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Original Article

Tissue and developmental distribution of *Six* family gene products

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ABSTRACT To examine the presence and distribution of Six family gene products in a variety of tissues at various developmental stages and in various cell types, we prepared specific antibodies against recombinant *Six* gene products. The distribution of Six2 and Six4 was examined by immunostaining in the developing mouse embryo. Production of Six2 was detected at E8.5 mainly in the mesenchyme, while Six4 was present in nuclei of neuronal cells in the peripheral region of the mantle layer of developing brain and spinal cord and in various ganglia at E10.5 and E11.5. Specific DNA binding activities of the Six proteins were analyzed by gel retardation super-shift assays using nuclear extracts from different rat tissues and cell lines. Six5 was the dominant isoform observed in the adult kidney, liver and lung but not in the brain. Six4 was not detected in all tested adult tissues, however, it was present in embryonic (FD21) lung nuclear extracts. In contrast, *Six4* was detected in a variety of cultured cell lines, including HeLa, 3T3, MDCK and C2C12. Our results suggest that Six4 plays a specific role in the differentiation or maturation of neuronal cells, while Six5 is an adult type *Six* gene isoform product and is distributed in the kidney, liver and lung.

KEY WORDS: *mouse embryo, immunohistochemistry, nuclear extracts, gel retardation assay, Six family genes*

Introduction

The *Six* family genes have been identified as murine homologs of *Drosophila sine oculis* gene, which is essential for the formation of a complex visual system (Cheyette *et al.*, 1994; Serikaku and O'Tousa, 1994). Five *Six* family genes (*Six1* to *Six5*) have been identified in the mouse (Oliver *et al.*, 1995a,b; Kawakami *et al.*, 1996a,b). The expressions of *Six1*, *Six2* and *Six3* genes are restricted to specific locations and at specific developmental stages as revealed by *in situ* hybridization analysis (Oliver *et al.*, 1995a,b). *Six4*/AREC3 was isolated as a regulatory factor that binds to the positive regulatory region of Na,K-ATPase α1 subunit gene (Kawakami *et al.*, 1996a), while *Six5* was isolated from mouse retina cDNA library (Kawakami *et al.*, 1996b) and also identified as a murine homolog of the myotonic dystrophy associated homeobox protein gene (*DMAHP*) which is located downstream of the causative CTG repeat (Boucher *et al.*, 1995). The conserved regions of the *Six* family genes are composed of 110 amino acid *Six* domain and 60 amino acid *Six* type homeodomain. Both domains are necessary for the sequence specific DNA binding activity (Kawakami et *al.*, 1996a,b).

To understand the biological function of these homeobox protein family genes, it is necessary to explore the regulatory mechanisms determining the distribution of gene products. In the first step, we analyzed the distribution of *Six2*, *Six4* and *Six5* gene products that share the same DNA binding specificity (Kawakami *et al.*, 1996b). For this purpose, we prepared specific antibodies against these products and tested the distribution of *Six2* and *Six4* gene products in the mouse embryo during early stages of development by using whole-mount and section immunohistochemistry. We also examined the presence of the protein in nuclear extracts in various adult rat tissues and a variety of cell lines.

Results

Specificity of antibodies

To confirm the mono-specificity of the affinity purified antibodies, we performed western blot analysis using C2C12 cell nuclear extracts, in which the presence of Six4 and Six5 proteins was confirmed by gel retardation super-shift assay (data not shown). Anti-Six2 antibody detected two proteins with an apparent molecular weight of 60 kDa and 44 kDa, the anti-SIX4 detected a 67 kDa protein consistent with the previous result using antiserum (Kawakami *et al.*, 1996a), while anti-Six5 detected a 71 kDa protein (data not shown). Although anti-Six5 and anti-SIX4 detected the

Abbreviations used in this paper: P3, postnatal day 3; FD21, foetal day 21; GST, glutathione-S-transferase.

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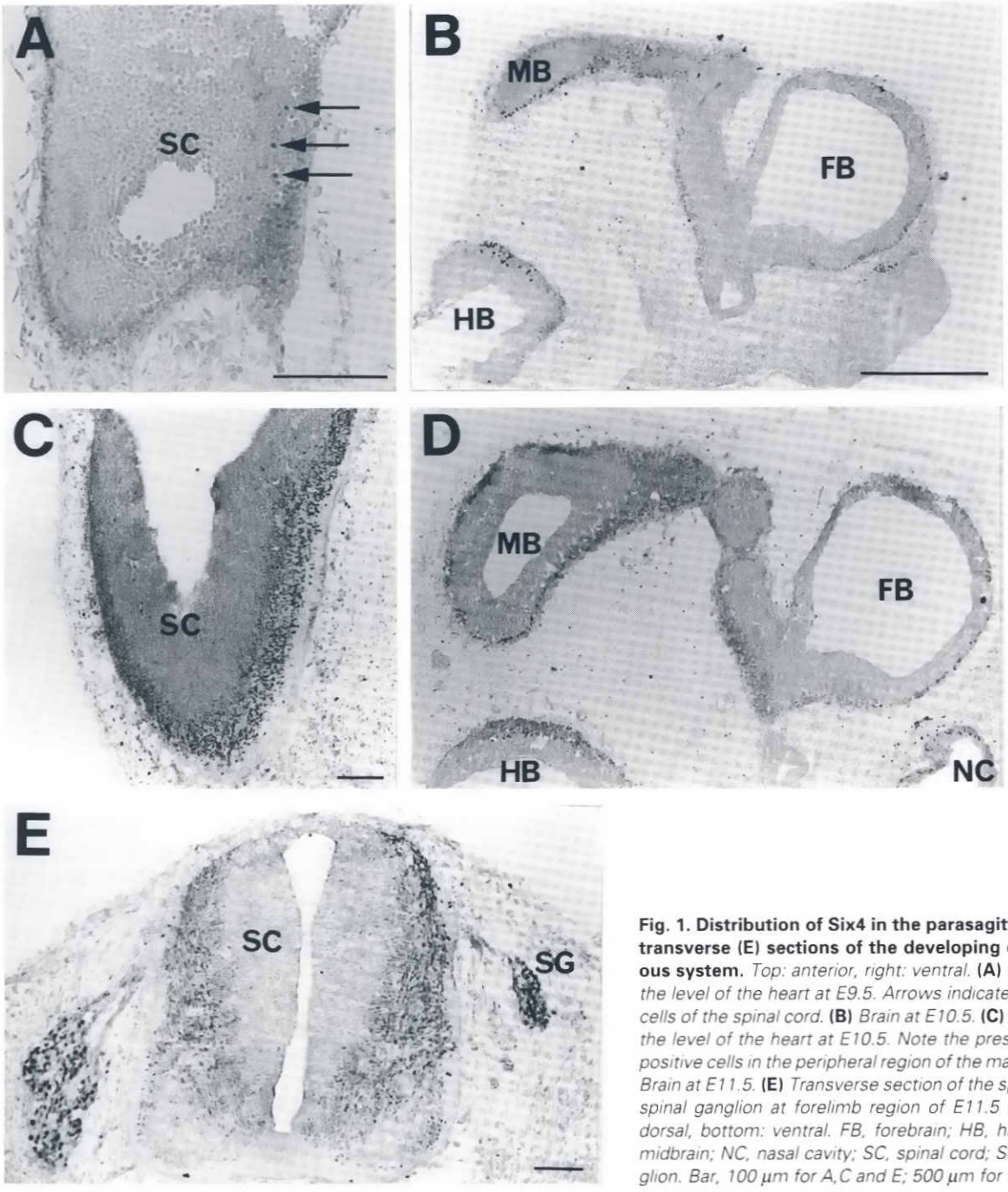

Fig. 1. Distribution of Six4 in the parasagittal (A-D) and transverse (E) sections of the developing central nervous system. *Top: anterior, right: ventral.* **(A)** *Spinal cord at the level of the heart at E9.5. Arrows indicate Six4-positive cells of the spinal cord.* **(B)** *Brain at E10.5.* **(C)** *Spinal cord at the level of the heart at E10.5. Note the presence of Six4-positive cells in the peripheral region of the mantle layer.* **(D)** *Brain at E11.5.* **(E)** *Transverse section of the spinal cord and spinal ganglion at forelimb region of E11.5 embryo. Top: dorsal, bottom: ventral. FB, forebrain; HB, hindbrain; MB, midbrain; NC, nasal cavity; SC, spinal cord; SG, spinal ganglion. Bar, 100 μm for A,C and E; 500 μm for B and D.*

proteins of the similar molecular weight, we never observed cross reaction by gel retardation super-shift assays (see Fig. 5). These results confirmed the mono-specificity of each antibody. Furthermore, no cross reactivity among these antibodies was observed with recombinant fusion proteins including the moiety for antibody production (data not shown).

Distribution of Six4 in mouse embryo

Previous studies have suggested that the *Six* family genes are involved in the developmental process in mice since the expression of *Six1*, *Six2* and *Six3* is restricted to specific regions and at specific stages of development (Oliver *et al.*, 1995a,b). To explore the function of *Six4*, we examined the site of the gene product produced during embryogenesis in mice. We prepared serial sections of the developing mouse embryo and identified the location of Six4 by immunostaining. Staining was observed only in the central and peripheral nervous systems. The distribution of Six4 in the central nervous system is summarized in Figure 1. The production of Six4 was first observed as early as E9.5 in a few cells in spinal cord periphery (Fig. 1A). The staining appeared in the mantle layer at E10.5 and E11.5 (Fig. 1C,E) but became weak at E12.5 (data not shown). At E10.5, most peripheral regions of the

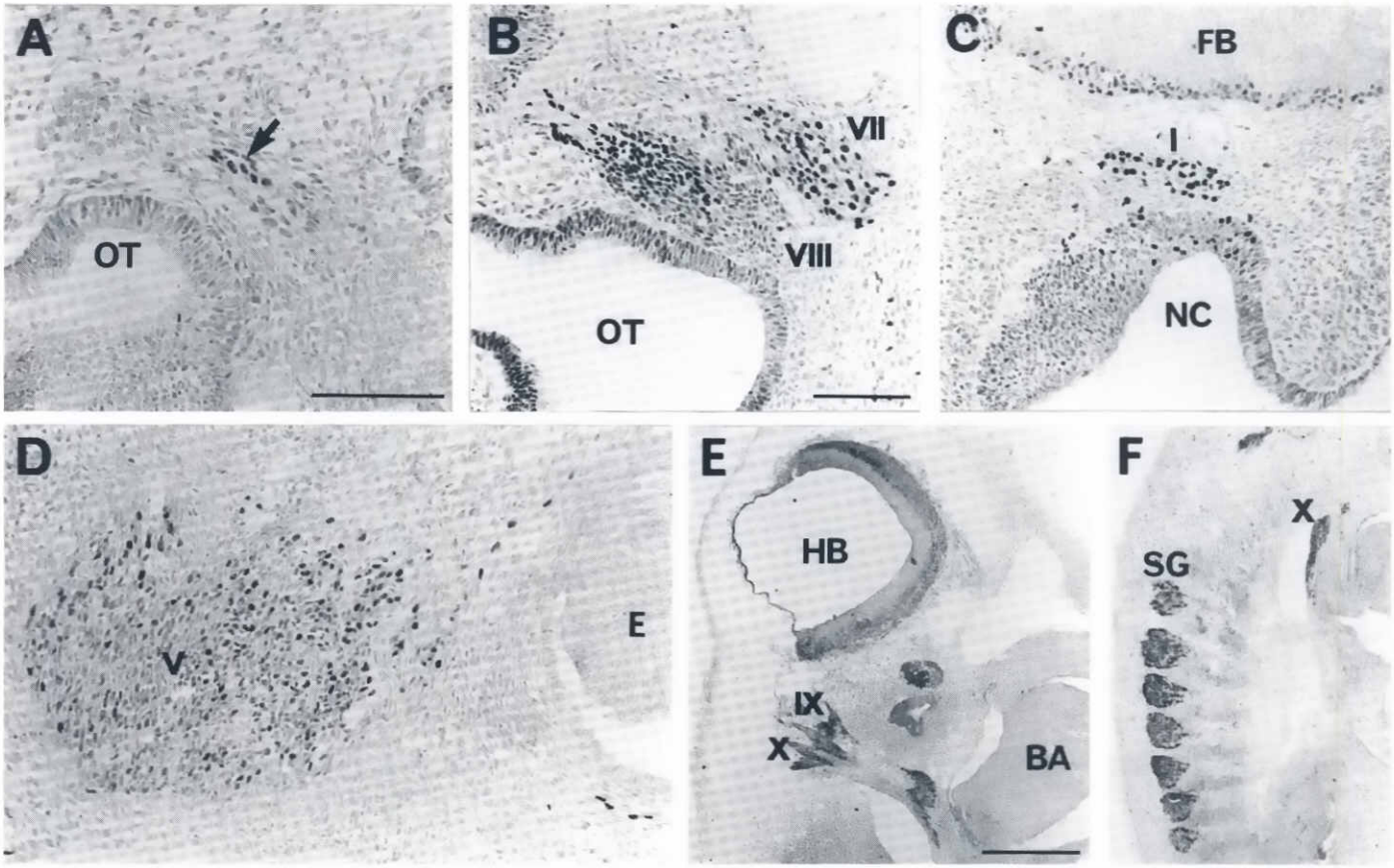

Fig. 2. Distribution of Six4 in parasagittal sections of various peripheral nervous system. *Top: anterior, right: ventral.* **(A)** *E9.5. Arrow indicates the staining in geniculate and acoustic ganglia.* **(B to D)** *E10.5.* **(E and F)** *E11.5. BA, branchial arch; E, eye; FB, forebrain; HB, hindbrain; NC, nasal cavity; OT, otic vesicle; SG, spinal ganglion; I, olfactory nerve; V, semilunar ganglion; VII, geniculate ganglion; VIII, acoustic ganglion; IX, superior and petrosal ganglia; X, jugular and nodose ganglia. Bar, 100 μm for A to D; 500 μm for E and F.*

mantle layer of forebrain, midbrain and hindbrain area were stained (Fig. 1B), but the staining became strong at E11.5 (Figs. 1D,2E). However, it disappeared at E13.5 in the midbrain and hindbrain (data not shown), although a weak staining was still evident in the forebrain at E14.5 (data not shown).

Figure 2 shows positive sites in peripheral nervous system. The production of Six4 was first observed as early as E9.5 in geniculate and acoustic ganglia (Fig. 2A). The staining was most prominent at E10.5 (Fig. 2B) and E11.5 but disappeared at E12.5 (data not shown). In other peripheral nervous regions, Six4 protein was initially detected at E10.5 in such as the olfactory nerve and olfactory epithelium (Fig. 2C), superior and petrosal ganglion, jugular and nodose ganglion (data not shown) and semilunar ganglia (Fig. 2D). The cells expressing Six4 protein was increased at E11.5, for example, in the superior and petrosal ganglion, jugular and nodose ganglion (Fig. 2E). Most staining of these regions disappeared at E13.5 and E14.5 (data not shown). On the other hand, staining in the spinal ganglion was noted at E10.5, but was most prominent at E11.5 (Figs. 1E, 2F), becoming weak at E14.5 (data not shown).

Distribution of Six2 during embryogenesis

In situ hybridization analysis of *Six2* mRNA in the mouse embryo have shown that the gene is expressed in the head and body mesenchyme, limb muscles and tendons (Oliver *et al.*, 1995b). To explore the mechanisms of translational control of the gene expression, we analyzed the location of Six2 by whole-mount and section immunohistochemistry.

Production of Six2 was not observed prior to E7.5. At E8.5, Six2 staining appeared in mesoderm cells at the hindbrain level in whole-mount analysis (Fig. 3A), and in the foregut region and a limited area in the heart by section immunostaining analysis (data not shown). At E9.5, Six2 protein was detected in the head mesenchyme, particularly in the frontal region, mesenchyme of the thoracic region, and first branchial arch. The esophageal-pharyngeal and midgut mesenchymes corresponding to stomach anlage were also stained at that stage (Fig. 3B). Production at Rathke's pouch was also detected (data not shown).

In addition to the regions described above, Six2 appeared at E10.5 in the mesenchyme of nephrogenic cords and thoracic somite (Fig. 3C), forelimb bud mesenchyme (data not shown), but the staining at Rathke's pouch became prominent (data not shown). As shown in Figure 4A, a small cell population at the anterior part of the hindbrain also produced Six2 protein. The region overlapped with Six4 positive region (see Fig. 1B). The epithelia of the otic vesicle and nasal cavity were also stained (data not shown).

At E11.5, the Six2 positive cells in the tip of the first branchial arch (data not shown), nasal cavity (Fig. 4B), the otic vesicle (Fig.

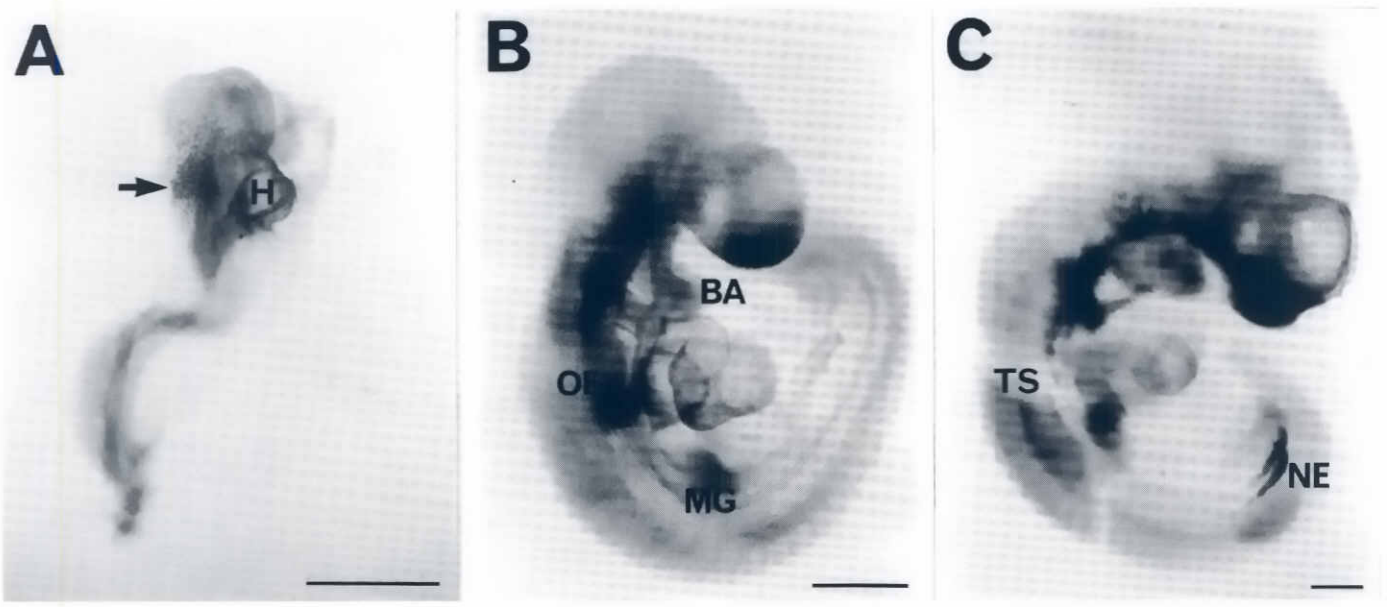

Fig. 3. Distribution of Six2 in the developing mouse analyzed by whole-mount immunostaining. *Top: anterior, right: ventral.* **(A)** *E8.5. Arrow indicates the head mesenchyme at the hindbrain level.* **(B)** *E9.5.* **(C)** *E10.5. BA, first branchial arch; H, heart; MG, midgut; NE, nephrogenic cords; OE, esophageal-pharyngeal area; TS, thoracic somites. Bar, 500 μm.*

4C) and the forelimb bud mesenchyme (Fig. 4D) were increased. Several Six2 positive cells were present in the same region containing Six4 positive cells in the olfactory epithelium (Fig. 1D). Staining at genital eminence and hindlimb bud began to appear (data not shown).

At E12.5, staining disappeared at midgut mesenchyme (corresponding to stomach anlage) and thoracic somites (data not shown). Staining at myotome and sclerotome appeared for the first time (Fig. 4E). The Six2 gene product was distributed in the mesenchyme of nephrogenic cords which forms the peripheral part of the kidney during development of the organ (Figs. 3C, 4E). At the same stage, the genital eminence became strongly stained (Fig. 4E). At E13.5, most of the staining observed at E12.5 disappeared. Staining in mesenchyme at the frontal region of head, thorax and at the tip of forelimbs and hindlimbs persisted (data not shown). However, at E14.5, most of the staining had disappeared.

Gel retardation analysis of Six family gene products in tissue nuclear extracts

To our knowledge, the production of the Six proteins has not yet been confirmed in the adult stage. To examine the presence of products as specific DNA binding proteins, we performed gel retardation super-shift assay of the Six proteins in nuclear extracts from four different tissues obtained from adult rats. We took advantage of using the C3 oligonucleotide probe containing the common binding sequence among Six2, Six4, and Six5 proteins to detect these products at the same time (Kawakami *et al.*, 1996b). The Six4 protein was originally identified as an ARE (*Atp1a1* Regulatory Element) binding protein of Na,K-ATPase α1 subunit gene in nuclear extracts from various cells (Suzuki-Yagawa *et al.*, 1992; Kawakami *et al.*, 1993). Figure 5A shows that the binding protein to the probe was present in the kidney, liver and lung (lanes 1,9,13). The formation of the major migrating complexes observed in the nuclear extracts of these tissues was specifically competed with excess wild type C3 oligonucleotide, while it was not competed with the mutation oligonucleotide (data not shown). The complex observed in the brain nuclear extract was not specific (lane 5) since both wild type and mutation C3 competitors did not interfere with the formation of this complex (data not shown). The addition of anti-Six5 serum inhibited the formation of the major retarded complex in the kidney, liver and lung nuclear extracts (lanes 4,12 and 16). In contrast, addition of anti-Six2 or anti-SIX4 serum produced little or no effect on the formation of the complex (lanes 2,3,6,7,10,11,14 and 15). These results indicated that *Six5* gene products are the major components of Six binding activities in the nuclear extracts of the adult kidney, liver and lung.

To analyze the developmental regulation of the Six proteins in the lung, we prepared nuclear extracts from neonatal (P3) and embryonic (FD21) lungs and compared the presence of Six proteins with that in tissues obtained from the adult rat. Figure 5B shows that the addition of anti-Six5 serum inhibited major complex formation in FD21 lung, as observed in the adult lung (lanes 4 and 12). Furthermore, complex formation was partially inhibited by the addition of anti-SIX4 serum (lane 11). A similar result was obtained using P3 nuclear extracts (lanes 5-8). These results indicated that the Six4 and Six5 proteins were produced in the embryonic lung, however, Six4 disappeared after tissue maturation.

Distribution of Six family gene products in nuclear extracts of cultured cell lines

cDNAs for *Six4* were obtained from mouse cDNA libraries from adult skeletal muscle and C2C12 myoblast cells, while those for *Six2* and *Six5* were obtained from the retina cDNA library (Kawakami *et al.*, 1996a,b). To investigate the exact type of Six proteins produced by each cell line, we examined the DNA binding activity of nuclear extracts obtained from several cell lines. Specific re-

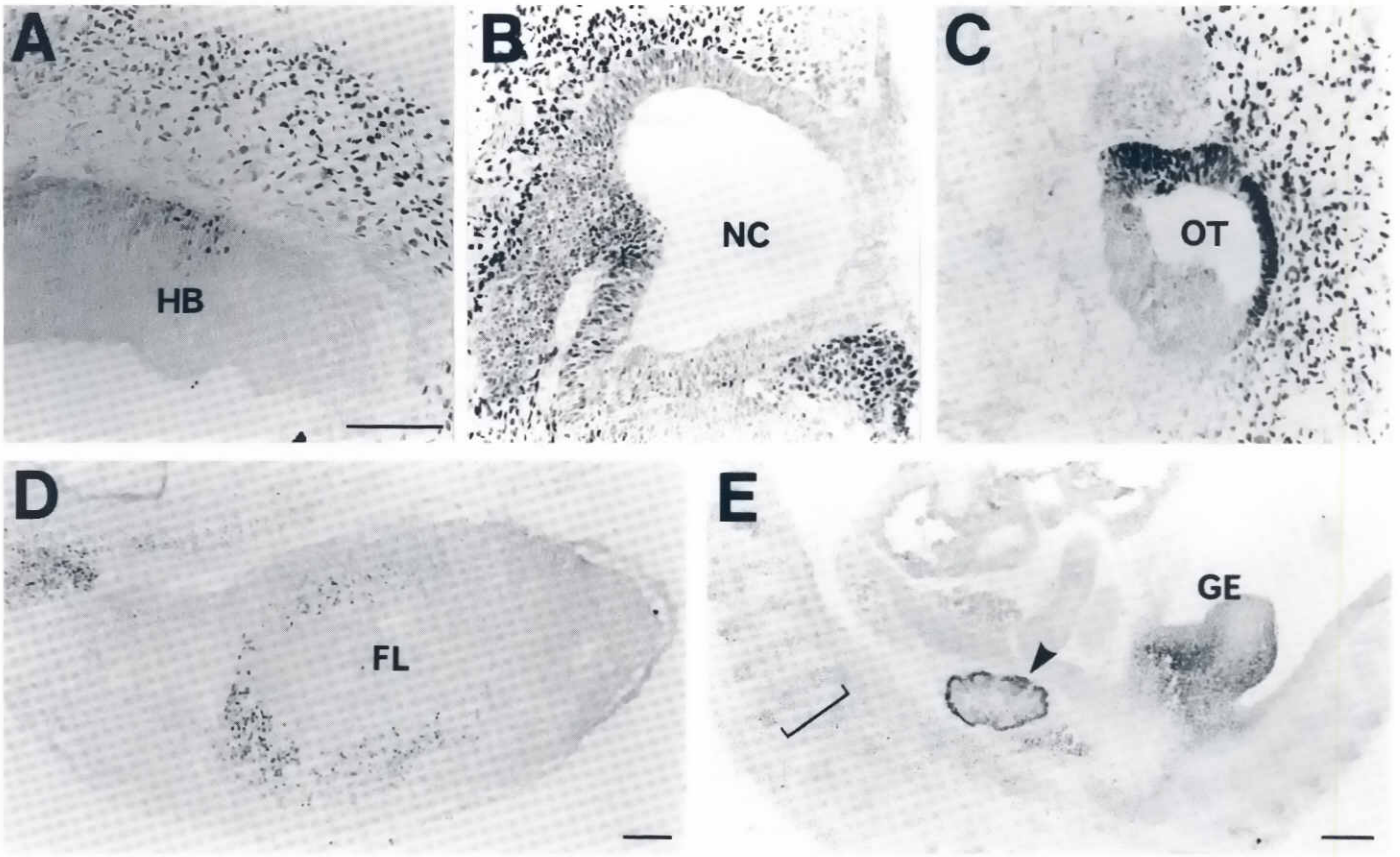

Fig. 4. Distribution of Six2 in parasagittal sections of developing mouse analyzed by immunostaining. *Top: anterior, right: ventral. Sagittal sections of the mouse embryo at* **(A)** *E10.5.* **(B,C,D)** *E11.5.* **(E)** *E12.5. Arrowhead indicates the staining in metanephros and bracket indicates that in myotome and sclerotome. FL, forelimb; GE, genital eminence; HB, hindbrain; NC, nasal cavity; OT, otic vesicle. Bar, 100 μm for A to D; 250 μm for E.*

tarded complexes were observed in HeLa from human uterine cancer, 3T3 from mouse fibroblast, and MDCK from canine kidney cells (Fig. 5C, lanes 1,5 and 9). The complex observed in B103 from rat neuroblastoma cells was not sequence-specific complex as observed in brain nuclear extracts since no specific competition was observed (data not shown). The addition of anti-SIX4 serum interfered with the formation of gel retardation complex in HeLa, 3T3 and MDCK cells (lanes 3 and 7 in longer exposure and lane 11) and super-shifted complexes were observed (in longer exposure, data not shown). The addition of anti-Six2 serum produced no effect, while the addition of anti-Six5 serum inhibited some of the retarded complex in HeLa and 3T3 cells (lanes 4 and 8 in longer exposure, data not shown). However, the addition of each serum produced no effect in B103 (lanes 13-16), consistent with the non-specific complex. A specific retarded complex was also observed in C2C12 cells which was inhibited by the addition of anti-SIX4 serum (data not shown). These results indicated that Six4 is the dominant isoform of Six proteins in all tested cultured cell lines except for B103.

Discussion

The present study shows that Six4 is present in the mouse embryo as early as E9.5 in the geniculate and acoustic ganglia as well as the spinal cord. In other ganglia, a similar production commenced at E10.5 and E11.5 but gradually disappeared at E13.5 or E14.5. Previous studies have also shown that many transcription factors of bHLH family are expressed in the developing nervous system. For example, *Mash1*, *Math1*, *neurogenin1*, *neurogenin2*, *neurogenin3* and *NeuroD* have similar temporal expression pattern with RNA expression as early as E8.5 or E9.5 (Guillemot and Joyner, 1993; Akazawa *et al.*, 1995; Ma *et al.*, 1996; McCormick *et al.*, 1996; Sommer *et al.*, 1996). However, the distribution of mRNA of *Math1* is restricted to the dorsal part of the central nervous system from E9.5 and transiently in the cranial ganglion at E9.5 (Akazawa *et al.*, 1995). *neurogenin1* and *neurogenin2* are expressed in the ventricular zone of the ventral neural tube between E8.5 and E10.5 (Sommer *et al.*, 1996). At E10.5, *neurogenin2* expression can be detected in a subset of cells in the trunk spinal ganglia, which also contain abundant *neurogenin1*-expressing cells. In contrast, *neurogenin3* is expressed in a very restricted region of the spinal cord, just dorsal to the floor plate from E9 to E14 (Sommer *et al.*, 1996). The distribution of *Mash1* mRNA is also restricted to the neuroepithelium of the midbrain and ventral forebrain as well as spinal cord between E8.5 and E10.5 but becomes widespread between E10.5 and E12.5 (Guillemot and Joyner, 1993). Our results showed that the distribution of the *Six4* gene product is widely distributed as shown by the positive staining of various regions of the central and peripheral nervous systems. This ubiquitous neural distribution suggests that the *Six4* is in-

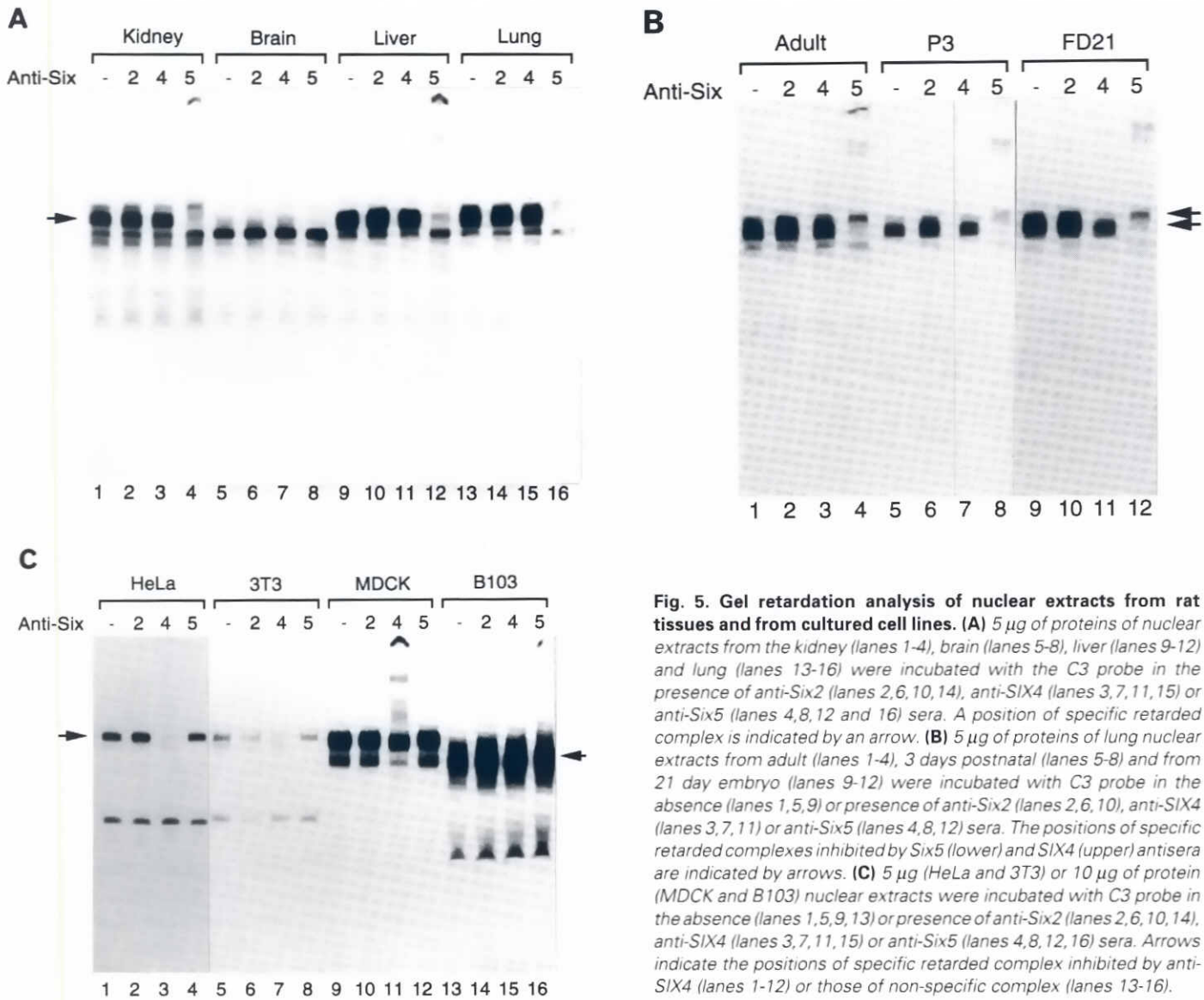

Fig. 5. Gel retardation analysis of nuclear extracts from rat tissues and from cultured cell lines. (A) *5 μg of proteins of nuclear extracts from the kidney (lanes 1-4), brain (lanes 5-8), liver (lanes 9-12) and lung (lanes 13-16) were incubated with the C3 probe in the presence of anti-Six2 (lanes 2,6,10,14), anti-SIX4 (lanes 3,7,11,15) or anti-Six5 (lanes 4,8,12 and 16) sera. A position of specific retarded complex is indicated by an arrow.* **(B)** *5 μg of proteins of lung nuclear extracts from adult (lanes 1-4), 3 days postnatal (lanes 5-8) and from 21 day embryo (lanes 9-12) were incubated with C3 probe in the absence (lanes 1,5,9) or presence of anti-Six2 (lanes 2,6,10), anti-SIX4 (lanes 3,7,11) or anti-Six5 (lanes 4,8,12) sera. The positions of specific retarded complexes inhibited by Six5 (lower) and SIX4 (upper) antisera are indicated by arrows.* **(C)** *5 μg (HeLa and 3T3) or 10 μg of protein (MDCK and B103) nuclear extracts were incubated with C3 probe in the absence (lanes 1,5,9,13) or presence of anti-Six2 (lanes 2,6,10,14), anti-SIX4 (lanes 3,7,11,15) or anti-Six5 (lanes 4,8,12,16) sera. Arrows indicate the positions of specific retarded complex inhibited by anti-SIX4 (lanes 1-12) or those of non-specific complex (lanes 13-16).*

volved in neuronal cell fate decision or common differentiation or maturation process of neurons rather than restricting neuronal cell types.

The expression of *neurogenin1* and *Mash1* is complementary and non overlapping from as early as E8.5 and appear collectively to account for most or all of the neuroepithelium in the central nervous system and ganglia of the peripheral nervous system (Sommer *et al.*, 1996). These genes are thought to regulate the expression of *NeuroD*, which is also widely distributed in the nervous system. *Six4* might be also regulated by these bHLH factors considering the location and timing of the expression.

The expressions of *Mash1*, *neurogenin1* and *NeuroD* were analyzed in the developing olfactory epithelium and each gene is thought to mark a distinct stage of olfactory neuron progenitor in the order. *Mash1* activates *neurogenin1* and *NeuroD* as confirmed in experiments using *Mash1* knockout mice (Cau *et al.*, 1997). The possible involvement of *Six4* gene in this cascade still needs to be determined in order to understand the regulatory mechanism of neural development.

The results of *in situ* hybridization analysis of *Six2* demonstrating a specific expression from E8.5 in the mesenchyme regions (Oliver *et al.*, 1995b) are to a large extent similar to the immunohistochemical findings described in the present study. Localization of Six2 in the cell nucleus suggests that the protein functions as a transcription factor to regulate target genes involved in the differentiation of tissues derived from the mesenchyme. There were, however, minor differences between the results of *in situ* hybridization and immunostaining. For example, the expression of *Six2* detected in the ectoderm between the mandibular and maxillary swellings and the mesonephric tubules was not observed in our staining. In contrast, staining at Rathke's pouch, epithelia of the otic vesicle and nasal cavity was only detected by immunostaining. These discrepancies could be due to differences in the translational efficiency of the mRNA.

The Six proteins have a specific DNA binding domain composed of Six domain and homeodomain (Kawakami et *al.*, 1996a). Six2, Six4 and Six5 have a common DNA binding sequence which is found in the ARE region of Na,K-ATPase α1 subunit gene (Kawakami *et al.*, 1996b). Using a probe containing a common binding sequence, we examined the presence of these Six binding activities in various tissues and in various cultured cell lines. The gel retardation super-shift assay clearly indicated the presence of Six5 in nuclear extracts from adult kidney, liver and lung but not in the brain. These results indicate that Six5 resides in nuclei of a wide variety of adult tissues. Consistent with our observation, *Six5* mRNA was recently detected in a variety of mouse adult tissues including liver and kidney by RT-PCR analysis (Heath *et al.*, 1997). In contrast, Six4 was barely detected in adult nuclear extracts, but was present in the developing lung. Six4 is the major isoform in cultured cell lines of HeLa, 3T3, C2C12 and MDCK. These findings indicate that Six4 is the embryonic protein that disappears after tissue maturation, at least in lung.

Materials and Methods

Preparation of specific antibodies

Antigens were prepared as GST-fusion proteins. For anti-Six2, *BsaHI*(957)-*Sau*3A1(1381) fragment of the *Six2* cDNA (Kawakami *et al.*, 1996b) was blunt-ended with Klenow and subcloned into the *Eco*RI site (blunt-ended with Klenow) of the pGEX3X. For anti-Six5, the *Sau*3A1(1485-2187) fragment of the *Six5* cDNA (Kawakami *et al.*, 1996b) was subcloned into *Bam*HI site of pGEX3X. The expression and purification of the fusion proteins were performed according to the protocol recommended by the manufacturer (Pharmacia, Uppsala, Sweden). For this purpose, rabbits were immunized with Freund's complete adjuvant and boosted twice with incomplete adjuvant. The anti-Six2 and anti-Six5 antibodies were affinity purified by thio-fusion proteins of Six2 and Six5 using HiTrap-NHS column (Pharmacia). For production of thio-Six2 protein, the blunt-ended *Bsa*HI(957)-*Sau*3A1(1381) fragment of *Six2* cDNA was ligated into the *Xba*I site of pTrxFus. For production of thio-Six5 protein, the blunt-ended *Sau*3A1(1485-2187) fragment of *Six5* cDNA was ligated into the *Xba*I site of pTrxFus. Expression and purification of thio-Six2 and thio-Six5 fusion proteins were performed as recommended by the manufacturer. Coupling of the thio-fusion protein and purification of the antibody were according to the protocol designed by the manufacturer. For anti-SIX4, the anti-human AREC3/SIX4 serum (Kawakami *et al.*, 1996a) was affinity purified by thio-fusion protein for the mouse Six4.

Immunohistochemistry of whole mount embryos

Immunostaining of whole-mount ICR mouse embryos (E7.5-E10.5) was performed as described previously (Davis, 1993) with the following modifications. The concentration of TritonX-100 in PBSMT and PBT was increased from 0.1% to 0.5%. We omitted $NiCl_2$ in the DAB solution. The embryos were then fixed in 4% paraformaldehyde (PFA) in PBS after color development. The affinity-purified anti-Six2 antibody (2.5 ng/μl) was used as the first antibody, and the peroxidase-conjugated swine anti-rabbit immunoglobulin (Dako, Glostrup, Denmark) diluted in 1/200 was used as the second antibody.

Immunohistochemistry of cryosections

ICR mouse embryos (E7.5-E14.5) were fixed in 4% PFA, 8% sucrose in PBS for 1.5-4 h at 4°C. After washing in 8% sucrose in PBS for 2 h at 4°C, the embryos were soaked over-night in 20% sucrose in PBS at 4°C. The embryos were then frozen in a tissue freezing medium (Leica, Nussloch, Germany). Sections (10 μm) were cut and mounted on aminopropyltriethoxy-silane coated coverslips followed by drying at 37°C for 2-4 h. The coverslips were washed in TBS (0.05M Tris-HCl, 0.15M NaCl pH 7.6) three times for 5 min. Samples were permealized with TBST (0.04% TritonX-100/TBS) for 30 min at room temperature. Immunostaining was performed using LSAB kit (DAKO) according to the instructions provided by the manufacturer except that the washing solution was TBST and the blocking reagent was 20% FBS/TBST. The anti-Six2 antibody (7.5 ng/μl) or anti-SIX4 antibody (5 ng/μl) was used as the first antibody.

Preparation of nuclear extracts from cell lines and tissues

Cell culture and preparation of nuclear extracts from HeLa, BALB/c-3T3, MDCK, B103 and C2C12 were performed as described previously (Suzuki-Yagawa *et al.*, 1992; Kawakami *et al.*, 1993; Ikeda and Kawakami, 1996). Nuclear extracts from the rat kidney, brain, liver and lung were prepared as described by Kobayashi and Kawakami (1995).

Gel retardation assay

5-10 μg protein of the nuclear extracts were used in each reaction. For C3 oligonucleotide probe, 5'-TCGAGCCGGTGTCAGGTTGCTCC and 5'-TCGAGGAGCAACCTGACACCGGC were annealed. The probe contains the S*ix* family protein binding sites (Kawakami *et al.*, 1996b). Gel retardation assays were performed as described previously by our laboratory (Kawakami e*t al.*, 1988) in the presence or absence of 0.03 μl of anti-Six2, anti-AREC3/SIX4 or anti-Six5 serum.

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