Published in final edited form as:

Dev Dyn. 2011 July ; 240(7): 1756–1768. doi:10.1002/dvdy.22675.

A combination of enhancer/silencer modules regulates spatially restricted expression of *cadherin-7* in neural epithelium

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Abstract

The spatially restricted expression of cadherin-7 to the intermediate domain of the neural epithelium and in migrating neural crest cells during early neural development is potentially regulated by multiple signaling inputs. To identify the regulatory modules involved in regulation of *cadherin-7*, evolutionary conserved non-coding sequences in the *cadherin-7* locus were analyzed. This led to the identification of an evolutionary conserved region of 606 bp (ECR1) that together with the *cadherin-7* promoter recapitulates endogenous *cadherin-7* expression in intermediate neural tube, spinal motor neurons, interneurons, and dorsal root ganglia. Deletion analysis of ECR1 revealed a 19 bp block that is essential for ECR1 enhancer activity, while two separate blocks of 10 bp and 12 bp were found to be essential for ECR1 silencer activity in the dorsal and ventral neural epithelium respectively. Together these data provide an insight into tissue-specific regulatory regions that might be involved in regulation of cadherin-7 gene expression.

Keywords

Cadherin; gene regulation; evolutionary conserved regions; enhancer; silencer; electroporation; transcription factor; neural epithelium; embryo

Introduction

Cadherin proteins mediating cell-cell recognition and adhesion play a fundamental role in the morphogenesis of the embryo (Hableib and Nelson, 2006). In the central nervous system (CNS), classical cadherins are expressed in distinctive spatiotemporal patterns and involved in delineation of boundaries, facilitation of axonal outgrowth and guidance, and selective cell sorting (Redies et al., 2003; Suzuki and Takeichi, 2008). These functions provide a combinatorial cadherin code for establishment of the complex circuitry of the nervous system (Price, 2002; Krishna-K et al., 2011). Both type I and type II classical cadherins are expressed in the developing spinal cord. N-cadherin is expressed throughout the neuroepithelium (Hatta and Takeichi, 1986), while type II cadherins such as cadherin -6B, 7, 8, 10, 11, 12, 20, and PB-cadherin/cadherin-22 are expressed in diverse and overlapping domains, including the motor neuron pools (Kimura 1995, Nakagawa and Takeichi 1995; Kitajima 1999; Price 2002; Suzuki 2007; Chalpe et al., 2010).

Cadherin-7 is a type II classical cadherin with a dynamic spatiotemporal expression pattern during neural development. At the trunk axial level, cadherin-7 is expressed in the dorsal domain of the basal plate (intermediate domain of the neural epithelium) and in migrating

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neural crest cells (Nakagawa and Takeichi, 1995, 1998; Ju et al., 2004). During later stages of development, cadherin-7 is expressed in clusters of motor neurons in the lateral motor column, the floor plate, and the dorsal root ganglia (DRG) (Nakagawa and Takeichi, 1995, 1998; Price, 2002; Ju et al., 2004). Functionally, cadherin-7 has been suggested to be involved in guiding neurons into appropriate tectofugal tracts in the brain (Treubert-Zimmerman, 2002), promoting the axonal outgrowth of cranial motor neurons (Barnes et al., 2010), and sorting Purkinje cell progenitors to the correct domain (Luo et al., 2004). As cadherins appear to be one of several classes of effector molecules for the signaling pathways that orchestrate patterning of the CNS, determination of the regulation of cadherin-7 expression pattern in the neural epithelium is of substantial interest. Transcription factors that specify neuronal cell fates in the developing spinal cord are expected to be involved in regulation of cadherin-7.

Following neural specification, dorso-ventral patterning of the neural tube involves the interplay of multiple inputs. BMP and Wnt signaling gradients from the dorsal neural tube interact with a Shh signaling gradient in the ventral neural tube that originates from the notochord and floorplate. These signaling pathways regulate the cell type diversity by interactions of the class I and II transcription factors that specify neural progenitor cell fates along the dorso-ventral axis (Jessell, 2000). In the neural epithelium, Shh and Pax7 have been implicated to affect cadherin-7 expression (Luo et al., 2006). Pax7 is a class I transcription factor expressed in the dorsal neural tube and its ventral border abuts the dorsal border of the cadherin-7 expression domain. Ectopic expression of Pax7 inhibits cadherin-7 expression in the neural epithelium (Luo et al., 2006). Ventrally, a gradient of Shh signaling has been associated with regulation of cadherin-7 expression in the intermediate domain of the neural epithelium. In the ventral neural tube, Shh signaling induces cadherin-7 expression at low concentrations while inhibiting its expression at high concentrations (Stamatakis et al., 2005; Luo et al., 2006). Interestingly, expression of cadherin-7 in the motor neurons was not affected by manipulations of Shh and Pax7, indicating that cadherin-7 expression in the neural epithelium is subject to complex regulation (Luo et al., 2006). Other factors known to be involved in patterning of the neural epithelium also have an impact on cadherin-7 expression. Cadherin-7 was upregulated in migrating neural crest cells in response to ectopic Wnt activation (Chalpe et al., 2010). Furthermore, the upregulation of cadherin-7 expression by ectopic expression of the FoxD3 and SoxE group transcription factors in the neural epithelium has been well documented (Dottori et al., 2001; Cheung and Briscoe, 2003; Cheung et al., 2005; McKeown et al., 2005). The studies described above have given some insight into the regulation of *cadherin-7*; however, a direct regulation of *cadherin-7* gene expression has not yet been identified.

To understand the regulation of cadherin-7 expression during neuroepithelial development, genomic regions of cadherin-7 with evolutionary conserved sequences containing conserved transcription factor binding sites (TFBS) were screened and characterized. Evolutionary conserved regions (ECRs) have previously been identified to play a functional role in regulation of N-cadherin (Inoue et al., 2008), cadherin-6 (Matsumata et al., 2005), and other developmentally important genes (Uchikawa et al., 2004; Bagheri-Fam et al., 2006; Sakai et al., 2005; Ishihara et al., 2008; Betancur et al., 2010). Using evolutionary conservation analysis, nine ECRs were screened for spatially-specific regulatory activity and ECR1 was identified as the primary regulatory region. A combination of ECR1 and the cadherin-7 promoter was able to recapitulate endogenous expression of cadherin-7 in the neural epithelium, DRG, and spinal motor neurons. Further characterization of ECR1 revealed the presence of a 19 bp enhancer sequence and two blocks of 10 bp and 12 bp silencer sequences that are required for its regulatory activity. These enhancer/silencer modules provide specific sites for identification of TFBS that are required for regulation of *cadherin-7* expression.

Results

The *cadherin-7* promoter is unable to recapitulate the endogenous gene expression pattern

To identify the regulatory regions required for chicken *cadherin-7* expression in the neural epithelium, we initially characterized the promoter region. Genomatix tools identified the putative promoter region as a 600 bp sequence upstream of *cadherin-7* exon-1 with conserved TFBS and essential promoter modules (Fig. 1A). A 1 kb sequence (Pro) encompassing the 600 bp predicted promoter region was functionally tested in the promoterless vector, pPL-EGFP, and the minimal essential promoter module was mapped to a proximal Pro1 (451 bp) subfragment upstream of exon-1. The promoter constructs were electroporated into trunk neural epithelium of stage 11–12 embryos and screened for EGFP fluorescence at 24 hrs and 48 hrs after electroporation. Pro1 drove EGFP expression in the entire neural epithelium and in a few migrating neural crest cells (Fig. 1B).

In the neural epithelium, the endogenous *cadherin-7* expression is spatially restricted to the intermediate domain of the neural epithelium (Fig. 1C) during stages 16–22. The *cadherin-7* expression pattern is consistent at both mRNA and protein levels in the neural epithelium and the migrating neural crest cells during these stages of development (Fig 1C). However, the identified *cadherin-7* promoter drives the reporter gene expression throughout the neural epithelium and is not restricted to the intermediate domain of the neural epithelium observed at stage 17 (compare figure 1B and 1C). Based on the promoter analysis, it is evident that additional regulatory modules are required for regulation of the promoter activity in the neural epithelium.

Identification of evolutionarily conserved non-coding sequences in the *cadherin-7* locus

To identify potential regulatory sequences that control tissue-specific expression of *cadherin-7*, conserved non-coding sequences were analyzed in the *cadherin-7* locus. Conserved non-coding sequences between mammals and other species such as chicken and zebrafish have been reported to possess tissue-specific regulatory activity (Uchikawa et al., 2003; Matsumata et al., 2005; Inoue et al., 2008; Ishihara et al., 2008). The conserved non-coding sequences were identified using Evolutionarily Conserved Region (ECR) Browser between chicken and seven other mammalian species (human, mouse, rat, monkey, dog, cow and possum). Nine ECRs conserved between chicken and at least three other species and containing conserved TFBS were selected for functional analysis. The nine ECRs are located in the intergenic, intronic, and untranslated regions (UTRs) of the *cadherin-7* locus (Fig. 2). The ECRs were screened for potential enhancer and silencer activity using two different reporter vectors. Enhancer activity was tested in a minimal promoter tk-vector, ptkEGFPv2 (Uchikawa et al., 2003) and silencer activity was tested in the *cadherin-7* promoter vector, pC7Pro1EGFPv2.

Identification of ECR activity in chicken embryo

Out of the nine ECRs identified above, regulatory activity was detected only in ECR1 and not detected in ECRs 2–9 in either the neural epithelium or in the migrating neural crest cells at the trunk level in embryos examined during stages 16–25 (data not shown). Table 1 summarizes the location of these ECRs on chicken chromosome 2 and their regulatory activity. ECR1 is a 606 bp fragment present 10.6 kb upstream of the exon-1 and highly conserved between chicken and seven other species. In the tk-vector, the enhancer activity of ECR1 was detected in the intermediate domain of the neural epithelium (Fig. 2B–a). ECR1 also demonstrated silencer activity, as it spatially restricted the *cadherin-7* promoter activity to the intermediate domain of the neural epithelium (Fig. 2B–b) that completely overlaps with the endogenous *cadherin-7* expression pattern (Fig. 2B–c). In addition, ECR1

and the *cadherin-7* promoter together drove EGFP expression in migrating neural crest cells (Fig. 2B–b).

ECR1 was further analyzed by a detailed fragment analysis to identify the enhancer and silencer modules. A schematic representation of the ECR1 fragment analysis is shown in Figure 3A and summarized in Table 2.

Identification of the fragment with enhancer activity—ECR1 was divided into three overlapping fragments ECR1(1–216), ECR1(195–427), and ECR1(401–606) that were analyzed for enhancer activity using the tk-vector. Only ECR1(195–427) showed enhancer activity and the enhancer sequence (ES) of ECR1(195–427) was mapped to an 85 bp fragment, ECR1ES (343–427) (Fig. 3B). The EGFP expression driven by ECR1ES (343–427) was observed in the entire neural epithelium suggesting the presence of the enhancer element and the absence of any silencer element in this 85 bp region. The other sub-fragments of ECR1(195–427) did not show enhancer activity (data not shown).

Identification of the fragment with silencer activity—From the three overlapping fragments of ECR1 [ECR1(1–216), ECR1(195–427) and ECR1(401–606)] the silencer activity was identified in the ECR1(195–427) fragment (Fig. 3C) using the *cadherin-7* promoter vector. The silencer sequence (SS) of ECR1(195–427) was mapped to a 60 bp ECR1SS (244–303) fragment with partial silencer activity in the ventral neural tube (Fig. 3C). The other sub-fragments of ECR1(195–427) did not show any silencer activity (data not shown). These data suggested that an additional ventral neural tube silencer element might be present in the ECR1(1–216) and/or the ECR1(401–606) fragment.

Deletion analysis of ECR1 to identify the minimum essential regulatory elements

To identify the additional silencer sequence and to establish the necessity of the enhancer and silencer sequences in the ECR1(195–427) fragment, the full length ECR1(1–606) was considered for deletion analysis. A schematic of deletion analysis is shown in Figure 4A and summarized in Table 3. Since there are ~500 overlapping TFBS identified by rVISTA in ECR1 that are conserved between chicken, human, mouse and rat, it is not possible to delete a particular sequence without disrupting the overlapping TFBS. Based on the maximum number of overlapping TFBS, four blocks of sequence were selected for deletion analysis. Each block contains a cluster of ~100 overlapping TFBS that are conserved between chicken, human, mouse, and rat. Individual deletions and combined deletions of all four blocks were designed to test their requirement for enhancer and silencer activity of the ECR1 fragment in tk-vector and *cadherin-7* promoter vector respectively.

Identification of enhancer block—Deletion of all four blocks (ECR1 del5) led to complete loss of activation by ECR1 in the tk-vector (Fig. 4B). Deletion of block1 (ECR1 del1) had no effect on the enhancer activity of ECR1 (Fig. 4B) while deletion of either block2 (ECR1 del2) or block4 (ECR1 del4) led to a reduction in enhancer activity of the ECR1, observed as reduced TagRFP fluorescence (Fig. 4B). Significantly, deletion of block3 (ECR1 del3) led to complete loss of enhancer activity of ECR1 (Fig. 4B) similar to the deletion of all four blocks (ECR1 del5). The presence of block3 in the core enhancer fragment, ECR1ES (343–427), also confirm the presence of minimal essential enhancer element of ECR1 in block3.

Identification of silencer block—Deletion of all four blocks (ECR1 del5) also led to a complete loss of the silencer activity of ECR1 in the *cadherin-7* promoter vector (Fig. 4C). Block2 (ECR1 del2) and block3 (ECR1 del3) deletions had no effect on the ECR1 silencer activity (Fig. 4C). Deletions of block1 (ECR1 del1) and block4 (ECR1 del4) led to loss of

ECR1 silencer activity in the dorsal and ventral neural tube, respectively (Fig. 4C). Block1 is located in the core silencer fragment, ECR1SS (244–304), that silences the *cadherin-7* promoter-driven EGFP expression in dorsal neural tube (Fig. 3C). This confirms block1 as the minimal essential dorsal neural tube silencer element of ECR1. Based on the fragment analysis of ECR1, the ventral neural tube silencer element was predicted to be present in the ECR1(1–216) and/or ECR1(401–606) fragment. The presence of block4 in the ECR1(401–606) fragment thus confirms block4 as the minimal essential ventral neural tube silencer element of ECR1. The loss of region-specific silencer activity by ECR1 due to deletion of block1 and block4 is further confirmed by co-electroporation with pCMV-EGFP to demonstrate the transfection of the deletion constructs in the entire neural epithelium (Fig. 4D).

Multiple conserved TFBS are present in ECR1 minimal essential blocks—

rVISTA analysis of the four deletion blocks identified ~100 overlapping TFBS in each of these blocks. The sequence alignment of ECR1 with some of the key TFBS in and around the minimal essential deletion blocks is depicted in Figure 5. Based on the expression patterns of transcription factors in the developing spinal cord and the TFBS that are present in these deletion blocks and ECR1, some of the transcription factors that could be functionally bound to ECR1 are FoxD3, Sox9, Pax3/7, Gli, Msx1, SMAD, TCF/LEF, and E-box binding basic helix-loop-helix transcription factors (Fig. 5). A list of TFBS in each of the four deletion blocks for neural tube specific transcription factors is given in supplemental Table 1.

***Cadherin-7* promoter and ECR1 are required to recapitulate endogenous cadherin-7 expression in DRG and spinal motor neurons**

To identify the regulatory potential of the *cadherin-7* promoter and the ECR1 regulatory region at a later developmental stage, embryos were electroporated at stages 14–15 and screened for fluorescence at stage 24 when cadherin-7 is expressed in the DRG and the spinal motor neurons. *Cadherin-7* promoter (Pro1)-driven EGFP expression was maintained in the entire neural epithelium and observed in the spinal motor neurons, interneurons, and a few EGFP-positive cells in the DRG (Fig. 6A). ECR1 in the tk-vector drove EGFP expression in the intermediate domain of the neural epithelium and the interneurons (Fig. 6A), but was unable to drive EGFP expression in the spinal motor neurons and DRG. However, the combination of ECR1 and *cadherin-7* promoter (Pro1) drove EGFP expression in a pattern that completely overlaps with the endogenous cadherin-7 expression in the intermediate domain of the neural epithelium, DRG, spinal motor neurons, and interneurons (Fig. 6A overlay). The EGFP expression driven by these regulatory regions in the spinal motor neurons was co-labeled with neuron-specific class III β -tubulin marker, Tuj-1, to identify the neurons (Fig. 6B).

The requirement of the ECR1 essential enhancer element in block3 was also tested during spinal motor neuron development. ECR1 with block3 deletion (ECR1 del3) in the tk-vector at stage 24 (data not shown) had no enhancer activity similar to stage 18 (figure 4B). In the *cadherin-7* promoter vector, the block3 deletion (ECR1 del3) did not affect the reporter gene expression and was similar to the expression driven by wild type ECR1 with *cadherin-7* promoter in the spinal motor neurons, interneurons, DRG, and neural epithelium. This further establishes the requirement of the *cadherin-7* promoter for expression in spinal motor neurons, ECR1 for regulating the *cadherin-7* promoter activity in neural epithelium, and a combinatorial enhancing activity of ECR1 and promoter in DRG.

Discussion

In this study, we coupled the evolutionary conservation of non-coding sequences with functional reporter analysis to identify the regulatory region of *cadherin-7*. ECR1 was identified as the evolutionarily conserved regulatory region that together with the *cadherin-7* promoter recapitulates endogenous *cadherin-7* expression in the developing neural epithelium, spinal motor neurons, and in DRG. Three essential regulatory blocks in ECR1 are required for enhancer and silencer activity of this region. The presence of multiple TFBS in the essential regulatory blocks of ECR1 suggests that the spatially restricted expression of *cadherin-7* requires multiple regulatory inputs.

Spatial regulation of *cadherin-7* promoter in neural epithelium requires additional regulatory elements

The *cadherin-7* promoter was active in the entire neural epithelium; however, during stages 16–22 of development, endogenous *cadherin-7* expression at both the RNA and protein level is spatially restricted to the intermediate domain of the neural epithelium. This suggested that the activity of the *cadherin-7* promoter was regulated by additional regulatory modules in the *cadherin-7* locus. This is consistent with the model of regulation for other cadherin genes in which the promoter alone was unable to recapitulate endogenous gene expression and distant regulatory regions were required. Five different regulatory regions are required for *N-cadherin* expression in chicken (Matsumata et al., 2005). Mouse *cadherin-6* is regulated by five distant regulatory regions spread across the *cadherin-6* gene locus (Inoue et al., 2008). In the case of the *cadherin-7* promoter, it was apparent that additional elements were required to silence the expression driven by the promoter in the dorsal and ventral neural epithelium and for enhancing expression in migrating neural crest cells. These silencer and enhancer sites could be regulated by signaling pathways such as BMP, Wnt, and Shh that are known to be involved in dorso-ventral patterning of the neural epithelium and neural crest cell development.

ECR1 contains both enhancer and silencer elements

Fragment analysis followed by deletion analysis was used to identify the enhancer and silencer elements that are sufficient and necessary for ECR1 regulatory activity. ECR1 contains an 85 bp enhancer region [ECR1ES (343–427)] with a 19 bp minimal essential enhancer block3. An insight into possible functional TFBS in block3 was obtained when the ECR1 block3 deletion (ECR1 del3) was tested for its regulatory activity in the *cadherin-7* promoter vector. Comparing ECR1 del3 in Figure 4B and 4C, it is clear that deletion of block-3 led to complete loss of ECR1 enhancer activity; however, in the presence of the *cadherin-7* promoter (Pro1) this activity was restored in the intermediate domain of the neural epithelium. This suggests that both block3 and the promoter have some of the same TFBS that complement each other endogenously to drive *cadherin-7* expression in the intermediate domain of the neural epithelium. The essential dorsal and ventral neural tube silencer elements of ECR1 are present in two separate blocks (block 1 and 4) about 238 bp apart. A combined effect of these two silencer blocks is required for complete silencer activity of ECR1 in dorsal and ventral neural tube. This result agrees with the known regulatory mechanisms that are involved in the dorso-ventral patterning of neural tube. The BMP signaling gradient defines the dorsal neural tube and Shh gradient defines the ventral neural tube patterning. The morphogen gradients regulate the class I and class II transcription factors which may regulate *cadherin-7* expression in dorsal and ventral neural tube by binding to the TFBS in block 1 and 4 of ECR1.

Regulatory activity of ECR1 and *cadherin-7* promoter in spinal motor neurons and DRG

During later stages of development (stage 24), the *cadherin-7* promoter was sufficient and necessary to activate reporter gene expression in spinal motor neurons. The requirement of the promoter for expression in spinal motor neurons was further supported by the fact that ECR1 was completely inactive in spinal motor neurons. However, ECR1 was still required to regulate the promoter activity in the neural epithelium. Additionally, the combination of ECR1 and promoter had enhanced activity in the DRG which cannot be replicated by ECR1 and promoter alone. This suggested an interaction between transcription factors bound to ECR1 and the promoter that could be mediated by looping of the chromosomal DNA *in vivo*. Further biochemical experiments will be needed to support these interactions. However, these experiments do establish the requirement of both ECR1 and promoter (Pro1) in regulation of *cadherin-7* gene expression at stages 16–24 in the neural epithelium, spinal motor neurons, interneurons, and DRG. Based on this analysis it can be concluded that ECR1 is a neural tube specific regulator and the combination of ECR1 and *cadherin-7* promoter is necessary and sufficient to recapitulate the endogenous *cadherin-7* expression in the DRG, spinal motor neurons, interneurons, and the intermediate domain of the neural epithelium.

Minimum essential enhancer and silencer blocks contain multiple conserved TFBS

The minimal essential enhancer and silencer blocks contain the largest clusters of TFBS in the ECR1 fragment with maximum number of conserved TFBS, providing an ideal sequence that might contain functional TFBS. All four deletion blocks contain highly conserved E-boxes. Based on previous studies of *cadherin* gene regulation, E-boxes were identified to play a role in regulating expression of *cadherin-6B* (Taneyhill et al., 2007) and *E-cadherin* (Hajra et al., 2002) by binding of Slug transcription factor to E-boxes in their respective regulatory regions. Although the E-boxes in the ECR1 may or may not play a role in regulation of *cadherin-7* gene expression, these four blocks of sequences also contain more than 100 conserved TFBS that form a cluster of TFBS in these blocks.

Based on the rVISTA analysis, some of the other transcription factors that could play a role in *cadherin-7* regulation are discussed here. Cath1, a basic helix-loop-helix transcription factor, is known to be expressed in the dorsal neural tube (Lee et al., 1998), and could act as a repressor for ECR1 by binding to the E-boxes. Pax7 is also expressed in the dorsal neural tube and its ventral border co-localizes with the dorsal border of the *cadherin-7* expression domain. Ectopic expression of Pax7 has been suggested to downregulate *cadherin-7* expression in the intermediate domain of the neural epithelium (Luo et al., 2006). Thus, Pax7 is a good candidate to test as a repressor protein that might bind to the silencer element in ECR1. BMP and Wnt signaling are known to regulate dorsal neural tube patterning (Chesnutt et al., 2004). The presence of LEF1/TCF11 and SMAD binding sites in the deletion blocks points toward a possible Wnt and/or BMP mediated regulation of ECR1 in the dorsal neural tube. Furthermore, BMP signaling induces expression of *Msx1* in the dorsal neural tube (Liu et al., 2004). There is an *Msx1* binding site in deletion block3 and *Msx1* may act as a repressor in the dorsal neural tube in combination with other repressors that have binding sites in block1. Ventral neural tube patterning requires Sonic hedgehog (Shh) signaling and gradients of Shh signaling activates different Gli activator and repressor transcription factors (Pachikara et al., 2007). Ectopic expression of different levels of Shh affects *cadherin-7* expression in neural tube (Stamatakis et al., 2005; Luo et al., 2006). There are Gli binding sites in ECR1; however, these sites are not present in the three deletion blocks. It can still be speculated that Gli transcription factors may play a co-operative or indirect role in repression of *cadherin-7* expression.

The current study focused on identifying the regulatory elements required for *cadherin-7* expression in the developing neural epithelium. Thus, we did not focus on specifically targeting the early migrating neural crest during enhancer analysis of ECR1. Given the presence of TFBS for neural crest-specific transcription factors such as AP2 α (Mitchell et al., 1991; Khudyakov and Bronner-Fraser, 2009), FoxD3 (Kos et al., 2001), and Sox9 (McKeown et al., 2005) in the deletion blocks of ECR1, a detailed characterization of the regulatory role of ECR1 in migrating neural crest is still needed. Ectopic expression of FoxD3 has been known to upregulate cadherin-7 expression in neural tube and neural crest cells (Dottori et al., 2001). Thus, FoxD3 might act as a transcriptional activator of ECR1 in migrating neural crest cells. To identify the functional occupancy by any of these transcription factors, a detailed mutational analysis of these TFBS with biochemical analysis to confirm the occupancy of these sites will be needed. This study provides an insight into the specific regulatory region of *cadherin-7* containing multiple TFBS that are regulated by multiple signaling inputs and that might regulate the endogenous *cadherin-7* expression in the developing neural epithelium and migrating neural crest cells.

Experimental Procedures

Genomic sequence analysis

The *cadherin-7* locus is present on chromosome 2 between 97,914,716 bp-97,993,225 bp on the reverse strand and consists of 12 exons. The genomic sequence containing the *cadherin-7* genomic locus along with the upstream and downstream sequence was obtained from Ensembl; *Gallus gallus* Ensembl release 49, March 2008. By comparing the cDNA sequence for *cadherin-7* with the genomic sequence, the ATG initiation codon is confirmed to be present in exon 2, 9109 bp downstream of the first predicted 5' exon (see Fig1). A putative transcriptional start site (TSS) is annotated in the 5'UTR of the first exon using Genomatix program (<http://www.genomatix.de/>). The promoter prediction tool of the Genomatix program was used to predict promoter modules containing conserved TFBS and TATA box motif. For identification of putative regulatory regions of *CDH7* gene, ECR Browser (Ovcharenko et al., 2004) linked with rVISTA (Loots and Ovcharenko, 2004) was used. ECR Browser uses multiple genome alignment to compare sequences between different species with the base sequence for a given genomic locus. rVISTA was used to identify the conserved TFBS in the conserved regions identified using ECR Browser.

Reporter constructs

The genomic regions of the *cadherin-7* gene locus selected for functional testing were amplified using Prime Star High Fidelity DNA polymerase (Takara Biosciences) from Bacterial artificial chromosome (BAC) clone (CH261-162G13) [BACPAC Resource, Oakland, CA] containing the chicken *cadherin-7* genomic sequence (*cadherin-7* locus with 50 kb upstream and 25 kb downstream regions) and/or chicken genomic DNA. BAC clones were confirmed for presence of the *cadherin-7* genomic locus by amplification of various exon-intron boundaries and by sequencing. For identification of the promoter region, a modified promoterless reporter vector pPL-EGFP (constructed by replacing d2EGFP from pd2EGFP; Clontech) was used to subclone putative promoter fragments between the *SacI* and *KpnI* sites. Identification of regulatory regions containing enhancer and silencer elements required two reporter vectors, ptkEGFPv2 (Uchikawa et al., 2004) and pC7Pro1EGFPv2 (tk-minimal promoter replaced by *cadherin-7* promoter) respectively. Putative regulatory regions (ECRs) identified using ECR Browser coupled with rVISTA were cloned into the pC7Pro1EGFPv2 vector using InFusion Advantage cloning kit (Clontech) and into the ptkEGFPv2 vector between the *KpnI* and *XhoI* sites. The DNA sequences for all the constructs were verified by sequencing. A pCMVTag-RFP vector was used as a positive control to confirm the electroporation efficiency.

Constructs for deletion analysis—For deletion analysis, EGFP was replaced by Tag-RFP (Merzlyak et al., 2007) in the pC7Pro1EGFPv2 and ptkEGFPv2 vectors. To create the desired deletion in the given reporter construct containing ECR1, primers were used to amplify the entire vector containing the ECR1 with the exception of the deleted sequence. The PCR product was further treated with *DpnI* to fragment the original template plasmid DNA, gel purified, treated with T4 Polynucleotide Kinase (NEB) followed by overnight ligation. All deletion constructs were verified for correct deletion by sequencing.

Chicken embryos

Fertile white Leghorn chicken eggs obtained from Sunnyside Farms, Inc., (Beaver Dam WI) were incubated at 38°C using a humidified force draft egg incubator (G.Q.F. Manufacturing) until reaching appropriate stages for experimental manipulation. Embryos were staged according to the criteria of Hamburger and Hamilton (Hamburger and Hamilton, 1951).

Electroporation

Chicken embryos were electroporated in the neural tube at HH stage 11–13 (Hamburger and Hamilton, 1951) as described in Kos et al., 2001. Briefly the DNA construct was electroporated using the Intracel TSS20 electroporator (9v/2mm, 90ms pulse width, 2 pulses). For targeting spinal motor neurons, embryos were electroporated at stage 14–16 with the 3 mg/ml reporter construct and 1 mg/ml control construct using the Intracel TSS20 electroporator (12v/3mm, 50ms pulse width, 3 pulses). During initial screening for promoter and enhancer activity, the DNA solution for electroporation consisted of 2.0–3.5 mg/ml of EGFP reporter vector construct along with 1.0–1.5 mg/ml of pCMVTagRFP as control, and 0.01% of Fast Green in 10mM Tris-HCl (pH7.5). Electroporations were verified by TagRFP fluorescence. For deletion analysis, 2.0–3.0 mg/ml of mutant constructs in TagRFP reporter vector were used with 2.0–3.0 mg/ml of wild type construct in EGFP reporter vector. Embryos were evaluated 24 hours (HH stage 17–19) and 48 hours (HH stage 23–24) after electroporation for reporter gene expression using a fluorescent stereomicroscope (Leica MZ16F) and recorded with CCD camera (QICAM, QImaging) for whole embryos. Embryos were embedded in Paraplast Plus (Fisher), sectioned at 10 µm, and imaged with a Laser Scanning Confocal Microscope (Olympus Fluoview 1000). Each reporter construct was electroporated in 10–20 embryos, and reproducible results were obtained for all the constructs in at least 8–10 embryos. Any embryos with developmental deformities or partial expression of EGFP due to non-homogenous distribution of electroporated DNA were discarded.

Immunofluorescence and in situ hybridization

Whole mount in situ hybridization for *cadherin-7* was done using DIG-labeled (Roche) anti-sense RNA probes as described in Nakagawa and Takeichi (Nakagawa and Takeichi, 1995), embedded in Paraplast Plus (Fisher), and sectioned at 10 µm. Whole mount immunostaining for cadherin-7 was done as described in Nakagawa and Takeichi (Nakagawa and Takeichi, 1998) (1:100 dilution, CCD7-1, Developmental Studies Hybridoma Bank, University of Iowa). Immunoreactions were identified using Cy5-labelled goat anti-mouse IgG (1:500 dilution, Jackson ImmunoResearch). Briefly, embryos were fixed in 4% paraformaldehyde (PFA) for 2 hours on ice, blocked with 5% heat-inactivated goat serum (Invitrogen) in Tris buffered saline (TBS) with 0.2% Triton X-100 (Sigma) for 4 hours and incubated with cadherin-7 primary antibody overnight at 4°C, washed with 1X TBS containing 0.2% Triton X-100, followed by incubation with Cy5-labeled goat anti-mouse IgG secondary antibody overnight at 4°C. Embryos were embedded in Paraplast Plus (Fisher), sectioned at 10 µm, and imaged with Laser Scanning Confocal Microscope (Olympus Fluoview 1000). Immunostaining for neuron-specific class III β -tubulin marker, TuJ-1 (5 µg/ml, R&D

systems), was done on 10 μm sections and detected with Cy5-labeled goat anti-mouse IgG secondary antibody (1:500).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

ECR	Evolutionary conserved regions
TFBS	transcription factor binding sites
NT	neural tube
UTR	untranslated region
DRG	dorsal root ganglia
SS	silencer sequence
ES	Enhancer sequence

Acknowledgments

This work was supported by a subproject from the NIH Center of Biomedical Research Excellence (COBRE) grant P20 RR-015567, NSF MRI Grant 0923419, USD Research Catalyst Grant, and USD Graduate Research Grant 2008. We thank Dr. Hisato Kondoh and Dr. Masanori Uchikawa for the generous gift of ptkEGFPv2 vector, Dr. Jeffrey J. Essner (Iowa State University, Iowa) and Dr. Roger Y. Tsien (University of California, San Diego) for TagRFP, Ms. Abha J. Chalpe for *in situ* hybridization images of Cadherin-7 and Dr. Fran Day (Sanford School of Medicine, University of South Dakota, Vermillion, SD) for help with confocal imaging. We thank Dr. Mark V. Reedy and Dr. Kathleen M. Eyster for critical reading of this manuscript. The cadherin-7 monoclonal antibody was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

Grant Information: NIH Center of Biomedical Research Excellence (COBRE) P20 RR-015567, NSF MRI 0923419

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Figure 1. Identification of *cadherin-7* promoter region

(A) Schematic representation of Genomatix output representing the predicted, tested, and identified promoter region. The putative transcriptional start site (TSS) predicted by Genomatix program is annotated in the 5' territory of exon-1, a part of the 5' UTR (untranslated region). (B) Images of neural tube sections from trunk level of stage 17 embryo co-electroporated with *cadherin-7* promoter (Pro1) in pPL-EGFP vector and pCMV-TagRFP as electroporation control. EGFP fluorescence driven by Pro1 is observed in the entire neural epithelium and few migrating neural crest cells (arrow). (C) In situ hybridization (a) and immunostaining (b) for endogenous *cadherin-7* expression at trunk level in stage 17 embryo. *Cadherin-7* is expressed in the intermediate domain of the neural epithelium and the migrating neural crest cells (marked by arrows) at both RNA and protein level. NT-Neural tube. Scale bar = 50 μ m.

Figure 2. Identification of ECRs in *cadherin-7* gene locus

(A) ECR Browser output comparing the chicken *cadherin-7* gene locus with seven other species. Peaks are generated based on 70% or more sequence conservation over 100 bp with red colored peaks denoting conservation in upstream and downstream regions, yellow in UTRs, salmon in introns, and blue peaks in coding exons. Nine ECRs selected for functional analysis based on the presence of conserved TFBS are denoted by boxes. The base pair scale line at the bottom corresponds to chicken chromosome 2, indicating gradations at every 10 kb. (B) Enhancer/silencer activity of ECR1 in ptk-EGFPv2 vector (a) and in *cadherin-7* promoter vector (pC7Pro1EGFPv2) (b) co-electroporated with pCMV-TagRFP as control. EGFP fluorescence is observed in the intermediate domain of the neural tube and few migrating neural crest cells (arrow). (c) An overlap of EGFP expression driven by ECR1 in the pC7Pro1EGFPv2 vector with cadherin-7 expression (immunostaining) in the intermediate domain of the neural tube. Images are from trunk neural tube sections of stage 17–19 embryos. NT-Neural tube. Scale bar = 50 μ m.

Figure 3. Fragment analysis to identify the essential regulatory region of ECR1
(A) A schematic representation of ECR1 fragment analysis to identify the essential regulatory region. Fragments with silencer and enhancer activity are denoted by green color, fragment with only silencer activity in black, and fragments without any silencer or enhancer activity are denoted by gray color. (B) Enhancer activity of ECR1 and its subfragments in ptk-EGFP vector. Enhancer activity of ECR1(1–606) and ECR1(195–427) restricted to intermediate domain of the neural epithelium observed as EGFP fluorescence. ECR1ES(343–427) enhancer activity is observed as EGFP fluorescence in the entire neural epithelium. (C) Silencer activity of ECR1 and its subfragments in pC7Pro1-EGFP vector. Silencer activity of ECR1(1–606) and ECR1(195–427) is observed in dorsal and ventral neural tube and restricts *cadherin-7* promoter activity to intermediate neural tube (brackets). ECR1(1–216) and ECR1(401–606) had no silencer activity as EGFP fluorescence was

observed in the entire neural epithelium. Silencer activity of ECR1SS(244–303) was observed in the dorsal neural tube and partially in the ventral neural tube indicated by much wider domain of EGFP fluorescence. The brackets indicate the EGFP expression domain in the neural epithelium. Images are from trunk neural tube sections from stage 17–18 embryos. pCMV-TagRFP co-electroporated as control. The number of embryos showing enhancer/silencer activity out of the total number of embryos electroporated is indicated at the bottom of each panel. NT-Neural tube. Scale bar = 50 μ m.

ECR1 [1-606]

ECR1 del1 [1-606 ; Δ246-255]

ECR1 del2 [1-606 ; Δ264-292]

ECR1 del3 [1-606 ; Δ365-383]

ECR1 del4 [1-606 ; Δ494-505]

ECR1 del5 [1-606 ; Δ246-255; Δ264-292; Δ365-383; Δ494-505]

ECR1 (195-427)

ECR1 (401-606)

ECR1 SS (244-303)

ECR1 ES (343-427)

Enhancer Activity	Overlay	EGFP	Tag-RFP
4/4
6/6
0/12
4/4
0/4

(A) Schematic representation of ECR1 deletion analysis denoting the four deletion blocks and their locations with respect to previously tested fragments. All the deletion constructs are represented with colors denoting their enhancer activity as normal (same as wild type) (red), reduced (pink) and no activity (grey). (B) ECR1 deletion constructs tested for

enhancer activity in ptk-TagRFPv2 vector and co-electroporated with wild type ECR1 in ptk-EGFPv2 vector. No effect on ECR1 enhancer activity was seen in ECR1 del1 observed as TagRFP fluorescence similar to EGFP fluorescence. ECR1 del2 and ECR1 del4 led to reduction in ECR1 enhancer activity observed as reduced TagRFP fluorescence. Complete loss of ECR1 enhancer activity was detected in ECR1 del3 and ECR1 del5 observed as no TagRFP fluorescence. (C) ECR1 deletion constructs tested for silencer activity in pC7Pro1-TagRFP vector and co-electroporated with wild type ECR1 in pC7Pro1-EGFP vector. ECR1 silencer activity was unaffected in ECR1 del2 and ECR1 del3 observed as TagRFP expression domain that overlaps with EGFP expression domain. ECR1 silencer activity was lost in dorsal neural tube in ECR1 del1 observed as TagRFP fluorescence. In ECR1 del4 the silencer activity of ECR1 was lost in ventral neural tube observed as TagRFP fluorescence. A complete loss of ECR1 silencer activity was observed in ECR1 del5 indicated by TagRFP fluorescence in the entire neural epithelium. EGFP and Tag-RFP expression domain in the neural epithelium are denoted by brackets. The number of embryos showing enhancer or silencer activity out of the total number of embryos electroporated is indicated at the bottom of the panels. (D) ECR1 del1 and ECR1 del4 in pC7Pro1-TagRFP vector were co-electroporated with pCMV-EGFP showing the total area of neural epithelium that was electroporated observed as EGFP fluorescence and the loss of silencer activity in dorsal neural tube (ECR1 del1) and ventral neural tube (ECR1 del4) observed as TagRFP expression. Images are from trunk neural tube sections of stage 17–19 embryos. NT-neural tube, DNT-dorsal neural tube, VNT-ventral neural tube. Scale bar = 50 μ m.

Figure 5. rVISTA analysis of deletion blocks in ECR1

rVISTA linked to ECR Browser was used for sequence alignment of ECR1 regions between chicken, mouse and human. The asterisk denotes an identical nucleotide between sequences. Location of ECR1 segment is shown for chicken at the beginning and end of the sequence. The four deletion blocks are highlighted in red and block numbers are indicated on top of each block. rVISTA-predicted transcription factor binding sites are indicated by underlined sequence.

Figure 6. *Cadherin-7* promoter and ECR1 drives EGFP expression in spinal motor neurons, interneurons, and DRG

(A) Section images of stage 24 embryos electroporated at stages 15–16 and immunostained for cadherin-7. pCMV-TagRFP was used as electroporation control. *Cadherin-7* promoter (Pro1) drives EGFP expression in the entire neural epithelium, spinal motor neurons, interneurons, and few cells in DRG. ECR1 drives EGFP expression in tk-vector in the intermediate neural tube and interneurons. EGFP expression driven by the combination of ECR1 and *cadherin-7* promoter (Pro1) in the intermediate neural tube, interneurons, spinal motor neurons, and DRG completely overlaps with the endogenous cadherin-7 expression (magenta). (B) EGFP expression driven by promoter, ECR1 and combination of ECR1 and

promoter in spinal motor neurons co-labeled with neuron marker, TuJ-1. SMN- spinal motor neurons, I-interneuron, DRG-dorsal root ganglia, NT-neural tube. Scale bar = 50 μ m.

Table 1
Summary of enhancer and silencer activity of ECRs with chromosomal locations

Constructs	Location on Chromosome 2	Enhancer Activity	Silencer Activity	Location on CDH7 Locus
ECR1	98003904–98004509	+ (6/6)	+ (6/6)	Intergenic
ECR2	97989416–97993225	– (0/4)	– (0/4)	Intronic
ECR3	97983860–97984900	– (0/4)	– (0/4)	Intronic/5'UTR
ECR4	97964721–97964861	– (0/4)	– (0/4)	Intronic
ECR5	97954074–97954692	– (0/4)	– (0/4)	Intronic
ECR6	97938026–97938598	– (0/4)	– (0/4)	Intronic
ECR7	97930712–97930945	– (0/4)	– (0/4)	Intronic
ECR8	97921686–97921987	– (0/4)	– (0/4)	3'UTR
ECR9	97912469–97915644	– (0/4)	– (0/4)	3'UTR/Intergenic

Table 2
Enhancer and silencer activity of ECR1 sub-fragments

Constructs	Enhancer Activity	Silencer Activity
ECR1	+ (8/8)	+ (16/16)
ECR1 (1–216)	– (0/4)	– (0/6)
ECR1 (195–427)	+ (6/6)	+ (7/7)
ECR1 (401–606)	– (0/4)	– (0/9)
ECR1 (195–262)	– (0/4)	– (0/4)
ECR1SS (244–303)	– (0/6)	+ (7/7)
ECR1 (294–370)	– (0/4)	– (0/4)
ECR1ES (343–427)	+ (6/6)	– (0/4)

Table 3
Effects of deletions on ECR1 enhancer and silencer activity

Numbers in brackets denote the number of embryos showing enhancer/silencer activity out of the total number of embryos electroporated.

Constructs	Block Number	Enhancer Activity	Silencer Activity
ECR1 del1	Block1	+ (4/4)	– (0/9)
ECR1 del2	Block2	+ (6/6)	+ (13/13)
ECR1 del3	Block3	– (0/12)	+ (8/8)
ECR1 del4	Block4	+ (4/4)	– (0/7)
ECR1 del5	All four	– (0/4)	– (0/5)