

## Supporting Online Material

### Methods

- **Identification of Gene Deserts in humans and mice.**

Gene deserts were characterized in the human genome using the April 2003 freeze of the human genome (<http://genome.ucsc.edu>). Using the union of the RefSeq and ENSEMBL gene collections as reference, the intergenic intervals between every two genes in the genome were calculated. Pericentromeric and sub-telomeric regions were excluded from the analysis.

A total of 818 intergenic intervals, ranging from 499kb to 4,780kb, were identified, using these criteria, and termed gene deserts. They represent 4% of all the intergenic intervals, while covering 30.1% of the euchromatic sequences of the human genome.

The mouse genomic regions orthologous to the human gene deserts were obtained from the ECR Browser tool (<http://ecrBrowser.dcode.org>). The February 2002 (mm2) freeze of the mouse genome assembly from UCSC was utilized for the alignment of the gene deserts between human and mice.

- **Identification of conserved non-coding sequences in *Xenopus tropicalis*, *Danio rerio*, *Tetraodon nigroviridis*, *Fugu rubripes* and *Ciona intestinalis*.**

The identification of orthologous conserved sequences in the remaining species used in this study was obtained by retrieving sequences from various databases:

- *Xenopus tropicalis* reads archives were downloaded from JGI web-site (<http://genome.jgi-psf.org/xenopus0/xenopus0.download.ftp.html>) on April 28, 2003.
- Danio rerio WGS Zv2 genome assembly from Ensemble (<ftp://ftp.ensembl.org/pub/assembly/zebrafish/Zv2release/>).
- *Tetraodon nigroviridis* version 6 genome assembly from Genoscope (<http://www.genoscope.cns.fr/externe/tetraodon/Ressource.html>).
- *Fugu rubripes* version v3.0 JGI genome assembly (<http://genome.jgi-psf.org/fugu6/fugu6.home.html>).

The identification of sequences in these vertebrates, orthologous to human sequences, was obtained using a combination of BLASTN and the ECR browser tool. An e-value of  $1e-7$  was used as a threshold to filter out low homology hits between queried and subject sequences.

All the alignments obtained represent comparisons between human sequences and that of a second species.

- **Vector design and cloning of CNS for enhancer assay.**

For the cloning of conserved noncoding sequence elements in the hsp68-lacZ vector, each element had its sequence conservation profile determined in all vertebrate

species studied. A human DNA fragment corresponding to the entire human-mouse conservation region was amplified by PCR. A HindIII or PstI linker preceded each primer used. The PCR products were digested for 24-hours with either HindIII or PstI and ligated to the hsp68-lacZ vector, also linearized with either HindIII or PstI. Following transformation, bacterial colonies were screened by PCR using the corresponding primers for each element being cloned. Directionality of the inserts was determined by restriction maps, assuring that the cloned elements were in the same direction in relation to the hsp68 promoter as to the neighboring DACH1 or DACH2 gene *in vivo*.

- **Generation of Transgenic Mice**

Selected colonies harboring each conserved sequence were grown in 200 ml LB medium, and plasmid DNA purified using chromatography columns in an endotoxin-free protocol (Qiagen). 500 µg of DNA was linearized with NotI, followed by purification in a CsCl gradient column. DNA concentration was determined both fluorometrically and in agarose gels and diluted to a final concentration of 2-5 µg, and used for pronuclear injections of FVB embryos, in accordance with protocols approved by the Lawrence Berkeley National Laboratory.

### **Embryo Staining**

Embryos were harvested at 12.5 or 13.5 dpc. Embryos were dissected in cold PBS, followed by 40-60 minutes of incubation with 4% paraformaldehyde at 4<sup>0</sup>C. Using a 27g needle, punctures were made in the head and trunk, to facilitate the penetration of

the staining solution, followed by 3-4 cycles of rinsing in wash buffer, 30 minutes each. A freshly made staining solution (0.8mg/ml X-gal; 4mM potassium ferrocyanide; 4mM potassium ferricyanide; 20mM Tris pH 7.5 in wash buffer) was used to stain the embryos for 16-24 hours. Stained embryos were rinsed 4-5 times in PBS, and post-fixed in 4% paraformaldehyde for at least 24 hours. Transgenic embryos were screened by PCR (lacZ primers) from tail DNA.

Details of each tested element is provided in table S1.

Element ID	Distance from Dach1 (Kb)	Human-mouse Length (bp)	Vertebrate conservation (%)	Expression pattern	Human Primers
<b>Dc1</b>	<b>+660</b>	<b>540</b>	<b>88</b>	<i>Negative</i>	F: GTTATGCCTGCTAATCAAGTGCTTT R: TTTCTTGTTTACCTTCTGTCTGTCC
<b>Dc2</b>	<b>+140</b>	<b>470</b>	<b>76</b>	<i>Hindbrain</i>	F: GCAATTTTGAAAAAGAAAACAATGG R: TAGACAGCTCATGCTGAGAAAACCTG
<b>Dc3</b>	<b>+10</b>	<b>390</b>	<b>71</b>	<i>Forebrain, Hindbrain, Spinal cord, retina</i>	F: CAAGTCCTGTCTTACTGCTTTTACACA R: AGAACAGATCCCTTAAATTCAAAT
<b>Dc4</b>	<b>-225</b>	<b>660</b>	<b>87</b>	<i>Retina</i>	F: ATGCATTCCAATGAAGAAGAACAA R: TCGCATTTTAAACAATCCTCAGAA
<b>Dc5</b>	<b>-250</b>	<b>330</b>	<b>70</b>	<i>Negative</i>	F: AGTCTGGAAGTCTGGGTACTGG R: TTTGTCTGATTAAGCATGCACTGA
<b>Dc6</b>	<b>-557</b>	<b>420</b>	<b>80</b>	<i>Midbrain, retina, dorsal root ganglia</i>	F: TGTACACTGCCAGAAGTCTTCCCTT R: CCTGTATTTTCTCATCCCACACAG
<b>Dc7</b>	<b>-637</b>	<b>380</b>	<b>82</b>	<i>Limb buds</i>	F: ATTGCTAGAGACCCCCTAAAAGCTA R: CCAAACGACCTCAGATTTTCTATAA
<b>Dc8</b>	<b>-643</b>	<b>820</b>	<b>78</b>	<i>Forebrain, neural tube</i>	F: TATCCCCCTACAGACAAAAATGCTA R: TCATTTTCCCAGCACAAAATAACATA
<b>Dc9</b>	<b>-780</b>	<b>305</b>	<b>88</b>	<i>Hindbrain, neural tube, genital eminence</i>	F: GAAAATGTAAGGAAGCTGCTCTTTG R: AACTTGAGTTAATGCAAACGGGTTA

**Table S1.** Profile of the elements tested *in vivo*. The location of each element is given (in kb) in reference to the TATA-box of human *DACHI*. The length of the conserved sequences between human and mouse is indicated, as well as the average degree of sequence conservation between the multiple vertebrates. The expression patterns of the elements shown in Fig.1 are described, as well as the human primers used to clone each element.