Appendix 2: Uchl1-mCherry/GFPgpi Allele Characterization

Authors: Nicole Fleming, Carrie B. Wiese, E. Michelle Southard-Smith
Generated: 3 March 2011 Version 6
Updated: 20 May 2011 Submitted: 20 May 2011

FINDINGS: VALIDATED

Our analysis confirmed expression of Uchl1-mCherry/GFPgpi in 15.5 dpc fetal gut and urogenital tracts in anatomical locations consistent with developing autonomic innervation. Uchl1 BAC transgene expression was comparable to wholemount immunohistochemistry for PGP9.5, the protein product of the Uchl1 gene.

Generation of Transgenic Lines

Uchl1-mCherry/GFPgpi animals were generated by introduction of a BAC modified to drive the mCherry/GFPgpi reporter from Uchl1 regulatory elements by insertion at the initiating methionine of Uchl1 coding sequences. Pronuclear injection of the circular construct into (C57BL/6xSJL)F2 eggs was performed using established methods (Camper et al., 1995). A total of 80 pups were born and were screened for three markers (two BAC flanking arms and one internal Histone2B PCR product).

Identification of BAC specific markers in transgenic progeny

Marker	No. pups positive
T7-BAC arm	10*
H2B - internal	10*
Sp6-BAC arm	8

Seven “complete” founders that were positive for all three BAC PCR markers used in the screening were identified. Two additional animals carried partial integrations of the BAC transgene, that is, they did not possess all three PCR markers*. One of the seven complete founders died before weaning. The remaining six founders (two males and four females) all transmitted the transgene to progeny. Progeny from male founders (M14116 and M14117) were examined to establish expression of the transgene in 14 dpc embryos. Female founders were bred to C3HeB/FeJ males to transmit the transgene and expression was subsequently evaluated in progeny derived from N1 males. Significant variation in expression among lines was observed. Three lines, hereafter referred to as Lines A, B, and F exhibited bright cranial ganglia expression and visible expression in the urogenital tract(see below). The other three lines either did not express or did not express in the tissues where Uchl1 expression was anticipated based on known patterns of immunohistochemical localization with PGP9.5 antibody.

Genotyping Tail biopsies were collected and incubated in tail digestion buffer overnight at 55ºC and then DNA was extracted by phenol/chloroform using routine methods. PCR was performed as described below and the PCR products were separated on 10% vertical polyacrylamide gels (see gel images below). Oligonucleotides used in the genotyping reactions to identify the transgenic allele included:

Internal Histone 2B region in the modified BAC (263 bp PCR product)
DNA sequence (forward): 5'- GTACTAAGGCCGTCACCAAG -3'
DNA sequence (reverse) 5'- GTACATGAACTGAGGGGACAG -3'

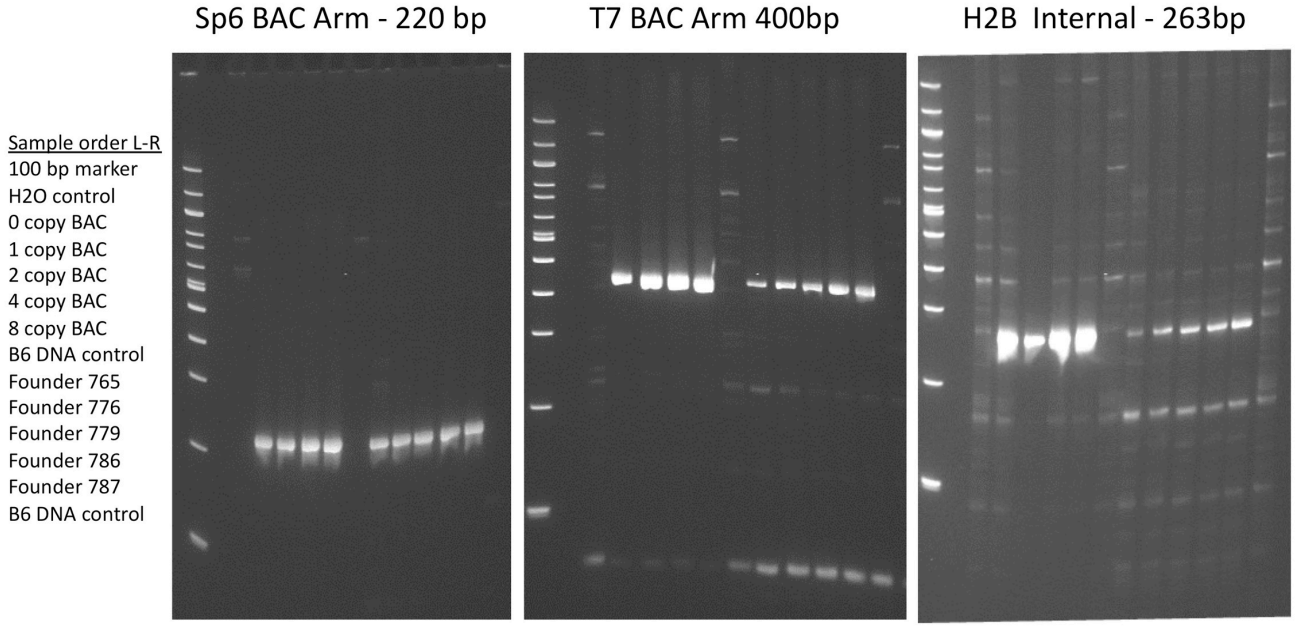
Sp6 Uchl1 BAC Arm primers that amplifyfrom the BAC vector arm to the flanking insert (220 bp PCR product)
DNA sequence (forward): 5'-GCCGTCGACATTTAGGTG-3'
DNA sequence (reverse): 5'-CCTACCCTTCGTCTTCTTTTG-3'

T7 Uchl1 BAC Arm primers that amplifyfrom the BAC vector arm to the flanking insert (400 bp PCR product)
DNA sequence (forward): 5'- GTCGAGCTTGACATTGTAGGA -3'
DNA sequence (reverse): 5'-GCCATCTCTTTAGCTCCAGTC-3'

Rxn Buffer and Conditions: (20µl reaction)

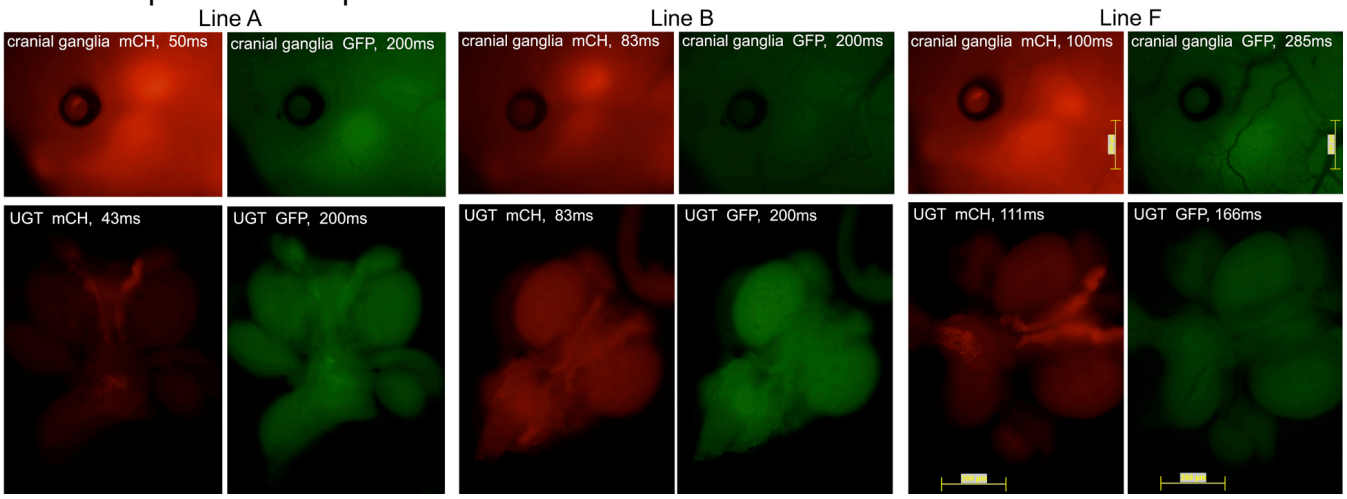
10X PCR B2.5	2 ul			
10mM dNTP	0.4ul	94°C	5min	1 cycle
6.6uM primer F	0.75ul	94°C	30sec	
6.6uM primer R	0.75ul	55°C	30sec	35 cycles
Taq Polymerase	(5u/ul)	72°C	30sec	
Genomic DNA	3ul (diluted 1:25)	72°C	10min	1 cycle
Total rxn volume	**25 ul**			

NOTE: 10x PCR B2.5 reaction buffer consists of: 100mM Tris pH 8.3, 500mM KCL, 20mM MgCl2

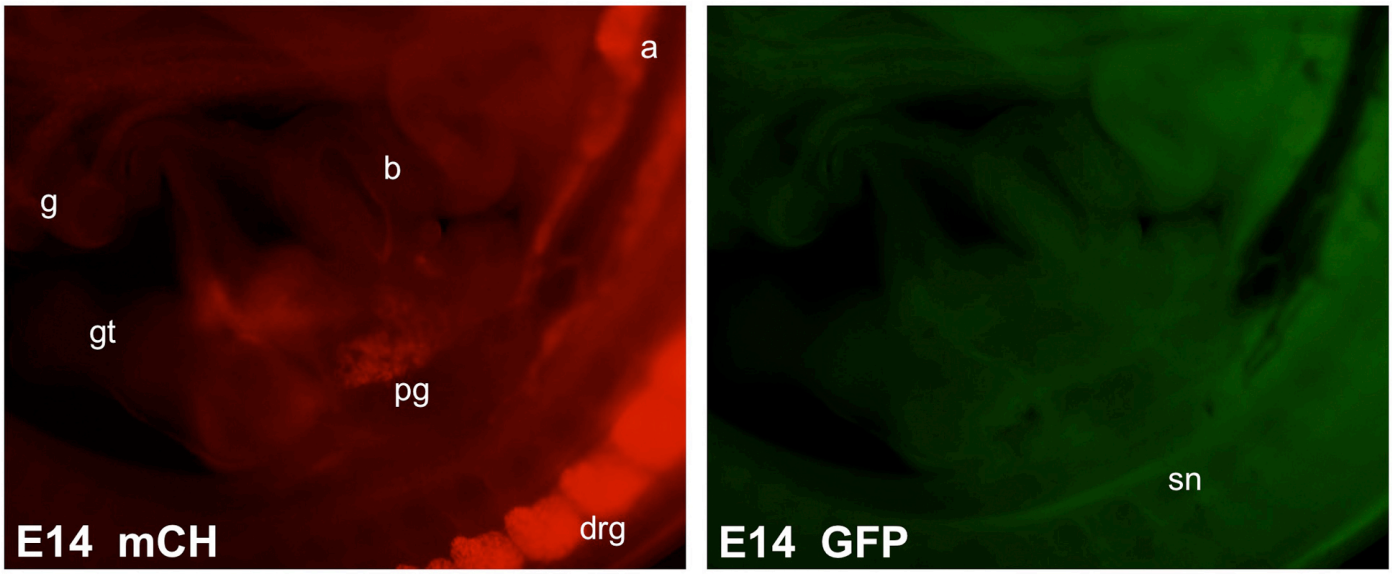


Analysis of Uchl1-H2BmCherry/GFPgpi BAC transgene Expression

Expression was examined in whole embryos and in subdissected tissues or vibratome sections viewed by direct fluorescence on an upright microscrope at 14 days post coitus (dpc). Both mCherry and GFP were visible under these conditions. Notable differences in fluorescence intensity were observed between Lines A, B, and F. Line F consistently exhibited brighter fluorescence in N2 embryos and was subsequently used for generation of vibratome sections to document patterns of expression.

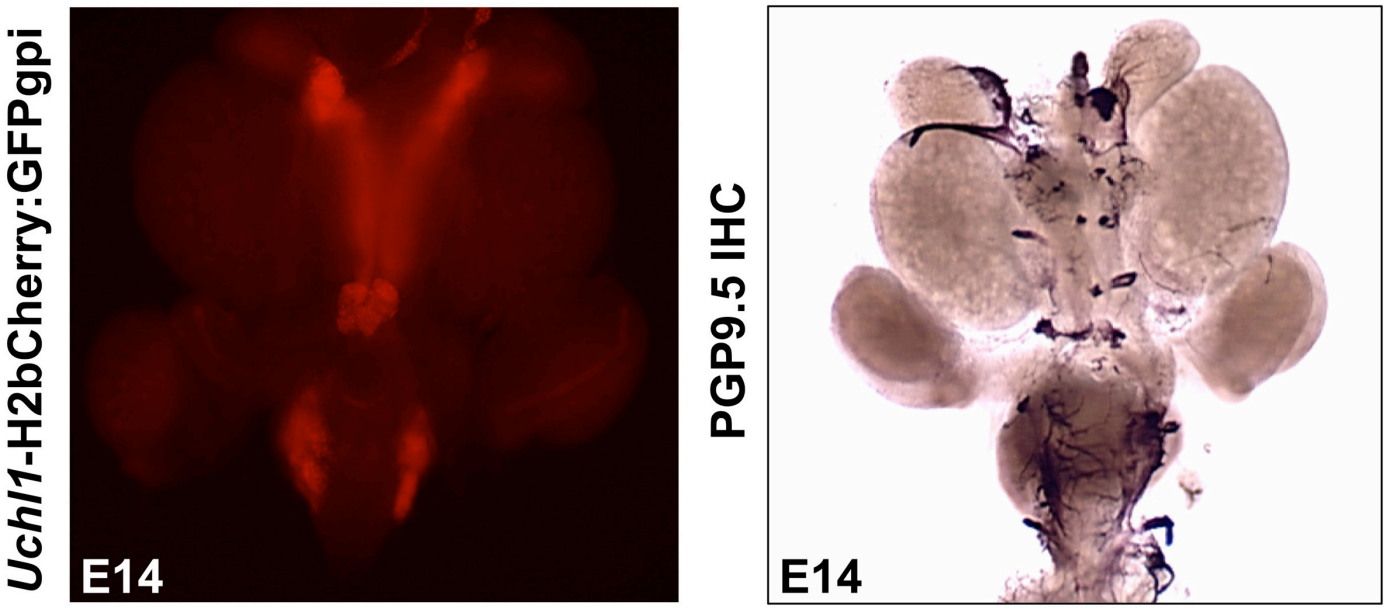


Mid-sagital vibratome sections from transgenic Line F taken at the sacral levels exhibit bright mCherry fluorescence in dorsal root ganglia (drg), adrenal (a), pelvic ganglia (pg). Intense GFP signal was also seen in spinal nerves (sn). mCherry fluorescence was also observed at higher magnifications in gut (g), bladder (b), and genital tubercle (gt) (data not shown).



Comparison of Uchl1 BAC transgenic expression with PGP9.5 IHC

Dorsal views of Uchl1 BAC transgene expression (Line F) show comparable localization to whole-mount immunohistochemistry for Uchl1 protein product, PGP9.5.



The immunohistochemical distribution of PGP9.5 was generated in whole-mount urogenital tracts (UGT) subdissected from 14 dpc fetal mice. UGT were fixed at 4˚C in 4% paraformaldehyde. Endogenous peroxidase activity was inactivated with 3% H_2O_2, 80% methanol, and 20% dimethylsulfoxide (DMSO) solution for 3 hours. Washes were performed with Tris-buffered saline (TBS) containing 1% Tween-20 and blocked in TBS containing 1% Tween-20 and 5% skim milk. Samples were incubated with rabbit anti-PGP9.5 (1:4000, Biogenesis) diluted in block containing 5% DMSO and 0.1% sodium azide for 3 days at room temperature. Secondary HRP-conjugated donkey anti-rabbit IgG (1:1000, Jackson ImmunoResearch) was applied in block to detect primary antibody. Visualization was achieved with 4-chloro-1-Naphtol solution (Sigma-Aldrich) according to the manufacturer's instructions.