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Convergence of Wnt and FGF signals in the genesis of posterior neural plate through activation of the *Sox2* enhancer N-1

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The expression of the transcription factor gene *Sox2* precisely marks the neural plate in various vertebrate species. We previously showed that the *Sox2* expression prevailing in the neural plate of chicken embryos is actually regulated by the coordination of five phylogenetically conserved enhancers having discrete regional coverage, among which the 420-bp long enhancer N-1, active in the node-proximal region, is probably involved directly in the genesis of the posterior neural plate. We investigated the signaling systems regulating this enhancer, first identifying the 56-bp N-1 core enhancer (N-1c), which in a trimeric form recapitulates the activity of the enhancer N-1. Mutational analysis identified five blocks, A to E, that regulate the enhancer N-1c. Functional analysis of these blocks indicated that Wnt and FGF signals synergistically activate the enhancer through Blocks A-B, bound by Lef1, and Block D, respectively. *Fgf8b* and *Wnt8c* expressed in the organizer-primitive streak region account for the activity in the embryo. Block E is essential for the repression of the enhancer N-1c activity in the mesendodermal precursors. The enhancer N-1c is not affected by BMP signals. Thus, Wnt and FGF signals converge to activate *Sox2* expression through the enhancer N-1c, revealing the direct involvement of the Wnt signal in the initiation of neural plate development.

KEY WORDS: Posterior neural plate, Node, *Sox2*, Enhancer N-1, Wnt, FGF, BMP

INTRODUCTION

The formation of the neural plate, the CNS primordium, is initially induced in response to signals derived from the organizer area. The organizer and associated tissues change their relative positions in the gastrulating embryo to the posterior side (Charrier et al., 1999; Kinder et al., 2001), and give rise to a continuous posterior addition to the neural plate. Recent investigations have indicated that this process is accomplished through a series of interactive steps involving multiple signaling molecules and transcription factors that segregate neural, mesodermal and endodermal lineages (Stern, 2005; Streit and Stern, 1999b; Wilson and Edlund, 2001).

Over the last decade, many signaling molecules and transcription factors variously involved in the genesis of the neural plate have been characterized (Munoz-Sanjuan and Brivanlou, 2002; Stern, 2005; Streit and Stern, 1999b; Wilson and Edlund, 2001). The participation of these molecules, either directly or indirectly, in the formation of the neural plate has been generally assessed either by examining the effect of disruption of their function using gene manipulation techniques or by analyzing the consequence of their ectopic activation. However, a clear view of how a signaling system participates in the specification and development of the neural plate has not been provided. Involvement of FGF activity in the neural plate specification has been indicated, for instance, by local administration of FGFs to an ordinarily non-neural domain of early-stage chicken embryos that provides and stabilizes certain, often posterior, pre-neural traits to the cells (Sheng et al., 2003; Storey et al., 1998; Streit et al., 2000). However, this condition alone is not sufficient for eliciting *Sox2* expression for the development of the neural plate, and the effects depend on the stage of the embryo employed in the study. In *Xenopus* eggs, the provision of Wnt signal before gastrulation promotes neural development (McGrew et al., 1997), but this condition also suppresses BMP signals that are otherwise inhibitory to neural development, by repressing BMP4 expression (Baker et al., 1999) and promoting the expression of BMP antagonists (Wessely et al., 2001). Meanwhile, a high Wnt signal is inhibitory to the neural development of early-stage chicken embryonic cells (Wilson et al., 2001). Thus, the outcome of these approaches tends to depend on the experimental system employed, and the distinction between direct and indirect effects is not always possible. The complete elucidation of the process of neural plate formation has remained elusive, and a more straightforward approach to identifying each regulatory step in the long-range process of inducing neural plate formation has long been awaited.

We analyzed the regulation of *Sox2*, a gene activated when neural plate formation is induced (Charrier et al., 1999; Rex et al., 1997; Uchikawa et al., 2003). *Sox2* is expressed in a manner that marks the neural plate in early-stage embryos (Darnell et al., 1999; Streit et al., 1997). To clarify the regulatory steps involved in the genesis of the neural plate, an extensive survey of the regulatory (enhancer) sequences of the *Sox2* locus of chicken was carried out (Uchikawa et al., 2003; Uchikawa et al., 2004). In the 50-kb *Sox2* region of the chicken genome, several enhancers directing *Sox2* expression in distinct domains of the embryonic neural plate were identified, which are also highly conserved in mammals. The wide coverage of *Sox2* expression in the neural plate is actually generated by piecing together the discrete activities of these enhancers (Uchikawa et al., 2003; Uchikawa et al., 2004). Importantly, the enhancer N-1, which is located 13 kb downstream of the *Sox2* gene, is activated in the tissue area of neural plate precursors (Brown and Storey, 2000), in response to signals emanating from the node area, whereas the enhancer N-2, located 4 kb upstream, appears to be responsible for anterior neural plate development (Uchikawa et al., 2003). The tissue area exhibiting the enhancer N-1 activity not only contains the precursor cells for the posterior neural plate, but also

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includes cells with multi-lineage (neural, epidermal and mesodermal) potentials (Brown and Storey, 2000; Catala et al., 1996; Diez del Corral and Storey, 2004), supporting the view that the activation of the enhancer N-1 is a prelude to the specification of the posterior neural plate.

In the present study, the enhancer N-1 was utilized for the identification of signaling and transcriptional regulatory systems that are involved in the genesis of the posterior neural plate. Within the 420-bp enhancer N-1, a 56-bp core enhancer N-1c was identified, which governs the spatiotemporal specificity of the enhancer N-1. Mutational analysis identified five Blocks, A to E, that regulate the enhancer. Functional analysis of these blocks indicated that Wnt and FGF signals synergistically activate the enhancer N-1c through Blocks A-B and D, respectively, and that Block E contributes by restricting the activity of the enhancer N-1c to superficial neural precursors. This orchestrated regulation of the enhancer N-1c establishes an essential step in the genesis of the posterior neural plate. This result clarifies how the FGF signal, long known to be involved in specification of the neural plate, and the Wnt signal, which in many contexts exhibits anti-neural activity, are directly involved in the activation of the *Sox2* expression, a step in posterior neural plate specification.

MATERIALS AND METHODS

Embryo electroporation

Chicken embryos at stage 4 were placed in New's culture conditions, and electroporated with tkEGFP-based DNA constructs from the dorsal side, as described previously (Uchikawa et al., 2003; Uchikawa et al., 2004). In most experiments, a trimerized N-1c sequence was placed 5′ of the tkEGFP cassette. The electroporated tissue area was marked by the co-electroporation of pDsRed1-N1 (Clontech). Effector cDNAs were expressed using a CAGGS vector (Sawicki et al., 1998).

Electrophoretic mobility shift assay

The assay was done as described previously (Kamachi et al., 1995). Recombinant cLef1 was synthesized in vitro using a TNT kit (Promega). Nuclear extracts from the organizer area were prepared from a rectangular area of tissue 1.1 mm long and 0.72 mm wide centered on the node from stage 8 embryos. Sixty pieces of tissue were combined and processed to yield 60 μl of nuclear extract, and 0.5 μl was used per lane. Probes were 60 bp long, including the specific sequences shown in Fig. 3A and flanking linker sequences. Anti-Lef1 antibodies were purchased from Santa Cruz (cat. no. SC8592X).

Transfection

Firefly luciferase constructs were prepared by inserting various enhancer sequences 5′ of δ51LucII (Kamachi et al., 1995). phRG-TK expressing *Renilla* luciferase (Promega) was mixed with δ51LucII constructs to control for transfection efficiency. 10T1/2 cells plated at 10^4 per 1-cm-diameter well 1 day before being transfected with 0.4 μg of total DNA and 1 μl of Fugene6 reagent (Roche). The DNA mixture for transfection contained 0.1 μg of firefly luciferase construct, 0.02 μg of phRG-TK, 0.2 μg each of CAGGS-based expression vectors for *Fgfs* or *Wnt3*, or cDNA-insert-free vectors. Recombinant FGFs (R&D Systems) were added to the culture medium at 100 ng/ml and SU5402 was added at 40 μg/ml immediately prior to transfection. Luciferase activities were measured after 24 hours.

cDNAs

The following cDNAs used as effectors or probes were previously reported and provided by other researchers: *cFgf8a/b* (H. Nakamura), human-mouse composite *Wnt3* (R. Behringer), *cLef1* (S. Nakagawa), stabilized *β-catenin* (S33A, S37A, Y41A, S45A) (A. Nagafuchi), *Dkk1* (C. Niehrs), and human *Sfrp1* (J. Rubin). Full-length *cWnt8c* and *cTbx6L* sequences were cloned from a λ-gt11 cDNA library of stage 8 chicken embryos, and the sequence data were deposited in DDBJ/EMBL/GenBank with accession numbers: *cWnt8c*, AB193181; *cTbx6L*, AB193180.

RESULTS

N-1c, a 56-bp core region of enhancer N-1

By electroporation of the chicken embryo with an enhancer-activated tkEGFP reporter gene (Fig. 1A), the enhancer N-1 was demonstrated to gain activity only in the tissue area surrounding the node located at the posterior end of *Sox2* expression domain. This tissue area continuously extends posteriorly in the primitive streak region (Fig. 1C), whereas in the neural plate anterior to the node, the enhancer activity is lost, resulting in a posteriorly moving comet-shaped region labeled by the activity of the enhancer N-1 (Uchikawa et al., 2003; Uchikawa et al., 2004).

The scanning of the 420-bp enhancer N-1 with 50-bp deletions identified a region essential for enhancer activity (Fig. 1B). The deletion of the N-1 sequence from either side delineated the minimum core region for the enhancer activity, i.e. 56-bp N-1c (Fig. 1B,C). The removal of the N-1c sequence from the 420-bp N-1 enhancer eliminated enhancer activity (Fig. 1B,C). The enhancer activity of N-1c was weaker than that of N- 1 (data not shown), but the activity of trimeric N-1c, occurring in the region surrounding the node was indistinguishable from that of N-1 (Fig. 1C). Thus, the basic activity of the enhancer N-1 is borne by the 56-bp N-1c sequence, and the flanking regions of the enhancer augment the activity.

Functionally defined sequence Blocks A to E regulate activity of enhancer N-1c

To characterize functional elements that make up the core enhancer N-1c, and also to identify the signaling system regulating this enhancer, block-wise base substitutions in three consecutive positions were introduced into the 56-bp N-1c sequence, which were then compared with wild-type N-1c in the activation of tkEGFP expression after electroporation of stage 4 chicken embryos. Most mutations affected the activity of the enhancer N-1c, determining functional Blocks A to E from the 5′ end (Fig. 2A,B).

The mutations of Blocks A and B (Mut-A and Mut-B, respectively) partially attenuated the enhancer activity. These Blocks are separated by sequences without mutational effect. These mutations decreased the enhancer strength without altering tissue domains for the activity (Fig. 2Bb,c), as confirmed by analysis of cross sections of the embryos (data not shown). Since Blocks A and B share the Lef1 binding sequences, as indicated below, the double mutant Mut-AB (Fig. 2A, M2&6) was tested, and found to have a very low enhancer activity that was barely above the background level (Fig. 2Bd), suggesting redundant functions of these blocks. Mut-C caused a less pronounced reduction of enhancer strength (Fig. 2Be). Mut-D completely eliminated the enhancer activity (Fig. 2Bf), indicating an essential function for this element.

Mut-E was unique in that it caused the expansion of the enhancer-active domain (Fig. 2Bg). The cross sections of these embryos revealed that whereas the wild-type enhancer was active primarily in the neural plate-forming superficial layer, the Mut-E enhancer activity extended to the underlying mesendodermal precursors (Fig. 2Bh,i). Even when using the wild-type N-1c, a low-level EGFP expression, which may be carried over from the expression in the preingression superficial cell layer, was detected (Fig. 2Bh), but in sharp contrast, the mesodermal EGFP expression using the Mut-E N-1c enhancer (Fig. 2Bi) was stronger than that in the superficial layer. The deletion of the Block E sequence from the N-1c sequence led to identical results (data not shown). These observations indicate that Block E is involved in the repression of the enhancer N-1c in the mesendodermal precursors.

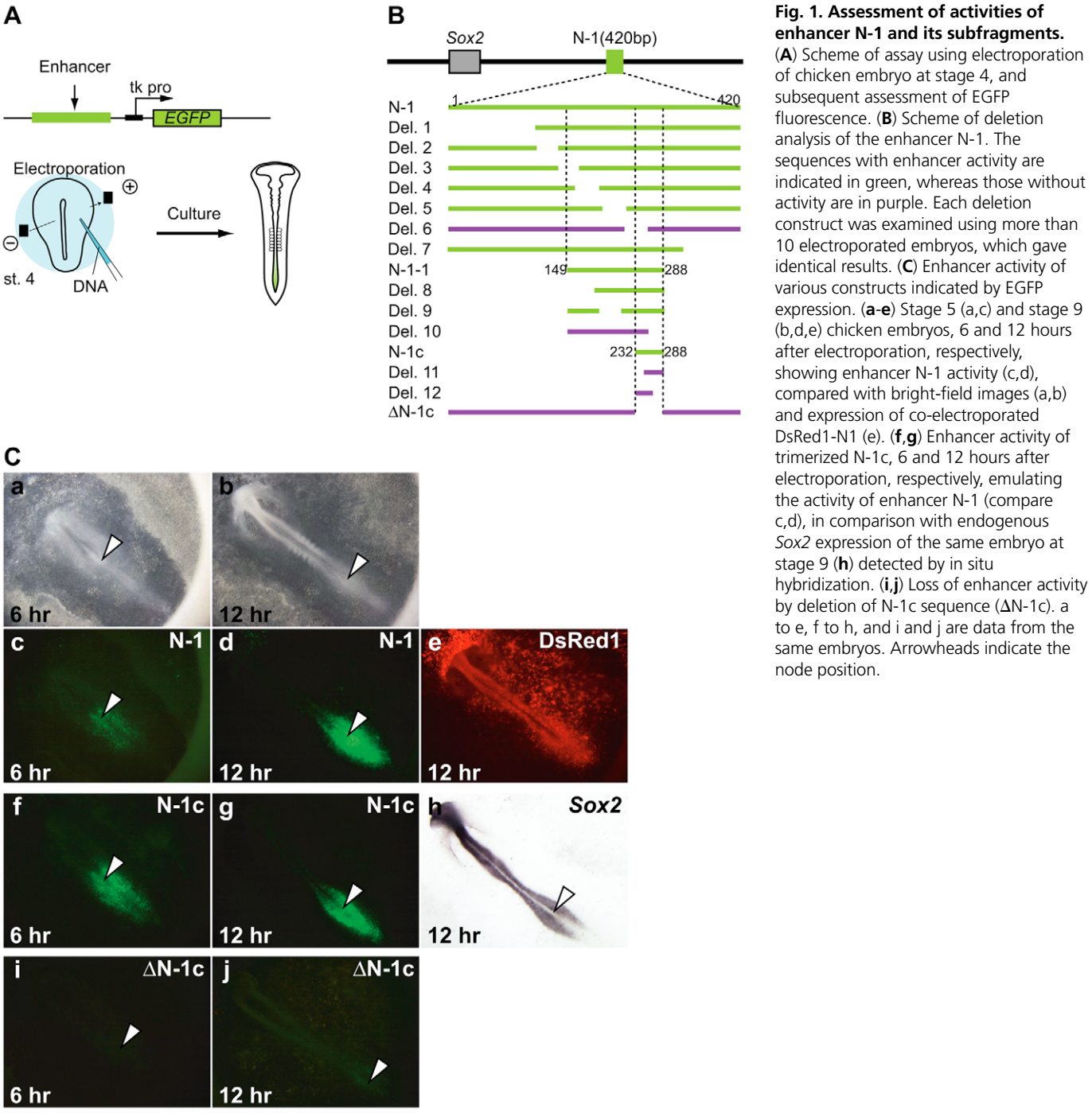

Fig. 1. Assessment of activities of enhancer N-1 and its subfragments. (**A**) Scheme of assay using electroporation of chicken embryo at stage 4, and subsequent assessment of EGFP fluorescence. (**B**) Scheme of deletion analysis of the enhancer N-1. The sequences with enhancer activity are indicated in green, whereas those without activity are in purple. Each deletion construct was examined using more than 10 electroporated embryos, which gave identical results. (**C**) Enhancer activity of various constructs indicated by EGFP expression. (**a**-**e**) Stage 5 (a,c) and stage 9 (b,d,e) chicken embryos, 6 and 12 hours after electroporation, respectively, showing enhancer N-1 activity (c,d), compared with bright-field images (a,b) and expression of co-electroporated DsRed1-N1 (e). (**f**,**g**) Enhancer activity of trimerized N-1c, 6 and 12 hours after electroporation, respectively, emulating the activity of enhancer N-1 (compare c,d), in comparison with endogenous *Sox2* expression of the same embryo at stage 9 (**h**) detected by in situ hybridization. (**i**,**j**) Loss of enhancer activity by deletion of N-1c sequence (ΔN-1c). a to e, f to h, and i and j are data from the same embryos. Arrowheads indicate the node position.

Canonical Wnt signal-dependent activation of enhancer N-1c through blocks A and B

Blocks A and B each contain a Lef1/Tcf factor-binding consensus sequence (G/C)TTTGA(A/T) (Giese et al., 1991) (Fig. 3A). In an electrophoretic mobility shift assay (EMSA) using recombinant chicken Lef1 (cLef1), Block A and Block B sequence probes formed a complex that was specifically competed by an excess of the same sequences but not by the mutated sequences (M2/M6), and was disrupted by anti-Lef1 antibodies (Fig. 3B, only data with probe B are shown). In contrast, the Block C sequence did not bind cLef1 in EMSA (data not shown). Using nuclear extracts from the node-proximal tissues of stage 8 chicken embryos, Block A and Block B probes also formed a complex of the same size, which was competed specifically by a normal sequence and largely eliminated by anti-Lef1 antibodies (Fig. 3B). Thus, the major portion of the specific binding protein of Blocks A and B in the node area tissue is accounted for by cLef1. This indicates redundant functions of Blocks A and B, which is supported by the results obtained using Mut-AB double mutations, which largely removed the enhancer activity (Fig. 2Bd).

The tissue area with the enhancer N-1c activity expresses *cWnt8c* (Hume and Dodd, 1993; Lawson et al., 2001; Skromne and Stern, 2001), and the Lef/Tcf factor genes *cLef1* and *cTcf1* (Schmidt et al., 2004; Skromne and Stern, 2001) (see Fig. S1 in

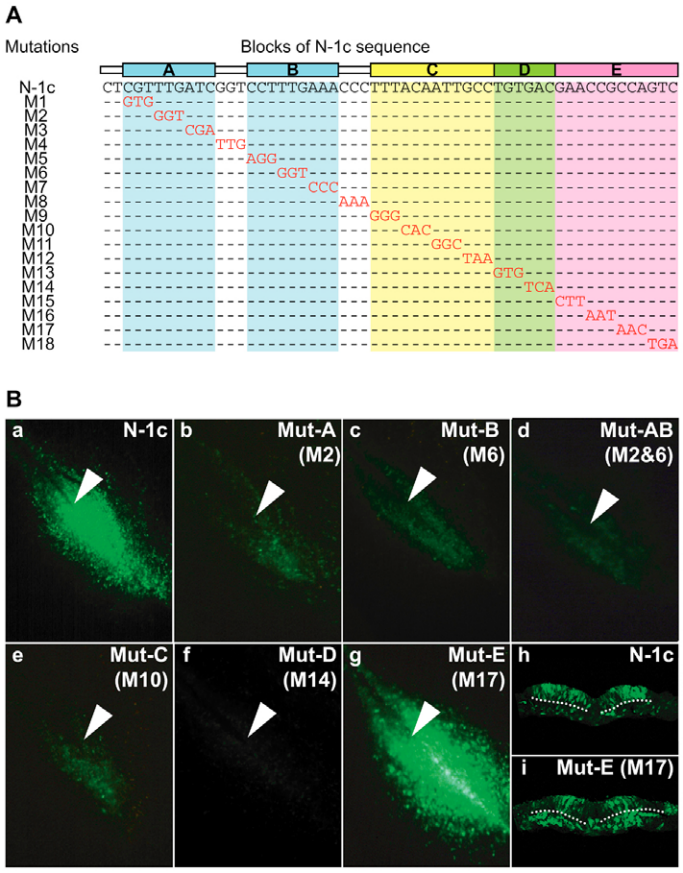

Fig. 2. Mutation analysis of the N-1c sequence. (**A**) Mutations introduced into the N-1c sequence and identified functional blocks. (**B**) (**a-g**) EGFP fluorescence indicating activity of wild-type and mutant enhancers in dorsal views of chicken embryos. Mutations are indicated in parentheses. Mut-A (b), Mut-B (c) and Mut-C (e) mutations resulted in a decrease in the enhancer activity and Mut-AB (d) and Mut-D (f) resulted in a large loss of the activity, whereas Mut-E (g) caused a widening of the area of tissue where the enhancer was active. (**h,i**) Cross section through the node of embryo, electroporated with wild-type N-1c and Mut-E constructs, respectively. Arrowheads indicate the node position. Each mutant enhancer was examined using more than 10 electroporated embryos, which all gave identical results.

supplementary material). *cTcf1* may account for the anti-Lef1-resistant fraction of the complex of the same mobility observed in EMSA, using tissue nuclear extract (Fig. 3B).

The above observations indicate that the activity of the enhancer N-1c depends on the canonical Wnt signaling pathway (Nusse, 1999), in which β-catenin is stabilized and allowed to interact with Lef1/Tcf1 bound to Blocks A and B. To test this model, a tkEGFP reporter vector carrying the enhancer N-1c trimer was co-electroporated with expression vectors for artificially stabilized β-catenin, or for the Wnt antagonist Dkk1 (Glinka et al., 1998) or Sfrp1 (Uren et al., 2000). The expression of stabilized β-catenin activated the enhancer N-1c throughout the neural plate (Fig. 3Cb), in contrast to the normal situation where the enhancer activity is quickly turned off as the neural plate is formed (Fig. 3Ca). The expression of Wnt antagonists, Dkk1 and Sfrp1, severely attenuated the activity of the enhancer N-1c (Fig. 3Cc,d), confirming the dependence of the enhancer on Wnt signal. The successful electroporation of DNAs throughout the embryo was confirmed by the expression of co-electroporated DsRed1 (Fig. 3Cc,d, insets).

Involvement of FGF signals in activation of enhancer N-1c

The possible involvement of FGF signals in the activation of the enhancer N-1c was investigated, given its implication in neural plate specification (Storey et al., 1998; Streit et al., 2000; Streit and Stern, 1999b; Wilson and Edlund, 2001; Wilson et al., 2000; Wittler and Kessel, 2004). *Fgf8* is expressed in the proximal streak region (Chapman et al., 2002; Charrier et al., 1999; Karabagli et al., 2002; Streit and Stern, 1999a) (see Fig. S1 in supplementary material), and it encodes multiple variant forms of FGF8 as a consequence of alternative splicing of the transcript, of which FGF8b is a strongly active form (Sato and Nakamura, 2004). cFGF8b was overexpressed in COS7 cells labeled by DsRed1 expression, and a clump of these cells was placed on electroporated chicken embryos (Fig. 4). The enhancer N-1c was activated in the area abutting the FGF8b source, in addition to the node-proximal region (Fig. 4A,B). This FGF8b-dependent activation of the enhancer N-1c was also observed using recombinant FGF8b-soaked beads, in various areas of the electroporated embryo including the area opaca (Fig. 4D). Normal COS7 cells, cells expressing the attenuated variant FGF8a, or an FGF-free bead had no such effect (Fig. 4C and data not shown). Thus, the activation of the enhancer N-1c involves FGF signals in addition to Wnt signals.

Synergy of Wnt and FGF signals in activation of enhancer N-1c

The interaction of Wnt and FGF signals in the activation of the enhancer N-1c was analyzed using transfection of 10T1/2 mesenchymal stem cells (Pinney and Emerson, 1989), where the Wnt- and FGF-dependent activation of the enhancer was clearly demonstrated. 10T1/2 cells were transfected with a firefly luciferase construct activated by the trimeric N-1 core enhancer. Cotransfection with the *Wnt3* expression vector activated the enhancer two- to threefold (Fig. 5A). The analogous activation of the enhancer N-1c was also observed using *cWnt8c* expression (data not shown). In mouse embryos, *Wnt3* is expressed in the area surrounding the node, in a pattern analogous to that of *Wnt8c* (Liu et al., 1999). The expression of *cFgf8b* by cotransfection also activated the enhancer threefold (Fig. 5A). Recombinant FGF2, FGF4 or FGF8b proteins added to the culture medium at 100 ng/ml activated the enhancer analogously, but EGF, even up to 400 ng/ml, had no such effect. Thus, the enhancer is activated by Wnt (e.g. Wnt3/Wnt8c) and FGF (e.g. FGF8b) signals.

The possible synergy of these two signals was examined by cotransfecting the *Wnt3* and *Fgf8b* expression vectors with the trimeric N-1c-bearing luciferase reporter. When the two signals acted together, the activation level was highly augmented (ninefold activation), indicating a strong synergistic effect (Fig. 5A).

The effect of mutations of each Block in response to Wnt and FGF signals of the trimeric N-1c enhancer was investigated, in order to clarify the molecular basis of synergy between these signals. When Wnt3 was expressed by cotransfection of the expression vector (Fig. 5Ba), the wild-type and Mut-C enhancers were activated by two- to threefold, whereas the Mut-AB double mutant enhancer did not respond to this exogenous Wnt signal, as expected from mutations in the Lef1/Tcf binding sequences. The response to exogenous Wnt3 was compromised in the Mut-E enhancer, and interestingly the Mut-D enhancer lost the Wnt response.

To determine the element responsible for FGF-dependent enhancer activation, trimerized subfragments of the N-1c sequence lacking Blocks A and B were examined to determine whether they act as an FGF-responsive enhancer (Fig. 5C). Subfragments for

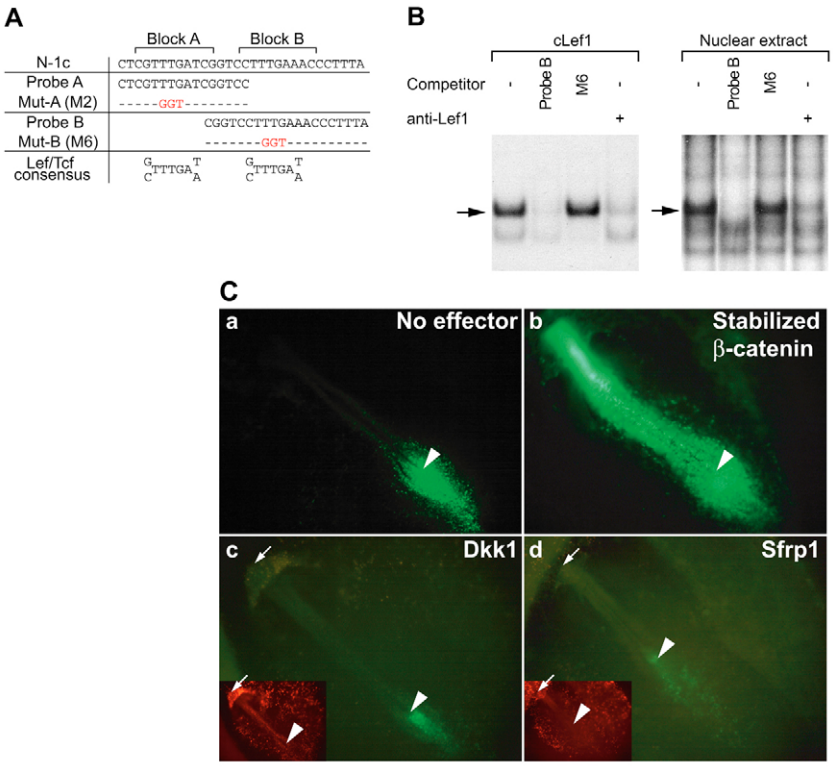

Fig. 3. Wnt signal-dependent regulation of enhancer N-1c. (**A**) Mutations and probe sequences for Blocks A and B, used for EMSA, and comparison with Lef1/Tcf-binding consensus. (**B**) EMSA performed using a sequence B probe, with recombinant cLef1 (left) or embryo nuclear extract (right). Arrows indicate cLef1-probe complex. (**C**) Effect of exogenous expression of stabilized β-catenin (**b**), Wnt antagonists Dkk1 (**c**) or Sfrp1 (**d**) on enhancer N-1c activity. Successful electroporation of a broad embryonic area was confirmed by DsRed1 expression (insets). Anterior margin of the embryo and the node position are indicated by an arrow and an arrowhead, respectively. At least six electroporated embryos were used to examine the effects of these molecules, which all gave identical results.

Blocks C-D-E (data not shown) or Blocks D-E were equally (10-fold) activated by exogenous FGF8b expression. When Block D was mutated in the D-E subfragment, the enhancer activity was lost, while Block E mutation only moderately decreased the activity. From these results, we identified Block D as the element responsible for the activation of the enhancer N-1c by FGF signals. In confirmation of this, the expression of FGF8b by transfection activated the wild-type and various mutated versions of N-1c by two- to threefold, except in the case of Mut-D, which showed no response (Fig. 5Bb).

The simultaneous supply of exogenous Wnt3 and FGF8b by the cotransfection of expression vectors activated the Mut-AB double mutant enhancer (trimeric N-1c) to the same level as FGF8b expression alone, confirming the Wnt response through Blocks A and B (Fig. 5Bc). Again, the Mut-D enhancer did not respond to the combined stimulation by Wnt3 and FGF8b. These observations indicate that although Wnt and FGF signals cooperate in the activation of the enhancer N-1c, the Wnt signal alone is not effective in inducing the activation. Mut-C and Mut-E enhancers responded to the combination of Wnt and FGF signals more weakly than the wild-type trimeric N-1c enhancer, suggesting the involvement of these blocks in functions enlarging the enhancer effect.

The addition to the culture medium of SU5402, a specific inhibitor of FGF receptor tyrosine kinases (Mohammadi et al., 1997), abolished the effect of exogenously expressed FGF (data not shown). The combined effect of exogenous Wnt3 and FGF8b derived from expression vectors, which otherwise activates the wild-type, Mut-C or Mut-E enhancers, was completely inhibited (Fig. 5Bd), strongly supporting the model that postulates the requirement of an FGF signal input for a Wnt signal to activate the enhancer. This also indicates that the activation of the enhancer by exogenous Wnt3 (Fig. 3A) depended on FGFs present in the culture medium or expressed endogenously.

Expression of Wnt and FGF signal components in chicken embryo

As described above, it has been reported that *cWnt8c* (Hume and Dodd, 1993; Lawson et al., 2001; Skromne and Stern, 2001) and *cFgf8* (Chapman et al., 2002; Charrier et al., 1999; Karabagli et al., 2002; Streit and Stern, 1999a) are expressed in the node-proximal streak region together with *cLef1* and *cTcf1* (Schmidt et al., 2004; Skromne and Stern, 2001) in the gastrulating chicken embryo. This was confirmed by the in situ hybridization of stage-matched embryos in comparison with the enhancer N-1c activity (see Fig. S1 in supplementary material). It is likely that cWnt8c and cFGF8b cooperate in the activation of the enhancer N-1c, and that the Wnt signal is mediated by cLef1 and cTcf1, rather than by cTcf3 or cTcf4.

Effect of BMP signals

As inhibitory effects of BMP signals on neural development and neural *Sox2* expression have been demonstrated in many experimental systems (Linker and Stern, 2004; Munoz-Sanjuan and Brivanlou, 2002; Stern, 2005; Streit and Stern, 1999b; Wilson and Edlund, 2001), we tested whether the modulation of BMP signals affects the activity of the enhancer N-1, although mutational analysis (Fig. 2) did not indicate a BMP-responsive element. In chicken embryos, BMP2, BMP4 and BMP7 are expressed in the streak region (Chapman et al., 2002; Linker and Stern, 2004; Streit and Stern, 1999a). The exogenous expression of the constitutive active (CA) form of the BMP receptor Alk6, mimicking a BMP signal, did not inhibit the enhancer N-1c, indicating that the activity of the enhancer N-1c is independent of BMP signals (Fig. 6C, lower panel). Under this condition, endogenous *Sox2* expression was severely down-regulated (Fig. 6C, upper panel), confirming the previous observation using BMP4 (Linker and Stern, 2004). The expression of either Noggin or the dominant-negative (DN) form of Alk6, lacking the cytoplasmic domain, did not affect the N-1c

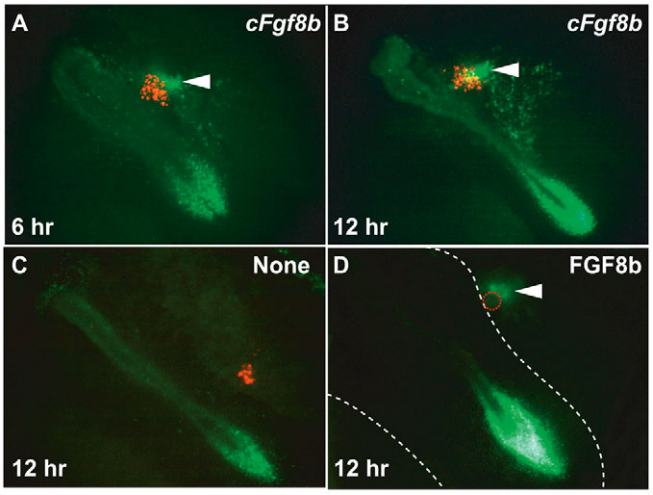

Fig. 4. Effect of ectopic administration of FGF8b on activity of trimeric N-1 enhancer in electroporated embryos. (**A,B**) A cFGF8b-expressing COS7 cell clump marked by DsRed1 expression was deposited in a distal location of an embryo immediately after electroporation. The same embryo 6 hours (A) and 12 hours (B) after electroporation, showing ectopic activation of the trimeric N-1c enhancer as indicated by the green fluorescence (arrowheads). (**C**) An embryo 12 hours after electroporation with deposition of normal COS7 cells. No ectopic activation of the enhancer was observed. (**D**) An FGF8b-soaked heparin bead (indicated by a red circle, soaked in 50 μg/ml FGF8b) deposited at a proximal site of area opaca of an electroporated embryo, activated the trimeric N-1c enhancer activity in the area opaca (arrowhead). The border between the area pellucida and area opaca is indicated by the white broken line. Six electroporated embryos were used for each experiment, which all gave essentially the same results.

enhancer activity (Fig. 6D,E, lower panels). Interestingly, however, the inhibition of BMP signals caused a posterior extension of the *Sox2*-expressing domain, which is otherwise arrested at the posterior margin, resulting in the matching of the domain with the activity of the enhancer N-1c (Fig. 6D,E, upper panels). This indicates that in the tissue posterior to the node, the activation of *Sox2* is inhibited by the BMP signal despite the activation of the enhancer N-1c. A corollary to this is that the activation of enhancer N-1c is a prerequisite for the activation of *Sox2* but this is not sufficient, and other conditions must be satisfied to induce the *Sox2* expression.

DISCUSSION

Multiple steps in genesis of posterior neural plate

In chicken embryos, *Sox2* expression is initiated concomitantly with node (organizer) formation at early stage 4, and marks the early neural primordium in chicken embryos (Chapman et al., 2003; Rex et al., 1997; Streit et al., 2000; Streit et al., 1997; Uchikawa et al., 2003). The enhancers N-1 and N-2 are first activated in the posterior and anterior domains, respectively, of node-proximal tissues (Uchikawa et al., 2003), followed by the activation of other *Sox2* enhancers including N-3 to N-5 presumably through autoactivation loops of *Sox2* expression (Uchikawa et al., 2004). Subsequently, the SOX2 protein cooperates with POU factors to establish (Kamachi et al., 2000; Tanaka et al., 2004) and maintain (Bylund et al., 2003; Graham et al., 2003) the neural primordial cell state. In this study, we analyzed the enhancer N-1, first focusing on signaling systems directly involved in the regulation of its activation.

The comparison of the enhancer N-1c activity with the posterior end of the *Sox2*-expressing neural plate indicates that there is a domain posterior to the node where the enhancer N-1c is active but *Sox2* is not expressed (Fig. 6). Earlier cell tracing experiments indicate that this domain contains multipotential precursors for neural, epidermal and mesodermal lineages (Brown and Storey, 2000; Diez del Corral and Storey, 2004), indicating that the activation of the enhancer N-1c and the initiation of *Sox2* expression mark two distinct steps leading to the specification of the posterior neural plate.

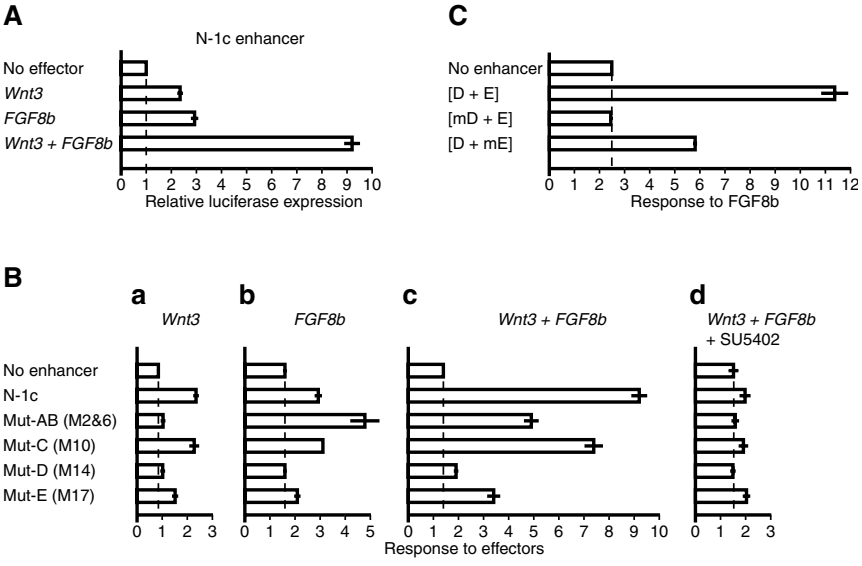

Fig. 5. Functional cooperation of Wnt and FGF signals demonstrated by transfection of 10T1/2 cells with firefly luciferase reporter bearing trimeric N-1c enhancer. (**A**) Activation of the trimerized N-1c enhancer by exogenous Wnt3 and cFGF8b. Wnt3 and cFGF8b were expressed by cotransfection of relevant expression vectors. Firefly luciferase expression was normalized using the expression of co-transfected *Renilla* luciferase. (**B**) Response of mutant forms of enhancer N-1c to FGF and Wnt signals. Luciferase expression level without stimulation with an effector was designated as 1. The unstimulated activity of trimeric N-1c enhancers compared with enhancer-less luciferase reporter were: N-1c (wild type), 5.0±0.4; Mut-AB, 6.4±0.3; Mut-C, 4.9±0.1; Mut-D, 2.8±0.2; Mut-E, 7.4±0.7 (mean ± s.e.m.). (**a**) Cotransfection with *Wnt3* expression vector. (**b** Cotransfection with *Fgf8b* expression vector. (**c**) Cotransfection with *Wnt3* plus *Fgf8b* expression vectors. (**d**) The same as c, but with addition of SU5402 immediately after transfection. (**C**) FGF-responsiveness of trimeric [D+E] subfragment of the enhancer N-1c. The activation levels with exogenous FGF8b derived from cotransfected expression vector are compared. The subfragment region of N-1c sequence is shown in Fig. 2A. Transfection was done at least in triplicate samples per experiment, and each panel shows a representative set of data derived from an experiment.

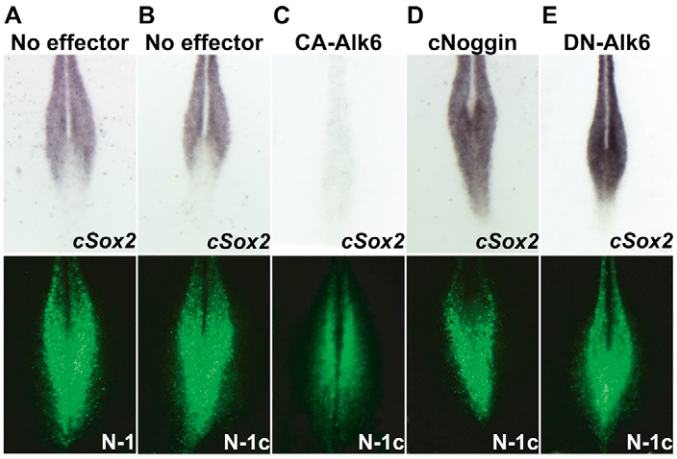

Fig. 6. Effects of BMP signals on activity of enhancer N-1c trimer and *Sox2* expression. (Top) Embryonic *Sox2* expression (in situ hybridization) and (bottom) activity of enhancer N-1/N-1c (EGFP fluorescence) are compared using the same embryos. (**A**) Enhancer N-1. (**B**) Enhancer N-1c (trimer). (**C**) Effect of misexpression of constitutive-active (CA) Alk6, which wiped out *Sox2* expression. (**D**,**E**) Effect of misexpression of BMP antagonists cNoggin (D) or dominant-negative (DN) Alk6 (E), posteriorly extending the *Sox2* expression in a manner matching the activity of enhancer N-1c.

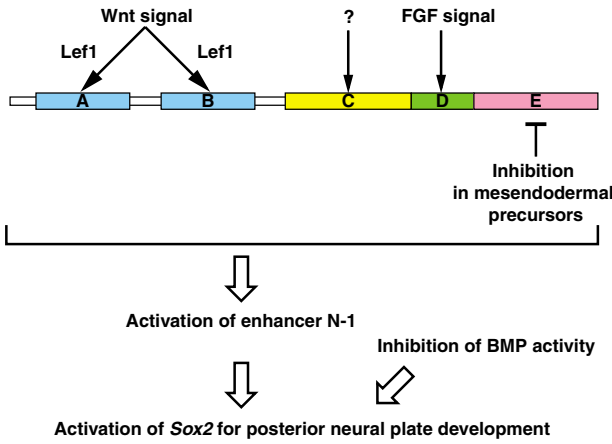

Fig. 7. Model of organization of functional Blocks of enhancer N-1c. Enhancer N-1c is activated by Wnt and FGF signals and repressed in mesendodermal precursors, and subsequent steps plus inhibition of BMP activity leading to genesis of posterior neural plate.

The signals regulating these steps, as indicated in this study are summarized in Fig. 7. As the first step in posterior neural plate generation, the enhancer N-1c is activated by the synergism of Wnt (e.g. Wnt8c) and FGF (e.g. FGF8b) signals derived from the node-primitive streak region (see Fig. S1 in supplementary material). This then leads to the activation of *Sox2* expression in the region surrounding the node, possibly as a consequence of relief from a BMP-dependent repressing effect, which occurs independently of the regulation of the enhancer N-1c (Figs 6, 7). An important point here is that these steps involving Wnt-dependent activation of the enhancer N-1c constitute a transient process for setting off *Sox2* expression, but do not participate in the widespread stable maintenance of the *Sox2* expression through the neural plate. Once *Sox2* expression is activated, the Wnt signal and Wnt-dependent activity of the enhancer N-1c are quickly turned off in the neural plate area after the node has migrated posteriorly. The Wnt antagonist Sfrp-1, expressed abundantly in the node-neural plate area (Esteve et al., 2000), can act as the major player for shutting down the Wnt signal. Therefore, the Wnt-independent *Sox2* maintenance mechanism must be in operation after the activating action of the enhancer N-1c. The enhancers N-3 to N-5 that are active in the later stages of neural plate development (Uchikawa et al., 2003; Uchikawa et al., 2004) may be responsible for the augmentation and maintenance of the once activated *Sox2* expression in the posterior end of the developing neural plate.

Thus, the activation of the enhancer N-1c marks the initiation of the process leading to the *Sox2* expression for the posterior neural plate development, but it is clear that other conditions must be satisfied for the stable *Sox2* expression to occur, including the inhibition of BMP signals and the proper regulation of Wnt signals. Indeed, the local administration of FGF8b at various ectopic sites of stage 4 embryos readily activates the enhancer N-1c (Fig. 4), but this does not necessarily activate the endogenous *Sox2* expression (Streit and Stern, 1999a).

Synergistic activation of core enhancer N-1c by FGF and Wnt signals

Through a series of deletion analyses of the enhancer N-1, a 56-bp core enhancer sequence, N-1c, was identified. The N-1c trimer emulated the activity of the enhancer N-1. Mutational analysis of the N-1c sequence identified five functional Blocks A to E, and provided clues to the mechanism initiating *Sox2* expression. Blocks A and B function as Wnt-responsive twin elements through the binding of cLef1/cTcf1. Block D serves as the FGF-responsive element. Block E is involved in inhibiting enhancer activity in the mesendodermal precursors. The expression of *cWnt8c* and *cFgf8* in the node and proximal streak area (Chapman et al., 2002; Charrier et al., 1999; Hume and Dodd, 1993; Lawson et al., 2001; Skromne and Stern, 2001) (supplementary Fig. S1) is consistent with their providing major signaling molecules for the activation of the enhancer N-1c.

Transcription factors binding to Block D and mediating the FGF signals have not yet been fully characterized. The Block D sequence contains a half site bZIP protein binding sequence TGAC (Kataoka et al., 1994), and DNA binding protein screening using a bacteriophage cDNA library (southwestern screening) also identified bZIP proteins binding to the Block D sequence (our unpublished results). As a subset of bZIP proteins are known to be regulated by FGF signals (Dailey et al., 2005), the proteins of this class are strong candidate mediators of the signals for the Block D-dependent enhancer regulation.

Although FGF and Wnt signals synergistically activate the enhancer N-1c, their interdependence is not equal, as indicated by transfection experiments using 10T1/2 mesodermal stem cells (Fig. 5). Under transfection conditions, although FGF signals by themselves show the potential to activate the enhancer, albeit at a low level, Wnt signals alone fail to activate the enhancer when FGF signal input is shut off, either by the receptor kinase inhibitor SU5402, or by mutations of Block D. This relationship between the FGF and Wnt signals in the activation of the N-1c enhancer is also reflected by the observations made using electroporated embryos. Mut-D mutant enhancers defective in response to the FGF signal

have no enhancer activity, whereas the Mut-AB enhancer defective in the Wnt response retains a low residual enhancer activity (Fig. 2B), and FGF8b alone is somehow capable of activating the enhancer N-1c locally, when applied at stage 4 (Fig. 4).

Segregation of neural and mesendodermal precursors

Mutational analysis of the enhancer N-1c identified Block E, the inactivation of which caused broadening of the enhancer-active cell population to mesendodermal precursors (Fig. 2Bi). After the ingression of the cells through the node-proximal streak area, the wild-type enhancer N-1c loses its activity, but the Block E mutant of the enhancer N-1c also displays its activity in the mesendodermal cell layers (Fig. 2Bi).

The Block E-dependent repression of the enhancer N-1c in mesendodermal precursors may inhibit neural development from these precursors. Particularly intriguing are the reports of mutant mouse embryos or chimeras lacking *Wnt3a* (Takada et al., 1994), *Tbx6* (Chapman and Papaioannou, 1998) or *Fgfr1* (Ciruna et al., 1997), where supernumerary neural tubes develop at the expense of mesodermal tissues. It is possible that in these mutant tissues, the Block-E-mediated repression of the enhancer N-1c fails. The presence of the cells having the capacity to produce either neural plate or mesoderm in the enhancer N-1c-active region (Brown and Storey, 2000; Catala et al., 1996; Diez del Corral and Storey, 2004) reinforces this model.

Not only in the *Tbx6* mutant, but also in other mutants, a connection with T-box factor activities is suggested in the production of extra neural tubes: Wnt3a regulating the *Brachyury* gene (Yamaguchi et al., 1999), and analogous cell motility defects shared by the *Brachyury* and *Fgfr1* mutant cells (Ciruna et al., 1997).

The DNA sequence of Block E, however, deviates from the T-box binding consensus AGGTGT (Conlon et al., 2001; Kispert and Herrmann, 1993), rendering unlikely the direct interaction of T-box proteins to the enhancer N-1c sequence. In the luciferase reporter assay, cotransfection of full-length *Tbx6L* (a chicken T-box protein expressed in mesendodermal precursors analogous to mouse *Tbx6* (Chapman et al., 1996; Knezevic et al., 1997) did not repress the enhancer N-1c (data not shown). T-box proteins may participate in Block-E-mediated repression of the enhancer N-1c through the regulation of a downstream gene.

Signals for anterior and posterior neural plate development

In this study, using *Sox2* expression as a landmark of neural primordium development in early-stage chicken embryos, the processes involved in the genesis of neural plate were investigated where previously identified *Sox2* enhancers (Uchikawa et al., 2003) provided the essential clues. It was clearly demonstrated that Wnt and FGF signals converge to synergize the activation of the enhancer N-1c that is involved in posterior neural plate development.

In previous studies, the broad involvement of FGF activity in the genesis of the neural primordium was demonstrated (Mathis et al., 2001; Sheng et al., 2003; Storey et al., 1998; Streit et al., 2000; Wilson et al., 2000; Wittler and Kessel, 2004). This study clearly demonstrated a direct interaction of the FGF signal with the molecular processes specifying the neural plate, namely the activation of *Sox2* expression through the regulation of the enhancer N-1 (Fig. 7). By contrast, the contribution of Wnt signals to neural development appears to be highly context-dependent in terms of assay system, timing of development and the level of Wnt activity (see Stern, 2005; Wilson and Edlund, 2001). The most highlighted effects of Wnt signals have been the inhibition of neural development and the posteriorizing effect, but this study clearly demonstrated a new important contribution of Wnt signals to neural development; Wnt signals are directly involved in the genesis of the neural plate, through the enhancer N-1-mediated activation of *Sox2* expression in cooperation with the FGF signal (Fig. 7).

In a study using epiblastic cells of chicken embryos isolated before gastrulation and cultured in vitro (Wilson et al., 2001), endogenous FGF activity that was sensitive to SU5402 was an absolute requirement for the expression of neural traits such as *Sox2*, but the high exogenous activity of Wnt (Wnt3A) was inhibitory, and instead promoted mesodermal differentiation. This emphasizes the inhibition of neural development by Wnt signals, despite the involvement of Wnt signals in the activation of *Sox2* expression demonstrated in the present study. An important factor here may be the strength of the Wnt signal, which may act differentially depending on its level. Indeed, Wilson et al. (Wilson et al., 2001) showed that a condition of low Wnt signal plus an endogenous FGF signal in the presence of Noggin promotes *Sox2* expression, in contrast to the neural inhibiting activity at a high Wnt level. Linker and Stern (Linker and Stern, 2004) also showed that mere removal of the Wnt signal, even in the presence of FGF signal, is not sufficient for initiating the program for the neural development of analogous cells. Despite this complexity, we focused the present study on the mechanisms underlying the activation of the enhancer N-1c, and we were successful in determining the step in which Wnt signals directly participate in the initiation of posterior neural plate development.

There are basically two different models for the deriving distinct characters of anterior and posterior neural plate. One model, the activation-transformation model, initially proposed for amphibian embryos by Nieuwkoop (Nieuwkoop et al., 1952; Nieuwkoop and Nigtevect, 1954), holds that the anterior character of the neural plate is induced first, and that of the posterior neural plate is then derived under the influence of 'caudalizing/posteriorizing factors'. However, the observations made in this study are generally in favor of the second model, which states that the process for inducing neural plate formation differs between the 'anterior' and 'posterior' CNSs (Chapman et al., 2003; Pera et al., 1999; Wilson and Houart, 2004; Withington et al., 2001). In this study, analysis of the enhancer N-1 revealed the signaling systems involved in the regulation of posterior neural plate development.

The Wnt-dependent posteriorizing effect is exerted in the context of an embryo body axis (Yamaguchi, 2001), and such an effect on an already established neural plate has been reported (Nordstrom et al., 2002). However, it is possible that some of the Wnt-dependent effects on early neural plate development, which were classified into the category of 'posteriorizing effect' are actually carried out through the activation of the enhancer N-1. The availability of the enhancer N-1 as a molecular probe will help unravel the complexity of Wnt effects in the early processes of inducing the formation and specification of the neural plate.

The direct involvement of FGF signal (a 'posteriorizing' signal in the activation-transformation model) in inducing the formation of the posterior neural plate reported for zebrafish embryos (Agathon et al., 2003; Kudoh et al., 2004) also corroborates the model presented here (Fig. 7).

As the analysis of the enhancer N-1 has revealed intricate interactions among signaling systems involved in the genesis of the posterior neural plate, an analogous analysis of the enhancer N-2, such as investigation of its interaction with nuclear factors

and signal responses (Catena et al., 2004), will contribute remarkably to our understanding of the regulation of anterior neural plate development. The use of knockout mouse embryos lacking either enhancer N-1 or N-2, or both, could further the analysis of the involvement of these enhancers and the *Sox2* gene in this process; this is a project currently under way in our laboratory.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/2/297/DC1

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