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Analysis of mouse *Cdh6* gene regulation by transgenesis of modified bacterial artificial chromosomes

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Abstract

Classic cadherins are cell adhesion molecules whose expression patterns are dynamically modulated in association with their diverse functions during morphogenesis. The large size and complexity of cadherin loci have made it a challenge to investigate the organization of *cis*-regulatory modules that control their spatiotemporal patterns of expression. Towards this end, we utilized bacterial artificial chromosomes (BACs) containing the *Cdh6* gene, a mouse type II classic cadherin, to systematically identify *cis*-regulatory modules that govern its expression. By inserting a *lacZ* reporter gene into the *Cdh6* BAC and generating a series of modified variants via homologous recombination or transposon insertions that have been examined in transgenic mice, we identified an array of genomic regions that contribute to specific regulation of the gene. These regions span ~350 kb of the locus between 161-kb upstream and 186-kb downstream of the *Cdh6* transcription start site. Distinct modules independently regulate compartmental expression (i.e. forebrain, hindbrain rhombomeres, and spinal cord) and/or cell lineage-specific expression patterns (i.e. neural crest subpopulations such as Schwann cells) of *Cdh6* at the early developmental stages. With respect to regulation of expression in neural crest cells, we have found that distinct regions contribute to different aspects of expression and have identified a short 79-bp region that is implicated in regulating expression in cells once they have emigrated from the neural tube. These results build a picture of the complex organization of *Cdh6 cis*-regulatory modules and highlight the diverse inputs that contribute to its dynamic expression during early mouse embryonic development.

Keywords: *Cdh6* (cadherin-6); Mouse; Gene regulation; BAC; Transposon; Homologous recombination; Forebrain; Hindbrain; Compartment; Neural crest cells

Introduction

Classic cadherins are cell adhesion molecules that are core elements of the rigid intracellular junctional complex called adherence junctions. As many as 20 classic cadherin subclasses have been identified in vertebrate model organisms and these are assigned into two groups, type I and type II, based on specific amino acid sequence similarities (Nollet et al., 2000). Each classic cadherin subclass confers selective adhesiveness to cells, whereby cells that express molecules of the same cadherin subclass have a tendency to form aggregates due to homotypic interactions between the extracellular domains of the subclass members (Nose et al., 1990; Patel et al., 2006). As a consequence, in a mixed population, groups of cells expressing a given cadherin subclass are capable of sorting from those expressing different subclasses in *in vitro* assays (Nose et al., 1988). Furthermore, quantitative differences in cadherin expression have been shown to generate a driving force for selective cell sorting, as cells that express a higher level of a given cadherin subclass compared with their neighbors segregate (Steinberg and Takeichi, 1994).

The developmental expression patterns and levels of classic cadherins dynamically change in a manner well correlated with

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their participation in morphogenetic processes. Several lines of evidence have indicated that active changes in expression of cadherins plays a crucial role in controlling the segregation and movement of distinct cellular groups, thereby sculpting cellular assembly during development. This is exemplified by the process of neurulation and neural crest formation in vertebrates. In the future neural plate ectoderm *E-cadherin* (an epithelial cadherin) is specifically down-regulated, while *N-cadherin* (a neural cadherin) is activated (Hatta and Takeichi, 1986). The *N-cadherin*-positive neural plate rounds up and fuses at the dorsal midline to form a tube which is segregated from the *E-cadherin*-positive epithelial cell sheet that comprises the surface ectoderm (Hatta and Takeichi, 1986). The initial population of neural crest cells emigrating from the dorsal midline of the neural tube also switch expression of cadherin subclasses, from *N-cadherin* to *cadherin-6B*. Subsequently these migrating neural crest cells come to express *cadherin-7* in association with a rapid down-regulation of *cadherin-6B* (Nakagawa and Takeichi, 1995). Proper regulation of *N-cadherin* or *cadherin-6B* is important for normal emigration of the neural crest cells (Nakagawa and Takeichi, 1998) and segregation between the surface ectoderm and neural tissue (Detrick et al., 1990; Fujimori et al., 1990). In humans, many of cancers with malignant and aggressive phenotypes tend to express abnormally low levels of cadherins (Foty and Steinberg, 2004; Takeichi, 1991) suggesting that modulation of cadherin expression plays a pivotal role in the formation and metastasis of tumors (Halbleib and Nelson, 2006; Redies, 2000; Takeichi, 1991, 1993). Hence, understanding the organization and mechanisms associated with regulation of cadherin gene expression in development and disease is an important problem.

Mouse *Cadherin-6* (*Cdh6*) is a type II classic cadherin, whose expression delineates subpopulations of the migrating neural crest cells, as well as discrete rhombomeric segments in the developing hindbrain (Inoue et al., 1997). This cadherin also demarcates the lateral ganglionic eminence (lge), which is neighbored by the *R-cadherin*-positive future cerebral cortex (ctx) from embryonic day (E) 10.5 onward (Inoue et al., 1997, 2001). We have previously demonstrated that these two adjoining telencephalic regions can be defined as lineage-restricted cellular compartments and that ectopic expression of *Cdh6* around the ctx/lge compartment boundary at E10.5 resulted in abnormal sorting of cells between compartments (Inoue et al., 2001). In contrast to these gain-of-function experiments, we have shown that abnormal sorting in these compartments is not observed in *Cdh6* loss-of-function mutant mouse embryos (Inoue et al., 2001). Together, these studies suggest that differential expression of cadherin classes at the interface between adjacent territories is responsible for the sorting influences that maintain the ctx/lge compartment boundary. At the later stages of neural development, restricted groups of neuronal cells are found to express *Cdh6* at the synaptic junctions and these *Cdh6*-positive neurons appear to constitute functional neuronal circuits (Suzuki et al., 1997; Inoue et al., 1998). Many of these regulated domains of *Cdh6* expression are tightly linked to developmental and/or functional units of the nervous system, which provides a means of marking the processes and elucidating the underlying molecular mechanisms that serve to regionalize the nervous system.

Most cadherin loci occupy a considerably large region in the genome (~400 kb), which has made it extremely difficult to investigate the nature and organization of *cis*-regulatory modules for these genes. Past studies on transcriptional regulation of cadherins have been primarily limited to type I subclasses. Analyses have identified a few genomic segments able to mediate *P-cadherin/Cdh3* expression in cell lines (Hatta and Takeichi, 1994), *E-cadherin/Cdh1* expression in early mouse embryos (Stemmler et al., 2003, 2005), and *N-cadherin/Cdh2* expression in the chicken nervous system (Matsumata et al., 2005). In this study, we have utilized bacterial artificial chromosomes (BACs) in combination with methodology that permits efficient modification via homologous recombination and/or random transposon insertions to generate an array of reporter constructs, which allowed us to systematically screen for *cis*-regulatory modules of the mouse *Cdh6* gene scattered over a large genomic section (~0.4 Mb). Our analysis of transgenic reporter mouse lines with these modified BACs revealed an array of genomic regulatory segments located both upstream and downstream of the *Cdh6* transcription start site that control distinct aspects of the restricted pattern of *Cdh6* expression in development. These results provide important insight into the spectrum of genetic elements and/or signals that govern *Cdh6* expression and provide a basis for comparing and contrasting the architecture of regulatory modules in other cadherin subclasses.

Materials and methods

Reporter constructs

To design our strategies for identifying the promoter and *cis*-regulatory modules of the *Cdh6* gene we first compared genomic and cDNA sequences (Supplemental Fig. 1). We confirmed that the mouse *Cdh6* gene on band qA1 of chromosome 15 consists of 12 exons, with the most 5′ predicted exon representing only untranslated DNA sequences positioned 81.5 kb upstream of the ATG initiation codon in exon 2 (see Fig. 1A; Supplemental Figs. 1A–C). This is in good agreement with the predicted organization of the human *CDH6* locus (hg18; http://genome.ucsc.edu/), which has a non-coding exon 73.5 kb upstream of the ATG codon in exon 2. By using the Genomatix program (http://www.genomatix.de/), a putative transcription start site (TSS) is annotated in the 5′ territory of the small mouse and human exon 1 non-coding sequence. In addition, by utilizing Genomatix prediction tools as well as the evolutionary conserved region (ECR) browser (http://ecrbrowser.dcode.org/) linked by the rVista 2.0 (http://rvista.decode.org/), promoter modules with a TATA box motif and binding sites for conserved transcription factors essential to activate promoter modules were found immediately upstream region of exon 1 (data not shown). This analysis suggests that the region immediately upstream of exon 1 harbors the minimal gene promoter of mouse *Cdh6* gene and we used this as a basis in our regulatory analysis.

A 721-bp fragment upstream of the *Cdh6* transcription start site (TSS), which corresponds to the nucleotide positions 13118308–13119028 of mouse chromosome (chr) 15 from the mm8-Feb'06 assembly, was amplified by KOD polymerase (TOYOBO, Japan)-mediated high-fidelity polymerase chain reaction (PCR) with a forward primer containing an *Apa*I linker (*720bpF*: 5′-ATATGGGCCCGCTTCTACACTCTCGGGGGC-3′) and a reverse one containing an *Nae*I linker (*720bpR*: 5′-ATATGCCGGCTTCTGCTCTGTGCTTCTTGC-3′). As the template, a BAC clone *RP23-190L12* that covers ~75 kb of 5′ upstream territory of the *Cdh6* TSS was utilized (CHORI, BACPAC Resources). The PCR product was then digested at the underlined *Apa*I and *Nae*I linker sites