Differential expression of Hox 3.1 protein in subregions of the embryonic and adult spinal cord

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Summary

Synthetic oligopeptides derived from the predicted Hox 3.1 protein coding sequence were used for the production of antibodies (anti-aa2) that specifically recognize Hox 3.1 protein in tissue sections. These antibodies were applied in immunohistochemical studies to monitor the expression of Hox 3.1 protein within the central nervous system (CNS) of embryonic and adult mice. We demonstrate congruency between the distinct Hox 3.1 RNA and protein expression patterns in the developing spinal cord by direct comparison of in situ hybridization and immunohistochemical staining in frozen sagittal sections from embryos of 12.5 days of gestation. A distinct pattern of spatially restricted expression of Hox 3.1 protein within the spinal cord was first detected at around 10.5 days of embryonic development. Within certain anteroposterior limits the geometries of this expression pattern change drastically during subsequent embryonic stages, concomitant with important cytoarchitectural changes in the developing spinal cord. Analyses on subcellular levels indicate predominant accumulation of Hox 3.1 protein within nuclei of neuronal cells. In addition to the nuclear localization in subsets of embryonic cells, persistent accumulation of Hox 3.1 protein was shown in nuclei of fully differentiated and mature neuronal cells of the adult CNS.

Key words: CNS, development, homeodomain protein, immunohistochemistry, Hox 3.1

Introduction

Recent progress in the molecular analysis of certain developmental control genes from the fruitfly Drosophila lead to the isolation of a cross-homologous protein coding DNA sequence known as homeobox (McGinnis et al. 1984a,b; Scott and Weiner, 1984). The fact that this highly conserved sequence has been found exclusively in Drosophila genes with developmental control functions, and the demonstration of its widespread occurrence in the genomes of other taxonomical animal groups including vertebrates (McGinnis et al. 1984b,c; Carrasco et al. 1984; Levine et al. 1984; McGinnis, 1985; Holland and Hogan, 1986), lead to the speculation that the homeobox sequence may serve as a molecular probe for the identification of developmental control genes in mammals (McGinnis et al. 1984b; Gehring, 1985, 1987). Provided that this assumption is correct, the murine homeobox-containing gene family may serve as a model system for studying genetic control mechanisms of mammalian development. This view is supported by the demonstration of distinct region-specific expression patterns of different mouse homebox genes during embryonic development (for review: Fienberg et al. 1987; Holland and Hogan, 1988b).

While different members of the murine homeobox gene family are expressed in a variety of tissues and organ primordia at certain periods during embryonic development, it is interesting to notice that most, if not all, of the homeobox genes tested so far are expressed at high levels in the developing central nervous system (CNS). The first example characterized in some detail in this respect was the Hox 3.1 locus mapped to chromosome 15 (Awgulewitsch et al. 1986). Using Northern blot and in situ hybridization analyses, predominant expression of the Hox 3.1 gene was shown to be localized in a specific subregion of the CNS that corresponds to the cervical and anterior thoracic spinal cord of newborn mice, as well as embryos of 13 days of gestation (Awgulewitsch et al. 1986; Utset et al. 1987). This expression in the central nerve cord persists into adulthood as demonstrated by RNA blot analysis (Awgulewitsch et al. 1986).

Further detailed studies on Hox 3.1 transcript expression during embryogenesis suggest its onset at about 7.5 days of gestation (LeMouellic et al. 1988; Gaunt, 1988). At this early stage, initial expression of Hox 3.1 encoded transcripts appears to be limited to the allantois, while at subsequent stages of 8–9.5 days post coitum (p.c.) this expression extends to most, if not all, tissues of the posterior region of the embryo including positively mesodermal and neuroectodermal layers. At stage 10.5 days p.c., Hox 3.1 expression begins to become spatially restricted within more anterior regions of the neural tube (LeMouellic et al. 1988), showing
highest levels in ventral subregions (LeMouellic et al. 1988; Breier et al. 1988). Thus, 10.5 days p.c. appears to represent a transitional stage in Hox 3.1 expression, which leads to the establishment of the precise pattern of transcript accumulation along the anteroposterior body axis within the spinal cord reported for later developmental stages (Auwgulewitsch et al. 1986; Usset et al. 1987; Holland and Hogan, 1988a; Gaunt et al. 1988; LeMouellic et al. 1988; Breier et al. 1988; Gaunt, 1988).

Recently, the putative primary structure of the Hox 3.1 protein derived from cDNA sequence analysis has been reported, indicating a protein of 242 amino acid residues with the homeodomain located at the carboxy-terminal end (LeMouellic et al. 1988; Breier et al. 1988). Based on these data, as well as on our own structural analyses of the Hox 3.1 coding region (Auwgulewitsch, Bogarad and Ruddle, unpublished data), synthetic oligopeptides derived from the predicted Hox 3.1 protein coding region were prepared and used for the production of polyclonal antibodies in rabbits. One of the antibody preparations specifically recognized Hox 3.1 protein in mouse tissue sections as determined by immunohistochemistry. Immunohistochemical analyses were performed to monitor Hox 3.1 protein expression in the developing CNS starting at stage 10.5 days p.c. We addressed the following questions: (i) does the expression of Hox 3.1 protein correspond to the reported patterns of transcriptional Hox 3.1 expression in the spinal cord? (ii) to what extent are morphological changes during spinal cord development reflected by changes in spatial patterns of Hox 3.1 protein expression? (iii) which cell types within the embryonic and adult spinal cord are expressing Hox 3.1 protein?

Materials and methods

In situ hybridization to tissue sections

In situ hybridization experiments with frozen sections (8 μm) were performed essentially as described (Auwgulewitsch et al. 1986; Usset et al. 1987). Hybridization conditions were: 50% formamide, 0.3 M NaCl, 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 50 mM DTT, 1× Denhardt’s solution (Denhardt, 1966), 0.5 mg/ml tRNA, 0.2 ng probe per μl and kilobase complexity, 50°C for 16 h. 35S-labelled (35S-UTP, =4.5×10^8 cts/min/μg) were synthesized as ‘run-off transcripts’ (Melton et al. 1984) from appropriately linearized cDNA plasmid DNA templates described in Fig. 1. Probe synthesis was carried out by using bacteriophage T7 (sense RNA) or T3 (antisense RNA) RNA polymerases (Stratagene, LaJolla CA) and components of the Riboprobe® system (Promega, Madison WI) as described by the manufacturer. After hybridization, washing (final washing conditions: 0.1× SSC, 0.1% SDS, 0.3 M NaCl, 0.1% sodium dodecyl sulfate at 60°C for 15–30 min) and ethanol-dehydration, slides were exposed to Kodak X-Omat/AR X-ray film overnight.

Peptide synthesis and preparation of antibodies

Oligopeptides derived from the predicted Hox 3.1 protein coding sequence (LeMouellic et al. 1988; Breier et al. 1988) described in Fig. 1 were synthesized on an Applied Biosystems model 430A peptide synthesizer using Boc (tert-butyloxycarbonyl)-amino acids and Pam-[4-(oxymethyl)phenyl]-acetamidomethyl]-resin (for review: Barany and Merrifield, 1980) and deprotected via the low HF/high HF procedure (Tam and Merrifield, 1985). Peptides were purified over a Sephadex G-25 superfine column equilibrated with 1 M acetic acid and lyophilized. For immunization of virgin New Zealand white rabbits, peptides were conjugated to keyhole limpet hemocyanin (KLH) via glutaraldehyde as described (Richardson et al. 1985; Odenwald et al. 1987). 5 mg of peptide and 5 mg of KLH were dissolved in PBS, and glutaraldehyde (Sigma, electron microscope grade, 25%) was added in small increments to a final concentration of 0.25% (vol/vol). After 30 min incubation at room temperature the peptide–KLH conjugates were dialyzed against PBS with two changes at 4°C overnight and stored at −70°C.

For each peptide, one rabbit was immunized with 1 mg peptide–KLH conjugate according to standard protocols (Johnstone and Thorpe, 1987) followed by booster immunizations with the same amount of conjugated peptide at 3, 5, 10, and 15 weeks after the first injection. Rabbits were bled (20–40 ml) via ear veins 3 weeks after each inoculation. The presence of peptide-specific IgGs was determined by enzyme-linked immunosorbent assays (ELISAs) as described (Johnstone and Thorpe, 1987) using peptide conjugated to bovine serum albumin (BSA, pentax fraction V) via l-ethyl-3(3-dimethylaminopropyl)carbodiimide as antigen. All three antisera were found to contain peptide-specific antibodies. Peptide-specific antibodies were affinity-purified by applying heat-inactivated antiserum to a peptide–Affigel 10 (Bio-Rad) column. Approximately 30 mg of peptide was coupled to 3 ml Affigel 10 according to the directions supplied by the manufacturer. The column was equilibrated with PBS before applying 12 ml of antiserum, which had been incubated at 56°C for 30 min. The column was washed with PBS until the A280 was at a baseline. Peptide-specific antibodies were eluted with 0.5 M NaCl, 0.2 M glycine (pH 2.8) and precipitated by addition of an equal volume of saturated ammonium sulfate. After an hour incubation at room temperature, antibodies were collected by centrifugation at 10,000 g for 10 min. Pellets were resuspended in 1 ml PBS, aliquoted and stored at −20°C.

Immunohistochemistry

Freshly dissected tissue obtained from CD-1 mice was embedded in OCT-compound 4583 (Miles, Elkhart IN), quick frozen on dry ice and stored at −70°C. 8 μm thick sections, cut at −20°C on a cryostat microtome, were picked up onto acid-cleaned, gelatin-coated (Gall and Pardue, 1971) slides, dried on a hot plate at 50°C for 1–2 min followed by additional drying at room temperature for 2 h. All the subsequent steps were carried out at room temperature. Sections were fixed in 3% paraformaldehyde in PBS for 10 min, rinsed in PBS 3 times for 10 min each, and incubated in blocking solution [10 mM Tris–HCl (pH 7.4), 100 mM MgCl2, 0.5% Tween-20 (Sigma), 1% BSA, 5% fetal calf serum]. Tissues were permeabilized by treatment with 0.05% Triton X-100 (Sigma) in TBS [10 mM Tris–HCl (pH 8.0), 150 mM NaCl] for 10 min and rinsed with TBST [10 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.05% Tween-20] 3 times for 10 min each. 100 μl of primary antibody solution diluted 1:100 in TBST was added per slide followed by an overnight incubation in a sealed chamber humidified with TBS. For competition experiments, the primary antibody solution contained peptide aa2 or aa3 (200 μg/ml−1). The next day, sections were rinsed in TBST 3 times for 5 min each. Excess buffer was removed by blotting followed by a 2 h incubation with 100 μl...
per slide of horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) diluted 1:50 in TBST. Slides were rinsed in TBST 3 times for 5 min each and subjected to enzymatic color development by the addition of 100 μl per slide of freshly made substrate solution containing 0.5 mg/ml 3,3'-diaminobenzidine (DAB) and 0.4 mg/ml NiCl in 0.2 M Tris-HCl (pH 7.6). Standard reaction time was 1 min. Reactions were stopped by immersing slides in deionized water with 2 changes over the course of an hour. Slides were dehydrated in a series of ethanol (30%, 60%, 80%, 95%, 100%) for 10 s each or stained with Giemsa [1:20 dilution of Giemsa Stain (Sigma) in 5 mM Tris (pH 7.5)] for 15 min prior to ethanol dehydration.

**Results**

**Preparation and characterization of antibodies**

According to recently reported cDNA sequencing data, the presumptive Hox 3.1 protein coding region spans 726 nucleotides coding for a protein of 242 amino acids (LeMouellic et al. 1988; Breier et al. 1988). The highly conserved homeodomain of 60 amino acids is located close to the 3' carboxy-terminal end followed by a stretch of 34 amino acid residues rich in glutamic acid. At least two additional conserved peptide regions of eight and six amino acid residues also found in certain other homeodomain proteins at the same relative positions (for references see Schughart et al. 1988) are located at the amino-terminal end of the protein, and just upstream of the homeodomain, respectively. Excluding any of these conserved regions, in order to avoid possible cross-reactivities with other homeodomain proteins, we synthesized three non-overlapping oligopeptides ranging from 15–19 residues, which were used for raising Hox 3.1 peptide-specific antibodies in rabbits. The amino acid sequences of these peptides referred to as aa1, aa2, and aa3, as well as their relative positions in the amino-terminal half of the predicted Hox 3.1 protein are shown in Fig. 1A. These peptides were conjugated to keyhole limpet hemocyanin for use as antigen in standard immunization protocols (see Material and methods). Enzyme-linked immunosorption assays (ELISAs) demonstrated that all the resulting antisera recognized their respective peptide with equal strength. However, after purification over peptide-specific affinity columns, only anti-aa2 antibodies reacted specifically with a localized antigen in mouse tissue sections, as determined by immunohistochemical staining using horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and 3,3'-diaminobenzidine (DAB) as a color reagent.

In order to determine whether the distinct staining pattern obtained with anti-aa2 antibodies within the central nerve cord of 12.5 days p.c. mouse embryos matched the spatially restricted expression pattern of Hox 3.1 encoded transcripts at this developmental stage, we performed in parallel in situ hybridization experiments. Hox 3.1-specific antisense RNA probe was hybridized to sagittal sections of 12.5 days p.c. embryos. The 35S-labelled probe was synthesized by using a subclone of the Hox 3.1-specific cDNA c210 (Awgulewitsch, Bogarad, and Ruddle, unpublished data), designated as c210-B (Fig. 1A), as a template for transcription in the Bluescript (Stratagene) vector system. Complete DNA sequence analysis of c210-B showed a perfect match to the Hox 3.1 cDNA sequence data previously reported (LeMouellic et al. 1988; Breier et al. 1988). The c210-B cDNA contains approximately 250 bp of untranslated leader sequence followed by about 450 bp of the putative Hox 3.1 protein coding region, which includes the sequences corresponding to all three synthetic peptides, and ends at the Sall restriction site located at the 5' end of the homeobox.

The specific hybridization signal obtained with this probe showed highest intensity within a precisely defined anteroposterior subregion of the central nerve cord positioned between the 4th and 10th prevertebrae (Fig. 1B,C), which is in agreement with previously reported expression data of Hox 3.1-encoded transcripts at this stage of development (Gaunt, 1988; Gaunt et al. 1988; Holland and Hogan, 1988; LeMouellic et al. 1988; Breier et al. 1988). Signal intensity decreased drastically in more posterior regions of the nerve cord. Important to notice is the weaker hybridization signal over cells of prevertebrae numbering 13–17 just posterior to the strongest signal in the spinal cord (Fig. 1C). This out-of-register pattern of transcript expression in mesodermally versus neuroectodermally derived tissues is characteristic for Hox 3.1 (Usset et al. 1987; Gaunt, 1988; Gaunt et al. 1988; Holland and Hogan, 1988; LeMouellic et al. 1988; Breier et al. 1988). Comparison to the anti-aa2 immunohistochemical staining pattern obtained with sagittal sections from the same developmental stage (Fig. 1D) showed congruency of both patterns. Combined with the fact that the reacting antibodies have been affinity-purified over a Hox 3.1 peptide (aa2)-specific column, these results strongly suggest that our anti-aa2 antibody preparation specifically recognizes Hox 3.1 protein in mouse tissue sections.

Further evidence for this specificity has been obtained by competition experiments in which the characteristic region-specific staining was completely abolished by addition of excess aa2 peptide during the incubation of sections with anti-aa2 antibodies (compare Fig. 2A and B). As a control, we used aa3 peptide derived from a different region of the predicted Hox 3.1 protein (see Fig. 1A), which did not abolish the anti-aa2-specific staining (Fig. 2C). These data establish proof that the unique staining patterns obtained with anti-aa2 antibodies are due to antigenic specificity of these antibodies against a defined subregion of the Hox 3.1 protein. Although we avoided any of the conserved regions known for homeodomain proteins when synthesizing our peptides, it still cannot be ruled out entirely that the aa2 region may be shared by one or several related proteins. However, the striking congruencies of the immunohistochemical staining to the unique Hox 3.1 in situ hybridization patterns strongly suggest that the antigenic determinant reacting with anti-aa2 belongs indeed to Hox 3.1 encoded protein.
Fig. 1. Sequences and map positions of synthetic peptides and comparative localization of Hox 3.1 encoded RNA and protein in frozen near-sagittal sections from 12.5 days p.c. mouse embryos. (A) Schematic structure of the c210-B cDNA containing approximately 250 bp of untranslated leader sequence (solid line) followed by about 450 bp of the predicted Hox 3.1 protein coding region (shaded box) is shown on top; the entire DNA sequence included in this cDNA has been reported (LeMouellic et al. 1988; Breier et al. 1988). The partial Hox 3.1 protein coding region shown ends at the Sal restriction site, which is located at the 5' end of the homeo box. The direction of transcription is from left to right (solid arrow) and the position of the presumptive first amino acid (+1) is indicated. This cDNA was used as a template for the synthesis of antisense RNA probe after cloning into the Bluescript (Stratagene) vector system. The regions selected for oligopeptide synthesis are represented as shaded boxes (aa1, aa2, aa3) with the numbers underneath indicating the limits for each peptide in terms of relative positions of amino acids within the protein coding region. Amino acid sequences of synthesized peptides are shown at the bottom. (B) 8 μm thick section stained with Giemsa after in situ hybridization. (C) X-ray film autoradiograph of the section shown in (B) after in situ hybridization with c210-B antisense RNA probe (specific activity: 4.5×10⁸ cts min⁻¹ μg⁻¹). (D) Frozen near-sagittal section of 12.5 days p.c. embryo after immunohistochemical staining with anti-aa2 antibodies. Positions of specific hybridization signals (C), or immunohistochemically stained regions (D) in the spinal cord (arrows) and prevertebrae (arrowheads) are indicated. Anterior is to the right, dorsal to the top. H=heart; SC=spinal cord; 4 and 13 indicate prevertebrae number; Scale bars=1 mm.

Differential anterior expression boundaries in the developing CNS

Having provided evidence that the anteroposterior patterns of Hox 3.1 protein and transcript expression within the central nerve cord of 12.5 days p.c. embryos are identical, we determined the protein expression patterns in the spinal cord at different developmental stages and compared them to each other, as well as to previously described transcriptional expression data (Awgulewitsch et al. 1986; Utset et al. 1987; Holland and Hogan, 1988a; Gaunt et al. 1988; LeMouellic et al. 1988; Breier et al. 1988; Gaunt, 1988). As mentioned earlier, 10.5 days of embryogenesis may be considered a transitional stage for Hox 3.1 expression (LeMouellic et al. 1988). At this stage, the diffuse transcriptional expression over the posterior embryonic region observed at stages 8–9.5 days p.c. changes to a precisely localized expression in a more anterior region of the neural tube (LeMouellic et al. 1988). Immunohistochemical data obtained with anti-aa2 antibodies indicate that Hox 3.1 protein expression displays the same changes during this period of development, showing strictly localized anti-aa2 staining within the central nerve cord of 10.5 days p.c. embryos (Fig. 3A). In contrast, the staining pattern at earlier developmental stages is diffusely distributed over more posterior regions, including mesodermal and neuroectodermal, as well as endodermal tissues (data not shown).

One important question we wanted to address was how the distinct anteroposteriorly restricted pattern of Hox 3.1 protein expression within the neural tube established at about 10.5 days p.c. changes during subsequent developmental stages. In addition to 12.5 days p.c. embryos (Fig. 1), four other stages of prenatal development including 10.5, 11.5, 14.5, and 19.5 days p.c. were selected for determining the distribution of
Hox 3.1 protein expression in spinal cord

415

Fig. 2. Test of anti-aa2-staining specificity in serial sagittal sections from a 12.5 days p.c. embryo by competition with aa2 and aa3 peptides. (A) Close-up view of anti-aa2-stained regions of the spinal cord (arrow) and prevertebral column (arrowhead) shown in Fig. 1D. (B) View of the same regions as shown in A in an adjacent section that was subjected to the same staining protocol with anti-aa2 antibodies (see Materials and methods), but in the presence of aa2 peptide (200 μg ml⁻¹). Note that the characteristic anti-aa2-specific staining pattern seen in A has been abolished completely. (C) Anti-aa2-specific staining in the next section in series in the presence of aa3 peptide (200 μg ml⁻¹). SC=spinal cord; Md=mesodermal tissue; scale bars=0.4 mm.

Hox 3.1 protein along the anteroposterior axis of the central nerve cord. The results are summarized in Fig. 3, which shows a stained sagittal section typical for each of these stages. At 10.5 days p.c. the staining is restricted to an anterior–posteriorly, as well as dorsal–ventrally, defined subregion of the neural tube (Fig. 3A). The staining is always localized at the most ventral cord regions with an anterior limit positioned at the level of the forelimb bud corresponding to the level of the 8th–12th somites (Theiler, 1989; Hogan et al. 1986). Taking into account that the first 4–5 somites contribute to the skull, then the 8th–12th somite region contributes to the formation of the 5th–9th, or 4th–8th prevertebrae, respectively. These data are entirely in agreement with the transcriptional expression studies for this stage reported by LeMouellic et al. (1988).

At 11.5 days p.c., when prevertebral structures are clearly visible, a more precise determination of the anterior Hox 3.1 expression boundary within the spinal cord is possible, locating it just posterior to the 6th prevertebra (Fig. 3B). Although there does not appear to be any significant change in the anterior expression boundary between 10.5 days and 11.5 days p.c. the immunohistochemical staining extends now to the dorsal region in the most anterior domain of expression. Interestingly, this staining is not uniformly distributed from ventral to dorsal, instead showing two horizontal bands or stripes, one on the dorsal, the other on the ventral side. As with sections of 12.5 days p.c. embryos described above (Fig. 1D), we also detected the so-called out-of-register staining of prevertebrae posterior to the most intense staining in the spinal cord. However, at this earlier stage, cells of more posterior prevertebrae numbering 14–17 appear to be stained compared to prevertebrae 13–17 in 12.5 days p.c. embryos.

At 14.5 days p.c., Hox 3.1 protein expression extends to the level of the 5th vertebra in the ventral most spinal cord region, and to the level of the 3rd vertebra in the middle layer of the spinal cord (Fig. 3C). The most dorsal spinal cord layer appears to be stained only in a very narrow anteroposterior subregion between the 5th and 7th vertebrae. Posteriorly, the domain of most intense staining in the CNS extends to the level of the 10th vertebra, corresponding to the anterior thoracic region. Prior to birth at 19.5 days p.c., specific staining within the central nerve cord becomes more diffuse, and the anterior boundary of expression is not as distinct as it is at earlier stages examined. However, the most intense labelling is noticed in the cervical cord region ranging roughly from the levels of the 2nd to the 8th vertebrae (Fig. 3D), which is again consistent with the distribution of Hox 3.1 encoded transcripts at a similar developmental stage, i.e. neonatal, (Awgulewitsch et al. 1986).

Close-up views of higher resolutions of the anterior expression boundaries within the spinal cord at the four different stages are shown in Fig. 4. The first two photographs compare the anti-aa2-stained nerve cord regions in near-sagittal sections taken from two separate 10.5 days p.c. embryo of the same litter with (a) and without (b) Giemsa-counter-staining; the section shown in Fig. 4A is identical to the one shown in Fig. 3A. Technically, this comparison demonstrates that counter-staining with Giemsa does not interfere with the detection of anti-aa2-specific immunohistochemical staining in conjunction with the protocols we employed (see Materials and methods), but rather enhances the distinction between specific and unspecific staining, as well as between morphological substructures. We be-
lieve that the uniformly dark staining of epidermal layers shown in both sections with and without Giemsa-counter-staining (Fig. 4A,B) is due to the higher cell densities in these tissues, and does not represent any Hox 3.1 specificity.

Fig. 4C and D show the posterior and anterior limits of the most intense anti-aa2 staining in the same section taken from an 11.5 days p.c. embryo. The posterior region clearly demonstrates specific staining of cells in prevertebrae 14–17, overlapping with the region of decreased staining intensity in the nerve cord. Considering the depth of the sectioning plane, we believe that the cells stained in the prevertebrae are sclerotome cells. The anterior limit of intense staining in the CNS shown in this section (Fig. 4D) indicates a cranially directed shift in Hox 3.1 protein expression for cells of the more central and dorsally located spinal cord layers from the level of the 6th to the 4th prevertebra. The staining in this section does not extend to the dorsal wall of the central nerve cord, because it is closer to the sagittal midline than the section shown in Fig. 3B, thereby leaving the narrow, laterally located clusters of Hox 3.1-expressing cells in the dorsal regions out of the plane of section. This will be demonstrated clearly by analyses of staining patterns in cross-sections.

Fig. 4E shows a close-up view of the anterior Hox 3.1 expression boundary at 14.5 days p.c. taken from the same section shown in Fig. 3C before counter-staining with Giemsa. The anti-aa2-staining pattern suggests that Hox 3.1 protein expression is abruptly, rather than gradually, turned on in cells posterior to this boundary. At 19.5 days p.c., the density of anti-aa2-stained cells is markedly decreased (Fig. 4F) versus earlier stages, concomitant with progressed cellular differentiation. Hox 3.1-expressing cells appear to be increasingly intermingled with non-expressing cells, apparently causing the anterior boundary of Hox 3.1 expression to lose some of its distinction and to become more diffuse.

Hox 3.1 protein expression in subsets of cells of the spinal cord and ganglia

In order to analyze the medio-lateral, as well as the dorsoventral arrangement of Hox 3.1 protein-accumulating cells within the developing spinal cord, we compared anti-aa2-stained cross-sections obtained from the four different prenatal developmental stages shown in Fig. 3. The cross-sections correspond to those levels along the longitudinal axis of the spinal cord that displayed most intense staining in sagittal sections at these different stages (see Fig. 3). The results presented in Fig. 5 indicate profound changes in the geometries of the Hox 3.1-specific staining patterns during this period of development.

At 10.5 days p.c., the most intense anti-aa2 staining is detected in two distinct ventro-lateral regions (Fig. 5A) corresponding to the developing ventral horns. Significantly weaker staining is detected in the more dorsal regions of the mantle layer, as well as throughout the layer of undifferentiated ependymal cells that make up the bulk of the neural tube at this developmental stage. Additional staining is also noticed in two medio-lateral clusters of neural crest-derived cells that contribute to the formation of the spinal ganglia. A close-up view of the ventral horn (A') suggests that most, if not all, of the densely packed cells in this region are stained intensely, while the more loosely packed cells of the primordial spinal ganglia show generally less intense staining of various degrees (A''). Our staining protocol does not allow us to distinguish histological differences between anti-aa2-stained cells and those that show only the bluish Giemsa-staining at this stage. However, comparison of the typical, nucleus-specific Giemsa-staining with the anti-aa2-staining pattern at a magnification of approximately 400× indicates that anti-aa2-specific antigen is primarily, although not exclusively, concentrated in cell nuclei. This is in agreement with the hypothesis of trans-acting regulatory DNA-binding functions suggested for Drosophila, as well as for vertebrate homeodomain proteins (for review: Gehring, 1985, 1987), and the previously demonstrated nuclear localization of several homeodomain proteins.
Fig. 5. Anti-aa2-immunohistochemical staining of spinal cord cross-sections from mouse embryos of four different stages. The sections were obtained from the anteroposterior positions that showed highest levels of Hox 3.1 protein expression. All sections were counterstained with Giemsa.

(A–D) Low power magnifications of approximately 80× (A–C) and 40× (D) of cross-sections through the spinal cord of 10.5 (A), 11.5 (B), 14.5 (C), and 19.5 (D) days p.c. embryos at levels of the hindlimb bud (10.5 d), the 7th/8th prevertebrae (11.5 d), the 7th/8th vertebrae (14.5 d), and the 4th/5th vertebrae (19.5 d), displaying the specific staining with anti-aa2 antibodies (brown color) and nuclear staining with Giemsa (blue). (A′–D′) Close-up views (400×) from the left ventral halves of the corresponding spinal cord regions shown in A–D. Large neuronal cells showing nuclear anti-aa2-staining can be distinguished in D′. (A′–D′) 400× magnification of spinal ganglia regions shown in A–D. Dorsal is to the top. C, spinal canal; DH, dorsal horn; EL, ependymal layer; FP, floor plate; Mg, marginal layer; ML, mantle layer; VH, ventral horn; SG, spinal ganglia. Scale bars=50 µm (A–D) or 25 µm (A′–D′).

Fig. 6. Spinal cord cross sections from 1 week postnatal and adult mice at the level of the 4th/5th vertebrae. (A) 1 week postnatal, and (B) 7 weeks postnatal, after staining with anti-aa2 antibodies (brown color) and Giemsa-counterstaining. Note that anti-aa2-specific staining reaches into the dorsal horn (DH) regions of the spinal cord at both stages. Note also the anti-aa2-specific staining of cells in the sympathetic ganglion (Y) displayed in A. (A′ and B′ and A″ and B″) Close-up views (400×) from the ventral halves of the spinal cord (A′ and B′) and the spinal ganglia (A″ and B″) shown in A and B. Note the non-uniform nuclear anti-aa2-staining in all four panels, leaving the nucleoli (arrowheads) unstained. Most of the smaller nuclei are only stained with Giemsa (blue). The larger nuclei stained with anti-aa2 belong to neuronal cells. Dorsal is to the top. DH, dorsal horn; EL, ependymal layer; Mg, marginal layer; SG, spinal ganglia; VH, ventral horn. Scale bars=100 µm (A and B), or 25 µm (A″ and B″).
These include, for example, those encoded by the *Drosophila* homeotic loci *Ultrabithorax* (White and Wilcox, 1984) and *Antennapedia* (Carroll et al. 1986), the mouse Hox 1.3 gene (Odenwald et al. 1987), and the *Xenopus* XlHbox 8 gene (Wright et al. 1988).

At 11.5 days p.c., differentiating cells of the mantle layer displace major parts of the central ependymal layer, particularly in the ventral and medio-lateral cord regions, thereby causing the V-shaped appearance of the ependymal region (Fig. 5B). Anti-aa2-staining continues to be present in cells of the mantle layer, while almost all the cells of the ependymal region are now negative for anti-aa2-staining. Interestingly, the anti-aa2-stained cells are mostly concentrated in two separate subregions of the mantle layer, located in the ventral most and middle areas on either side. Between these two subregions, as well as in the thinner dorsal mantle layer, significantly less cells are stained. The ventral subregions are known to contain motorneuromasts (for review: Weiss, 1971), which we believe are among those cells expressing Hox 3.1 protein based on studies of subsequent stages (see following data), when it becomes possible to distinguish between neuronal cells and neuroglial cells (Figs 5D, D' and 6). However, we do not know whether the clusters of anti-aa2-stained cells in the mid-region will give rise to a functionally distinct subset of neurons or neuroglial cells. Anti-aa2-staining of cells in the spinal ganglia appears to be of a distinct subset of neurons or neuroglial cells. Anti-aa2-stained cells. In the outer mantle layers of the ventral horns, however, the majority of the large neuronal cells are stained with anti-aa2, while most of the smaller cells, probably representing neuroglial cells, show Giemsa-staining only. Most of the large neuronal cells of the spinal ganglia show nuclear anti-aa2-staining of similar intensity as the cells of the ventral horns (Fig. 5D').

### Hox 3.1 protein expression persists into adulthood

We have previously demonstrated that Hox 3.1 transcripts continue to be expressed at high levels in the adult spinal cord (Augsulewitsch et al. 1986). Highest signal intensities were detected with RNA samples obtained from the cervical and thoracic spinal cord regions while RNA from the lumbar/sacral regions showed only low signal intensities upon Northern blot hybridization with a Hox 3.1-specific probe. No hybridization signal was detected with the RNA sample from the brain. These data indicate that Hox 3.1 encoded transcripts continue to be differentially expressed along the anteroposterior body axis during adulthood. Based on these data, we analyzed the patterns of Hox 3.1 protein expression in the cervical spinal cord at two different postnatal stages by immunohistochemical staining of cross-sections from this region. The data obtained with cervical spinal cord cross-sections from mice of one and seven weeks of age show no major changes in the overall patterns of anti-aa2-specific staining (Fig. 6). However, comparison of the one week postnatal stage (Fig. 6A) to the prenatal stage of 19.5 days p.c. (Fig. 5D), which is close to birth, indicates a more even distribution of less densely packed anti-aa2-stained nuclei at the postnatal stage. This is probably due to further maturation of neuronal and neuroglial cells allowing now clear distinctions between certain cell types based on their cytoarchitectural differences and sizes. Generally, the majority of cells with neuronal appearance of the ventral and middle spinal cord layers, as well as of the spinal ganglia, show nuclear Hox 3.1-specific staining, while the majority of the smaller cells, which we believe represent mostly neuroglial cells, do not show detectable levels of Hox 3.1 protein expression at the two postnatal stages. It is interesting to point out that the anti-aa2-staining within the nuclei themselves is not uniform, displaying in many cases faintly stained or whisht substructures of round or oval shape, which presumably represent nucleoli. This dif-
ferential nuclear staining suggests that Hox 3.1 protein is located preferentially, if not exclusively, in the euchromatin. A similar distribution has previously been reported for the murine Hox 1.3 homeodomain protein in nuclei of cultured embryonic mouse fibroblasts (Odenwald et al. 1987). The residual ependymal layer in the center of the spinal cord remains negative for Hox 3.1 protein expression.

Throughout postnatal development and in adulthood, anti-aa2-specific staining was also detected in nuclei of certain cells located in the sympathetic ganglia of the cervical region (for an example, see Fig. 6A). The corresponding cells appear to be mostly neurons based on their morphological appearance (data not shown). Transcriptional Hox 3.1 expression in sympathetic ganglia of 12.5 days p.c. embryos had been reported earlier (Breier et al. 1988). Since we did not systematically analyse Hox 3.1 protein expression in sympathetic ganglia during this period of prenatal development, we do not know the timepoint of its initiation.

Discussion

Important information concerning the functions of certain homeobox genes during development of the fruitfly Drosophila has been gained by comparing their distinct expression patterns in mutant and wildtype embryos (for reviews: Gehring, 1985, 1987; Akam, 1987; Scott and Carroll, 1987). An essential outcome of these studies was that the domains of highest expression levels of a particular homeobox gene usually correspond closely to the body regions being most severely affected when the gene was mutated. Accordingly, an important step towards determining the functions of vertebrate homeobox genes is a detailed analysis of their spatial and temporal patterns of expression during development. Although up to the present date numerous vertebrate homeobox genes have been isolated and many of them have been analyzed with respect to their transcriptional expression patterns by Northern blot and in situ hybridization methods, very little is known about the precise localization of their protein products.

The few cases where the distributions of homeodomain proteins have been analyzed during embryogenesis by immunohistochemical methods include the Xenopus genes XHbox 8 (Wright et al. 1988) and XHbox 1 as well as its mouse homolog (Oliver et al. 1988a,b), and homologs to the human Hox 5.2 gene in mouse, chicken and frog (Oliver et al. 1989). We have prepared antibodies that specifically react with a defined peptide region (aa2, see Fig. 1) of the predicted Hox 3.1 protein (LeMouellic et al. 1988; Breier et al. 1988) in mouse tissue sections and used these anti-aa2 antibodies to monitor the localization of Hox 3.1-specific antigen in the developing and adult murine CNS.

Direct comparison between the characteristic, spatially restricted expression pattern of Hox 3.1 transcripts as determined by in situ hybridization to sagittal sections from 12.5 days p.c. embryos and the anti-aa2-specific staining pattern in analogous sections demonstrate virtual identity of both patterns (Fig. 2). In addition, the anti-aa2 staining patterns determined for earlier and later stages of prenatal development are also consistent with previously reported transcriptional expression data for Hox 3.1 (Awgulewitsch et al. 1986; Utset et al. 1987; Holland and Hogan, 1988a; LeMouellic et al. 1988; Breier et al. 1988; Gaunt, 1988; Gaunt et al. 1988). The strong correspondence between the distribution of Hox 3.1 RNA and protein suggests that Hox 3.1 expression is controlled on the level of transcription.

Our data suggest that the distinct anterior boundary of Hox 3.1 protein expression within the central nerve cord established in 10.5 days p.c. embryos shifts cranially during subsequent stages of prenatal development (Figs 3, 4). As the expression extends towards dorsal regions of the spinal cord subsequent to 10.5 days p.c., the initially precise anterior limit at the level of the 5th prevertebra is no longer uniform along the dorsoventral cord axis. At these later stages, expression in middle and dorsal spinal cord layers extends two to three vertebrae further towards the cranium than in ventral layers and, prior to birth at 19.5 days p.c., the diffuse anterior limit of expression extends to the second and first (atlas) vertebrae. It would be interesting to know whether this apparent shift of Hox 3.1 expression towards the cranial direction is due to anterior movement of Hox 3.1 protein-expressing cells, or whether it is the consequence of de novo expression in more anteriorly located cells of the spinal cord. This question is difficult to address, since to our knowledge the positional relationships between neural segments formed during neurulation at earlier stages, including 10.5 days p.c., and the developing vertebrae, ribs and limbs, distinguishable as topographical landmarks at later stages, are not established (Sakai, 1987).

Anti-aa2-staining of spinal cord cross-sections shows that the profound cytoarchitectural changes occurring in the developing central nerve cord during midgestation are accompanied by similarly drastic changes in the medio-lateral and dorsoventral patterns of Hox 3.1 antigen expression (Fig. 5). In 10.5 days p.c. embryos, the neural tube consists largely of a thick inner layer of undifferentiated neuroepithelial cells, known as the ependymal layer. During subsequent developmental stages most of the centrally located ependymal layer is replaced by the surrounding, progressively growing mantle layer, which harbors differentiating neuroblasts. The first distinct, spatially restricted anti-aa2-staining has been detected in the more rapidly growing ventral regions of the mantle layer forming the ventral horns, concomitant with weaker, diffusely distributed staining of the ependymal layer. It is interesting to point out that the anti-aa2-specific staining of individual cells in the ependymal layer disappears completely during subsequent stages, while the intense staining in the mantle layer becomes spatially more extended (Fig. 5). There are at least two possible explanations for these changes in the anti-aa2 staining patterns. The first one is based on the suggested cell movements from the centrally
located ependymal layer to the outer mantle layer occurring during cellular differentiation and maturation (for review: Weiss, 1971). Accordingly one may suspect that individual undifferentiated cells expressing Hox 3.1 protein in the ependymal layer are predetermined to move laterally to the mantle layer, where they undergo terminal differentiation and maturation, resulting in the depletion and final loss of Hox 3.1-expressing cells in the ependymal layer. An alternative explanation would be that cells originally positive for Hox 3.1 protein expression in the ependymal layer simply cease to synthesize Hox 3.1 protein at a certain timepoint during development and that the cells expressing Hox 3.1 protein in the mantle layer are not direct descendants from those expressing it in the ependymal layer, but rather initiate de novo Hox 3.1 protein synthesis.

Although the cell morphologies retained in frozen sections are generally not ideal, they were sufficient enough in our case to distinguish between neuronal cells and neuroglial cells, particularly in sections from late prenatal and postnatal stages. Based on differences in size and shape, it appears that the vast majority of anti-aa2-stained cells are neuronal cells, while most of the non-stained cells seem to be neuroglial cells (P. Garen, personal communication). The fact that no anti-aa2-staining has been detected in marginal layers, which are known not to contain neuronal cell bodies, is consistent with this finding. The anti-aa2-staining of middle and/or dorsal spinal cord regions subsequent to 10.5 days of gestation suggests that the expression of Hox 3.1-specific antigen comprises several, if not all, of the ten horizontal spinal cord laminae described in mammals, (for a review: Brichta, 1985), although highest levels of expression are found mostly in the ventral half of the spinal cord. These data indicate that the expression of Hox 3.1 protein is not strictly limited to certain functionally distinct dorsoventral subregions of the spinal cord.

The expression of Hox 3.1 protein in neuronal cells is not limited to the central nerve cord. Hox 3.1 protein has also been detected in cells of primordial and mature spinal ganglia of all developmental stages examined. In individual cases without any systematic investigation, expression has also been found in sympathetic ganglia, particularly at postnatal stages. These data are consistent with previous transcriptional expression data reporting the detection of Hox 3.1 encoded RNA in spinal ganglia as well as sympathetic ganglia of 12.5 days p.c. embryos (Breier et al. 1988). Our data demonstrate that the expression of Hox 3.1 protein is mainly restricted to neuronal cells of the spinal ganglia.

We have demonstrated the persistent expression of Hox 3.1 protein within the adult murine CNS which is consistent with the transcriptional expression of Hox 3.1 encoded RNAs in the spinal cord of adult mice (Awgulewitsch et al. 1986). Several other murine homeobox genes have been reported to be transcriptionally expressed in the embryonic, as well as in the adult CNS, i.e. Hox 1.2 (Toth et al. 1987), Hox 1.3 (Odenwald et al. 1987), Hox 1.5 and Hox 2.1 (Ruddle et al. 1985), Hox 2.2 (Schughart et al. 1988), Hox 2.5 (Bogard et al. 1989), and En-2 (Davis et al. 1988). Hox 1.3 encoded proteins have been localized in specific cell types of the adult brain including Purkinje neurons of the cerebellum and pyramidal and dentate neurons of the hippocampus (Odenwald et al. 1987). The continued expression of murine homeobox genes in the adult CNS is not in contradiction with their proposed functions as developmental control genes. One supporting argument for this view is that certain Drosophila homeobox genes with known morphogenetic control functions during embryonic and larval development of the fruitfly, as for instance Antennapedia and Deformed, continue to be expressed within the CNS of the adult fly (Scott et al. 1983; Chadwick and McGinnis, 1987). A possible function of homeobox gene expression in adult tissues might be to maintain the differentiated status including functional and positional identities of certain cells. It would be important to know whether the mature neuronal cells showing the apparent accumulation of Hox 3.1 protein in their nuclei are the direct descendants of those differentiating neuroblasts expressing Hox 3.1-specific antigen during embryogenesis. The use of a second independent cell marker would be helpful in addressing this difficult question of cell lineage.

We gratefully acknowledge the generous support of Dr Frank Ruddle, in whose laboratory the c210 cDNA clone has been isolated. We would like to thank Drs Christian Schwabe and Erika Bullesbach for their help in peptide synthesis, and Jim Nicholson for invaluable photographic assistance. We thank Drs Claudia Kappen, Klaus Schughart, Gerado Vasta, Dominick DeLuca, Christopher Wright and Sam Schlüter for technical discussions concerning antibody production and immunohistochemical methods. Finally, we acknowledge our appreciation to Drs William McGinnis, Paul Garen, and Barry Ledford for discussion and critical review of the manuscript. This work was supported by grant GM 43334-01 awarded by the National Institute of General Medical Services, Department of Health and Human Services to A.A., as well as MUSC Institutional Research Funds, and a corporate contribution by Westvaco to the Health Sciences Foundation.

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(Accepted 8 December 1989)