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Developmental Control of N-CAM Expression by Hox and Pax Gene Products

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Developmental control of N-CAM expression by Hox and Pax gene products

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SUMMARY

A mounting body of evidence suggests that cell–cell adhesion molecules (CAMs) play critical roles in morphogenetic patterning. Perturbations of CAM binding can lead to altered tissue pattern and interruption of tissue interactions can lead to altered patterns of CAM expression. These observations focus attention on the factors responsible for the place-dependent expression of adhesion molecules such as N-CAM, the neural cell adhesion molecule. Our recent experiments *in vitro* indicate that transcription factors encoded by Hox and Pax genes bind to specific DNA sequences in the N-CAM promoter and activate that promoter. In particular, a region of the N-CAM promoter designated the homeodomain binding site (HBS) interacts with a variety of different homeodomain proteins. A different region of the N-CAM promoter binds to the paired domain of Pax proteins. These transcription factors differentially regulate the N-CAM gene. Such *in vitro* studies suggest that the N-CAM gene may be an *in vivo* target for homeobox and Pax gene products. Recent experiments on transgenic mice carrying normal and mutated segments of the N-CAM promoter linked to a lacZ reporter gene suggest that the N-CAM regulation observed *in vitro* actually has counterparts *in vivo*. The significance of these observations is that they connect gene products capable of morphoregulation (such as CAMs) to pattern-forming genes.

1. INTRODUCTION

N-CAM, the neural cell adhesion molecule, mediates adhesion in the nervous system and in a variety of embryonic tissues (Edelman & Crossin 1991). This CAM has a precise pattern of place-dependent expression during development (Crossin *et al.* 1985; Prieto *et al.* 1989). Perturbation experiments have shown that blockage of N-CAM binding or expression leads to alterations of morphogenetic patterning (Fraser *et al.* 1988). Furthermore, disruption of tissue interactions such as those between nerves and muscles by either nerve crush or cut leads to specific alterations of N-CAM expression at distant sites in both the spinal cord and muscle (Rieger *et al.* 1985, 1988; Daniloff *et al.* 1986). These findings are in accord with the notion that CAMs have a key mechanochemical function in directly regulating tissue form and cell migration during development.

Elaborate regulatory mechanisms must govern place-dependent N-CAM expression at specific times of development and regeneration and thereby affect the mechanochemical guidance of tissue pattern. One clue to the possible control of N-CAM expression is suggested by our observations (Jones *et al.* 1992, 1993; Edelman & Jones 1993; Holst *et al.* 1994) that the N-CAM promoter is responsive *in vitro* to the products of Hox and Pax genes. Another clue is that products of several homeobox and Pax genes (Akam 1989; Chalepakis *et al.* 1993), which are related to well-known pattern-forming genes in *Drosophila melanogaster*, appear to be co-localized with N-CAM during development of the vertebrate embryo. These findings and those based on more recent work (Jones *et al.* 1992, 1993; Valarché *et al.* 1993; Chalepakis *et al.* 1994*a*; Holst *et al.* 1994) raise the possibility of a link between the appearance of transcription factors encoded by Hox genes and Pax genes and the spatiotemporal patterns of expression of N-CAM during development.

In this paper, we review some findings indicating that the N-CAM promoter contains DNA sequences that are targets for regulation by homeobox and Pax gene products. These findings open the possibility of relating the mechanochemical control of tissue architecture to specific epochs of gene expression during development.

2. PLACE-DEPENDENT EXPRESSION OF N-CAM DURING DEVELOPMENT

Various cell and substrate adhesion molecules have been shown to be expressed in specific combinations at definite locations during neurogenesis. An example involving N-CAM in early development is shown in figure 1. Following gastrulation and coincident with neural induction, there is a marked alteration in the distribution of two primary CAMs, L-CAM (the liver cell adhesion molecule) and N-CAM. N-CAM appears in the region of the neural plate and groove as L-CAM disappears. Concurrently and in conjugate fashion, L-CAM is expressed intensely in the somatic ectoderm while N-CAM expression diminishes (Crossin *et al.*

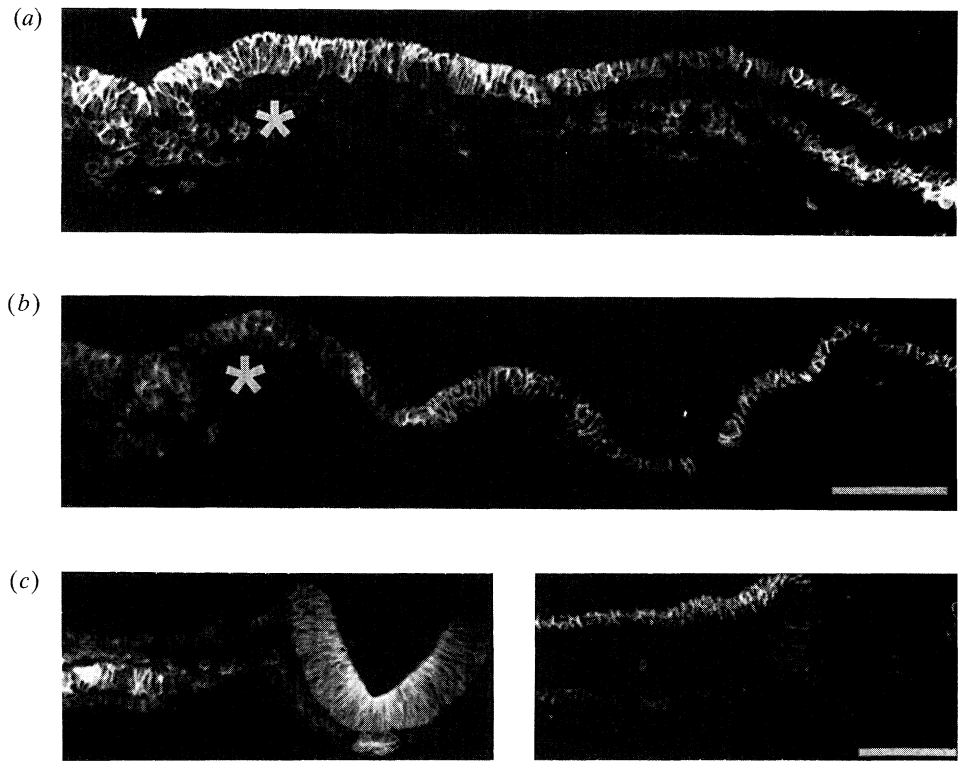

Figure 1. Immunocytochemical staining of early ectodermal epithelial structures showing transition from the distribution of N-CAM and L-CAM together on the blastoderm to their segregation after neural induction. (*a*,*b*) Transverse section of a head fold-stage chick embryo stained with N-CAM (*a*) or L-CAM (*b*) antibodies. Arrow indicates location of the primitive streak, asterisk denotes ingressing cells. (*c*) Transverse sections through a five somite-stage embryo at a position posterior to the last somite were stained either with N-CAM (left) or L-CAM (right) antibodies.

1985). Such polarization of CAM expression in cells of different embryonic fates prompts the investigation of the local signals that regulate the expression of the genes encoding CAMs.

3. THE MORPHOREGULATOR HYPOTHESIS: A SPECIFIC APPLICATION

In a previous publication (Edelman 1992), it was suggested that the place dependence of CAM expression results from an interplay between historegulatory genes, morphoregulatory genes such as those for CAMs and selector genes such as Hox and Pax. A specific application of a key part of this hypothesis is shown in figure 2. In this form, the

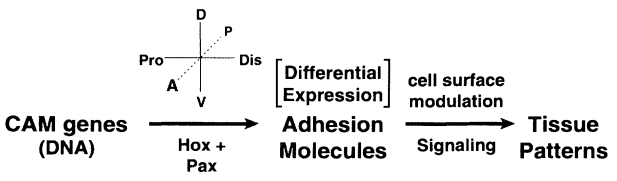

Figure 2. Specific application of the morphoregulator hypothesis showing the connection between local expression of pattern forming genes such as those encoded by Hox and Pax and the place-dependent regulation of CAM gene expression. Coordinate expression of Hox and Pax genes in various axes of the body plan leads to differential expression of particular adhesion molecule genes. The set of adhesion proteins will determine adhesive and signalling properties of cells at a particular place leading to specific tissue patterns.

proposal is that the promoters for a variety of CAM and SAMs (substrate adhesion molecules) are targets for homeobox and Pax gene products. These genes are known to be important elements in pattern formation during embryogenesis of a variety of species. Different subsets of such transcriptional regulators may be employed for the various contexts of place dependent adhesion molecule gene expression (reviewed in Edelman 1992; Edelman & Jones 1993). A diversity of these transcriptional cues may be central, for example, in the local specification of neuronal migration pathways and tissue modelling. In that specification, a continual interplay must occur between cell–cell interactions modulated by adhesion molecules and gene regulatory elements. In the following sections, we briefly review evidence to support the hypothesis that *cis*-acting DNA regulatory elements that respond to signals from different Hox and Pax gene products elements control N-CAM expression.

4. BINDING AND REGULATION *IN VITRO* OF THE N-CAM PROMOTER BY HOX GENE PRODUCTS

In a series of studies *in vitro*, a link has been found between the expression of homeobox genes and the expression of specific CAMs. We found (figure 3) that N-CAM promoter activity is controlled in a concentration-dependent manner by HoxB9 and HoxB8 proteins. Expression of the HoxB9 (Hox 2.5)

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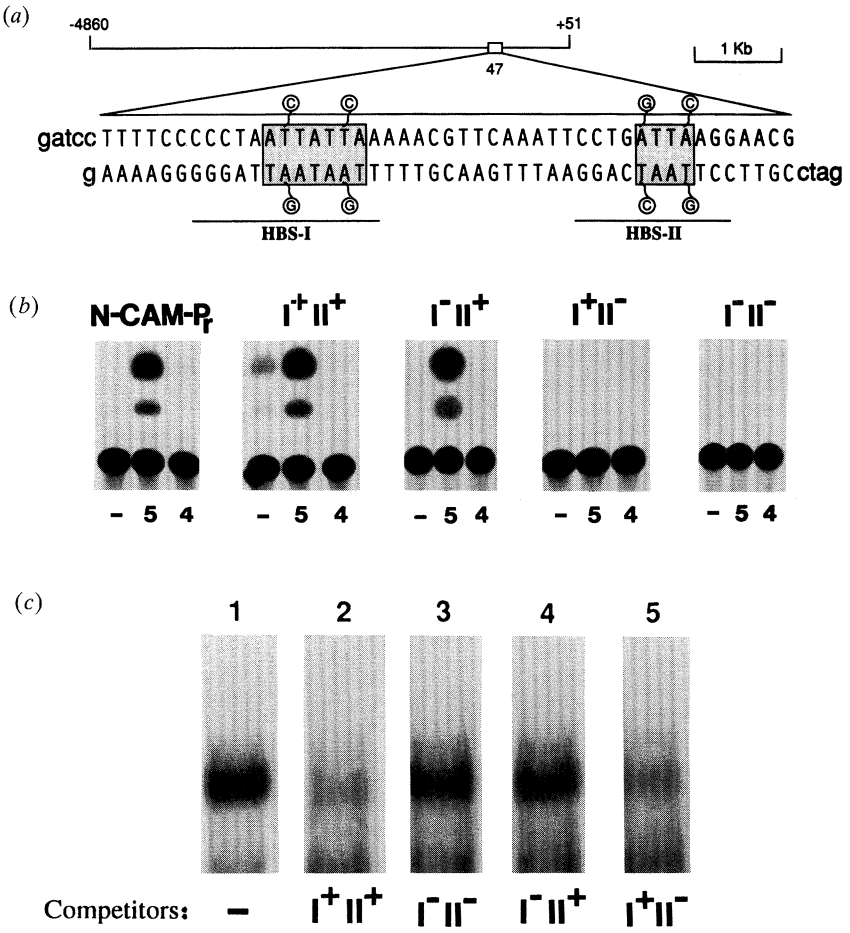

Figure 3. Co-transfection of NIH 3T3 cells with plasmids driving the expression of Xenopus HoxB9 (Hox 2.5) and HoxB8 (Hox 2.4) genes modulates the activity of a chloramphenicol acetyltransferase (CAT) reporter gene driven by the N-CAM promoter. (*a*) A 4.9 kb *Pst* I fragment including the first exon (black box) and the 4860 b.p. of 5′ flanking sequence from the mouse N-CAM gene was cloned upstream of a promoterless CAT gene. A 47 b.p. region (between 512 and 559 b.p. upstream of the ATG codon in the first exon) containing two potential homeodomain binding sites (designated HBS-I and HBS-II) was cloned upstream of a minimal SV40 promoter and a downstream CAT gene. In addition, multiple base pair substitutions were made in the TAAT sequence cores of HBS-I and HBS-II. These plasmids were tested in co-transfection experiments to determine whether the HBSs participate in the modulation of N-CAM promoter activity by Hox gene products and, if so, to determine whether specific HBSs mediated the response. (*b*) The N-CAM-P_r Plasmid gave no detectable activity when transfected without the Hox gene expression plasmid (N-CAM-P_r, lane —). However, transfection with HoxB9 stimulated CAT activity driven by the N-CAM promoter (N-CAM-P_r, lane 5) while transfection with HoxB8 had little or no effect (N-CAM-P_r, lane 4). The wild type HBS sequence (HBS-I^+II^+) had significant basal activity in NIH 3T3 cells. Co-transfection with HoxB9 elevated expression from basal levels significantly, but co-transfection with HoxB8 repressed the basal activity. Mutation of HBS-I (HBS-I^-II^+) had no effect on HoxB9 activation. However, a mutation either of HBS-II (HBS-I^+II^-) or both HBSs (HBS-I^-II^-) completely abolished activation by HoxB9. These data suggest that Hox 2.5 gene product activation of the N-CAM promoter is mediated by HBS-II, and that Hox 2.4 is a potent inhibitor of Hox 2.5 activation. From Jones *et al.* (1992). (*c*) A HoxC6 (Hox 3.3) fusion protein directly binds to HBS region of the N-CAM promoter. Electrophoretic mobility shift assays were done by using the ^{32}P-labelled 47 b.p. HBS region of the N-CAM promoter and HoxC6 fusion protein. HoxC6 bound to the HBS probe and produced a prominent band (lane 1). Inclusion of a 200-fold molar excess of unlabelled HBS competitor (I^+II^+) competed effectively for formation of this DNA/HoxC6 complex (lane 2). Oligonucleotides containing mutations in both HBS-I and HBS-II (I^-II^-) or in HBS-I (I^-II^+) failed to compete for HoxC6 binding to the ^{32}P-labelled HBS probe (lanes 3 and 4). An oligonucleotide containing a mutation in only HBS-II (I^+II^-) competed for binding although not as effectively as the unmutated HBS (I^+II^+). These data suggest that HoxC6 binds to the N-CAM promoter through HBS-I. From Jones *et al.* (1993).

gene activated N-CAM promoter activity. Co-transfection with a neighbouring gene HoxB8 (Hox 2.4) mitigated this effect (Jones *et al.* 1992; not shown). A 47 base pair (b.p.) DNA segment in the N-CAM promoter containing two potential homeodomain binding sites (HBSs) with TAAT motifs was found to be sufficient for promoter modulation by these homeobox gene products. Mutation of the HBS motifs completely abolished these effects (Jones *et al.* 1992). The HBS regions of the N-CAM promoter not only affected responsiveness to homeobox genes in transfected cells but also bound to homeodomain proteins

in a DNA sequence-specific manner. For example, a homeoprotein encoded by the HoxC6 (Hox 3.3) gene, which activates the N-CAM promoter, bound to the specific TAAT-containing sequence (CCTAATTATTAA) in the N-CAM promoter, designated HBS-I (Jones *et al.* 1993).

The mouse N-CAM promoter has also been shown to be modulated by two other homeobox gene products, cux (cut-like homeobox) and Phox-2 (paired-like homeobox 2) (Valarché *et al.* 1993). Expression vectors for these genes were transiently co-transfected with a construct containing a 2.6 kb N-CAM promoter fragment driving the expression of the CAT gene into a neuroblastoma cell line (N2A) that endogenously expresses N-CAM. The cux gene product behaved as a repressor and Phox-2 was able to relieve this repression. These factors were also shown to bind to the promoter using DNase footprinting and gel mobility shift assays. Collectively, our findings and these data strengthen the hypothesis that homeodomain proteins may participate in a cascade of transcriptional regulation resulting in the place-dependent expression of N-CAM.

5. REGULATION BY PAX GENE PRODUCTS

Recently, we have found that transcription factors encoded by the Pax gene family affect N-CAM promoter activity. Pax genes are expressed widely by cells undergoing neural development (Chalepakis *et al.* 1993). Mutations in some Pax genes result in phenotypes that may be due to aberrant cell migration, a process that has been shown to be affected by CAMs and SAMs. Mutation in Pax-6 is associated with the *small eye* mutation in mice and rats (Hill *et al.* 1991; Matsuo *et al.* 1993) and that in Pax-3 is associated with the *splotch* mutation in mice (Epstein *et al.* 1991). Both of these phenotypes appear to be caused by aberrant neuronal migration (Schmahl *et al.* 1993).

Eight different Pax genes have been identified in the genome of the mouse (Chalepakis *et al.* 1993), and counterparts of many of these have been found in the genomes of the chick, zebrafish, and human (Goulding *et al.* 1991; Krauss *et al.* 1991; Burri *et al.* 1989). All of the Pax genes described thus far encode a paired domain of 128 amino acids, originally described in the *Drosophila* segmentation gene, *paired* (Bopp *et al.* 1986). The paired domain is a DNA binding structure that is predicted to have three alpha helices, but does not have the helix-turn-helix motif characteristic of the homeodomain family of transcription factors (Chalepakis *et al.* 1991). It has been shown to mediate sequence-specific binding to DNA, and recognizes target sequences quite different from those that bind to homeodomains (Chalepakis *et al.* 1991; Treisman *et al.* 1991; Epstein *et al.* 1994).

To identify factors in addition to Hox gene products that may influence place-dependent N-CAM gene expression, we studied the binding and activation of the N-CAM promoter by Pax-8 (figure 4). Pax-8 strongly increased N-CAM promoter activity in cellular co-transfection experiments. We found that a short segment of the promoter (−143 to −15) mediated the response. The −143 to −15 region produced a DNA–protein complex when incubated with extracts from COS-7 cells transfected with the Pax-8 expression vector or a Pax-8/GST fusion protein. Pax-8 bound to the N-CAM promoter through two **TGCTCC** motifs (designated PBS-1 and PBS-2) that resemble paired domain binding sites. Mutation of PBS-1 and PBS-2 eliminated Pax-8 activation of the N-CAM promoter. Transfection of N2A neuroblastoma cells with the Pax-8 expression vector resulted in a fivefold increase in the transcription of the endogenous N-CAM gene (Holst *et al.* 1994). The combined results suggest that Pax-8 activates transcription of the N-CAM gene through binding of sequences resembling paired domain binding sites (PBS) that are present in the N-CAM promoter.

6. *IN VIVO* FINDINGS

Different members of the Hox and Pax gene families are expressed in defined patterns, some of which overlap along the anterior–posterior and dorsal–ventral axes of the developing nervous system (Goulding *et al.* 1991). For example, the expression of Pax genes becomes more restricted toward the anterior region of the embryo. In many cases, the molecular pattern of Pax gene expression correlates with morphological borders in the embryo. N-CAM expression *in vivo* corresponds in several places to zones and borders of Pax gene expression. For example, the posterior border of Pax-6 expression in the diencephalon corresponds to the border between neuromeres D1 and D2 and delineates the anterior boundary of a tight zone of N-CAM expression (Figdor & Stern 1993). Similarly, particular Hox genes are expressed in the hindbrain and posterior regions such as the spinal cord in definite overlapping patterns (Hunt *et al.* 1991). Their spatiotemporal order of expression is coordinate with their position in paralogous clusters. While vertebrates do not show segmentation such as that of *Drosophila*, the expression of many of these genes is seen to have distinct borders.

According to our hypothesis (figure 2), different combinations of Pax gene products may regulate the place-dependent expression of a unique set of CAM target genes, which in part determine the properties of cell collectives creating these morphological borders. Combinatorial interactions of homeodomain- and paired domain-containing transcription factors with the N-CAM promoter could result in different levels of CAM expression. Modulation of levels of expression of CAMs may occur at positions where different expression domains of Pax genes intersect. Such variations in the expression of adhesion molecules at the cell surface could alter cell interactions and thereby define morphological borders during tissue formation.

These ideas and the findings we have summarized require the further experimental demonstration that alterations in Hox and Pax gene binding elements of the N-CAM promoter will alter the place-dependent expression of this adhesion molecule. To fulfill this requirement, we have constructed N-CAM promoter

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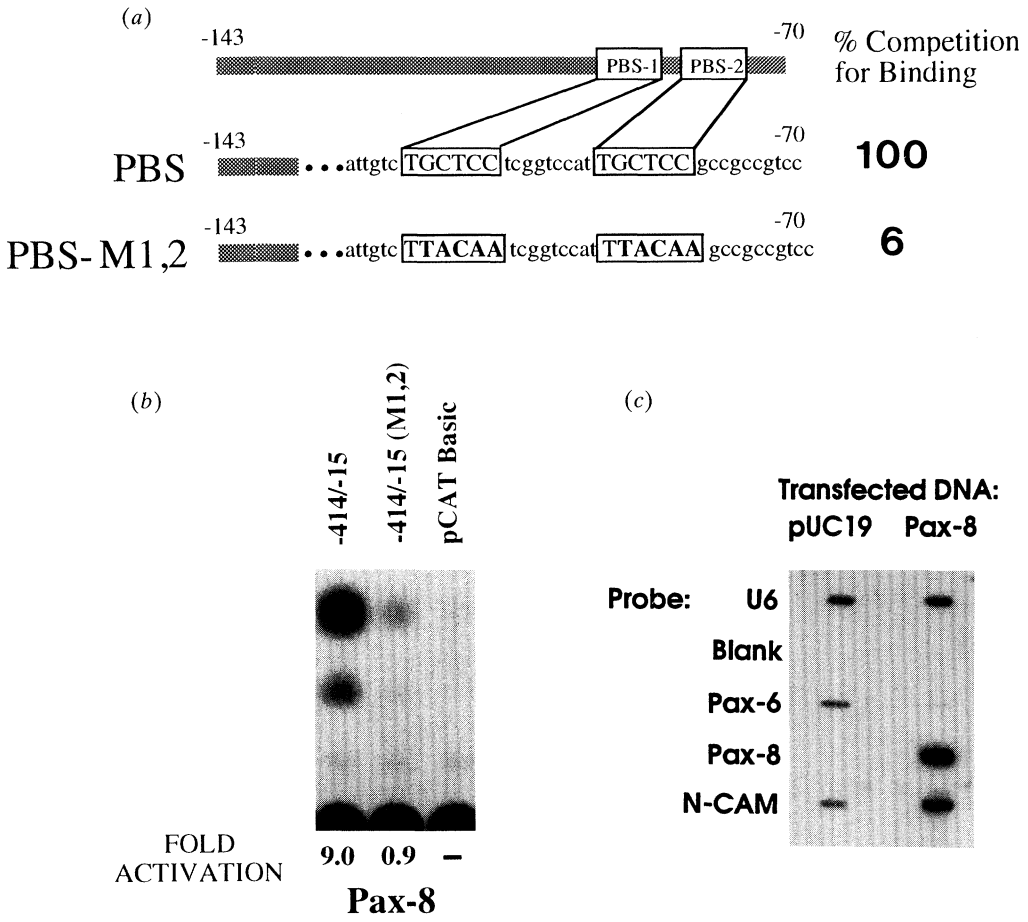

Figure 4. The N-CAM promoter contains binding sites for Pax-8. (*a*) Schematic representation of the −143 to −70 region of the 5′-flanking region of the mouse N-CAM gene (negative numbers refer to the number of base pairs upstream of the translational initiation site, which is designated +1). The locations of two sequences that resemble paired domain binding sites (PBS), designated PBS-1 and PBS-2 are boxed. Drawn below is the actual DNA sequence of the region of the N-CAM promoter shown to bind the Pax-8 gene product in electrophoretic mobility shift assays. PBS-1 and PBS-2 contain two common TGCTCC motifs and are shown in upper case and are boxed. The PBS region of the N-CAM promoter competed 100% with the binding of Pax-8 protein to a larger ^{32}P-labelled N-CAM promoter DNA fragment. However, when mutations were made in both the PBS-1 and PBS-2 motifs, as in the mutated N-CAM promoter region PBS-M1,2, the altered fragment no longer competed effectively (only 6%) for binding of Pax-8 to the native promoter. These data suggest that the PBS-1 and PBS-2 sequences are critical for the binding of Pax-8 to the N-CAM promoter. (*b*) Role of the PBS-1 and PBS-2 sequences in the activation of the N-CAM promoter by Pax-8 in co-transfection experiments. A segment of the mouse N-CAM promoter containing the PBS-1 and PBS-2 sequences and a portion of the first exon (−414/−15) was inserted upstream of a chloramphenicol acetyltransferase (CAT) reporter gene. This construct showed a 9.0-fold activation of CAT activity in NIH 3T3 cells after co-transfection of Pax-8. An N-CAM promoter construct containing the PBS-1 and PBS-2 mutations shown to affect binding in (*a*) no longer showed a significant elevation in CAT activity in cells after co-transfection with Pax-8. Thus, mutations that impair binding of Pax-8 to the N-CAM promoter affected the ability of the promoter to respond to Pax-8 as well. (*c*) Expression of Pax-8 in N2A neuroblastoma cells increases transcription of the endogenous N-CAM gene in nuclear run-on experiments. Nuclei were prepared from N2A cells (cells which normally express N-CAM RNA) that were either mock-transfected with the pUC19 vector or transfected with the Pax-8 expression vector. ^{32}P-labelled run-on transcripts were prepared from these nuclei used as probe in slot-blot hybridizations of filters containing equal amounts of cDNA corresponding to the genes of interest: U6, Pax-6, Pax-8, and N-CAM. U6 RNA showed no change in expression after expression of Pax-8. Pax-6 RNA was expressed in mock-transfected N2A cells and its level appeared to decrease slightly upon expression of Pax-8. Pax-8 RNA was virtually absent in mock-transfected N2A cells and showed a 66-fold increase upon transfection with the Pax-8 expression vector. N-CAM RNA showed a moderate level of expression in mock-transfected cells and was induced approximately fivefold upon transfection with the Pax-8 expression vector. From Holst *et al.* (1994).

segments driving a lacZ reporter gene and containing mutations in PBS or HBS or both (figure 5). These constructs were used to prepare transgenic mice. Preliminary analysis of transgenic mice containing a wild type N-CAM promoter construct at embryonic day 12 has revealed a pattern that appears to be specific to the nervous system and occurs only in places where N-CAM is normally expressed. We have obtained five transgenic lines (F1 generation), and have analysed E12 embryos and adult tissues from the F2 generation by histochemical staining for β-galactosidase. Out of the five lines established as F2,

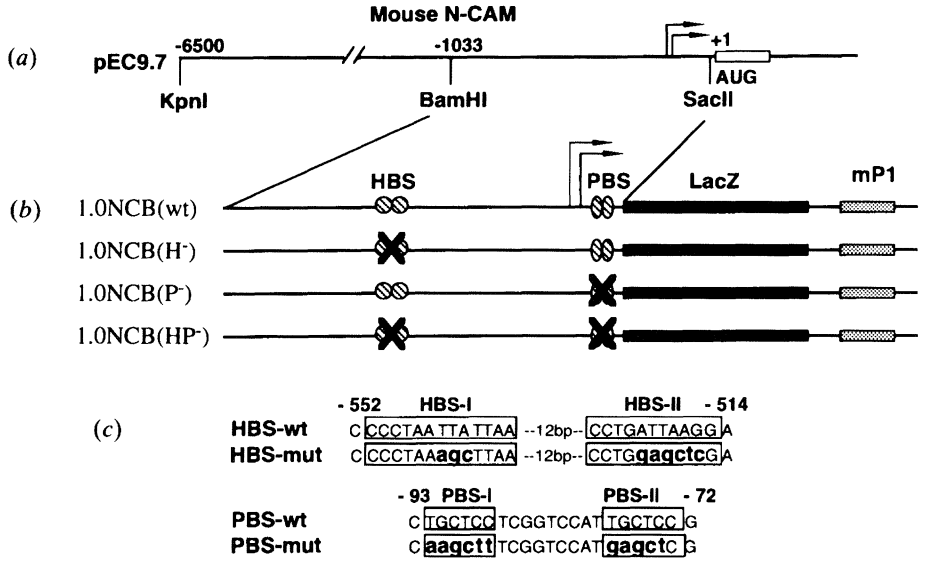

Figure 5. Diagram of the mouse N-CAM gene promoter (*a*). Linear representation of the N-CAM promoter region containing 6.5 kb of 5′ flanking sequence, the first exon and the 5′ portion of the first intron of the N-CAM gene. RNA start sites are indicated by rightward arrows and translation start codon is depicted by AUG. (*b*) Diagram of the 1.0 kb BamHI/SacII proximal N-CAM promoter region containing the homeodomain (HBS) and the paired domain (PBS) binding sites. All reporter constructs were prepared as described in the text. Reporter constructs containing mutations in either the HBS, PBS, or both sequences are shown for the 1.0 kb N-CAM promoter. (1.0 NCB(HBS$^-$), 1.0 NCB(PBS$^-$), and 1.0 NCB(H/P$^-$), respectively.) The corresponding mutations were introduced into the 6.5 kb N-CAM promoter fragment to give 6.5 kb NCB(HBS$^-$), 6.5 NCB(PBS$^-$), and 6.5 NCB(HP$^-$), respectively (not shown). (*c*) Exact base pair substitutions made in the HBS, PBS, and HP$^-$ promoter mutants and comparison with the wild type N-CAM promoter sequence.

three expressed the β-galactosidase gene. All three lines showed expression of the reporter gene in the nervous system, two lines expressing it at high levels, one at a low level.

An example of the neural staining from the wild type N-CAM promoter lacZ construct (1.0 NCB(wt)) is shown in figure 6A, B. Staining was observed in the nervous system and showed a discontinuous pattern along the anterior–posterior axis. Staining was particularly prominent in the forebrain and midbrain. Proceeding posteriorly, there was a gap in the expression of the reporter gene in the region between the posterior midbrain and hindbrain. This was followed by high expression in the hindbrain which continued down through the spinal cord. The reporter gene was expressed in the dorsal regions of the spinal cord, and staining was detected in dorsal root ganglia. These zones of lac-z expression driven by the wild type N-CAM promoter correspond to the expression domains of the several different homeobox and Pax genes that we have found to regulate activity of the N-CAM promoter in *in vitro* and cellular transfection studies. These include Pax-6, Pax-3, Pax-6, HoxC6, HoxB9, and HoxB8 (Jones *et al.* 1992, 1993; Chalepakis *et al.* 1994*b*; Holst *et al.* 1994).

The pattern of expression in the nervous system directed by the 1.0 N-CAM(wt) promoter fragment provides a useful standard of comparison for the patterns obtained with the N-CAM reporter constructs containing mutations in the HBS and PBS sequences. To analyse the effect of mutations in these regulatory elements on the pattern of expression, we have so far prepared F_0 embryos by injection of mouse eggs with three mutant N-CAM promoter constructs

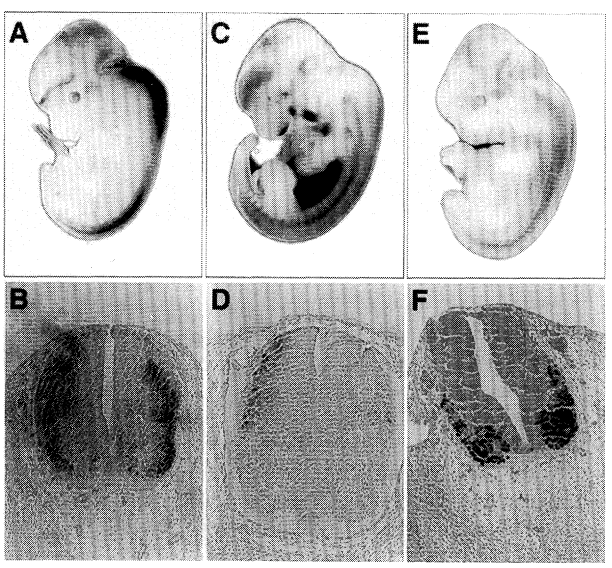

Figure 6. Expression patterns of reporter gene expression in transgenic mice carrying different N-CAM promoter/lacZ minigenes. (Black and white print of a color negative.) Whole mount (A, C, E) and their transverse sections of day 11–12 mouse embryos showing β-galactosidase activity produced by three N-CAM promoter minigene constructs: the wild type 1.0 kb N-CAM promoter (1.0 NCB(wt), A, B), an N-CAM promoter containing mutations in PBS-1/PBS-2 (1.0 NCB(P$^-$), C, D), and an N-CAM promoter containing mutations in both HBS-I/HBS-II and PBS-1/PBS-2 (1.0 NCB(HP$^-$), E, F). Sites of β-galactosidase activity appear as dark grey or black regions.

1.0 NCB(H$^-$), 1.0 NCB(P$^-$) and 1.0 NCB(HP$^-$) (see figure 5). Preliminary analysis was carried out on F_0 embryos generated from the 1.0 NCB(P$^-$)(see figure

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6C,D) and 1.0 NCB(HP$^-$)(see figure 6E,F) constructs which contained mutations respectively in the PBS element or in both HBS and PBS elements. There were consistent differences in the expression pattern of the lacZ reporter in the spinal cord and in the limbs as compared with the pattern obtained with 1.0 NCB(wt). The pattern in the spinal cord for both the 1.0 NCB(HP$^-$) and 1.0 NCB(P$^-$) constructs showed an expansion of lacZ stained area, from a predominantly dorsal to a more ventral expression. In addition, the mutant showed a continuous staining pattern in the cervical region of the spinal cord, whereas the wild type showed a more discontinuous pattern in this region.

The changes in patterns produced in F_0 individual embryos will require rigorous confirmation in independent stable transgenic lines before firm conclusions can be drawn. Nevertheless, the preliminary evidence provides support for the hypothesis that the HBS and PBS elements in the N-CAM promoter are important for place-dependent expression of this adhesion molecule gene *in vivo*. Mutations in these elements lead to definite altered expression patterns *in vitro* and to suggestive changes *in vivo*. The altered pattern in the spinal cord and neural tube is particularly striking in view of the observation that Pax-3 and Pax-6 have differential (Goulding *et al.* 1993) dorsal–ventral expression patterns in neuroepithelia in the neural tube and during differentiation and migration of cells in the spinal cord. Moreover, Pax-3 and Pax-6 have two independent DNA binding domains – a paired domain and a homeodomain (Chalepakis *et al.* 1993, 1994b; Holst *et al.* 1994) – that together may engage in combinatorial binding modes with the PBS and HBS sequences in the N-CAM promoter.

7. CONCLUSION

While it is too early to conclude that N-CAM expression patterns depend solely upon Hox and Pax genes, it is clear that these genes play a major role in modulating these patterns. These conclusions may apply to other adhesion molecules. We have shown, for example, that a consensus HNF-1 motif – a TAAT-containing sequence characteristic of several homeodomain binding sites – is found in a part of a cell type-specific enhancer within the second intron of the gene encoding L-CAM, the liver cell adhesion molecule (Sorkin *et al.* 1993; Goomer *et al.* 1994). More recently, we have found that the promoter region for Ng-CAM, a secondary immunoglobulin-like CAM which is restricted to the nervous system appears to be activated by a variety of Pax gene products.

It is a likely extension of our hypothesis that the regulatory regions of genes encoding other adhesion molecules will be found to contain HBS and PBS elements that are targets for cues provided by the wide variety of homeobox and Pax gene products. A somewhat daunting challenge brought up by these experiments is to understand how specific combinations of transcriptional regulators in the Hox and Pax families recruit the expression of a set of adhesion molecules appropriate for the formation of a tissue at a particular place in the embryo.

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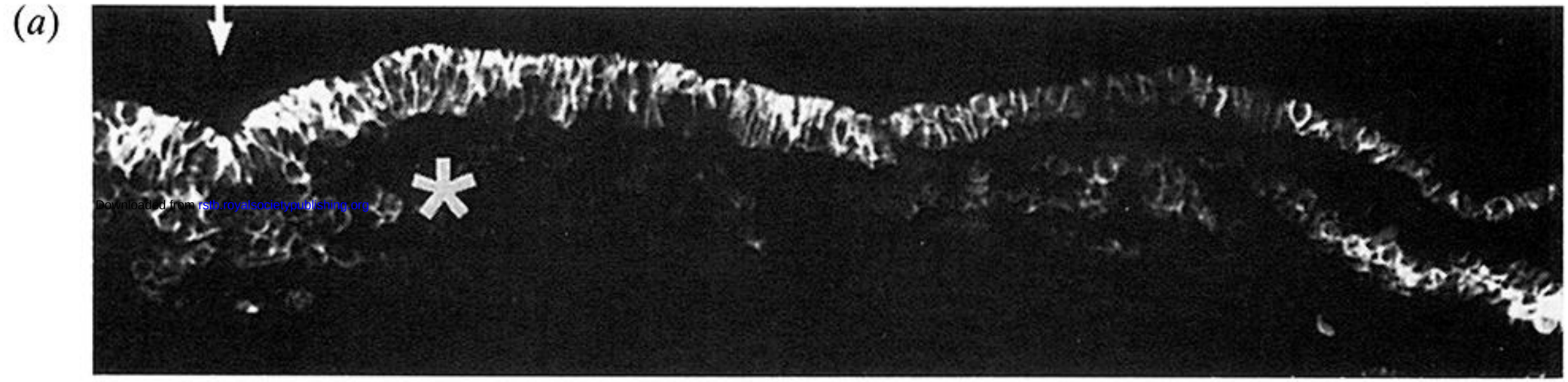
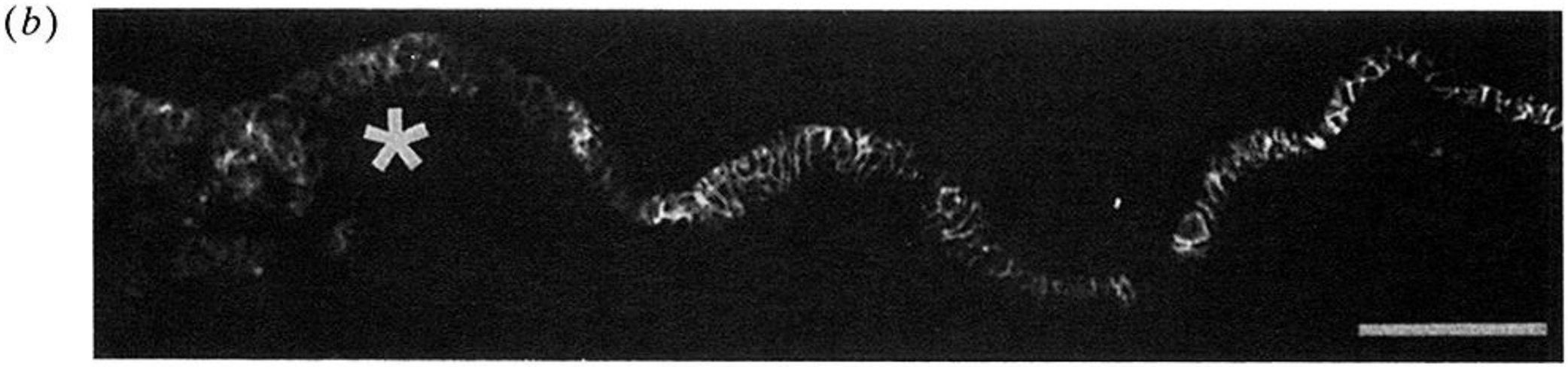
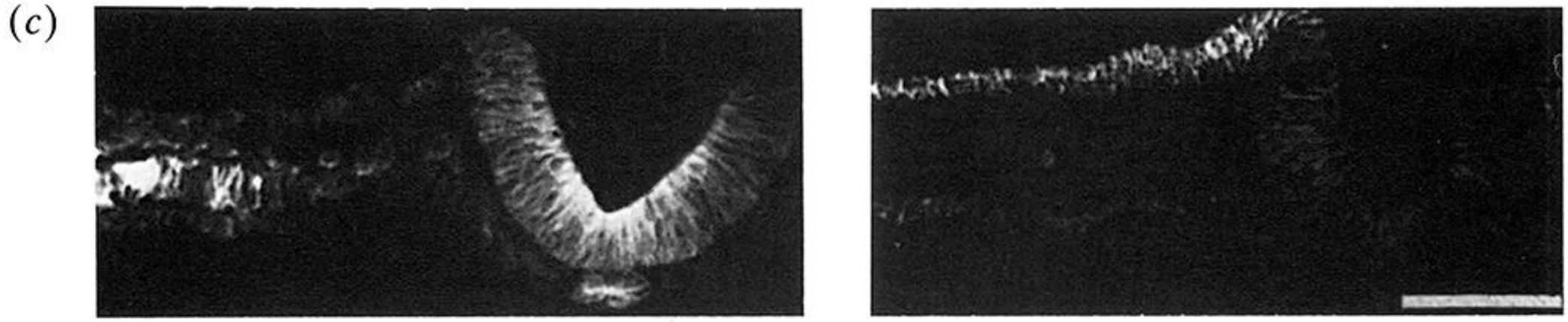
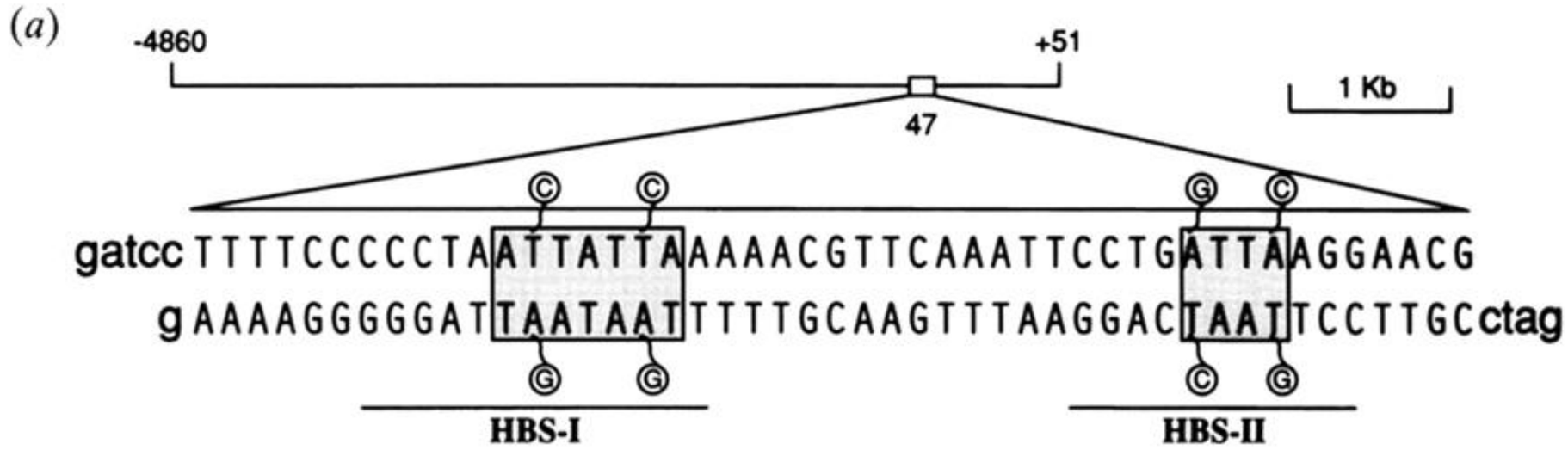


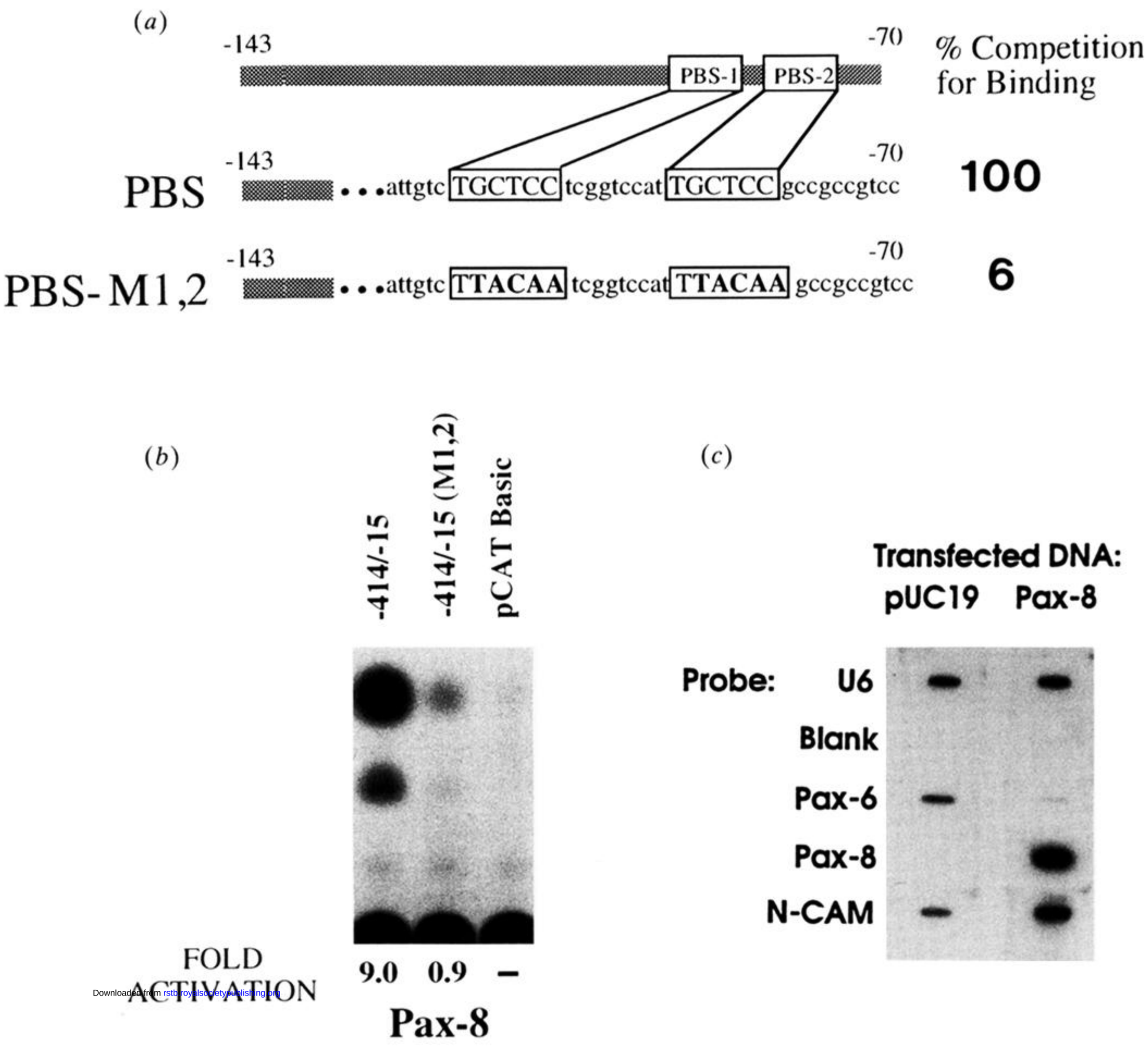




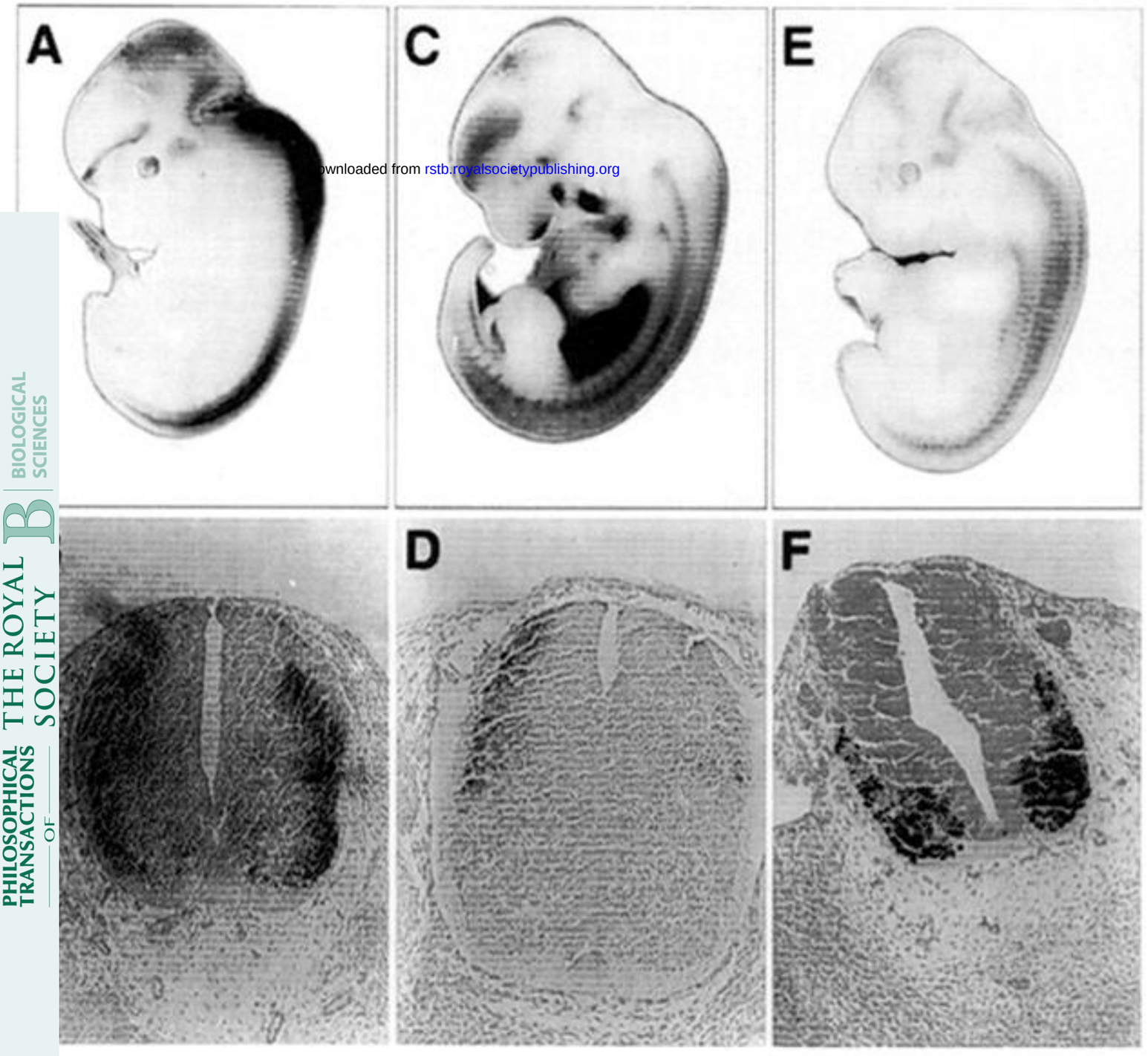
gure 1. Immunocytochemical staining of early ectodermal epithelial structures showing transition from the stribution of N-CAM and L-CAM together on the blastoderm to their segregation after neural induction. (*a*,*b*) ransverse section of a head fold-stage chick embryo stained with N-CAM (*a*) or L-CAM (*b*) antibodies. Arrow dicates location of the primitive streak, asterisk denotes ingressing cells. (*c*) Transverse sections through a five mite-stage embryo at a position posterior to the last somite were stained either with N-CAM (left) or L-CAM ght) antibodies.



igure 3. Co-transfection of NIH 3T3 cells with plasmids driving the expression of Xenopus HoxB9 (Hox 2.5) and loxB8 (Hox 2.4) genes modulates the activity of a chloramphenicol acetyltransferase (CAT) reporter gene driven y the N-CAM promoter. (*a*) A 4.9 kb *Pst* I fragment including the first exon (black box) and the 4860 b.p. of 5′ anking sequence from the mouse N-CAM gene was cloned upstream of a promoterless CAT gene. A 47 b.p. region between 512 and 559 b.p. upstream of the ATG codon in the first exon) containing two potential homeodomain inding sites (designated HBS-I and HBS-II) was cloned upstream of a minimal SV40 promoter and a downstream AT gene. In addition, multiple base pair substitutions were made in the TAAT sequence cores of HBS-I and HBS-I. These plasmids were tested in co-transfection experiments to determine whether the HBSs participate in the odulation of N-CAM promoter activity by Hox gene products and, if so, to determine whether specific HBSs ediated the response. (*b*) The N-CAM-P_r Plasmid gave no detectable activity when transfected without the Hox ene expression plasmid (N-CAM-P_r, lane —). However, transfection with HoxB9 stimulated CAT activity driven y the N-CAM promoter (N-CAM-P_r, lane 5) while transfection with HoxB8 had little or no effect (N-CAM-P_r, lane). The wild type HBS sequence (HBS-I^+II^+) had significant basal activity in NIH 3T3 cells. Co-transfection with loxB9 elevated expression from basal levels significantly, but co-transfection with HoxB8 repressed the basal activity. lutation of HBS-I (HBS-I^-II^+) had no effect on HoxB9 activation. However, a mutation either of HBS-II (HBS-$^+II^-$) or both HBSs (HBS-I^-II^-) completely abolished activation by HoxB9. These data suggest that Hox 2.5 gene roduct activation of the N-CAM promoter is mediated by HBS-II, and that Hox 2.4 is a potent inhibitor of Hox .5 activation. From Jones *et al.* (1992). (*c*) A HoxC6 (Hox 3.3) fusion protein directly binds to HBS region of the -CAM promoter. Electrophoretic mobility shift assays were done by using the ^{32}P-labelled 47 b.p. HBS region of ne N-CAM promoter and HoxC6 fusion protein. HoxC6 bound to the HBS probe and produced a prominent band ane 1). Inclusion of a 200-fold molar excess of unlabelled HBS competitor (I^+II^+) competed effectively for formation f this DNA/HoxC6 complex (lane 2). Oligonucleotides containing mutations in both HBS-I and HBS-II (I^-II^-) or HBS-I (I^-II^+) failed to compete for HoxC6 binding to the ^{32}P-labelled HBS probe (lanes 3 and 4). An ligonucleotide containing a mutation in only HBS-II (I^+II^-) competed for binding although not as effectively as the nmutated HBS (I^+II^+). These data suggest that HoxC6 binds to the N-CAM promoter through HBS-I. From Jones *al.* (1993).



gure 4. The N-CAM promoter contains binding sites for Pax-8. (*a*) Schematic representation of the −143 to −70 gion of the 5′-flanking region of the mouse N-CAM gene (negative numbers refer to the number of base pairs)stream of the translational initiation site, which is designated +1). The locations of two sequences that resemble ired domain binding sites (PBS), designated PBS-1 and PBS-2 are boxed. Drawn below is the actual DNA sequence the region of the N-CAM promoter shown to bind the Pax-8 gene product in electrophoretic mobility shift assays. 3S-1 and PBS-2 contain two common TGCTCC motifs and are shown in upper case and are boxed. The PBS region the N-CAM promoter competed 100% with the binding of Pax-8 protein to a larger ^{32}P-labelled N-CAM promoter NA fragment. However, when mutations were made in both the PBS-1 and PBS-2 motifs, as in the mutated N-CAM omoter region PBS-M1,2, the altered fragment no longer competed effectively (only 6%) for binding of Pax-8 to e native promoter. These data suggest that the PBS-1 and PBS-2 sequences are critical for the binding of Pax-8 to e N-CAM promoter. (*b*) Role of the PBS-1 and PBS-2 sequences in the activation of the N-CAM promoter by Pax- in co-transfection experiments. A segment of the mouse N-CAM promoter containing the PBS-1 and PBS-2 quences and a portion of the first exon (−414/−15) was inserted upstream of a chloramphenicol acetyltransferase :AT) reporter gene. This construct showed a 9.0-fold activation of CAT activity in NIH 3T3 cells after co- ansfection of Pax-8. An N-CAM promoter construct containing the PBS-1 and PBS-2 mutations shown to affect nding in (*a*) no longer showed a significant elevation in CAT activity in cells after co-transfection with Pax-8. Thus, utations that impair binding of Pax-8 to the N-CAM promoter affected the ability of the promoter to respond to ax-8 as well. (*c*) Expression of Pax-8 in N2A neuroblastoma cells increases transcription of the endogenous N-CAM ne in nuclear run-on experiments. Nuclei were prepared from N2A cells (cells which normally express N-CAM NA) that were either mock-transfected with the pUC19 vector or transfected with the Pax-8 expression vector. ^{32}P- belled run-on transcripts were prepared from these nuclei used as probe in slot-blot hybridizations of filters ntaining equal amounts of cDNA corresponding to the genes of interest: U6, Pax-6, Pax-8, and N-CAM. U6 RNA owed no change in expression after expression of Pax-8. Pax-6 RNA was expressed in mock-transfected N2A cells id its level appeared to decrease slightly upon expression of Pax-8. Pax-8 RNA was virtually absent in mock- ansfected N2A cells and showed a 66-fold increase upon transfection with the Pax-8 expression vector. N-CAM NA showed a moderate level of expression in mock-transfected cells and was induced approximately fivefold upon ansfection with the Pax-8 expression vector. From Holst *et al.* (1994).



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gure 6. Expression patterns of reporter gene expression in nsgenic mice carrying different N-CAM promoter/lacZ nigenes. (Black and white print of a color negative.) Whole ount (A, C, E) and their transverse sections of day 11–12 ouse embryos showing β-galactosidase activity produced three N-CAM promoter minigene constructs: the wild pe 1.0 kb N-CAM promoter (1.0 NCB(wt), A, B), an CAM promoter containing mutations in PBS-1/PBS-2 0 NCB(P^-), C, D), and an N-CAM promoter containing utations in both HBS-I/HBS-II and PBS-1/PBS-2 0 NCB(HP^-), E, F). Sites of β-galactosidase activity pear as dark grey or black regions.