DEVELOPMENTAL BIOLOGY 144, 215-219 (1991)

Alternate Expression of the HNK-1 Epitope in Rhombomeres of the Chick Embryo

SHIGERU C. KURATANI

Department of Anatomy, Medical College of Georgia, Augusta, Georgia 30912-2000

Accepted November 20, 1990

Rhombomeres are regarded as the manifestation of innate segmentation within the vertebrate CNS. To investigate developmental changes occurring in the CNS and PNS, a series of chick embryos were immunostained with several monoclonal antibodies. The HNK-1-immunoreactivity (IR) appeared in rhombomeres (r) 3 and r5 around stage 15, when r2 and r4 were not stained. This alternate pattern is similar to the Krox-20 gene expression in the mouse embryo. At levels of r2 and r4, HNK-1$^+$ neural crest cell masses were attached to the CNS forming cranial sensory ganglia. In these rhombomeres, an accumulation of neuroepithelial cells near the cranial nerve root and early development of neuroblasts in the basal plate were observed. The above observations seem to suggest that the alternate HNK-1-IR in rhombomeres might be related to the expression of cell adhesion molecules, and therefore also to the adhesion of the cranial ganglion precursors to the CNS, which takes place every other rhombomere in the preotic region. Thus, the alternate pattern of the HNK-1-IR seems to be related to the morphogenesis of preotic branchial nerves.

INTRODUCTION

Metamerism within the vertebrate CNS, when considered from a developmental perspective, is an intriguing topic. It has been suggested that the apparent segmentation of the spinal cord is established through induction by the paraxial mesoderm (Keynes and Stern, 1984). In the hindbrain, on the other hand, an innate segmentation is apparent, which is represented by the arrangement of reticulospinal neurons in the zebrafish larva (reviewed by Kimmel *et al.*, 1988). In higher vertebrates, the earliest neurons in the hindbrain develop in a segmental arrangement in accordance with rhombomeres as revealed by Lumsden and Keynes (1989). Furthermore, homeobox-containing genes are expressed in a rhombomere-related pattern (reviewed by Holland and Hogan, 1988; Wilkinson *et al.*, 1989b). Thus, the hindbrain is conspicuous, when compared with the spinal cord, in its possession of an innate segmental pattern.

The study described here was designed to investigate segmentation in the CNS and PNS of the chick embryo. The results show a unique expression pattern of the HNK-1 epitope in rhombomeres which seemed topographically related to cranial sensory ganglion primordia, and thus to neurogenesis within the CNS. This suggests that the HNK-1 epitope in rhombomeres may be a part of a molecule involved in the morphogenesis of CNS and PNS.

MATERIALS AND METHODS

Fertilized Arbor Acre (for HNK-1 and E/C8 staining) and White Leghorn (for neurofilament protein (NFP) staining) chicken eggs were incubated at 37°C and constant humidity. Embryos were staged after Hamburger and Hamilton (1951) and fixed for 1 hr at room temperature in either Bouin's fixative for HNK-1, Carnoy's fixative for E/C8, or Zamboni's fixative for NFP immunolocalization. The embryos were embedded in paraffin and sectioned at 15 μm. The sections were deparaffinized and treated with 2% periodic acid for 10 min. The incubation with primary monoclonal antibodies (MAbs) was as follows: for HNK-1, Leu-7 (Becton Dickinson, diluted 1/20) was applied for 1 hr at room temperature; E/C8 (gift of Gary Ciment, diluted 1/20) was applied for 1 hr at room temperature; anti-NFP MAb (IT-0168, all neurofilaments, 70 K + 160 K + 210 K, DP5 + 43 + 12, Cosmo Bio., diluted 1/100) was applied for 1 day at 4°C. all the primary MAbs were diluted in PBS containing 0.3% Triton X-100, 0.2% BSA, 0.1% sodium azide. Secondary antibodies were diluted in PBS containing 0.3% Triton X-100, 0.2% BSA and applied for 30 min for each primary antibody. For HNK-1, HRP-conjugated anti-mouse IgM, diluted 1/400, was used and for E/C8 and anti-NFP MAb, HRP-conjugated anti-mouse IgG, diluted 1/200, was used. The peroxidase reaction was performed in 100 ml Tris-HCl buffer containing 1.5 mg 3,3′-diaminobenzidine and 10 μl of 30% hydrogen peroxide.

RESULTS AND DISCUSSION

An alternating pattern of HNK-1-IR in rhombomeres was recognized by stage 13 in this study, where a slight reaction was observed near the ventricular surface in all

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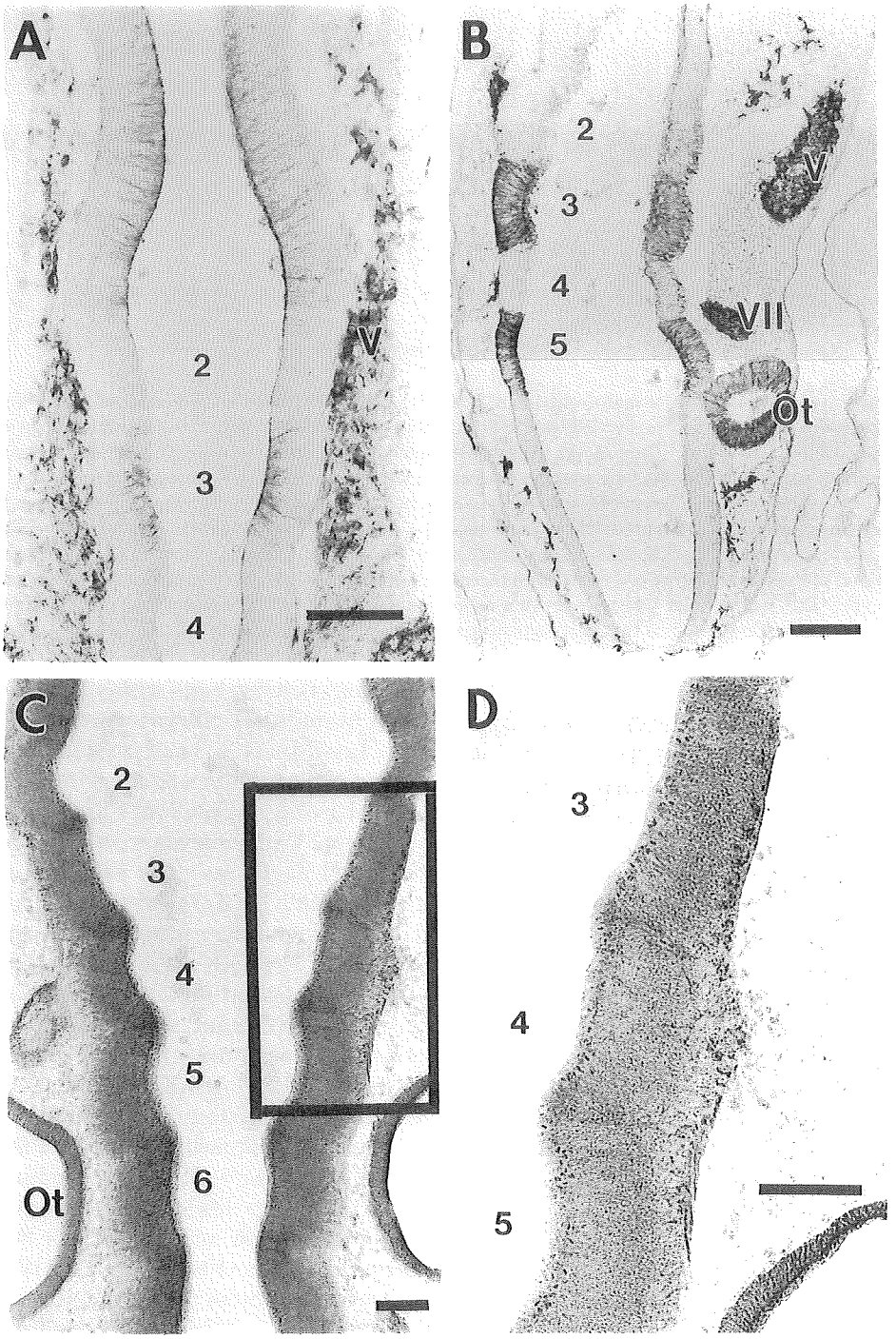

FIG. 1. HNK-1-IR in developing chick embryos. Sections are cut horizontally. At stage 13 (A), the alternate pattern of the HNK-1-IR is detected from r2 to r4. At stage 15 (B), an alternate pattern is seen in r2 through r5. At stage 19 (C, D, counterstained with hematoxylin), the alternating pattern of the HNK-1-IR becomes indistinct below the level of nerve roots. Ot, otocyst; V, neural crest representing the trigeminal ganglion precursor; VII, neural crest population representing the acusticofacialis ganglion precursor. Numbers designate the number of the rhombomeres. Bar = 100 μm.

the rhombomeres except r2 and r4 (Fig. 1A). The staining pattern of the HNK-1 antibody within the neuroepithelium resembles that of neural cell adhesion molecule (NCAM) expression reported by Thiery *et al.* (1982) and by Lumsden and Keynes (1989). However, in these studies, an alternating pattern was not reported. Antibody labeling increased with development and became most intense in r3 and r5 at stage 15 (Fig. 1B), when the

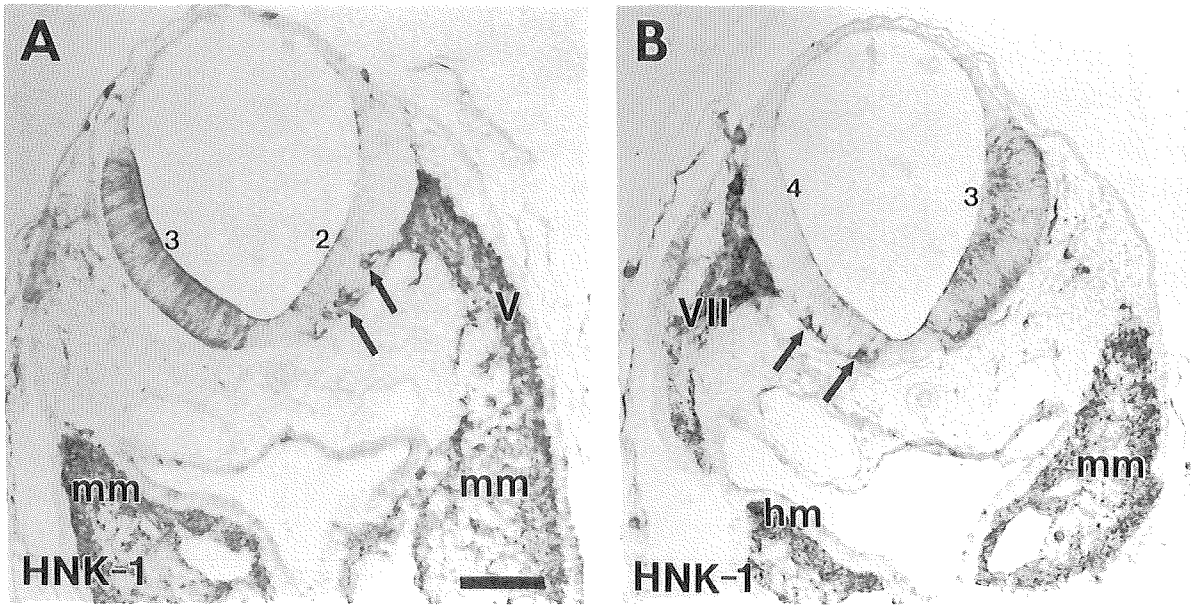

FIG. 2. Stage 15 chick embryo stained with HNK-1. Transverse sections have been cut somewhat obliquely. In r2 and r4, which do not express the HNK-1 epitope except for several motoneurons (arrows), cranial sensory ganglion connect with the CNS. Note that the HNK-1-IR is restricted to the area between the floor and roof plates in r3. Ventrally, ectomesenchymal cells within the mandibular and hyoid arches are HNK-1$^+$. hm, hyoid arch ectomesenchyme; mm, mandibular arch ectomesenchyme. Bar = 100 μm.

HNK-1-immunoreactivity (IR) was rather even within the neuroepithelium except for the roof and floor plates. After stage 15, this alternate staining pattern gradually became indistinct (Figs. 1C, 1D) and by stage 25 was barely observed except for a small less stained area in the dorsal portion of the alar plate in r2 and r4 (data not shown).

At stage 15, the HNK-1-IR was most intense in the ventricular surface of the neuroepithelium (Figs. 2A, 2B). Up to this stage, the HNK-1-IR was almost absent in r2 and r4 (Figs. 2A, 2B) except for the primitive medial column neurons described by Covell and Noden (1989) (Figs. 2A, 2B). Neural crest cell populations representing the sensory ganglion primordia of trigeminal and acusticofacialis nerves were attached extensively to the lateral aspect of these nonreactive rhombomeres (Figs. 2A, 2B).

Neurogenesis within the CNS had already begun by stage 14. As observed by the E/C8 immunostaining (Ciment *et al.*, 1986), E/C8$^+$ neuroepithelial cells were accumulated in r2 and r4 at the level where the cranial sensory ganglia were attached to the CNS (Fig. 3A). In these rhombomeres, neuroblasts in the basal plate appeared to be comparatively mature and possessed neurites (Fig. 3A). In r3 and r5, on the other hand, E/C8$^+$ neuroepithelial cells were scattered evenly within the neuroepithelium (Fig. 4A). Neurogenesis was barely detectable within the sensory ganglion primordium at this stage (Fig. 3A). Similar results were observed in the immunolocalization of NFP at stage 15 (Figs. 4A, 4B). The accumulation of the NFP$^+$ cells was observed in association with the attachment of the cranial sensory ganglion primordia (Fig. 4B). Thus, HNK-1-IR in rhombomeres shows an alternating segmental pattern which is seemingly related to cranial sensory ganglion development and neurogenesis in the CNS.

The pattern I have observed could be related to differential gene expression. Of the genes whose expression within rhombomeres has been examined to date, Krox-20 in the 9.5-day-old mouse embryo (Wilkinson *et al.*, 1989a) shows almost the same pattern as the HNK-1-IR, i.e., the gene is transcribed in r3 and r5 and not in r2 and r4. Unlike the expression of the HNK-1 epitope in chick, Krox-20 is expressed even before the appearance of the rhombomeric boundaries, and it is seen in all parts within the neuroepithelium (Wilkinson *et al.*, 1989a). The HNK-1-IR was restricted to an area between the floor and roof plates, with the alternating pattern appearing after the rhombomeres become visible (Figs. 1A, 2). It is interesting that, at this stage of development, cranial sensory ganglion primordia opposed to r2 and r4 also express the Krox-20 gene product (Wilkinson *et al.*, 1989a). Wilkinson and others (1989a) have noted the coincidence between the Krox-20 gene expression and later development of motoneurons whose pattern is also alternating in the hindbrain (Lumsden and Keynes, 1989). It is still unclear, however, whether this gene is directly involved in neurogenesis. Because it appears

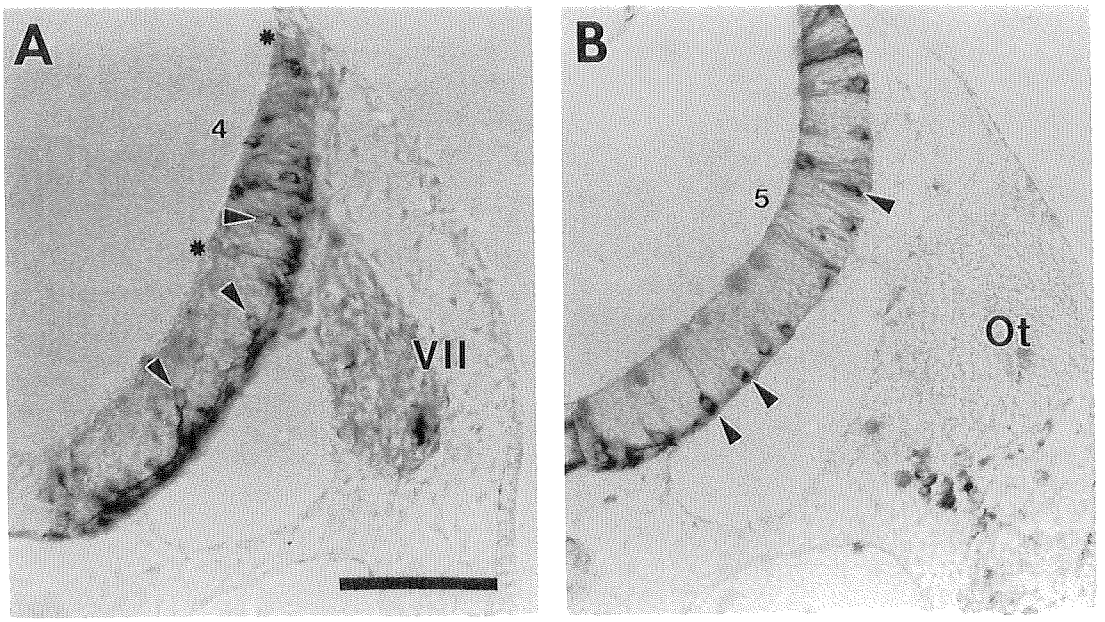

FIG. 3. E/C8-IR in stage 14 chick embryo. In r4 (A), E/C8$^+$ neuroblasts are observed in the basal plate (arrowheads). E/C8$^+$ neuroepithelial cells accumulate near the facial nerve root (between asterisks), while at the level of r5 (B), they are evenly scattered within the neuroepithelium (arrowheads). Bar = 100 μm.

early in the development, Wilkinson *et al.* (1989a) have assumed that Krox-20 plays a role in determining CNS segmentation, in a manner similar to pair-rule genes found in the development of *Drosophila.* This idea is very attractive in light of the fact that such an alternating pattern is found only in the preotic region, where two rhombomeres are assigned to one pharyngeal arch. However, it remains to be determined whether there is a gene in the chick homologous with Krox-20 and whether it might be related to an alternate expression or modulation of cell adhesion molecules (CAMs) in rhombomeres. Such a two-rhombomere-based segmentation is less clear caudal to the otic placode. Interestingly, expression of other Hox-2-cluster genes is also limited at two-segment intervals (Wilkinson *et al.*, 1989b). However, the limits of expression are located in the anterior sulci of r3 and r5, and do not correspond in their motoneuron assignment to cranial nerves V and VII.

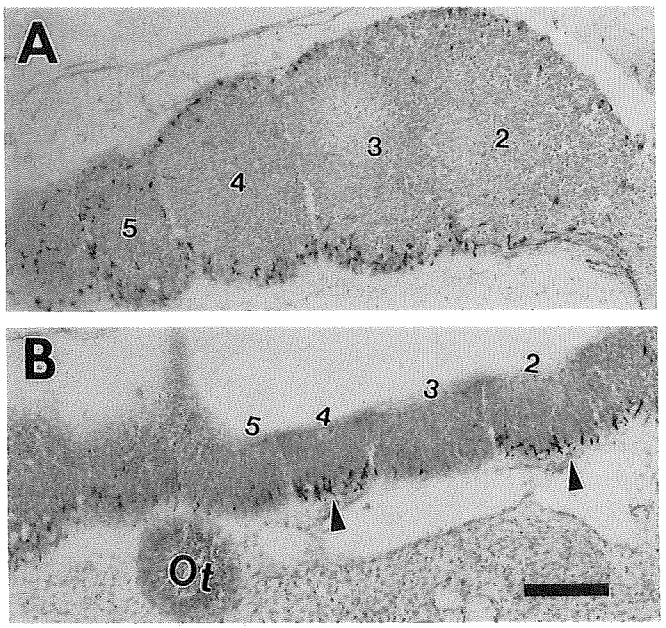

FIG. 4. NFP-IR in stage 15 chick embryo. The plane of sections is approximately horizontal. In the dorsal portion of the CNS (A), no alternating pattern is apparent during development of the marginal zone, while ventrally (B), near the level of cranial nerve roots (arrowheads), neurons accumulate in r2 and r4. Bar = 100 μm.

The HNK-1 is known to recognize a sulfated glucuronic acid-containing carbohydrate epitope, a determinant which is shared by several adhesion proteins (Chou *et al.*, 1986; Shashoua *et al.*, 1986). Of these glycoproteins, NCAM appears early in the chick development (Hoffman *et al.*, 1982) and seems to be involved in morphogenesis of the nervous system (Thiery *et al.*, 1982). In the present study, it was not determined whether the HNK-1-IR in the CNS represents NCAM. Still, like HNK-1-IR, NCAM-IR is also intense in the ventricular surface of the earliest neuroepithelium (Thiery *et al.*, 1982). This type of distribution of CAMs might be correlated with the regulation of the neuroepithelial cell cycle by anchoring the foot plates of these cells to the luminal surface. Lumsden (1990) has suggested that an alternate expression of CAMs could play a role in the formation of

boundaries in the hindbrain, inhibiting the intermingling of cells from one rhombomere to another.

It is significant that the alternate expression of the HNK-1 epitope becomes evident at a time when the neural crest-derived cranial ganglion precursors become adherent to the hindbrain. This process is important in the proper sprouting of the motoneurons into each of the cranial nerve roots, as suggested by Moody and Heaton (1983a,b). In all vertebrates, the cranial ganglion precursors V and VII adhere to the CNS at the levels of r2 and r4, respectively (Kuhlenbeck, 1935). The alternate expression of different kinds of CAMs would allow adhesion of ganglion precursors in an alternate fashion as well.

I thank Gary Ciment for the gift of E/C8 antibody, Shigeki Hirano for valuable discussions and technical advice, and Margaret Kirby for critical reading of the manuscript.

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