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Developmental Biology 286 (2005) 601 – 617
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

Genomes & Developmental Control

Multiple *N-cadherin* enhancers identified by systematic functional screening indicate its Group B1 SOX-dependent regulation in neural and placodal development

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Received for publication 21 April 2005, revised 23 July 2005, accepted 1 August 2005
Available online 16 September 2005

Abstract

Neural plate and sensory placodes share the expression of *N-cadherin* and Group B1 *Sox* genes, represented by *Sox2*. A 219-kb region of the chicken genome centered by the *N-cadherin* gene was scanned for neural and placodal enhancers. Random subfragments of 4.5 kb average length were prepared and inserted into tkEGFP reporter vector to construct a library with threefold coverage of the region. Each clone was then transfected into *N-cadherin*-positive (lens, retina and forebrain) or -negative embryonic cells, or electroporated into early chicken embryos to examine enhancer activity. Enhancers 1–4 active in the CNS/placode derivatives and non-specific Enhancer 5 were identified by transfection, while electroporation of early embryos confirmed enhancers 2–4 as having activity in the early CNS and/or sensory placodes but with unique spatiotemporal specificities. Enhancers 2–4 are dependent on SOX-binding sites, and misexpression of Group B1 *Sox* genes in the head ectoderm caused ectopic development of placodes expressing *N-cadherin*, indicating the involvement of Group B1 *Sox* functions in *N-cadherin* regulation. Enhancers 1, 2 and 4 correspond to sequence blocks conserved between the chicken and mammalian genomes, but enhancers 3 and 5 are unique to the chicken.

Keywords: *N-cadherin*; *Sox2*; Group B1 *Sox*; Enhancers; Genome scan; Electroporation; tkEGFP; Conserved sequence

Introduction

The primordia of CNS and sensory organs, the neural plate and sensory placodes, share many features in common. They start as a thickened epithelial sheet formed in the ectodermal compartment of the embryo and then invaginate. A prominent molecule that is commonly expressed among these tissues and involved in morphogenetic cellular actions is N-cadherin. Cells expressing N-cadherin segregate from bulk ectodermal cells expressing other cadherins and organize the neural plate and placodes (Hatta and Takeichi, 1986; Hatta et al., 1987; Takeichi, 1988). It has been demonstrated that interference with N-cadherin functions affects later histogenesis of the CNS (Bronner-Fraser et al., 1992; Detrick et al., 1990; Fujimori et al., 1990; Ganzler-Odenthal and Redies, 1998; Matsunaga et al., 1988; Radice et al., 1997). Thus, N-cadherin expression in the sensory placodes and early CNS is coupled with the morphogenesis of these tissues. However, regulation of the *N-cadherin* gene underlying this process has remained largely uninvestigated.

A set of transcription factor genes expressed in tight association with the genesis of the early CNS and sensory placodes is the Group B1 *Sox* genes, consisting of the major *Sox2* and related *Sox3* and *Sox1* (Ishii et al., 2001; Rex et al., 1997; Uchikawa et al., 1999, 2003; Uwanogho et al., 1995). In early chicken embryos, strong *Sox2* expression occurs throughout the neural plate, in sensory (nasal, lens and otic) placodes and their derivatives and in branchial arches (Uchikawa et al., 1999, 2003). Not only in the neural plate (Rex et al., 1997), but also in lens and otic placodes (Groves and Bronner-Fraser, 2000; Kamachi et al.,

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0012-1606/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2005.08.005

1998; Uchikawa et al., 1999), does expression of *Sox2* and *Sox3* overlap, and later all Group B1 *Sox* genes (*Sox1/2/3*) are expressed in derivatives of these tissue primordia, neural tube, nasal pit, lens and inner ear (Uchikawa et al., 1999).

Comparison of the expression pattern of Group B1 *Sox* genes with that of *N-cadherin* indicates a remarkable resemblance except that *N-cadherin* expression has mesodermal component, somites and heart (Hatta and Takeichi, 1986; Hatta et al., 1987) (see also Fig. 2A). These observations suggest the possibility that Group B1 SOX transcription factors are involved in the regulation of *N-cadherin* expression.

While keeping the possible involvement of Group B1 *Sox* functions in the regulation of *N-cadherin* expression in mind, we nevertheless took an unbiased systematic approach to the investigation of *N-cadherin* regulation. The *N-cadherin* gene spans over 200 kb in mammals (Miyatani et al., 1992), containing 16 exons and a long intron that occupy half the length of the gene, as is the case with other cadherin family genes (Hatta et al., 1991). Among higher vertebrate species, the chicken genome is more compact than the mammalian genome, and so is the *N-cadherin* gene. We successfully identified a single BAC clone covering from upstream to downstream of the *N-cadherin* gene, prepared random subfragments of the BAC insert and constructed a subfragment library in the tkEGFP expression vector (Uchikawa et al., 2003), which in total covers the *N-cadherin* gene three times. Each clone was transfected to three representative types of cells expressing endogenous N-cadherin (lens, retina and forebrain) in primary cultures, and another set of cells (lung, gizzard and liver) without N-cadherin expression. One-tenth of the clones exhibited enhancer activity in a subset of cells, and mapping of the N-cadherin genomic inserts in these clones identified five distinct enhancers of separate locations, three of which were clearly responsible for *N-cadherin* regulation in early embryos as demonstrated by embryo electroporation. Two of these enhancers are active both in placodes and the CNS, while another is active only in the placodes in early embryos. These findings indicate that although *N-cadherin* is expressed throughout the placodes and the early CNS, its regulation depends on regionally and temporarily specific mechanisms.

Three of the enhancers investigated in this study depend on a SOX-binding site for their activity, and in the case of Enhancer 4 the site is bound by Group B1 SOX proteins in the lens nuclear extract. Furthermore, misexpression of Group B1 *Sox* genes in the embryonic head ectoderm caused ectopic expression of N-cadherin and the development of placodal tissue. Collectively, the results obtained in this study indicate that *N-cadherin* expression is regulated by multiple Group B1 SOX-dependent enhancers in neuro-placodal development and suggest that SOX-dependent regulation plays a pivotal role in placodal development.

Materials and methods

Identification of a BAC clone carrying the entire chicken N-cadherin genomic sequence and construction of its subfragment library

Using the chicken 5′ and 3′ sequences derived from *N-cadherin* cDNA (Hatta and Takeichi, 1986) as probes, a filter set of chicken genomic BAC clones (Seven Chicken-*Hin*dIII BAC clones, Genefinder Genomic Resources, Texas A&M University) was screened for clones harboring both ends of the N-cadherin coding sequence. One of these clones, pBeloBAC#81-E14, carrying a 219-kb long insert was positive for both probes and characterized further. The whole BAC clone DNA was suspended at 0.2 μg/μl in 100 μl of 10 mM Tris–HCl, 1 mM EDTA and fragmented by mechanical shearing caused by the pushing and sucking of the solution through a 27-gauge needle of 19 mm length attached to a hypodermic syringe 350 times at the rate of 2 s per cycle, until an average fragment length of 4.5 kb was attained. The fragments were further size selected by agarose gel electrophoresis and purified using QIAquick Gel Extraction Kit (Qiagen). After flushing the sheared DNA ends using sequential treatment with T4 DNA polymerase and Klenow's fragment in the presence of NTPs and 5′ phosphorylation with T4 polynucleotide kinase, the fragments were inserted by T4 DNA ligase treatment into *Eco*RV-cleaved and alkaline phosphatase-treated ptkEGFP(RV) vector at the insert/vector molar ratio of 1. ptkEGFP(RV) is a derivative of ptkEGFP (Uchikawa et al., 2003), in which an *Eco*RV site is introduced immediately downstream of the tkEGFP transcriptional unit. 188 clones were randomly chosen and expanded for DNA preparation, and DNAs purified using QIA Plasmid Midi Kit (Qiagen) were used for DNA sequence determination at both ends of the inserts, and for transfection and electroporation. Among the 188 clones, 4 carried only BAC vector sequences, 5 had multiple inserts and the remaining 175 clones identified as single insert clones were used for further analysis.

Preparation of primary cultures and transfection

Lens epithelial cells were prepared from E14 chicken embryo and cultured as described previously (Kamachi et al., 2001). Retinal and forebrain cells of E7 chicken embryo and gizzard, liver and lung cells of E10 embryos were dissociated by treatment with 0.25% trypsin in 1 mM EDTA and cultured in Dulbecco's modified MEM (Gibco) (Kamachi et al., 2001) in 6-well plates by inoculation at 2.4×10^6 cells per well for retinal and forebrain cells and 10^5 cells per well for gizzard, liver and lung cells. After 3 days in culture, the cells were transfected with 2.2 μg DNA per well using Fugene 6 reagent (Roche). 48 h after transfection, EGFP fluorescence was photo-recorded using a CCD camera (DP70, Olympus)-equipped *epi*-fluorescence dissecting

microscope (SZX12, Olympus) and/or quantified for intensity by scraping, suspending and lysing the cells in 300 μl 100 mM potassium phosphate (pH 7.8), 1 mM DTT containing 1% NP40 and by dispensing 100 μl aliquots into a 96-well plate and measurement for EGFP fluorescence using an LB940 Mithras Multilabel Reader (Berthold Technologies).

Electrophoretic mobility shift assay (EMSA)

The assay was done using nuclear extracts essentially as described previously (Kamachi and Kondoh, 1993), employing polydGdC as non-specific competitors to detect SOX–DNA complexes.

Electroporation of chicken embryos

Electroporation of chicken embryos in ovo was done essentially as described previously (Kamachi et al., 2001; Nakamura et al., 2000). A pair of platinum electrodes was placed on both sides of the head of a stage 10 chicken embryo at an interelectrode distance of 4 mm. Plasmid DNA solution of ca. 1 μl at 2–6 μg/μl in 10 mM Tris–HCl at pH 8.0 was delivered to the cathode side of the head ectoderm and an electric pulse at 5 V/mm for 50 ms was applied 5 times at 100-ms intervals using an Electroporator CUY21 (BEX Co. LTD). Electroporation of the embryos at stages 4–5 under New's culture condition was done as described previously (Uchikawa et al., 2003, 2004).

In situ hybridization and immunohistology

Chicken embryos were staged according to Hamburger and Hamilton (1951). Whole mount in situ hybridization of embryos with *Sox* probes was done as described by Kamachi et al. (1998). The *N-cadherin* probe was a 5′ fragment of the cDNA (positions 1–845; DDBJ database accession number X07277). For immunohistological analysis, anti-GFP polyclonal rabbit antibodies (CLONTECH), anti-N-cadherin monoclonal rat antibody (TaKaRa) and anti-N-CAM monoclonal mouse antibody 5e (Developmental Studies Hybridoma Bank) were used as the primary antibodies. Chicken embryo was fixed with 4% paraformaldehyde made in PBS-Ca (PBS containing 1 mM $CaCl_2$) for 4 h at 4°C, soaked in 25% sucrose/PBS-Ca and embedded in OCT compound for preparation of frozen sections. Cross sections at a thickness of 7 μm were prepared using a cryomicrotome. The sections were blocked using 2% skim milk in TBS-Ca (100 mM Tris–HCl at pH 7.5, 150 mM NaCl and 10 mM $CaCl_2$), reacted with the primary antibodies and washed with TBS-Ca. For immunofluorescence, the sections were reacted with Alexa Fluor™ 488-anti-rabbit IgG (Molecular Probes) and biotin-conjugated anti-mouse/rat IgG (Jackson/CAPPEL), then with Alexa Fluor™ 568-streptavidin (Molecular Probes).

Results

Identification of multiple enhancers regulating N-cadherin expression in the early CNS and placodes

Using a full-length chicken *N-cadherin* cDNA (Hatta and Takeichi, 1986), a chicken genomic BAC library was screened for a clone carrying both 5′ and 3′ end sequences, leading to the identification of pBeloBAC#81-E14. Alignment of the cDNA sequence with a partial sequence of the clone determined by ourselves and with recently published whole genome data (Hillier et al., 2004) indicated that the *N-cadherin* gene of the chicken consists of 16 exons, as in the case of mammalian *N-cadherin* gene (Miyatani et al., 1992), and covers a region of 113.7 kb including the largest intron 2 of 60 kb. Although gene organization is similar to mammalian *N-cadherin* genes, the size of the chicken gene, including the largest intron, was half that of mammalian counterparts.

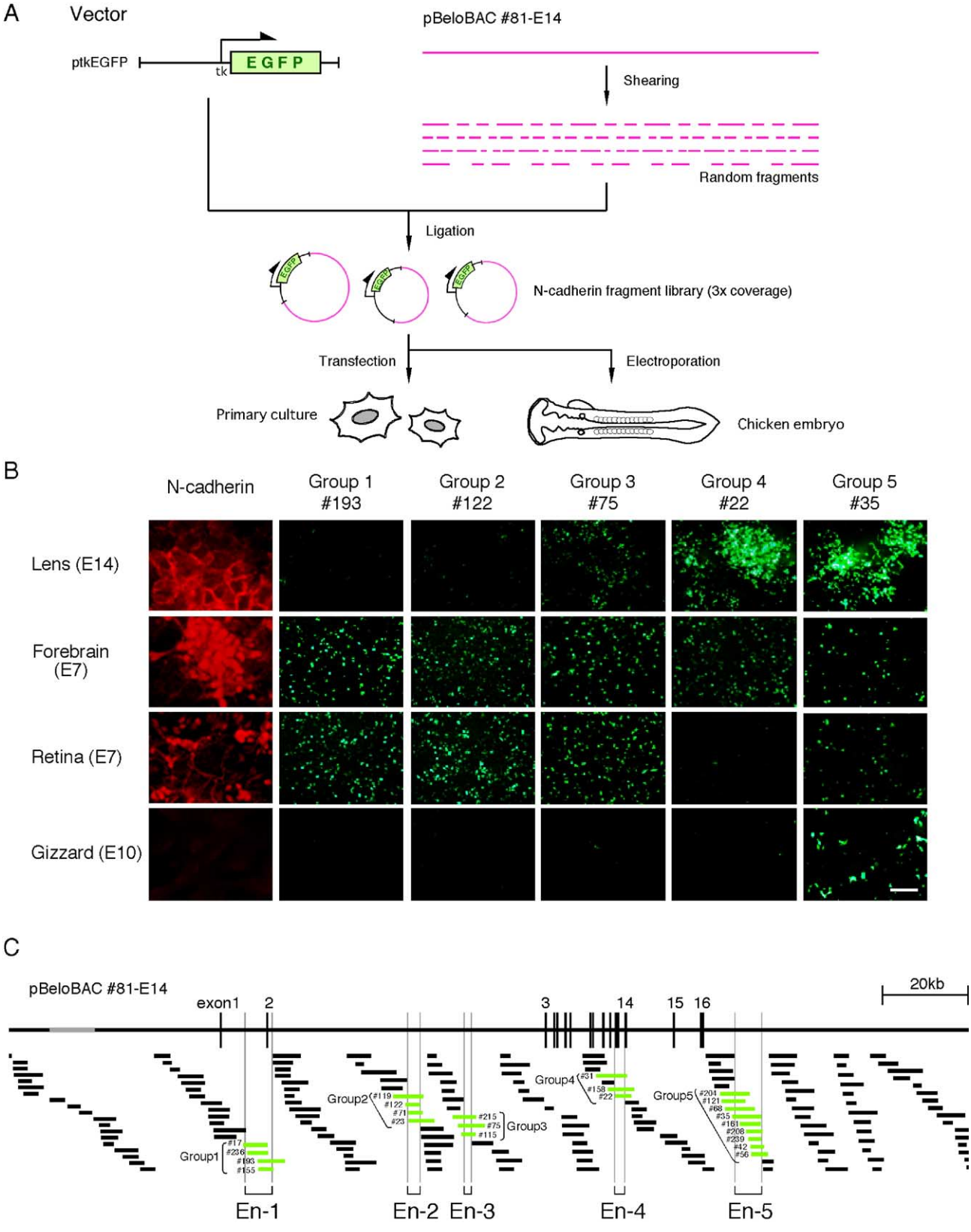
Enhancers regulating *N-cadherin* expression in the early CNS and sensory placodes were explored in the 219-kb insert region of pBeloBAC#81-E14, in which the 43-kb upstream sequence and the 62-kb downstream sequences are both included. The total BAC clone DNA was randomly fragmented by mechanical shearing to an average size of 4.5 kb, flush-ended, and each fragment was inserted in the tkEGFP(RV) reporter vector to comprise a library which fully covers the *N-cadherin* gene region (Fig. 1A). A total of 175 clones derived from single subfragment insertion was used, which should cover the *N-cadherin* gene three times. As these clones were constructed using a tkEGFP reporter vector (Uchikawa et al., 2003), individual inserts can be directly tested for their enhancer activities, either by transfection or by embryo electroporation (Fig. 1A).

We prepared primary cultures of several different cell types from chicken embryos, three with endogenous N-cadherin expression and another three without, and transfected them with all individual clones in the library. If enhancer activity is associated with the insert of a clone, then activity and specificity of the enhancer can be assessed by EGFP expression (Figs. 1B and C). EGFP expression was photo-recorded as a routine (Fig. 1B), but it was also quantified measuring fluorescence intensity. Lens epithelium of E14 embryo, forebrain of E7 embryo and neural retina of E7 embryo were used as representative N-cadherin-expressing cells, while gizzard, lung mesenchyme and liver of E10 embryo were as those without N-cadherin expression (Fig. 1B).

Among the 175 clones tested, 23 exhibited enhancer activity in at least a subset of the cultures, which were grouped into 4 classes, namely, those showing activity in the forebrain and retina (8 clones); in the lens, forebrain and retina (3 clones); in the lens and forebrain (3 clones); and in all cultures (9 clones).

All inserts were determined for nucleotide sequences at both ends and positioned in the entire *N-cadherin* genomic

sequence derived from the chicken whole genome data (Hillier et al., 2004) (Fig. 1C). This revealed that the inserts of clones exhibiting enhancer activity of the same cell specificity class were grouped in the same regions of the *N-cadherin* sequence (green lines in Fig. 1C). Those inserts showing enhancer activity in the forebrain and neural retina



but not in the lens were further divided in two regions of the *N-cadherin* gene, the intron 1–exon 2 region (group 1) and in the middle of intron 2 (group 2). The group 3 inserts showing enhancer activity in the lens, forebrain and neural retina were mapped to a 3′-proximal region of intron 2, while group 4 inserts showing activity in the lens and forebrain were mapped to the short region covering exons 13 and 14. The group 5 inserts active in all cell types were positioned in a region 3′ of the gene. Within a group, the inserts overlap extensively sharing a common region, indicating that these groups represent discrete enhancers, 1–5 regulating the *N-cadherin* gene (Fig. 1C).

Assessment of enhancers using primary tissue cultures was successful in identifying specific enhancers, but these cultures were derived from mid-stage embryos. We examined activity of these specific enhancers in early embryos by electroporating the tkEGFP-bearing clones in stages 4–5 embryos and observing them at stage 12 (Uchikawa et al., 2003). An expression pattern of *N-cadherin* in an embryo at stage 12 is shown in Fig. 2A. Its comparison with those of *Sox2* and *Sox3* indicates a similarity of their expression patterns (Fig. 2A), except that *N-cadherin* is also expressed in the mesodermal tissues, e.g., heart and somites (Hatta and Takeichi, 1986; Hatta et al., 1987). Strong expression of N-cadherin in the lens placode is not activated yet and occurs only after stage 13 (see Fig. 6).

Activity of the enhancers in nasal and lens placode derivatives was examined by electroporating head surface ectoderm of stage 10 embryos and following EGFP expression up to stage 21 (Kamachi et al., 2001). Enhancer 1 was inactive in the assay using early embryo, indicating that it is active in the brain and neural retina only in the later stages of development, and was not studied further. Enhancer 2 was active throughout the CNS at stage 12, with prominent activity in the anterior CNS up to rhombomere 1, in rhombomere 6 and remarkably in the otic placodes (Fig. 2B). Enhancer 2 also showed activity in the lens placode after stage 13 and in the nasal placode after stage 14 (Fig. 2C). Enhancer 3 also exhibited enhancer activity widely throughout the CNS, particularly highly in rhombomeres 2–6, and in the lens placode (after stage 14) and nasal placode (after stage 15) (Fig. 2). This enhancer had no activity in the otic placode, however (Fig. 2B). Enhancer 4 was unique in that it showed activity in electroporated embryos only in the lens placode (after stage 14) and nasal placode (after stage 15) (Fig. 2C), without any activity in the CNS or otic placode (data not shown), in spite of its exhibiting enhancer activity in forebrain cells derived from older embryos (Fig. 1B) (Table 1).

Molecular dissection of Enhancer 4

From our interest in placodal development, we first analyzed Enhancer 4 in great detail (Fig. 3A). Inserts of clones 22, 31 and 158 overlap in the common 2-kb span, which is included in an *Eco*RI fragment, and this *Eco*RI fragment had the identical enhancer activity in transfected primary cultures (data not shown). The essential region in the fragment was narrowed down to the AS fragment of 254 bp. As short enhancer subfragments tend to loose enhancer strength, dimeric forms of AS subfragments were prepared and tested for enhancer activity in the lens epithelial cells (Fig. 3Aa). Among four overlapping subfragments of AS, only AS3 subfragment of 87 bp showed activity, as shown by photorecording (Fig. 3Ab) and EGFP fluorescence measurement (Fig. 3Ac). Subfragments of AS3 were further prepared and octamerized for assessment of enhancer activity. The minimum element was the 30-bp AS38 fragment (Figs. 3A and B).

To characterize functional domains of the AS38 sequence for interaction with DNA-binding proteins, a series of 3-bp base substitutions was introduced into the sequence (Fig. 3Ba), and their effect on enhancer activity in lens cells was examined (Fig. 3Bb). Three domains were recognized by the effect of mutations. Block 1 corresponded to the 5′-most 9 bp, where the mutations (M1, 2 and 8) abrogated the enhancer activity. Block 2 was the adjacent 6 bp, where mutations (M12, 10 and 9) reduced the enhancer activity. Block 3 was the 3′-most 4 bp, where mutations (M14 and 7) severely reduced enhancer activity.

Nuclear factors binding to the block 1 sequence were characterized using EMSA (Fig. 3C). As this block contains the SOX factor-binding sequence ATTGTG (Kamachi et al., 2000; Pevny and Lovell-Badge, 1997; Wegner, 1999) (Fig. 3Ba), and as Group B1 *Sox* genes are widely expressed in the CNS and sensory placodes (Fig. 2A) (Groves and

Fig. 1. Systematic screening of placodal and neural *N-cadherin* enhancers. (A) A schematic illustration of screening of various *N-cadherin* gene subfragments for enhancer activity using transfection. Random subfragments of genomic *N-cadherin* locus DNA were inserted into the tkEGFP (RV) reporter vector to construct a subfragment library. (B) Representative transfection data which in combination with mapping classified the enhancer-bearing fragments into 5 groups. Panels in the "N-cadherin" column show anti-N-cadherin immunostaining of the cultures used for transfection. Other panels show EGFP fluorescence reflecting enhancer activity of the inserted subfragments. Inserts of groups 1 and 2 show enhancer activity in the forebrain and retinal cultures, group 3 inserts in the lens, forebrain and retinal cultures, and group 4 inserts in the lens and forebrain cultures. Group 5 inserts showed in all cultures including lung and dermal fibroblasts (not shown here). The scale bar indicates 100 μm. (C) Alignment of *N-cadherin* locus subfragments indicated by bars on the genomic sequence. Green bars indicate subfragments with enhancer activity of assigned groups, while black bars indicate those without enhancer activity in cultures used for assay. The genomic sequence is based on contigs 51.100–51.104 of Release 30.1f (Ensembl database), which originally had four unsequenced gaps in the 5′ flanking region of the *N-cadherin* gene. However, examination of the sequence indicates that three gene-proximal gaps are due to mis-assembly of the already overlapping sequences and corrected in this figure. The most distal gap remains and is shown in gray. Inserts of the three clones (#12, #105 and #135), which were unassigned to any of other regions, are presumed to correspond to the unsequenced region.

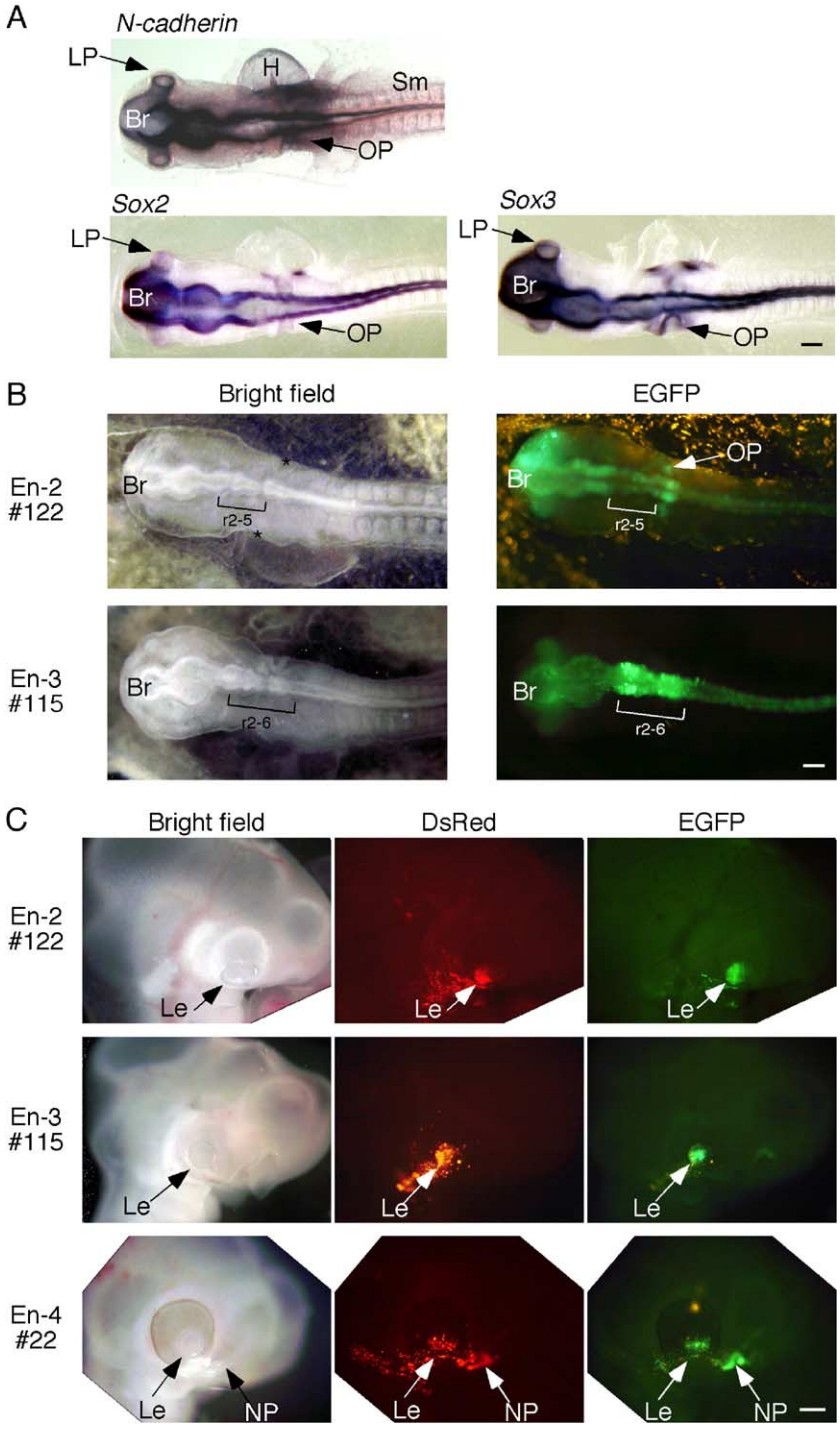


Fig. 2. Embryonic *N-cadherin* expression and the activity of *N-cadherin* enhancers. (A) Expression of *N-cadherin*, *Sox2* and *Sox3* in the sensory placodes and CNS of chicken embryos at stage 12, showing similarity of their expression patterns, except that *N-cadherin* is also expressed in the mesodermal tissues, e.g., heart and somites. LP, lens placode; OP, otic placode; Br, brain; H, heart; Sm, somites. (B and C) Activity of *N-cadherin* enhancers in electroporated chicken embryos. (B) Embryos in New's culture electroporated at stage 4 with tkEGFP carrying Enhancer 2 (En-2, Clone 122) or Enhancer 3 (En-3, Clone 115) and observed at stage 12. Br, brain; r2-5, region of rhombomeres 2–5, where En-2 activity is low; OP, otic placode, where En-2 shows significant activity; r2-6, region of rhombomeres where En-3 is particularly high. (C) Embryos electroporated in ovo at stage 10 with tkEGFP carrying Enhancer 2 (En-2, Clone 122), Enhancer 3 (En-3, Clone 115) or Enhancer 4 (En-4, clone 22), together with pDsRed, and observed at stage 21. DsRed fluorescence indicates the ectodermal area of electroporation, and EGFP fluorescence indicates activity in the lens (Le) and nasal pit (NP). The En-2 and En-3 panels in this figure demonstrates lens activity only, but other samples show activity in the nasal pit. Under visual inspection, enhancer activity in the lens placode became detectable at stage 14 and that in the nasal placode at stage 15 (Table 2). The scale bars indicate 200 μm.

Table 1
Enhancer activity of the clone groups in electroporated embryos and transfected cells

Clone group	Clones[a]	Activity in electroporated embryo				Activity in transfected cells			
		Brain, spinal cord (stage 12)	Otic vesicle (stage 12[b])	Lens placode (stage 14[b])	Nasal placode (stage 15[b])	Lens (E14)	Forebrain (E7)	Retina (E7)	Gizzard, etc. (E10)
Group 1	17, 155, 193, 236	–	–	–	–	–	+	+	–
Group 2	23, 71, 119, 122	+[c]	+	+	+	–	+	+	–
Group 3	75, 115, 215	+[d]	–	+	+	+	+	+	–
Group 4	22, 31, 158	–	–	+	+	+	+	–	–
Group 5	35, 42, 56, 68, 121, 161, 204, 208, 239	n.d.[e]	n.d.	n.d.	n.d.	+	+	+	+

[a] All clones were tested by transfection, and those indicated by underlines were used for electroporation as representative clones.
[b] Earliest stage of detection.
[c] Activity was high in the brain, rhombomere 1 and rhombomere 6.
[d] Activity was high in rhombomeres 2–6.
[e] Not determined.

Bronner-Fraser, 2000; Ishii et al., 2001; Kamachi et al., 1998; Rex et al., 1997; Uchikawa et al., 1999, 2003; Uwanogho et al., 1995), interaction of these SOX protein factors with AS38-U probe DNA carrying block 1 and block 2 sequences was investigated (Fig. 3B).

When recombinant SOX1, SOX2 or SOX3 proteins of the chicken were synthesized in COS7 cells, their nuclear extracts were active in forming each unique bands of probe–protein complex showing similar but slightly different electrophoretic mobilities reflecting molecular sizes of the SOX proteins (Kamachi et al., 1998) (Fig. 3Ca, lanes 2–4), which was not formed by using an extract of normal COS7 cells (Fig. 3Ca, lane 1). These complexes were supershifted by anti-Group B1 SOX antibodies (Tanaka et al., 2004), confirming that Group B1 SOX proteins bind strongly to the AS38-U probe sequence (data not shown).

When a nuclear extract prepared from E14 embryonic lenses was analyzed using EMSA, the extract produced a group of strong bands which showed electrophoretic mobilities corresponding to those formed with recombinant SOX1, SOX2 and SOX3 (Fig. 3Ca, lane 5, the bands marked by an asterisk). Indeed, anti-Group B1 SOX antibodies supershifted all bands of this group (Fig. 3Cb, lanes 4–5).

These Group B1 SOX–probe complexes were competed out by excess of wild-type AS38-U sequence (Fig. 3Ca, lane 6), but this competition did not occur when the AS38-U sequence contained the mutations M1, M2 or M8, which alter the SOX-binding sequence (Fig. 3Ca, lanes 7–9) and abolish enhancer activity of AS38 (Fig. 3B). By contrast, the AS38-U sequences carrying the mutations of block 2 (M12, 10 and 9) were fully active as the competitors to the probe (Fig. 3Ca, lanes 10–12). Finally, the complex was efficiently competed by the known SOX-binding site sequence (γF-SOX) of γF-crystallin promoter (Kamachi et al., 1995) (Fig. 3Ca, lane 13). The bands which were not competed by block 2 mutant competitors may represent proteins binding to the blocks 2 sequence, but they have not been characterized. Taken together, activity of the minimum core sequence AS38 of Enhancer 4 is dependent on binding of the Group B1 SOX proteins and two other factors binding to blocks 2 and 3.

When the M2 mutation of block 1 was introduced in the larger AS3 dimeric enhancer, enhancer activity was severely compromised in transfected lens cells, with the 40-fold activation observed using the wild-type sequence (Fig. 3Ac) reduced to fourfold activation (data not shown). In addition, the M2 mutation destroyed enhancer activity in the lens and nasal placodes in electroporated head ectoderm (Fig. 5A). These findings indicate that the binding of a SOX protein factor to the minimum core sequence AS38 is essential to the activation of Enhancer 4.

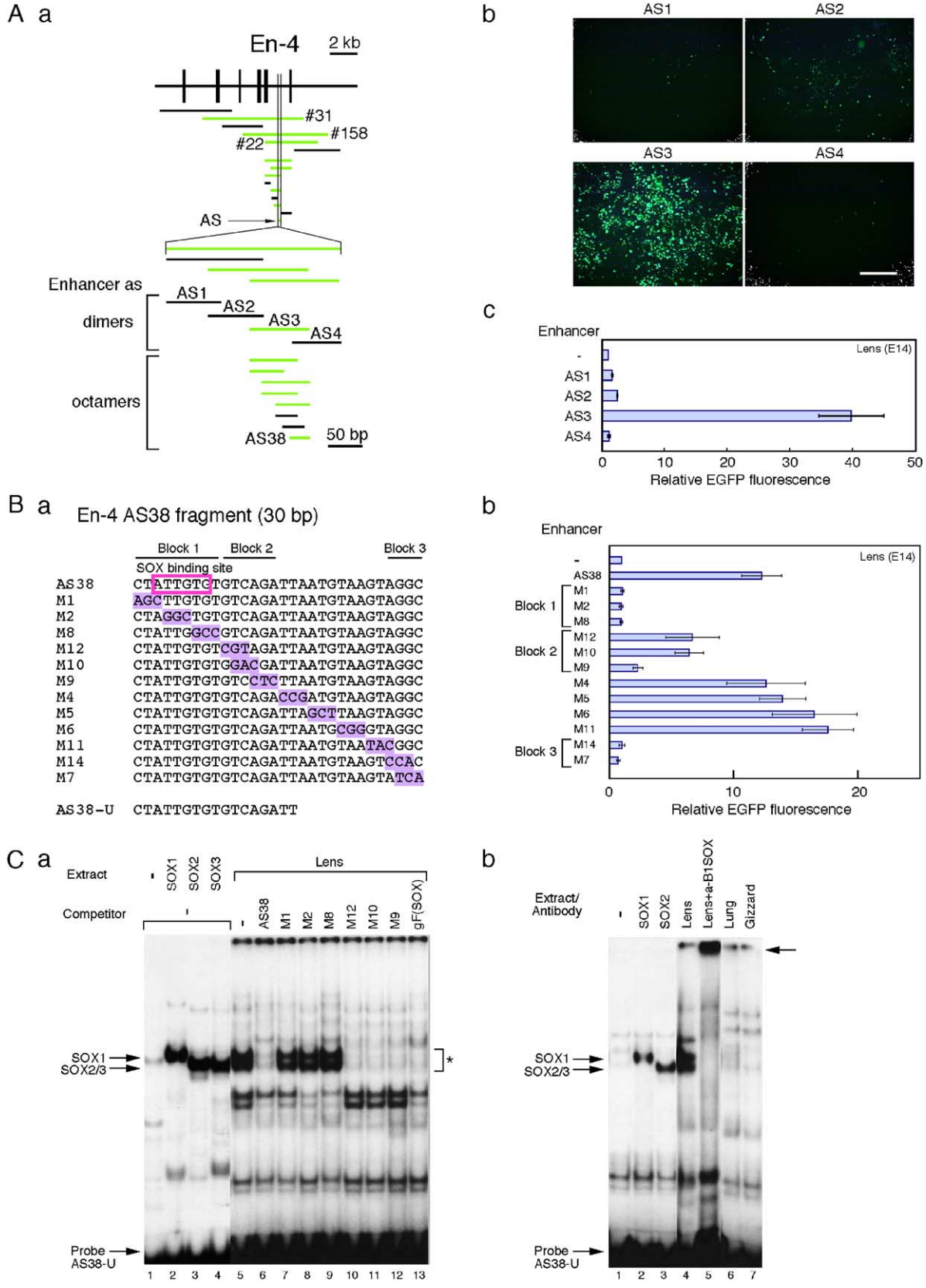
Analysis of Enhancer 3

Enhancer 3 is included in the shortest group 3 clone, 3453-bp insert carried by clone 115 (Fig. 4Aa). Testing various subfragments of Enhancer 3 identified its subfragment MS of 484 bp as carrying the enhancer activity (Fig. 4Ab). Examination of the sequence of Enhancer 3 MS fragment indicated a single candidate SOX-binding site, and mutation of this site abrogated the enhancer activity in transfection assay (Fig. 4Ac). When the same mutated enhancer-bearing tkEGFP was electroporated in stage 10 head ectoderm, enhancer activity of the MS fragment in the lens and nasal placodes was lost (Fig. 5B). Thus, the activity of Enhancer 3 is also dependent on binding of a SOX protein.

Analysis of Enhancer 2

Enhancer 2 is represented by the shortest group 2 clone, 3518-bp insert carried by clone 122 (Fig. 4Ba). Comparison with the mammalian *N-cadherin* genomic sequence (see Fig. 8) indicates a region with a high degree of conservation covered by a fragment DP of 694 bp. This DP sequence showed full enhancer activity in transfected brain cells (Fig. 4Bb) and in electroporated embryonic head ectoderm (data not shown). The sequence contains many potential SOX-binding sites, suggesting the multiple and possibly redun-

dant involvement of SOX functions in this enhancer. When the single SOX-binding sequence with a perfect match with the consensus ATTGTG was mutated, the mutation significantly reduced the enhancer strength in transfection assay (Fig. 4Bc), indicating that this SOX site is essential to the enhancer activity in late brain cells. However, the same



mutation only slightly attenuated the placodal enhancer activity in electroporated embryos (Fig. 5C), suggesting that multiple SOX-binding sites differentially regulate this enhancer.

Sox2 and N-cadherin expression in the sensory placode development

The analysis of Enhancers 2–4 described above indicated that Group B1 SOX or related activities are involved in the activation of *N-cadherin* expression. As the earliest-expressed *Sox* gene in the placode development is *Sox2*, we compared time course of the expression of *Sox2* and *N-cadherin* in the placode development. The data shown in Fig. 6 are from representative paraffin sections of embryo specimens hybridized in whole mount, which provide superior signal-to-noise ratio and histology than those hybridized after sectioning. However, the observations described below were confirmed using the serial sections of same embryos hybridized with different probes (data not shown).

In development of the lens placode, *Sox2* expression was activated in the optic vesicle-contacted surface ectoderm at stage 12, confirming the previous report (Kamachi et al., 1998), 4 h ahead of the *N-cadherin* expression that initiates at stage 13 when the placode thickens (Fig. 6A). In the nasal placode, expression of *Sox2* was initiated at stage 13, while that of *N-cadherin* occurs at stage 14 (Fig. 6B). In the otic placode, strong *Sox2* expression initiated at stage 10 (concomitant with *Sox3*) (Fig. 2A), followed by strong *N-cadherin* expression at stage 11 (Fig. 6C). *N-cadherin* mRNAs were consistently localized in the apical side of placodal cell layers, suggesting their specific subcellular localization mechanism (Cohen, 2005; Kloc et al., 2002). Thus, *Sox2* expression always precedes that of *N-cadherin* in sensory placode development, and the observations support the model that Group B1 *Sox* functions are involved in the regulation of *N-cadherin* expression.

Ectopic placode development by misexpression of Group B1 Sox in the head ectoderm

The analysis of Enhancers 2–4 described above indicated that Group B1 SOX or related activities are indeed involved in the activation of *N-cadherin* expression. As formulated in previous studies (Kamachi et al., 2000, 2001; Kondoh et al., 2004; Tanaka et al., 2004), Group B1 SOX proteins function only by forming heterodimers with specific partner factors that depend on the DNA sequence adjacent to the SOX-binding site. Thus, it is expected that if Group B1 *Sox* genes are expressed ectopically, it would cause expression of *N-cadherin*, as far as relevant partner factors are available.

Sox2 expression vector was electroporated into surface ectoderm of stage 10 chicken embryo, when placodes are not well developed, together with a Cytomegalovirus enhancer-driven EGFP expression vector to visualize the electroporated cell population (Figs. 7A and B). EGFP fluorescence was observed 3 h after electroporation, providing a rough estimate of the period for exogenous gene expression after electroporation. Ectopic *N-cadherin* expression became detectable in the ectoderm after 6 h of electroporation. This time course of ectopic *N-cadherin* expression is consistent with the model that *N-cadherin* gene is directly activated by SOX2. On the other hand, electroporation of the EGFP expression vector alone did not elicit *N-cadherin* expression (Fig. 7C).

This *N-cadherin*-activating potential was common to Group B1 *Sox* genes, as similar rates of ectopic N-cadherin expression were observed using *Sox1* or *Sox3* expression vectors (Table 2). By contrast, *Sox* genes belonging to other groups, *Sox9* (Group E), *Sox11* (Group C) and *Sox21* (Group B2), were inactive (Table 2), indicating Group B1-specific action in the induction of *N-cadherin* expression.

It was interesting to note that the cells of head ectoderm that received vectors for Group B1 *Sox* and marked by EGFP expression not only expressed *N-cadherin* but also

Fig. 3. Determination of the core region of Enhancer 4 and demonstration of SOX-dependent activity of the enhancer. (A) Determination of a 30-bp AS38 core fragment of Enhancer 4 (En-4). (a) Starting from the common region of three clones (#22, #31 and #158), a series of subfragments was tested for enhancer activity by transfection of lens cells. For subfragments shorter than 100 bp, the sequences were dimerized before assessing enhancer activity. (b and c) Assessment of enhancer activity by EGFP expression, by photorecording (b), and by fluorescence intensity measurement (c), taking comparison of the four fragments AS1 to AS4 as an example. The scale bar in panel b indicates 100 μm. In panel c, relative fluorescence intensity in transfected lens cells is shown relative to enhancer-less (−) tkEGFP(RV). (B) Dependence of Enhancer 4 on the SOX-binding site. (a) The AS38 sequence of Enhancer 4 and the series of mutations introduced. Identified blocks 1–3 are indicated at the top, and the probe sequence AS38-U used for EMSA in C is shown at the bottom. The SOX-binding sequence in block 1 is also indicated. (b) Effect of mutations in AS38 sequence on its enhancer activity. Octamerized AS38 sequences were placed downstream of tkEGFP(RV) gene and the effect of the mutations was evaluated. Relative fluorescence intensity in transfected lens cells is shown relative to enhancer-less (−) tkEGFP(RV). (C) EMSA using the AS38-U probe. (a) Binding of Group B1 SOX proteins to the probe sequence. Lanes 1–4, nuclear extracts of COS7 cells expressing Group B1 SOX proteins of the chicken (Kamachi et al., 1995). Lane 1, without SOX; lane 2, SOX1; lane 3, SOX2; lane 4, SOX3. Lanes 5–13, analysis of chicken lens nuclear extract using various competitors. Lane 5, no competitor; lane 6, wild-type AS38-U; lanes 7–12, with AS38-U sequences carrying mutations M1, M2, M8, M12, M10 and M9, respectively; lane 13, SOX-binding site sequence of γF-crystallin promoter (−63 to −44) (Kamachi et al., 1995). Competitors with mutations M1, M2 or M8 disrupting the SOX-binding sequence failed to compete out the complex formation of the probe with Group B1 SOX proteins. The autoradiogram for lanes 5–13 was taken after sevenfold longer exposure than for lanes 1–4 in order to adjust band intensities. (b) Comparison of nuclear extracts of various embryonic tissues. Lanes 1–3, nuclear extracts of COS7 cells with no (lane 1), SOX1 (lane 2) and SOX2 (lane 3) expression. Lane 4, lens (E14) nuclear extract; lane 5, lens nuclear extract plus anti-Group B1 SOX antibodies (Tanaka et al., 2004); lane 6, lung fibroblast (E10) nuclear extract; lane 7, gizzard (E10) nuclear extract.

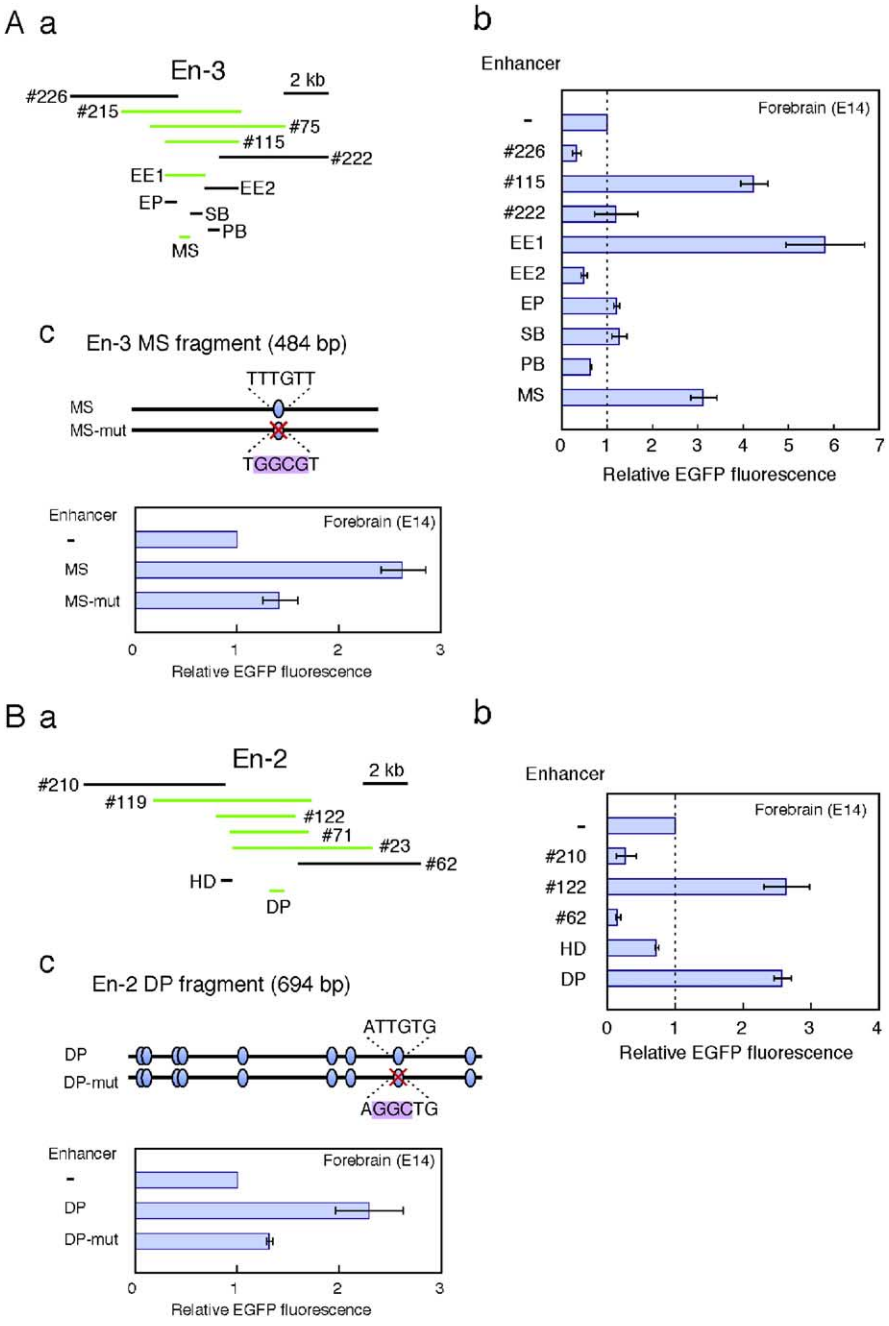


Fig. 4. Determination of the core region of Enhancers 3 and 2 by assessment of enhancer activity of various subfragments, and dependence of their activity on SOX-binding sequences. (Aa) Determination of the core sequence of Enhancer 3 (En-3). The enhancer activity-bearing region was narrowed down to the MS fragment of 484 bp by stepwise fragmentation. (b) Enhancer activity of various fragments in transfected forebrain cells. (c) Effect of the SOX-binding site mutation (MS-mut) on the activity of Enhancer 3 MS fragment in transfected forebrain cells. The SOX-binding site is indicated by an oval, and original and mutated sequences are shown. Relative EGFP fluorescence reflecting enhancer activity in transfected brain cells is shown. (Ba) Enhancer activity-bearing fragments of Enhancer 2 (En-2). Initial overlap of group 2 clones (#119, #122, #71, #23) was roughly 4 kb. A region with a high degree of sequence conservation found between the chicken and human genomes, represented by a DP fragment of 693 bp length (see Fig. 8), was taken as a candidate core sequence. The DP fragment showed full enhancer activity in transfected forebrain cells (data not shown) and in lens/nasal placodes of electroporated embryos (see Fig. 6). (b) Enhancer activity of various fragments in transfected forebrain cells. (c) Effect of mutation in one of the SOX-binding sites (DP-mut) on the activity of Enhancer 2 DP fragment in transfected forebrain cells. Candidate SOX-binding sites are indicated by ovals.

gathered to form tight clusters (Fig. 7B), indicating that all these cells expressed functional N-cadherin molecules. The cells that received only EGFP expression vector remained scattered in the head ectoderm.

In the head ectoderm electroporated with Group B1 *Sox* genes and labeled with EGFP expression (Figs. 7E and H), expression of N-cadherin proteins was confirmed using a monoclonal antibody (Fig. 7F), and in addition thickening

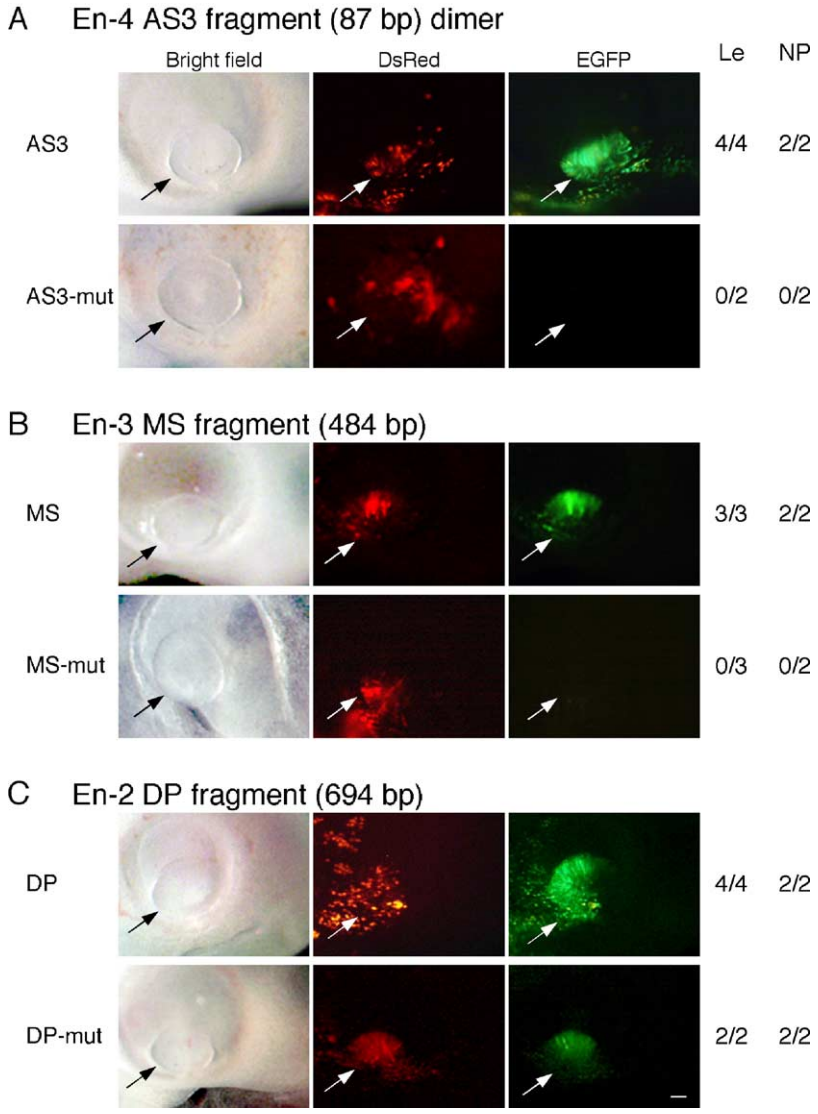


Fig. 5. Activity of subfragments of Enhancers 4, 3 and 2 in the lens placode in electroporated embryos. Stage 10 embryos were electroporated in ovo in the head surface ectoderm in the area of forming lens placode, and enhancer activity at stage 21 was photorecorded. Electroporated areas are marked by DsRed fluorescence, and enhancer activity is indicated by EGFP fluorescence. Arrows point to the ventral aspect of lenses. The numbers to the right of the panels indicate enhancer active samples in lens (Le) and nasal pit (NP) among successfully electroporated samples. (A) AS3 fragment and its M2 mutant version (Fig. 3) of Enhancer 4 (En-4) in dimeric form. The mutation inactivated the enhancer in the lens. (B) MS fragment of Enhancer 3 (En-3) and its SOX site mutant MS-mut (Fig. 4Ac). The mutation inactivated the enhancer in the lens. (C) DP fragment of Enhancer 2 and its mutant version (Fig. 4Bc). Although the mutation inactivated the enhancer in transfected forebrain cells (Fig. 4Bc), it only slightly decreased the enhancer activity in the lens. The scale bar indicates 10 μm.

of the cell layer (Figs. 7G and J) and expression of N-CAM (Fig. 7I) characteristic of sensory placodes were observed. These observations indicate that Group B1 *Sox* not only activates *N-cadherin* expression in the cells but also directs a series of molecular events which lead to the establishment of the placodes.

An interesting observation was that ectopic *N-cadherin* expression occurred efficiently when electroporation of the Group B1 *Sox* expression vectors was targeted to head ectoderm, but that efficiency was significantly lower when electroporation was done on the trunk ectoderm (Table 2). This probably indicates that the major partner factors of Group B1 SOX for the activation of *N-cadherin* enhancers are available only in the head ectoderm.

Discussion

Systematic screening for N-cadherin enhancers

In this study, we took a systematic approach in identifying enhancers regulating the *N-cadherin* gene, evenly scanning the entire *N-cadherin* locus using a BAC subfragment plasmid library constructed in a tk-EGFP expression

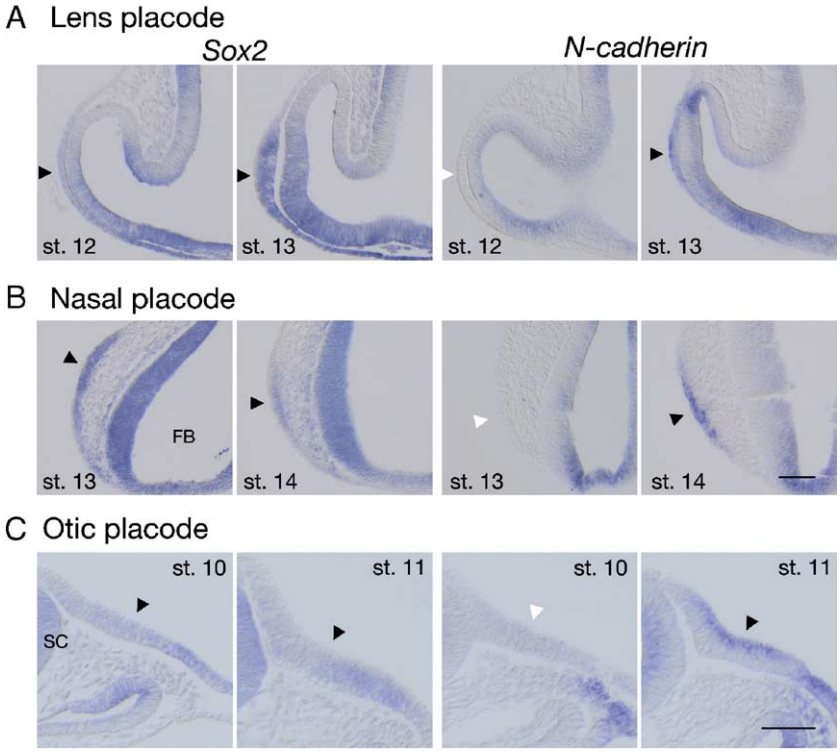


Fig. 6. Expression of *Sox2* and *N-cadherin* in the sensory placodes and CNS of chicken embryos. Cross sections of embryos at the initial stages of placode development are shown. (A) Lens placode at stages 12 and 13. (B) Nasal placode at stages 13 and 14. (C) Otic placode at stages 10 and 11. Paraffin sections were made after whole mount in situ hybridization. Hybridization of serial frozen section yielded similar results (data not shown), but for better signal-to-noise ratio and histology, these sections are shown. The arrowheads in black indicate expression of the gene, while those in white indicate low expression. Expression of *Sox2* precedes that of *N-cadherin*. The scale bars indicate 50 μm.

vector, and taking advantage of the transfection of culture-specific cells and electroporation of chicken embryos using the same plasmids. An earlier view held that regulatory sequences (enhancers) are localized in the vicinity of the promoter. However, cumulative evidence indicates that enhancers are widespread around the gene, as exemplified by our previous study of *Sox2* enhancers (Uchikawa et al., 2003, 2004). Many enhancers are utilized differentially in the spatiotemporal order, responding to diverse signals and different sets of transcription factors, but the functions of these enhancers are organized to satisfy consistent expression of a gene in a tissue. In the present study, the *N-cadherin* gene was also shown to harbor discrete enhancers active in the CNS and placodal development.

It is a generally accepted notion that a substantial fraction of candidate regulatory sequences is predictable as conserved sequences across the phyla. Our previous study on widely scattered *Sox2* enhancers determined by functional assay indicated that such regulatory sequences in amniotes are predictable with reasonable accuracy as the sequence blocks highly conserved between mammalian and chicken genomes (Uchikawa et al., 2003). This empirical rule appears to hold true for *N-cadherin* enhancers (Fig. 8, green stripes), as Enhancers 1, 2 and 4 match a subset of conserved sequence blocks, other than exons (Fig. 8, blue stripes), found between the chicken and human *N-cadherin* sequences. Comparison of the chicken and human *N-cadherin* genomic sequences also indicates there are six more blocks clearly conserved between the chicken and human, three in intron 2 (Fig. 8, pink stripes). It is likely that these represent regulatory sequences for mesodermal *N-cadherin* regulation (Hatta and Takeichi, 1986; Hatta et al., 1987) (Fig. 2A), which were not investigated in this study. When human and mouse sequences were compared those sequence blocks conserved between the chicken and human *N-cadherin* genes are obscured by being buried within longer stretches of sequence similarity, as we experienced with the *Sox2* enhancers (Uchikawa et al., 2003). These findings confirm the significance of the conserved sequence blocks common to chicken and mammalian genes.

However, the present study also raises a warning on the approach of relying solely on sequence conservation. As exemplified by neuro-placodal Enhancer 3 and non-specific Enhancer 5 of chicken *N-cadherin*, which were not found in mammalian *N-cadherin* locus (Fig. 8), enhancers exist that may be unique to a branch of phylogenic development.

A reliable way to determine regulatory sequences may be to systematically test various genomic regions, and then to identify the foci of important sequences with the aid of sequence conservation across animal species, as carried out in the analysis of Enhancer 2. In any event, the approach taken here, namely, to construct a random fragment library of a BAC insert in a reporter vector, and then to assess

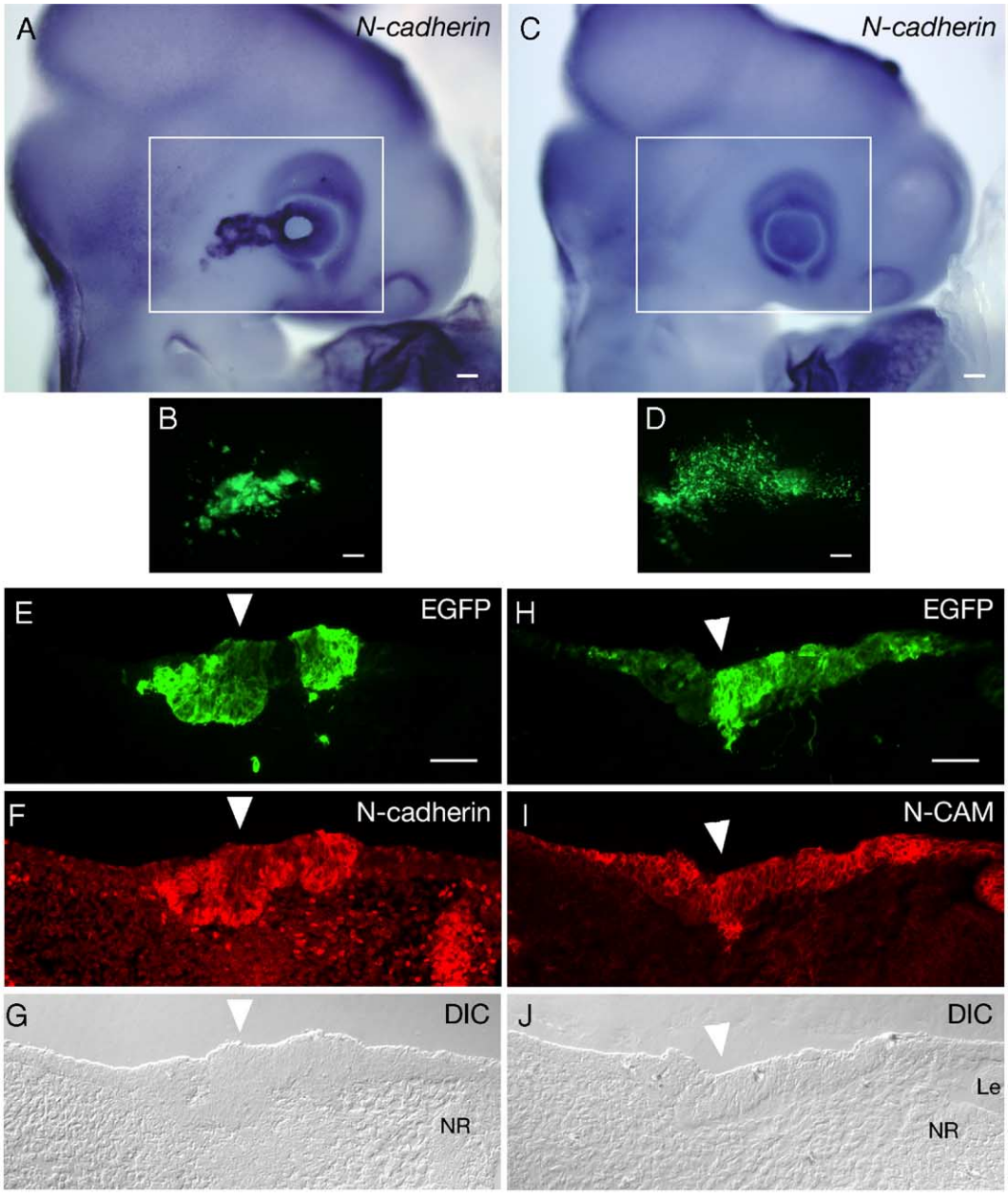


Fig. 7. Ectopic induction of *N-cadherin* expression and placode development by in ovo electroporation of chicken embryo with *Sox2* expression vector. Embryos at stage 10 were electroporated with *Sox2* expression vector (pCMV/SV1-*Sox2*; Kamachi et al., 1999) or insert-free vector at 5 μg/μl, and 1 μg/μl EGFP expression vector in the region of the surface ectoderm caudal to the eye. Analysis was done at stage 21. (A) An embryo electroporated with *Sox2* expression vector and hybridized in whole mount with *N-cadherin* probe. Strong *N-cadherin* expression is observed in the region of the electroporation of the surface ectoderm. (B) EGFP fluorescence of the same embryo showing the region of electroporation. The region boxed in panel A is shown. Note that EGFP-positive cells form coherent clusters. (C and D) An embryo analogous to panels A and B but without the *Sox2* expression vector in which no ectopic expression of *N-cadherin* occurs (C). Note also that electroporated EGFP-expressing cells remain scattered (D). (E–J) Embryos electroporated analogous to panel A at stage 23 were cut into serial frozen sections (E–G and H–J) and immunohistologically analyzed: (E and H) EGFP immunofluorescence; (F) N-cadherin immunofluorescence and (I) N-CAM immunofluorescence; and (G and J) differential interference contrast (DIC) images of the sections. The cluster of electroporated cells marked by EGFP expression (E and H, arrowheads) becomes thickened columnar epithelium and assumes placodal morphology (G and J) and express N-cadherin (F) and N-CAM (I). NR, neural retina; Le, lens. The scale bars in panels A–D indicate 100 μm, and those in panels E and H 50 μm.

enhancer activity directly by transfection or embryo electroporation, is highly efficient. Analogous approaches should be taken in the investigation of various genes of developmental interest to gain a precise view of how the gene is regulated.

Among the five *N-cadherin* enhancers identified in this study, Enhancer 1 is located in the intron 1-exon 2 region, and broadly active Enhancers 2 and 3 are located in the long intron 2, while Enhancer 4 is in intron 13 and Enhancer 5 is downstream of the gene. Recent investigation of mouse

Table 2
Frequency of ectopic *N-cadherin* expression observed after electroporation of *Sox* expression vectors (Kamachi et al., 1999; Uchikawa et al., 1999) in the ectoderm of stage 10 embryos

Ectodermal region		Head			Trunk		
N-cadherin expression		−	+/−	+	−	+/−	+
Sox2	2 μg/μl		1/12	12/13			
	5 μg/μl			8/8	3/6	3/6	
Sox1	2 μg/μl		1/14	13/14			
	5 μg/μl			6/6	4/5	1/5	
Sox3	2 μg/μl	1/5	1/5	3/5			
	5 μg/μl			5/5			
Sox21	5 μg/μl	2/2					
Sox9	2 μg/μl	4/4					
	5 μg/μl	3/3					
Sox11	2 μg/μl	2/2					
	5 μg/μl	4/4					
Non-*Sox* DNA	2 μg/μl	4/4					
	5 μg/μl	2/2					

DNAs at different concentrations were delivered on embryos and electroporated. Embryos were fixed at 18, 24 and 36 h after electroporation for examination of *N-cadherin* expression by whole mount in situ hybridization. Since data of the three stages are consistent, they are compiled to give the table: −, No ectopic *N-cadherin* expression; +/−, *N-cadherin* expression at the marginal level of detection; +, definite *N-cadherin* expression as seen in Fig. 7A. The number of cases in each experimental group is indicated.

E-cadherin enhancers (Stemmler et al., 2003, 2005) indicates that the major regulatory elements are included in intron 2, while *E-cadherin* expression in some specific tissues (e.g., yolk sac and apical ectodermal ridge) are independent of the intron 2 sequence. This characteristic of widely scattered enhancer elements through the gene locus may be common to *cadherin* genes.

Differential spatiotemporal regulation of N-cadherin enhancers

The observations made in this study clearly indicate that *N-cadherin* expression is temporally regulated by reliance on discrete, stage-dependent activity of the enhancers (Table 1). In transfection assay, Enhancer 1 was active in the forebrain and retinal cells, but the same enhancer showed no activity in the electroporated early embryos, suggesting that Enhancer 1 regulates *N-cadherin* gene in later development of the CNS. Enhancer 2 was active in the forebrain and retina cells in transfection assay without activity in the lens epithelium, but in electroporated embryos this enhancer showed prominent activity not only in the CNS, but also in all nasal, lens and otic placodes. Thus, Enhancer 2 regulates *N-cadherin* in the early placodes, and in the early to late CNS. Analogously, Enhancer 4 was active both in the lens epithelial and forebrain cultures but showed no activity in the early embryonic CNS, indicating that it also functions late in the CNS. It was also noted that although Enhancers 2 and 3 were active throughout the early CNS, there were regional differences in their activity, particularly in the rhombomere region (Fig. 2B).

It is also to be noted that while Enhancer 2 is active in all three sensory placodes, Enhancers 3 and 4 are active only in nasal and lens placodes (Fig. 2C). It is known that sensory placode precursors are derived from a similar, anterior embryonic location as indicated by cell tracing analysis, and are interchangeable to a certain extent, as indicated by experiments using heterotopic and heteroplastic grafts of embryonic ectoderm, and that the unique developmental fate of individual placodes appears to be determined by local inductive cues originated from surrounding tissue (Baker and Bronner-Fraser, 2001; Bhattacharyya et al., 2004; Streit, 2004; Vogel and Davies, 1993; Webb, 1993). It is possible that Enhancer 2 is activated by a mechanism pertinent to global placode development, while the activation of enhancers 3 and 4 is linked to lens and nasal specificity.

This study also demonstrated that early neural/placodal Enhancers 2–4 are all dependent on a SOX-binding site, where Group B1 SOX proteins, exemplified by SOX2, likely act. It is important to note that Group B1 SOX proteins do not function by themselves, but act by forming complexes with a partner factor binding to a DNA sequence in proximity of the SOX site (Kamachi et al., 2000, 2001; Kondoh et al., 2004; Tanaka et al., 2004). It is possible that availability of the SOX partner factors that depend on the enhancers limits the activity to certain developmental stages and to subsets of neuro-placodal tissues. Availability of the SOX partner factors for activation of placodal enhancers may also delimit placodal development to the cephalic region, as induction of ectopic placodes by exogenous Group B1 *Sox* genes occurs only in the corresponding region of the embryos (Table 2). Identification of these partner factors and investigation of their regulation would clarify how the specificity of these enhancers is determined.

Not only SOX2 protein regulates *N-cadherin* Enhancers 2–4 active in the sensory placodes, but Group B1 *Sox* genes themselves are also under placode-specific regulation. Our previous study (Uchikawa et al., 2003) has shown that *Sox2* expression in the nasal and otic placodes is regulated by the same enhancers, namely, NOP-1 and NOP-2, while lens placodal *Sox2* expression is regulated by enhancer N-3. This different coverage of enhancers for sensory placodes between the *N-cadherin* and *Sox2* genes must reflect a multitude of gene regulation in placode development. Further analysis of these placodal enhancers for interacting partner factors and upstream signaling systems would be highly informative in understanding the development of the common placodal nature, as well as the individual characteristics of the sensory placodes.

Involvement of Group B1 Sox functions in neuro-placodal development

The essential involvement of Group B1 *Sox* functions in *N-cadherin* expression and neuro-placodal development gained a strong support from expression analysis and was confirmed by *Sox* misexpression experiments.

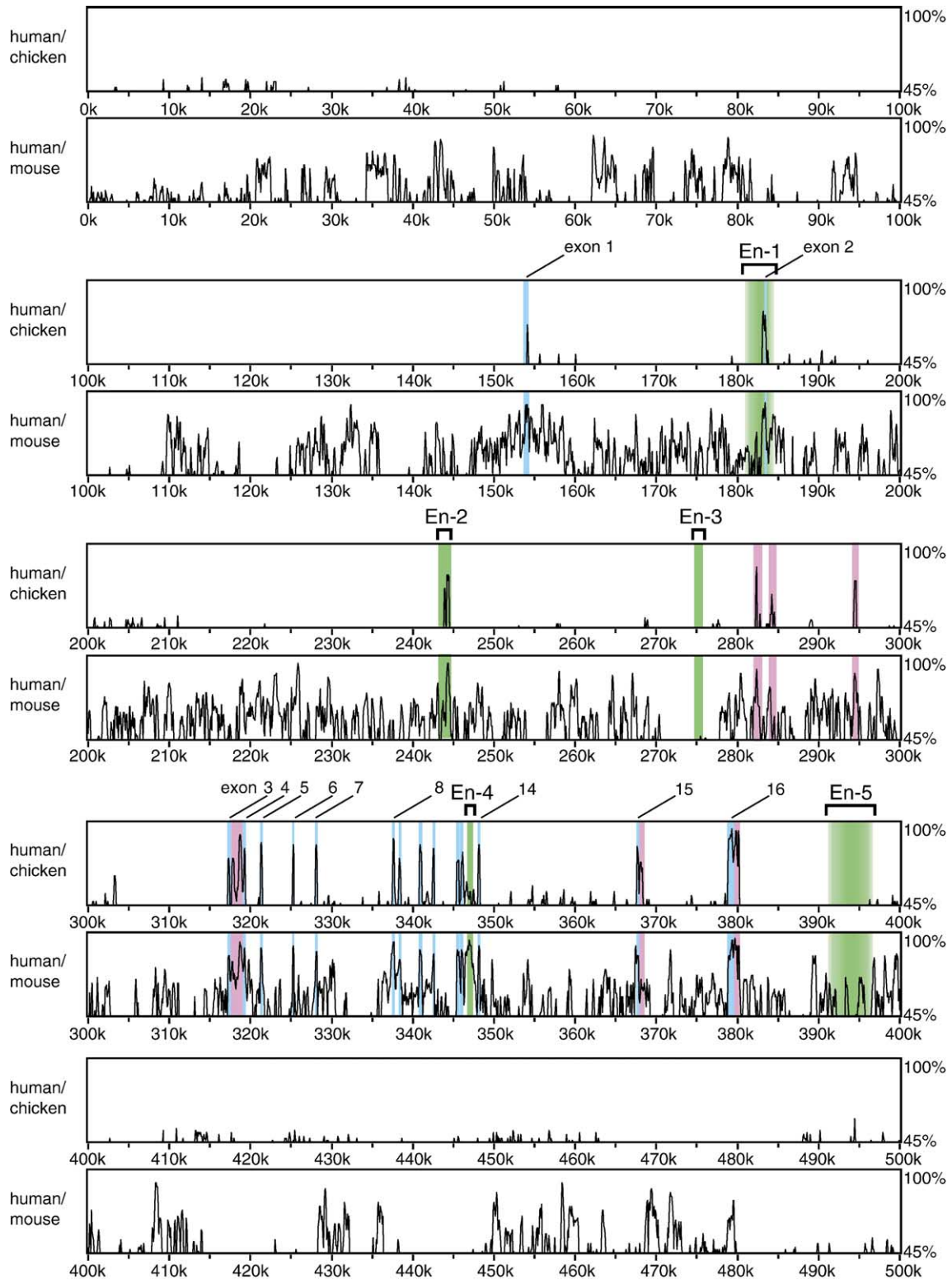


Fig. 8. Analysis of sequences conserved between chicken and mammalian *N-cadherin* genes using the VISTA program. Upper rows: Comparison of chicken versus human *N-cadherin* sequence, evaluating every 50 bp alignment and plotting regions over 45% sequence identity. The abscissa represents human sequence coordinates. Exons are indicated by blue stripes, enhancers identified in this study by green stripes, and other highly conserved region found between the human and chicken sequence by pink stripes. Lower rows, comparison of mouse and human *N-cadherin* genomic sequences based on the same criteria. Conservation of Enhancer 4 sequence between human and chicken is less pronounced compared with Enhancers 1, 2 or 5, but significance of conservation is highlighted by remarkably high score of this region between human and mouse. Enhancers 3 and 5 sequences determined in the chicken genome does not have a corresponding sequence in mammalian genomes and are positioned roughly in the way to reflect their coordinates in the chicken sequence. Note three pink-shaded prominent peaks in intron 2, which may correspond to mesodermal enhancers or sensory/neural enhancers of later development.

Sensory placodes and the prospective neural plate are both marked by expression of *Sox2*, or *Sox2* plus *Sox3*, in the chicken and mouse embryos (Rex et al., 1997; Uchikawa et al., 2003; Uwanogho et al., 1995; Wood and Episkopou, 1999). During neural plate development, high *N-cadherin* expression is then activated and a dramatic change in tissue architecture involving thickening of the cell sheet (neural plate) is initiated (Hatta and Takeichi, 1986; Hatta et al., 1987; Takeichi, 1988). In the present study, it was clearly shown that N-cadherin expression and thickening of placodal cell sheet occur synchronously in three sensory placodes and are preceded by *Sox2* expression (Fig. 6).

Misexpression of Group B1 *Sox* genes in the head ectoderm of stage 10 chicken embryo caused ectopic development of placodal structures (Fig. 7, Table 2), where not only was *N-cadherin* expression activated, but cell clustering, cell sheet thickening and N-CAM expression also took place. *N-cadherin* gene activation is thus a prominent example of the many processes of placodal development that are under the regulation of Group B1 *Sox* activity.

Involvement of Group B1 *Sox* function was suggested by an earlier study (Koster et al., 2000), in which overexpression of exogenous *Sox3* in whole Medaka embryo caused frequent development of ectopic lenses and otic vesicles, but due to complex interactions in the process, a cell-autonomous effect of exogenous *Sox3* was not always demonstrated. The present study, however, demonstrated that ectopic placodes develop as a direct consequence of misexpression of Group B1 *Sox* genes in the head ectoderm.

Many genes involved in placodal development are thus expected to bear multiple SOX-dependent enhancers, as was determined in the *N-cadherin* gene. These multiple enhancers may function in concert to provide the general placodal nature, and variability of the SOX partner factors will determine the characteristics of individual placodes or specific domains of the CNS. Although the *N-cadherin* gene is just one example case, further characterization of the enhancers determined in this study should provide deep insight into the process of neuro-placodal development.

Acknowledgments

We appreciate the stimulating discussions with members of the Kondoh laboratory. We thank Professor M. Takeichi for the provision of N-cadherin cDNA, and Dr. H. Zhang and other members of Genefinder Genomic Resources of Texas A&M University for BAC clones. This work was supported by grants (16770162 to MU, 16570173 to YK, and 12CE2007 and 17018025 to HK) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and from the Human Frontier Science Program (RGP0040/2001-M) to HK.

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