotome.
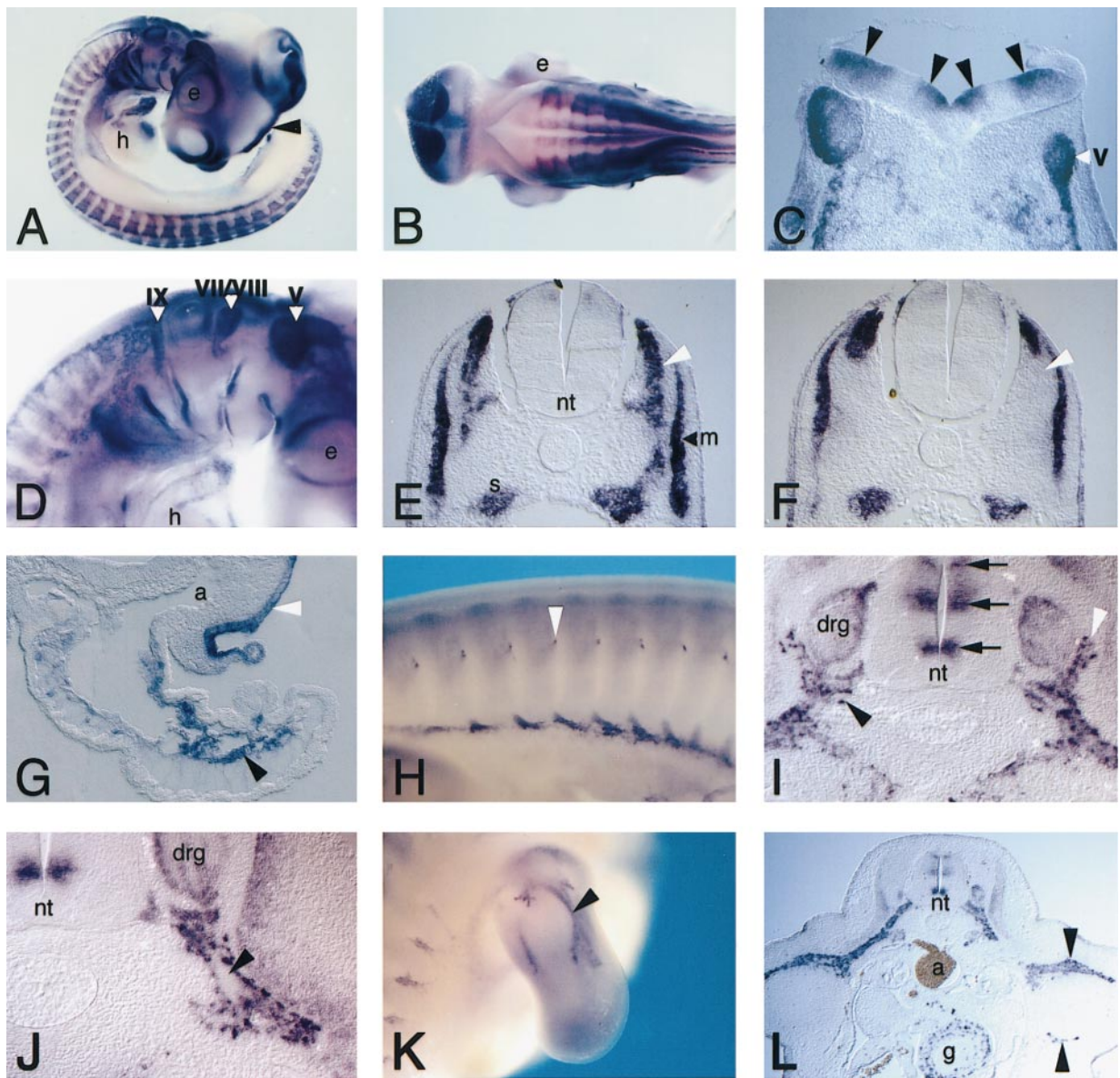
FIG. 3.   DBHR expression in later stages (stage 19 and stage 26). All sections are transverse, with dorsal toward the top. (A) Side view of a 3-day (stage 19) embryo, showing DBHR expression in the dorsal diencephalon (arrowhead), the cranial ganglia, the heart (h), and the myotome of the somites. [Staining in the telencephalon, mesencephalon, and around the eye (e) represents background due to trapping.] (B) Dorsal view of the hindbrain at the same stage, with multiple rostrocaudal and dorsoventral stripes of DBHR expression. Anterior is to the left. (C) Section through the hindbrain and trigeminal ganglion (V) at the same stage. Dorsoventral stripes of DBHR expression in the hindbrain are indicated by arrowheads. (D) Higher magnification side view of the same stage, demonstrating staining in the trigeminal (V), fused geniculate/vestibular acoustic (VII/VIII), and petrosal (IX) ganglia (arrowheads), as well as the margins of the branchial arches. Anterior is to the right. (E) Section through the trunk of a stage 19 embryo. DBHR is expressed in the ventrally migrating neural crest (white arrowhead), developing sympathetic ganglia (s), and myotome (m). (F) Slightly more caudal section through the same region as in (E), showing the same pattern except that DBHR expression is excluded from the sclerotomal region (white arrowhead). (G) Section through the heart of a similar-staged embryo, specifically the outflow tract extending down from the aorta (a) into the truncus arteriosus. DBHR is expressed in the endocardium of the truncus (black arrowhead), as well as in the body wall ectoderm lateral to the aorta (white arrowhead). (H) Side view of the trunk of a 5-day (stage 26) embryo, with dorsal toward the top, showing weak DBHR expression in the myotome, and strong expression in the ventral roots (including the dorsal rami, white arrowhead). (I) Section through the trunk of a stage 26 embryo, with spotty staining in the dorsal root ganglia (drg) and all along the ventral root (black arrowhead), including the dorsal ramus (white arrowhead). DBHR is also expressed in three medial domains (arrows) in the neural tube (nt). (J) Enlarged section similar to (I), showing absence of DBHR expression in the center of the ventral root (arrowhead). (K) Dorsal view of a stage 26 wing bud, showing staining in the nerves that project from the ventral root into the limb bud (arrowhead). (L) Cross-sectional view of these dorsal and ventral nerve projections (arrowheads); DBHR is also expressed in enteric ganglia in the gut (g). [Red staining in the dorsal aorta (a) is due to blood.]
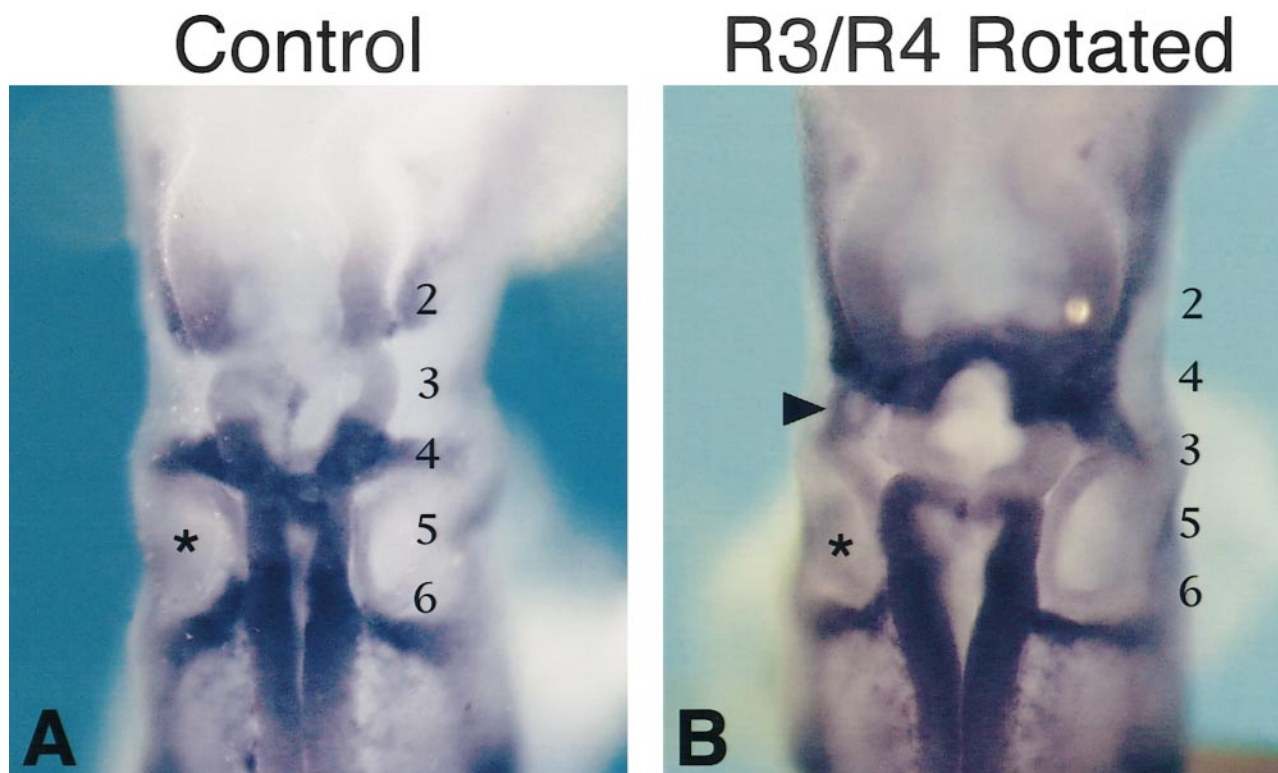
FIG. 4. Expression of DBHR in rotated rhombomeres at stage 14–15. Dorsal views of the hindbrain are shown, with anterior toward the top; the otic vesicle is indicated by an asterisk. (A) In a control embryo, r3/r4 was excised but then reimplanted in its original orientation. DBHR is expressed in the normal pattern (absent in r3, present in r4). (B) In a rotated embryo, DBHR is still expressed in r4, despite its ectopic position, while DBHR expression is still lost from r3. The arrowhead indicates an ectopic otic vesicle, produced by the accidental inclusion of some r5 dorsal ectoderm during dissection of r3/r4. Of 16 rotated embryos that survived and healed properly, 15 displayed the altered pattern shown in (B).

sively more caudal (younger) somites, this expression extends more weakly into the ventral portion of the somite, or prospective sclerotome (black arrowhead, Fig. 2H). Thus, it is likely that DBHR is initially expressed diffusely through the ventral somite, but is progressively restricted to the myotome during the early stages of its formation. DBHR expression in the myotome is maintained for several days of development (Figs. 2I and 3), with staining only declining at stage 26 (Figs. 3H–3L).

DBHR Continues to Be Expressed in the Hindbrain and in Neural Crest Derivatives

After 3 days of development (stage 19), DBHR is highly expressed at multiple locations in the embryo (Fig. 3A). In the diencephalon, DBHR expression in maintained at the dorsal midline (arrowhead, Fig. 3A). In the hindbrain, DBHR expression no longer varies between odd and even rhombomeres, but there remain six thin stripes of expression across the hindbrain which may represent rhombomere boundaries (Fig. 3B). Perpendicular to these stripes are wider bands of expression that subdivide the hindbrain dorsoven-

trally: a weak band just adjacent to the nonexpressing floorplate, and a stronger band further laterally (Figs. 3B and 3C). Thus, DBHR expression forms a complex grid in the developing hindbrain, reflecting both rostrocaudal and dorsoventral organization.

At this stage, DBHR expression is also maintained in neural crest cells which are coalescing and differentiating to form a wide variety of ganglia. In the head, DBHR strongly stains many of the cranial ganglia, including both lobes of the trigeminal ganglion (V, Figs. 3C and 3D), the fused geniculate (VII) and vestibular acoustic (VIII) ganglia, and the petrosal (IX) ganglion (white arrowheads, Fig. 3D). In the trunk, DBHR expression is observed in the ventrally migrating neural crest (white arrowhead, Fig. 3E), including the forming dorsal root ganglia (DRG) and sympathetic ganglia (s, Fig. 3E). The staining pattern observed in transverse section in Fig. 3E alternates with the pattern shown in Fig. 3F, in which DBHR-expressing cells are absent from the sclerotomal region (white arrowhead). This alternating pattern is consistent with DBHR expression marking migrating neural crest, which is excluded from the caudal half of each somite. DBHR is additionally expressed in the heart (h,

Fig. 3A), in the endocardium of the truncus arteriosus (black arrowhead, Fig. 3G), and at the junction of the atrium and ventricle.

After 5 days of incubation (stage 26), DBHR is strongly expressed in all branches of the ventral roots (or spinal nerves; arrowheads, Figs. 3H–3L), including lateral projections (ventral stripes in Fig. 3H), and dorsally projecting dorsal rami (white arrowhead, Fig. 3I), which, in whole embryos, are visible as small patches between the somites (white arrowhead, Fig. 3H). The entire extent of the ventral root is strongly stained, including dorsal and ventral projections into the wing bud (arrowheads, Figs. 3K and 3L). However, this staining is not solid and uniform, but spotty and uneven. Furthermore, staining is not observed in fibers after they enter into the neural tube, or in the center of the nerve (arrowhead, Fig. 3J). Thus, we propose that, at this stage, DBHR is expressed not in these neurons but rather in their supporting Schwann cells. DBHR is also expressed at this stage in a spotty manner in the DRG (Figs. 3I, 3J, and 3L), sympathetic ganglia, and enteric ganglia of the gut (g, Fig. 3L); such staining may represent neural or glial cells, or both. In the spinal cord, DBHR is expressed in the ventricular zone, in three transverse stripes: dorsal, intermediate, and ventral (arrows, Fig. 3I). These dorsoventral domains echo the dorsoventral stripes observed previously in the hindbrain (Figs. 3B and 3C) and maintained at this stage (data not shown).

DBHR Is Not Expressed in All Neural Crest Cells

We have shown that DBHR is a strong marker of much of the neural crest. However, DBHR is not expressed in all neural crest cells at all stages. For example, no staining was observed in dorsally migrating melanocyte precursors (Fig. 3L). In the head, DBHR is expressed at the margins of the branchial arches (Fig. 3D), but not in the neural crest-derived skeletogenic mesenchyme within the arches and face. Thus, DBHR represents a very useful marker for most, but not all, neural crest cells and their derivatives.

DBHR Expression in Specific Rhombomeres Is Maintained after Rotation

The pattern of DBHR expression is highly dynamic, especially in the hindbrain: initially DBHR is expressed throughout the dorsal hindbrain; then expression is up-regulated in even rhombomeres; and finally a complex grid of rostrocaudal and dorsoventral stripes is established. As a preliminary investigation into the signals that regulate DBHR expression, we explored the tissue interactions responsible for DBHR down-regulation in r3 and up-regulation in r4 between stages 10 and 15. This segmented pattern could be produced by cues intrinsic to the rhombomeres themselves; alternatively, external cues, such as signals from the adjacent mesoderm, might control DBHR expression.

To distinguish between these possibilities, we microsur-

gically excised a piece of hindbrain containing r3 and r4 (as well as the overlying ectoderm and underlying notochord), from 9- to 12-somite embryos (stage 10). We chose this stage because the segmented pattern of DBHR expression, as well as rhombomere boundaries in general, is forming but not yet completed, and thus is likely to be subject to regulation. The r3/r4 piece was rotated 180° and reimplanted, thereby establishing a new order of rhombomeres: r2, r4, r3, r5 (Fig. 4B; note that this surgery often causes fusion across the lumen of the neural tube at the cut sites, though this fusion does not otherwise affect the development of the hindbrain). After incubating these embryos for 16 h (until stage 14–15), we performed whole-mount *in situ* hybridization to determine the resulting pattern of DBHR expression. We found that r4 continues to express DBHR in the ectopic r3 position, and that r3 does not express DBHR even in the r4 position (Fig. 4B). These results show that DBHR expression is controlled autonomously, within rhombomeres (with possible contribution from ectoderm or notochord), and that this pattern cannot be overridden by local environmental signals during the stages following rotation.

DISCUSSION

We have identified and characterized chick DBHR, a gene with weak homology to DBH. Chick DBHR is expressed in neural crest through a wide range of stages, beginning with neural crest induction at the lateral border of the neural plate, continuing through neural crest migration, and persisting in differentiating ganglia and glia. Thus, DBHR is a potentially useful neural crest marker, though it is not expressed in all neural crest cells, and it is additionally expressed in myotome and some ectodermal regions. We have examined regulation of segmented DBHR expression in rhombomeres of the hindbrain; we found that the original expression pattern is maintained in rhombomeres placed ectopically, suggesting that DBHR expression during these stages is not responsive to signals from the surrounding tissues.

DBHR/MOX, DBH, and DBHL Form a Family of DBH-Related Genes

Given the low but clear homology between DBHR/MOX, DBHL, and DBH, these genes appear to represent divergent branches of a family of DBH-related genes, which may share a common evolutionary origin. A common origin would also account for similarities in expression patterns, such as the coexpression of DBHR and DBH in the sympathetic ganglia. There is some evidence that DBH is also expressed during development, perhaps only transiently, in other areas of DBHR expression, such as cranial and enteric ganglia (Mercer *et al.,* 1991). One model to explain this transient coexpression would be that the common ancestor of DBH-related genes was expressed broadly throughout the neural crest lineage, and that this original expression pat-

tern was subsequently modified differently in different branches.

Biochemical Activity of DBHR

Although MOX was named for its homology to the monooxygenase DBH, the biochemical activity of DBHR/ MOX genes has not been determined. DBHR is likely to be structurally similar to DBH, given the conserved positions of cysteines involved in intramolecular disulfide bonds. Furthermore, DBHR does contain conserved copper-binding residues, as well as additional active site residues necessary for DBH activity. Thus, DBHR may possess monooxygenase activity, though it may act upon substrates quite different from those of DBH; for example, the same residues critical for DBH activity, and conserved in DBHR, are also conserved in the monooxygenase domain of PAM, which hydroxylates peptidylglycine.

Given the overall low similarity (about 30%) between DBHR and DBH, it is also possible that DBHR is not a functional monooxygenase but rather an evolutionarily derived protein of novel function. One critical difference in DBHR is the nonconservation of cysteines involved in intermolecular disulfide bonds in DBH dimers. Absence of these cysteines in DBHR may prevent dimerization, which in turn could affect efficiency or specificity of action. Other changes may affect DBHR processing and localization; residues that are glycosylated in DBH are not conserved in DBHR (data not shown), and the apparent lack of a signal sequence in human MOX may prevent its secretion or membrane localization. In summary, while the similarity to DBH may provide some clues about DBHR, much remains to be determined about its function.

Biological Activity of DBHR

As described, DBHR could not be assigned a function based on sequence analysis. Furthermore, our attempts to examine DBHR function by overexpression/ectopic expression in chick embryos (by viral infection and electroporation) were inconclusive (data not shown). Nonetheless, the localized and highly regulated expression of DBHR in embryos suggests that this gene plays a specific role in development. If DBHR does function as a monooxygenase, that role could be to synthesize a neurotransmitter. Since the expression of neurotransmitter catabolists often correlates with neuronal differentiation, the reduced expression of DBHR in rhombomeres 3 and 5 (relative to high expression in r2, r4, and r6) at stage 13 is consistent with delayed neuronal differentiation in odd-numbered rhombomeres at this stage (Lumsden and Keynes, 1989). However, DBHR is expressed in the neural crest lineage significantly (2 days) earlier than DBH and TH (stage 18; Ernsberger *et al.,* 2000), and prior to neuronal differentiation as marked by neurofilament or Hu staining (stage 17–18; Marusich *et al.,* 1994). Similarly, DBHR is expressed in the mainly undifferenti-

ated ventricular zone of the spinal cord at stage 26. Finally, DBHR is expressed in nonneuronal as well as neuronal cell types. Thus, the function of the putative DBHR product is unlikely to be limited to neurotransmission.

Instead, DBHR may synthesize a neurotransmitter-like signal with broader cell–cell signaling roles. Certainly, the only commonality in the sites of DBHR expression, in multiple tissues undergoing different developmental processes, is the importance of cell–cell signaling in all these locations. Neurotransmitter molecules may have roles outside the nervous system; for example, it has been suggested that serotonin can act as a developmental signal (Whitaker-Azmitia *et al.,* 1996), including a proposed role in regulating the migration of cranial neural crest (Moiseiwitsch and Lauder, 1995). Similarly, the putative product of DBHR may represent a novel cell–cell signaling molecule in early development.

Regulation of DBHR Expression

DBHR expression in complex, changing patterns is clearly highly regulated. As a first exploration into the mechanisms of this regulation, we concentrated on tissue interactions in the hindbrain, where perhaps the most complex patterns of DBHR expression were observed. Specifically, we asked whether the segmented pattern of expression in the rhombomeres was established and maintained by signaling from the surrounding tissues, or positional information within each rhombomere. We found the latter to be true, as rotated rhombomeres express the level of DBHR that is appropriate to their original location.

In previous r3/r4 rotation experiments (Sechrist *et al.,* 1994), neural crest cells from both r3 and r4 were found to migrate toward the otic vesicle and into the second branchial arch (see Fig. 4B, right side); however, in cases where a small ectopic otic vesicle formed (arrowhead, Fig. 4B, left side), neural crest cells migrated toward that vesicle and into the first branchial arch. The streams of neural crest visualized by expression of DBHR in these experiments are consistent with these previous results, demonstrating that neural crest cells, and their expression of DBHR, can be rerouted by environmental signals.

A formal possibility in these experiments is that DBHR expression in the hindbrain is regulated by previously uncharacterized signals from the dorsal ectoderm or notochord, as both tissues were rotated along with the neural tube. However, there are certainly sufficient mechanisms within the neural tube to explain maintenance of the DBHR expression pattern. In particular, the Hox genes become expressed in their characteristic patterns in the hindbrain during roughly the same period that the DBHR pattern is established (Hunt *et al.,* 1991a). Thus, regulation of DBHR expression may be a downstream consequence of Hox gene expression and the establishment of rhombomere identity. Further analysis, perhaps using electroporation to misexpress putative upstream regulators, will be necessary to

explore DBHR regulation in finer detail, and in additional tissues.

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