The neural cell adhesion molecule (NCAM) is one of the most abundant cell adhesion molecules expressed in vertebrates and it is thought to play important roles as a regulator of morphogenetic processes, but little is known of its expression pattern in mammalian embryos. In this study, we have examined the developmental profile of NCAM gene expression in mouse embryos from gestational day 7.5 to 12.5, focusing on the developing neural tube. NCAM transcripts were first detected around day 8.5 in the somites and the forming neural tube. NCAM transcripts were expressed in the neuroepithelium throughout the width of the neural groove and tube up to a rostral boundary within the hindbrain, whereas NCAM mRNA levels were very low or undetectable in the neuroepithelium of the head region. The positional restriction of NCAM expression was confirmed by immunohistochemistry at the protein, and by polymerase chain reaction analysis at the RNA level. Expression in the neuroepithelium was transient as the level of NCAM transcripts declined in the germinal layer beyond day 8.5. By day 9.5, strong NCAM expression had appeared on the earliest postmitotic neurones along the entire neuraxis, and this pattern of expression in all regions with differentiating neurones was maintained until day 12.5. We conclude that NCAM expression in the neural tube occurs in two spatiotemporal distinct waves: a first wave in the proliferating neuroepithelium showing positional dependence along the rostrocaudal axis, and a second wave on essentially all neurones that have become postmitotic.

Key words: NCAM, mouse neural tube, regional specification.

INTRODUCTION

During embryonic development, cell-cell interactions mediated by cell adhesion molecules play key roles in the control of cell movements, aggregation and migration, and may also influence cell proliferation and differentiation. The NCAMs (neural cell adhesion molecules) are a family of structurally closely related cell surface proteins that arise from a single gene by differential processing of the primary transcript and mediate adhesive interactions among neural and other cells mainly by a self-binding mechanism. During embryonic development, the expression of NCAM proteins is precisely regulated at both the transcriptional level (with concomitant changes in the overall abundance of all forms of NCAM) and at the level of alternative splicing of the premRNA (resulting in changes in the relative amounts of the different isoforms; for reviews, see Edelman, 1986; Rutishauser and Jessell, 1988; Goridis et al., 1989; Thiery, 1989; Walsh and Doherty, 1991; Edelman and Crossin, 1991; Goridis and Brunet, 1992).

In the amphibian and avian embryo, NCAM has been shown to appear early in development on derivatives of all three germ layers. A recurrent theme is that, with the possible exception of the pregastrulation chick embryo, NCAM is never uniformly expressed and that its spatiotemporal expression pattern is highly dynamic. Drastic changes in the abundance of NCAM proteins or transcripts are often seen at times and places of embryonic induction or of important morphogenetic events such as epithelial-mesenchymal transformations or neural crest cell migration (Thiery et al., 1982; Crossin et al., 1985; Duband et al., 1987; Kintner and Melton, 1987; Levi et al., 1987; Keane et al., 1988; Klein et al., 1988). On the basis of its adhesive function and its evocative expression patterns, NCAM is believed to be critically involved in controlling a variety of morphogenetic events. This conjecture receives some support from recent results suggesting that NCAM is a likely target for homeodomain proteins (Hirsch et al., 1991; Jones et al., 1992), important regulators of morphogenesis throughout the animal kingdom (for reviews, see Kessel and Gruss, 1990; McGinnis and Krumlauf, 1992). Moreover, there is increasing evidence that NCAM homophilic binding activates second messenger pathways (Schuch et al., 1989) which in turn may influence cell differentiation.

SUMMARY

The neural cell adhesion molecule (NCAM) is one of the most abundant cell adhesion molecules expressed in vertebrates and it is thought to play important roles as a regulator of morphogenetic processes, but little is known of its expression pattern in mammalian embryos. In this study, we have examined the developmental profile of NCAM gene expression in mouse embryos from gestational day 7.5 to 12.5, focusing on the developing neural tube. NCAM transcripts were first detected around day 8.5 in the somites and the forming neural tube. NCAM transcripts were expressed in the neuroepithelium throughout the width of the neural groove and tube up to a rostral boundary within the hindbrain, whereas NCAM mRNA levels were very low or undetectable in the neuroepithelium of the head region. The positional restriction of NCAM expression was confirmed by immunohistochemistry at the protein, and by polymerase chain reaction analysis at the RNA level. Expression in the neuroepithelium was transient as the level of NCAM transcripts declined in the germinal layer beyond day 8.5. By day 9.5, strong NCAM expression had appeared on the earliest postmitotic neurones along the entire neuraxis, and this pattern of expression in all regions with differentiating neurones was maintained until day 12.5. We conclude that NCAM expression in the neural tube occurs in two spatiotemporal distinct waves: a first wave in the proliferating neuroepithelium showing positional dependence along the rostrocaudal axis, and a second wave on essentially all neurones that have become postmitotic.

Key words: NCAM, mouse neural tube, regional specification.
(Doherty et al., 1991) and proliferation (Aoki et al., 1991), inviting speculations that NCAM is involved in regulatory circuits that control cell fate in the embryo.

Low levels of NCAM immunoreactivity have been detected in the blastoderm of the chick embryo before gastrulation (Crossin et al., 1985; Keane et al., 1988). In amphibian embryos, NCAM transcripts have not been detected by in situ hybridization prior to neural tube formation (Kintner and Melton, 1987), but low levels of NCAM immunoreactivity were found at earlier stages in two other studies (Levi et al., 1987, Saint-Jeanett et al., 1989). Comparably early stages in the chick have not been analyzed by in situ hybridization (Priot et al., 1989). However, all reports agree on the point that NCAM is dramatically up-regulated in the neural plate and forming neural tube in response to neural induction, and NCAM is said to be ubiquitously distributed throughout the neural tube and in the developing central nervous system at later stages (Daniloff et al., 1986; Rutishauser and Jessell, 1988; Prieto et al., 1989 Slack and Tannahill, 1992). In early embryos, another domain of strong NCAM expression are the somites and the developing myotome (Thiery et al., 1982; Levi et al., 1987).

The spatiotemporal expression patterns of NCAM in mammalian embryos have been much less well studied. Published immunohistological studies in the mouse focused on localized regions or specific developmental processes such as the neocortex (Fushiki and Schachner, 1986), the olfactory system (Miragall et al., 1989; Chung et al., 1991) or spinal cord motor neurones (Chen and Chiu, 1992). More complete information on in vivo expression sequences of the NCAM gene are needed in the light of the recent reports indicating that the NCAM gene might be a primary target of homeodomain proteins (Hirsch et al., 1991; Jones et al., 1992; Valarché, Hirsch, Tissier-Seta, Goridis and Brunet, unpublished data) and of the possibility of altered NCAM expression in mouse mutants (Moase and Trasler, 1991). In this study, we analyzed the expression patterns of NCAM transcripts during mouse neural tube formation and early neurogenesis by in situ hybridization using a probe that recognizes all NCAM mRNAs. We present evidence for a biphasic expression pattern of NCAM and show that NCAM gene expression in the neuroepithelium is position-dependent at early stages.

MATERIALS AND METHODS

Mice

Embryos were obtained from timed matings of outbred OF1 mice (IFFA-Credo, Lyon, France). Gestational ages were based on detection of a vaginal plug on the morning following mating and midday of the same day was designated as day 0.5 p.c. (E0.5).

Probes

The DW3-le cDNA described in Barthels et al. (1987), contained in the pGEM-2 vector (Promega) was used as a probe. This cDNA covers part of exon 2, exons 3 to 9 and part of exon 10, which are present in all NCAM mRNAs. The linearized clone was transcribed from the corresponding promoter by T7 RNA polymerase to produce an anti-sense probe, and by SP6 RNA polymerase to produce a sense probe. For hybridization on tissue sections, the cRNA probes were labelled by incorporation of [35S]UTP (Amersham, 1000 Ci/mmol) and were then hydrolyzed to generate 150 nt fragments (Fontaine and Changeux, 1989). For hybridization of whole-mount embryos, the probes were synthesized by T7 or SP6 RNA polymerase in the presence of 0.17 mM digoxigenin-UTP (Boehringer Mannheim), 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP and 0.33 mM UTP and were not hydrolyzed after synthesis. For in situ hybridization on tissue sections, the labelled probe was applied at 5×10^3 cts/minute/µl digoxigenin-labelled probes were used at 2 ng/µl on whole mounts.

In situ hybridization

For hybridization on tissue sections, embryos were removed from the uterus (except for E7.5 embryos) and fixed by immersion in 4% paraformaldehyde in PBS overnight at 4°C. They were then dehydrated and embedded in 1:1 xylene:paraffin (Paraplast plus). Serial sections, 7.5 µm thick, were mounted on gelatin-coated slides. Hybridizations were done as described by Fontaine and Changeux (1989) with minor modifications (Bally-Cuif et al., 1992).

Hybridization on whole-mount embryos was done following a protocol kindly provided by D. G. Wilkinson exactly as described (Bally-Cuif et al., 1992). For subsequent sectioning, stained embryos were incubated 2 hours in 100% methanol, 2 hours in xylene, 1 hour in Paraplast-plus at 60°C and finally 6 hours in Paraplast at 60°C. 30 µm sections were cut and dried down on gelatin-coated slides. Paraffin was then dissolved in xylene and the sections mounted in Eukitt.

Immunocytochemistry

Embryos were fixed overnight in 4% paraformaldehyde in 0.12 M phosphate buffer at 4°C, cryoprotected in 15% sucrose in 0.12 M phosphate buffer, frozen in isopentane in liquid nitrogen and cryostat-sectioned at 10 µm. Sections were degelatinized and washed in PBS, and then incubated in 1/5000-diluted rabbit anti-mouse NCAM antibodies (Gennarini et al., 1984) overnight at 4°C. Antibody binding was revealed by the peroxidase-antiperoxidase method of Sternberger et al. (1970). After washing, the sections were incubated with sheep anti-rabbit IgG (diluted 1/100, Cappel) for 1 hour, and then incubated with peroxidase-anti-peroxidase-coupled rabbit IgG (diluted 1/200, Sternberger). All incubation solutions were prepared in PBS containing 2 g/l gelatin and 0.25% Triton X-100. Peroxidase activity was revealed by a 30 minute incubation in 0.03% diaminobenzidine tetrahydrochloride (Sigma), dissolved in 0.1 M Tris buffer (pH 7.6) containing 0.005% H2O2. Control sections were treated as above except for omission of the first antibody.

Amplification of NCAM mRNA sequences

The head region and the posterior part caudal to the last somite of E8.5 embryos were dissected and total RNA extracted according to Chomczynski and Sacchi (1987). RNA prepared from N2a neuroblastoma cells served as positive control. 6 µg RNA from each sample was analyzed by polymerase chain amplification after reverse transcription into cDNA (RT/PCR) as described (Sherman et al., 1989) using primers derived from sequences present in all NCAM mRNAs and positioned in exon 1 and 3 to preclude amplification of genomic DNA (position 221-240 and position 537-557 (Barthels et al., 1987) for the sense and anti-sense primers, respectively.) The RNA was reversed transcribed using 50 ng sense primers in the presence of 200 U MMLV reverse transcriptase (BRL) and 0.2 mM dNTPs in 2× Taq DNA polymerase buffer (Promega) for 1 hour at 42°C. One third of each reaction product
was amplified by PCR with 500 ng each of the sense and antisense primers (30 seconds at 93°C, 1 minute at 61°C and 1 minute at 72°C) in the presence of 2.5 U Taq DNA polymerase (Promega) and 0.4 mM dNTPs in 1× Taq DNA polymerase buffer. The reaction was stopped after different cycles to check for the logarithmic nature of the response. 10 µl of the reaction mixture was analyzed by electrophoresis through 2% agarose gels and the bands visualized by ethidium bromide staining. No reaction product could be detected when reverse transcriptase was omitted. From densitometry of the gels, we estimated that amplification proceeded for up to 23 cycles in a logarithmic fashion.

RESULTS

An NCAM anti-sense cRNA probe, which detects all splice variants, was used in the present study to examine the distribution of NCAM transcripts in the mouse neural tube. Mouse embryos were analyzed by in situ hybridization either on tissue sections at one-day intervals between E7.5 and E12.5, or on whole-mount preparations at E8.5 and E8.75.

NCAM expression is restricted to the caudal neural tube early in development

We did not study stages before day 7.5. At E7.5, no NCAM transcripts were detected in sections of several embryos (Fig. 1A). NCAM expression was seen at E8.5, in neuroectoderm- and mesoderm-derived structures: the neural plate and neural tube (Fig. 1B, C) and the somites (Fig. 1C). Surprisingly, NCAM expression in the neural tube, which at this stage is composed of proliferating neuroepithelial cells that form a pseudostratified germinal epithelium (Nornes and Carry, 1978; Altman and Bayer, 1984), was not uniform along the rostrocaudal axis. It was strong in the caudal region of the neural tube and neural plate, but the grain density in the headfolds did not exceed back-
Fig. 2. (A-E) Detection of NCAM transcripts in whole-mount E8.5 (A-D) and E8.75 (E) mouse embryos. (A) The two embryos at the top were hybridized with an NCAM antisense probe (+) and the embryo at the bottom is a negative control hybridized with a sense probe (−). Ventral views are shown, rostral is to the right and caudal to the left. (B) A higher magnification of a positive embryo shown in A. NCAM is expressed in all somites, in the neural plate and the presumptive spinal cord up to the middle of the hindbrain (arrows in B and on the schematic sagittal view of the same embryo). Note that the headfolds are not stained. Sections at different levels (numbered 1 to 4 from rostral to caudal) of an E8.5 embryo, whole-mount-hybridized with the NCAM antisense probe are shown in C and D. At E8.75 (E), the head region is still NCAM-negative while the rostral limit of NCAM expression in the germinative neuroepithelium has shifted caudally (arrow). (F-I) Immunohistological detection of NCAM protein on cryostat sections of E8.5 (F, transversal section), E8.75 (G, sagittal section) and E9 (H, frontal section) mouse embryos. At all stages, staining is much more intense in caudal than in rostral regions of the neural tube (in F and G, caudal is to the right and rostral to the left). A negative control where the anti-NCAM antibody has been omitted is shown in I (same embryo as in H). np, neural plate; n, neural tube; hf, head folds; s, somites. Scale bars: A, 600 µm; B, 300 µm; C,D, 200 µm; E,F, 300 µm; G,H,I, 200 µm.
NCAM in neural tube

Detection of NCAM transcripts by RT/PCR. Total RNA (6 μg) from N2a cells, and from the caudal part (posterior, P) and the head region (anterior part, A) of E8.5 embryos was reverse transcribed and amplified with NCAM-specific primers for 27 (lanes 1, 4, and 7), 23 (lanes 2, 5 and 8) and 19 (lanes 3, 6 and 9) cycles. The products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. Already after 19 cycles, a band of the expected size of 336 bp became detectable in RNA from the caudal region of the embryo, but could not be detected in the head region even after 27 cycles.

ground levels (Fig. 1B,C). In the neural tube, the stained region extended from the caudalmost up to the rostral part of the future spinal cord (Fig. 1B). Similarly, the absence of labelling in the head mesenchyme contrasted with the intense staining in the somites (Fig. 1C).

To obtain a global view of NCAM expression and to determine its approximate rostral limit in the neural tube, we performed whole-mount in situ hybridization experiments on mouse embryos 8.5 (8 somites) to 8.75 days (14 somites) of age using a digoxigenin-labelled NCAM probe. NCAM transcripts were detected in all somites and in the caudal neuroectoderm but not in the head region as shown in Fig. 2A,B for an 8.5 day embryo. At this stage, the signal in the neural tube extended up to a rostral limit beyond the first somite, i.e. approximately halfway into the hindbrain. The signal was most intense in the neural folds around the rostral part of the posterior neuropore, weaker around its caudal part and rostrally in the recently closed neural tube, till it faded out in the middle of the hindbrain. Slightly later, at the 14-somite stage, NCAM expression has retracted caudally to the level between somites 12 and 14 (Fig. 2E). Staining of the somites changed according to the rostrocaudal gradient of development (Theiler, 1989; Rugh, 1990), with labelling of the caudal somites throughout their width and progressive concentration in the newly formed dermomyotome rostrally (Fig. 2E). Transverse sections of whole mount-stained embryos demonstrated that NCAM was expressed throughout the entire width of the neural tube and plate (Fig. 2C,D). The hybridization signal was stronger near the lumen of the tube in the pseudostratified single-layered epithelium as if the transcripts accumulated near the luminal pole of the cells. Expression in the presumptive spinal cord was more intense where the tube was not yet closed, confirming what was seen in the unsectioned embryos. In the caudalmost section, the hybridization signal extended laterally beyond the neuroepithelium, showing that NCAM transcripts are expressed in the still unsegmented paraxial mesoderm.

To confirm the caudally restricted expression of NCAM transcripts in the neural tube by an independent method, we applied the extremely sensitive RT/PCR technique. Identical amounts of RNA extracted from the head region and from the caudal part (caudal to the last visible somite to minimize contribution from these structures) of E8.5 embryos were reverse transcribed and the PCR-amplified cDNA visualized after electrophoretic separation on agarose gels. As shown in Fig. 3, even after 27 amplification cycles, no band corresponding to NCAM transcripts could be detected in the head samples, whereas 19 cycles were sufficient to produce a band of the expected size in samples from the caudal region. Although RT/PCR is a semi-quantitative method, it can be estimated that NCAM mRNA is at least 1000-fold more abundant in the caudal than in the head samples.

Similar results were obtained at the protein level. NCAM protein expression was analyzed by immunocytochemistry on sections of E8.5 to E9 embryos (Fig. 2F-H). At all stages, NCAM immunoreactivity was strong in the caudal neural tube and neural plate as opposed to the very weak, diffuse labelling in the headfolds. In the light of the RT/PCR data, we interpret these low, uniform levels of labelling as non-specific background staining. In line with the in situ hybridization data, the head mesenchyme was also negative, whereas the somites were clearly labelled.

**NCAM expression in the neural tube is restricted to postmitotic neurones at later stages**

The maturation of the vertebrate neural tube occurs temporally in a rostrocaudal sequence and different stages of development can be observed in the same embryo along the craniocaudal axis. Hence, at E9.5, the caudal neural tube still consists only of the germinal epithelium, whereas neuronal differentiation is already under way in the rostral spinal cord and the brain, giving rise to subdivisions into ventricular, intermediate and marginal layers. Postmitotic neurones generated in the ventricular layer, consisting of the dividing neuroepithelial cells, migrate through the intermediate layer to populate its ventrolateral parts, where the neurones start growing axons that form the marginal layer (Nornes and Carry, 1978; Altman and Bayer, 1984). At this stage, NCAM transcripts were still detected throughout the entire caudal tube. Concomitantly, NCAM transcripts appear now rostrally in the primitive brain (Fig. 4A,B) on neurones of the synencephalon (later becoming the interstitial nucleus of Cajal) and of the mesencephalic and motor nuclei of the trigeminal nerve, that is in the first postmitotic neurones to appear in the head region (Pierce, 1973; Fig. 4B). Similarly, the earliest born neurones in the rostral spinal cord, the motor neurones in the incipient ventral horn, are also stained at this stage (not illustrated).

Two distinct expression domains coexisted also at E10.5 as shown by the intense, uniform labelling of the caudal neural tube and staining of those parts of the rostral spinal cord and brain containing postmitotic neurones (Fig. 4C-E). In the cervical spinal cord, the hybridization signal did not exceed background levels in the germinal epithelium, while NCAM expression was strong in the developing ven-
tral horn containing the differentiating motor neurones (Fig. 4C). To analyze these expression sequences in more detail, we processed transverse sections of the spinal cord at different levels of E10.5 embryos, examples of which are shown in Fig. 4D and E (sections 1 to 4, from rostral to caudal). As one moves rostrally, the strong staining of the proliferating neuroepithelium seen caudally becomes weak at lumbar levels and virtually undetectable further rostrally. Concomitantly, NCAM-positive cells, which populate the ventral horn in the cervical spinal cord and should represent differentiating motor neurones, become first visible at thoracic levels. Hence, the down-regulation of NCAM transcripts in the germinal layer seems to precede the appearance of NCAM-expressing differentiating neurones in the future spinal cord, in agreement with the whole-mount experiments, showing that NCAM expression had receded to caudal regions already at E8.75.

By E9, NCAM expression also appears in a variety of other tissues, such as heart and spleen, and remains intense in the somites, particularly in the forming myotome (not shown).

By E11.5, the entire nervous system is undergoing cellular differentiation. At this stage, high levels of NCAM transcripts were detected in the differentiating neurones along the entire neuraxis, while NCAM expression was very low or absent in the dividing cells of the ventricular layer and in the inner parts of the intermediate layer (Fig. 5A). The ventral parts of the spinal cord containing the differentiating motor neurones were intensely stained (Fig. 5B). Because of the curvature of the embryo shown in Fig. 5A, the spinal cord has been cross-sectioned both at lumbar and cervical levels. Caudally, the ventral parts of the neural tube are still only sparsely populated by postmitotic neurones; yet, the germinal layer has already become negative. In the rostral section, the lateral parts of the tube, where the later-born relay neurones are about to settle (Altman and Bayer, 1984), are already strongly NCAM-positive. In the head, differentiating, postmitotic neurones, which are now present in all brain vesicles, were also heavily labelled. Curiously, the labelling along the ventral aspects of the neural tube was interrupted in the isthmic region, which separates the mesencephalic and metencephalic vesicles (Fig. 5B,C).

Additional sites of NCAM expression were seen in the olfactory epithelium and in the olfactory roots, in cranial
NCAM in neural tube

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DISCUSSION

We have used in situ hybridization to analyze the localization of NCAM mRNA in the mouse embryo focusing on the early development of the neural tube. Contrary to a widely held belief (Edelman, 1986; Rutishauser and Jessell, 1988), we did not find NCAM expression to be uniform throughout the neural tube. At 8.5 days of gestation, we found NCAM mRNA expression in the neuroepithelium to be confined to the posterior neural groove and neural tube with a rostral limit in the hindbrain. The caudally restricted expression of NCAM at this stage was confirmed by two independent methods. Even with the extremely sensitive PCR technique, we could not detect NCAM mRNA in the head, and immunohistochemistry also revealed dramatic differences in NCAM protein levels between the rostral and caudal neural tube and plate. During further development, there was a striking transition in the expression of NCAM. By day 8.75, the rostral limits of expression in the neuroepithelium had receded caudally and, at E9.5, NCAM transcripts appeared in the earliest born neurones along the entire neuraxis. We can thus distinguish two distinct modes of NCAM expression in neuroectoderm-derived cells: a first phase in the neuroepithelium followed by a second phase in differentiating neurones that have emigrated from the then negative germinal layer. Whereas expression in the neuroepithelium shows regional specificity along the cranio-caudal axis, expression in postmitotic neurones seems to be dictated only by the differentiation state of the cells. A schematic overview of the NCAM expression sequences in the neural tube is given in Fig. 6.

Previous studies on NCAM expression in the embryo have relied mainly on immunohistochemistry, the most complete results being obtained in the chick. In this species, low levels of NCAM were detected already in the blastoderm before gastrulation, where it persisted up to neural groove formation (Thiery et al., 1982; Crossin et al., 1985; Keane et al., 1988). We did not detect NCAM transcripts in mouse embryos at E7.5, but would have missed transient expression at an earlier stage. High levels of NCAM expression have also been observed in the caudal neural tube of early chick embryos, but anterior sections were not shown (Thiery et al., 1982). Our results are consistent with NCAM immunostaining of the chick spinal cord revealing intense labelling of the areas containing postmitotic neurones (Daniloff et al., 1986). In contrast to our data, however, these authors detect a low level of staining in the ventricular zone and NCAM is said to be expressed on all cells of the neural tube. The only published in situ hybridization data on NCAM expression in the developing spinal cord are from a somewhat later stage of chicken development than we have studied in the mouse and show a rather uniform labelling throughout all layers (Prieto et al., 1989).

Fig. 5. Localization of NCAM transcripts on sagittal sections of E11.5 (A, B) and E12.5 (C) mouse embryos. Postmitotic neurones are stained over the whole length of the neural tube, but this expression is interrupted on the basal plate at the metencephalomesencephalic border (arrow in B and C). m, motor neurones; olf, olfactory epithelium; g, dorsal root ganglia; s, somites. Scale bars: A, 250 µm; B, 500 µm; C, 800 µm.

Fig. 6. Summary of the spatiotemporal pattern of NCAM gene expression during development of the mouse neural tube. Schematic cross-sections at different levels of the neural tube are shown at each stage and NCAM transcripts are presented in black. The level considered is identical for all sections of the same column: A, caudal spinal cord; B, thoracic level; C, cervical level; D, metencephalon.
Much less is known on NCAM expression sequences in the mammalian embryo. In agreement with our results, a study on NCAM expression in the early mouse embryo describes immunostaining throughout the neuroepithelium in cross-sections of the E8 and E9 neural tube at the level of the future spinal cord (Moase and Traslser, 1991). In a study on the development of the mouse cerebral cortex (Fushiki and Schachner, 1986), it is said that NCAM immunoreactivity is detected throughout the width of the telencephalic anlage by E9, in contrast to our data. However, the results are documented only from E11 onwards. They reveal dramatic differences in staining intensity with much higher levels of expression in the marginal zone and in the outer part of the intermediate zone, which contain the differentiating neurones and their processes, than in the inner layers. In complete agreement with our data, a recent immunohistological study on motor neurone development (Chen and Chiu, 1992) shows absence of NCAM immunoreactivity in the E10.5 rat cervical spinal cord and immunoreactivity restricted to motor neurones at E11.

In all species examined, important morphogenetic events, such as neural induction, placode formation, epithelial-mesenchymal transformations or neural crest cell migration are correlated with up- or down-regulations of NCAM (Edelman, 1986; Rutishauser and Jessell, 1988; Thiery, 1989; Edelman and Crossin, 1991) and, mainly for this reason, NCAM is thought to be an essential regulator of morphogenesis. Our results represent another striking example of the dynamic changes in NCAM expression during embryonic development. Moreover, they provide the first evidence for position-dependent regulation of NCAM expression. At E8.5, the mouse neural tube consists of a single layer of dividing cells without obvious cellular differences along the rostrocaudal axis. Yet, a hybridization signal was only found in those parts that differentiate later into hindbrain and spinal cord and its rostral boundary within the hindbrain does not correspond to any morphological cue at this stage. Hence, NCAM may specify positional information in the developing neural tube along the rostrocaudal axis.

Recent evidence (Storey et al., 1992) supports earlier results (Nieuwkoop, 1955) indicating that the neural tube of the trunk CNS is generated by mechanisms different to those in the anterior CNS. In this respect, the results of Keane et al. (1988), showing that in explants of chick neuroectoderm, NCAM-expressing groups of cells are linked through gap junctions that fail to develop in the presence of anti-NCAM antibodies, may be of particular relevance. When considered in the light of our data, these results invite speculations that the neuroepithelial cells in the posterior tube establish junctional communications among themselves but not with those of the head region. Mechanisms that link posterior neuroectodermal cells to the exclusion of anterior ones may facilitate the passage of signals within the posterior neuroepithelium which have been implicated in the induction and/or regionalisation of posterior neural primordia (Dixon and Kintner, 1989; Ruiz i Altava, 1990; Storey et al., 1992).

As the labelling was always strongest around the posterior neuropore prior to neural tube closure, an alternative possibility would be that NCAM plays a role in the latter process. If this were true, we might expect NCAM expression to be strong also at anterior points of fusion of the neural folds (Sakai, 1989), which was not observed. However, we cannot exclude that neural tube closure proceeds differently in the anterior and posterior neural plate.

Another important question concerns the factors responsible for regulating NCAM expression along the rostrocaudal axis. Several members of the *Hox*-1- and *Hox*-2- and *Hox*-4-cluster genes have rostral expression limits in the hindbrain at or near the anterior border of NCAM expression (Gaunt et al., 1989; Kessel and Gruss, 1990, Wall et al., 1992). Moreover, recent results suggest that the NCAM gene may be a downstream target of *Hox* gene products (Hirsch et al., 1991; Jones et al., 1992). Our results showing regional specificity of NCAM expression in the neural tube add further weight to the notion that the NCAM gene is a target for *Hox* proteins, which may be instrumental in initiating NCAM expression in the early neural tube. NCAM expression starts to disappear in the proliferating cells lining the ventricle beyond E8.5 suggesting that NCAM may be involved in initiating rather than in the maintenance of positional information. Most *Hox* genes, by contrast, remain expressed in the germinall layer beyond this stage (Dony and Gruss, 1987; Graham et al., 1991).

An intense hybridization signal was detected along the entire neural tube over areas containing postmitotic neurones indiscriminately of neuronal type. This expression pattern persisted up to E12.5, the latest stage studied. In the head region, where we did not detect NCAM mRNA in the neuroepithelium, NCAM may serve as a convenient marker for the earliest born neurones in the mesencephalon and synencephalon. Down-regulation of NCAM expression in the neuroepithelium appears to precede the appearance of the earliest postmitotic neurones (Nornes and Carry, 1978), as seen best by the caudally receding expression as early as E8.75. This second wave of NCAM expression is thus temporally and spatially quite distinct from the expression pattern in the neuroepithelium, and we suggest a second, independent induction of the gene in the neurones that have emigrated from the germinall layer. NCAM may also subserve a function in the neurones that is distinct from the one that it assumes in the precursor cells. All early born neurones start growing axons as soon as after they have reached their final localisation (Altman and Bayer, 1984; Chen and Chin, 1992). That NCAM may be involved in this process receives support from in vitro studies showing that NCAM-NCAM interactions promote neurite outgrowth via transmembrane signallling (Doherty et al., 1991). Together with other adhesion receptors, high levels of NCAM expression at this stage may thus act as a signal for initiating and maintaining rapid axonal growth.

NCAM expression in the ventral part of the neural tube was found to be interrupted at the level of the isthmic region, the met-mesencephalic border. This region is distinguished by the localized expression of the Wnt-1 developmental control gene (Wilkinson et al., 1987; Bally-Cuif et al., 1992) and the fact that the axons of the IVth cranial nerve, which have their exit point in this area, are the only motor axons that exit from the dorsal face of the neural tube. The absence of NCAM expression at this location may signify that no postmitotic neurones are present in this area,
but close inspection of our sections revealed that neuronal cell bodies were present in the ventral tube at this level at E12.5 (not shown). It will be interesting to determine the timing and extent of axonal outgrowth by these neurons.

In conclusion, we have demonstrated that NCAM gene expression in the neural tube and spinal cord follows a biphasic pattern with an early, positionally restricted component and a later phase in essentially all postmitotic neurons. We would like to propose that the two waves of expression are regulated by different transcriptional control mechanisms and that NCAM subserves different functions in the early tube and in differentiating neurons.

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