Gene bashing of ceh-6 locus identifies genomic regions important for ceh-6 rectal cell expression and rescue of its mutant lethality

Arnaud Ahier\textsuperscript{1,2}, Shashi Kumar Suman\textsuperscript{1} and Sophie Jarriault\textsuperscript{1,8}

\textsuperscript{1}IGBMC, Development and Stem Cells Department, CNRS UMR7104, INSERM U1258, Université de Strasbourg, Illkirch CU Strasbourg, 67404 France

\textsuperscript{2}Current address: Clem Jones Centre for Ageing Dementia Research, Queensland Brain Institute, The University of Queensland, Brisbane, Australia

\textsuperscript{8}To whom correspondence should be addressed: sophie@igbmc.fr

Abstract

Strong loss-of-function or null mutants can sometimes lead to a penetrant early lethality, impairing the study of these genes’ function. This is the case for the ceh-6 null mutant, which exhibits 100% penetrant lethality. Here, we describe how we used gene bashing to identify distinct regulatory regions in the ceh-6 locus. This allowed us to generate a ceh-6 null strain that is viable and still displays ceh-6 mutant Y-to-PDA transdifferentiation phenotype. Such strategy can be applied to many other mutants impacting viability.

Figure 1. Bashing and identification of a cis-regulatory region responsible for the rectal cell-specific ceh-6 expression and rescue of ceh-6 mutants transdifferentiation defects. (A) Schematic view of the ceh-6 genomic fragment used for dissection of rectal cell-specific regulatory sequences. The in-frame insertion site of GFP, as well as the location of ceh-6 ATG and TAG, and each region size are indicated. Deleted regions are represented as closed brackets containing their name in blue (e.g. “a”, “b”, “c”, or the names of the deleted intron i1 to i5 (intron 1-5) and 3’UTR deletion). The location of the deletions with respect to the ATG is also indicated under brackets on the right. Orange: the ceh-6 3’UTR was swapped with unc-54 3’UTR. (B) The ability of the various ceh-6 constructs to i) rescue ceh-6(gk665) lethality; ii) drive expression in rectal cells and iii) rescue ceh-6(gk665) defects in transdifferentiation of the Y rectal cell into a PDA neuron is indicated. (n), total number of L3 animals and older scored. Note, constructs Δ3’UTR led to transgenic lines that did not show any expression nor rescue of the lethality, and no lines could be obtained with construct i1+i4. (C) Expression pattern of ceh-6 constructs in the Y and rectal cells. (i), expression pattern of syIs63 (cog-1::gfp) alone for reference; (ii) WT control construct; (iii) “c” construct; (iv), schematic representation of the rectal cells; Red arrowhead, Y cell. Dotted white line, rectal slit. All images were acquired at the L1 larvae stage. Scale bar represents 10 µm.

Description

Loss of the activity of certain genes, such as ceh-6, can lead to lethality at early developmental stages, precluding the study of their function later on during development. Indeed, it was reported that more than 80% of ceh-6(mg60) animals died during embryogenesis...
exhibiting various phenotypes, including an abnormal rectal area and absent excretory canal cell (Bürglin and Ruvkun 2001). *mg60* is a 1.4kb deletion allele that removes ceh-6 second exon and is believed to cause the null phenotype. The expression pattern of ceh-6 is complex and matches the reported defects. Most of the ceh-6 expressing cells, which include head neurons, dividing Pn.a cells in the ventral cord, the excretory cell, and rectal cells, are not related by cell lineage nor by function (Bürglin and Ruvkun 2001).

We have previously shown that knock-down of ceh-6 activity results in a loss of Y cell transdifferentiation (Td) (Kagias et al. 2012), and that ceh-6 RNAi inactivation at low dsRNA concentration leads to low penetrance Td defects (Kagias et al. 2012). Since the early lethality associated with strong loss-of-function or null ceh-6 alleles precludes the study of its role during Td, we sought to engineer a viable ceh-6 mutant that lacks ceh-6 activity in the Y cell. One strategy is to drive expression of ceh-6 in the cells where its activity is needed for viability, but not in the cell where it acts to promote Td. However, the cellular focus for the lethality is unknown, precluding a strategy where expression of ceh-6 WT cDNA would be specifically targeted to these cells. Since our previous results suggested that ceh-6 could act cell-autonomously in the Y cell (Kagias et al. 2012), we sought to identify the genomic region(s) within the ceh-6 locus that are necessary for expression in the Y or the rectal cells. Removing these regions from an otherwise ceh-6 rescuing construct should help to generate a viable ceh-6 mutant that lacks ceh-6 expression in the Y or rectal cells. To do so, we have initiated a gene bashing of the ceh-6 locus and have assessed the ability of the fragments to i) rescue the lethality of ceh-6 mutants; and ii) still result in Td defect, correlated with a loss of ceh-6 expression in Y or all rectal cells.

The ceh-6 gene consists of six exons intervened by five introns, spanning a 3.8kb region (https://wormbase.org/species/c_elegans/gene/WBGene00000431#0-9fb-10). A 13kb fragment for the ceh-6 locus that includes 5.6kb of upstream sequences and 3.6kb of downstream sequences was used as a template (Fig.1A, control construct). This fragment is able to rescue the phenotypes of ceh-6(gk665) mutants, including its lethality and Y Td defect (Fig.1B). In this construct, the GFP sequence was fused in frame with the ATG of the ceh-6 gene to follow ceh-6 expression in transgenic animals during rescue experiments (Fig. 1A, C), and we have found it to be expressed in the rectal cells (Fig.1C panel (ii)). A PCR-based approach was used to delete several different regions of the ceh-6 locus (Fig. 1A). We focused on the first intron of the ceh-6 gene, by far the largest, as well as on the upstream region as they contain several conserved sequence elements when compared with other Caenorhabditis species (ceh-6 UCSC browser). In addition, promoter regions and long first introns have been shown to bear different transcription factor binding sites that may act as additive regulatory regions (Fuxman Bass et al. 2013).

Rescue experiments were performed by injecting these constructs in ceh-6(gk665) deletion mutants, which bear a 1.5kb deletion encompassing the first exon and most of the first intron (Bürglin and Ruvkun 2001; The C. elegans Deletion Mutant Consortium 2012) and exhibit defects similar to the ceh-6 allele, including an early lethality (see Methods). No rescue of ceh-6(gk665) lethality was obtained when ceh-6 3'UTR sequences were altered or when both the first and fourth introns were removed (Fig.1A, constructs Δ3'UTR and Δ1+4). In addition, removal of a large upstream region (-4102 to -215, Fig.1A, construct b), or removal of intron 1 plus swapping of ceh-6 3'UTR (Fig.1A, construct1+u-54 3'UTR) led to poor worm survival. These constructs were not further pursued. Constructs with the simultaneous deletion of two or all five introns (Fig.1A) were able to rescue the lethality, suggesting that these regions are dispensable for expression in the cells where lack of ceh-6 activity causes lethality. However, these constructs still led to expression in the rectal cells and, in large part, result in the Td defect of ceh-6 null mutants (Fig. 1B). Large deletions in the upstream region (3887bp and 2631bp resp., Fig.1A, constructs a, b) did not affect expression of the construct in rectal cells either and led to significant rescue of the Td defect. Interestingly, eliminating an additional 113bp closer to the ATG (Fig.1A, construct c) resulted in the complete loss of ceh-6 expression exclusively in rectal cells (Fig. 1B, Fig. 1C panel (iii)), while its expression appeared normal in other tissues, like the excretory cell and head neurons. Importantly, while construct “c” successfully rescued the lethality of ceh-6 mutants, transgenic animals exhibited a very penetrant Y Td defect (93%). Thus, most of our constructs rescued ceh-6(gk665) lethality. Two constructs, “c” and “1-3’utr”, resulted in no visible expression in the rectal cells and a corresponding highly penetrant Y-to-PDA transdifferentiation defect, confirming that ceh-6 activity is necessary in the rectal cells for Y identity swap. Of these two constructs, “c”, which lacks an upstream region, resulted in a relatively healthy transgenic strain.

In summary, we dissected the ceh-6 gene regulatory sequence in the upstream, intronic and 3'UTR regions. We have identified a small regulatory sequence, located upstream and close to the ATG, that is necessary to drive expression in the rectal cells. A deletion encompassing this region allowed us to build a genomic constructs to be tested were injected in IS2581 [ceh-6(gk665) I / hT2[qsIs48]; syIs63[coq-1::gfp; unc-119(+)]] animals (5ng/µl), together with a co-injection marker odr-1p::RFP (pSJ6106, 50ng/µl) and pBSK+ (200 ng/µl). hT2[qsIs48] animals are recessive lethal; we found that homozygotes ceh-6(gk665) animals are 100% lethal before the L2

Methods
Request a detailed protocol

All strains were cultured using standard conditions (Brenner 1973). The ceh-6 genomic loci, encompassed by fosmid WRM0633dB02, was tagged in-frame at the N-terminus with a GFP as described earlier (Turun et al. 2009). To create a GFP::ceh-6 rescuing construct, GFP-tagged fosmid WRM0633dB02 was used as a template to PCR-amplify a 13993bp fragment using custom-made oligos (table 1), which encompasses the ceh-6 gene as follows: 5664bp of the ceh-6 upstream region, ceh-6 ORF and 3587bp of the downstream sequence. This 13993bp genomic region was cloned into the pSCB vector using the StrataClone Blunt PCR cloning kit (Agilent Technologies) yielding pSJ6255, which was further used as a parent template to generate all specific deletions as highlighted in Figure 1A. All deletion constructs were made using custom oligos (Table 1) through standard reverse polymerase chain reactions with Phusion® High-Fidelity DNA Polymerase (M0530, NEB) and a Bio-Rad T100 Thermocycler. PCR fragments were phosphorylated using T4 Polynucleotide Kinase (M0201, NEB), and religated using T4 DNA Ligase (M0202, NEB). The ceh-6 3’utr was altered by digesting the plasmid with Sph1 and re-ligation on itself.

To generate ceh-6 transgenic lines, the ceh-6 genomic constructs to be tested were injected in IS2581 [ceh-6(gk665) I / hT2[qsIs48]; syIs63[coq-1::gfp; unc-119(+)]] animals (5ng/µl), together with a co-injection marker odr-1p::RFP (pSJ6106, 50ng/µl) and pBSK+ (200 ng/µl). hT2[qsIs48] animals are recessive lethal; we found that homozygotes ceh-6(gk665) animals are 100% lethal before the L2
stage [as such, all growing progeny from ce-6(gk665) / hT2 mother is heterozygote for ce-6(gk665): 43/43 adults, 33/33 L4 and 53/53 early L3]. Viability was assessed by scoring transgenic ce-6(gk665) adult worms in our transgenic lines. Transgenic ce-6 homozygous animals (L3 and older) were scored for the presence of a PDA neuron using the cog-1p::GFP marker as previously documented (Richard et al. 2011; Zuryn et al. 2014).

Reagents

Table 1:

| Plasmid (construct name) | Primer | Strain | Extrachromosomal array | Genotype |
|--------------------------|--------|--------|-------------------------|----------|
| pSJ6255                  | ceh-6pF ataaagaGCGGCGGCGCGtgtgtgttagcaacctctctactccttc | IS2577 | [fpEx902[GFP::ceh-6 locus 13kb; odr-1::rfp]] | ceh-6(gk665)I; [fpEx902; syIs63[cog-1::gfp;unc-119(+)]] IV |
|                          | ceh-6 UTR-R tgtgtagaagtagagaggattg | IS2639 | [fpEx940[GFP::ceh-6 13kb locus(3-2464 to -215); odr-1::rfp]] | ceh-6(gk665)I; [fpEx940; syIs63[cog-1::gfp;unc-119(+)]] IV |
|                          | ceh-6pmidR gcacacgcctgataataagccac | IS2691 | [fpEx958[GFP::ceh-6 13kb locus(3-4102 to -215); odr-1::rfp]] | ceh-6(gk665)I/hT2[qIs48]; [fpEx958; syIs63[cog-1::gfp;unc-119(+)]] IV |
| pSJ6321 (a)              | ceh-6pmidR2 gcatactgtagatgagattatc | IS2670 | [fpEx955[GFP::ceh-6 13kb locus(3-2464 to -102); odr-1::rfp]] | ceh-6(gk665)I; [fpEx955; syIs63[cog-1::gfp;unc-119(+)]] IV |
|                          | 6gk769extF gtggcctagagacgcgagaag | IS2628 | [fpEx938[GFP::ceh-6 13kb locus(A175-2173); odr-1::rfp]] | ceh-6(gk665)I; [fpEx938; syIs63[cog-1::gfp;unc-119(+)]] IV |
| pSJ6341 (b)              | ceh-6pmidR gcacacgcctgataataagccac | IS2648 | [fpEx945[GFP::ceh-6 13kb locus(Δ175-2173 & Δ2629-2848); odr-1::rfp]] | ceh-6(gk665)I; [fpEx945; syIs63[cog-1::gfp;unc-119(+)]] IV |
|                          | ceh-6PROMmF cttttatcgctagaaaataacatctac | IS2651 | [fpEx948[GFP::ceh-6 13kb locus(A175-2173 & Δ2952-3042); odr-1::rfp]] | ceh-6(gk665)I; [fpEx948; syIs63[cog-1::gfp;unc-119(+)]] IV |
| pSJ6324 (i1)             | ceh-6exon2f atacaccaagcagatagaggtg | IS2645 | [fpEx944[GFP::ceh-6 13kb locus (A3326-3667); odr-1::rfp]] | ceh-6(gk665)I; [fpEx944; syIs63[cog-1::gfp;unc-119(+)]] IV |
| pSJ6318  | U54swapF  | IS2603                        |
|-----------|-----------|-------------------------------|
| (i1 + 3\'utr unc-54 UTR) | ctcacagagccccgagacaacaatagcaacttgagcccgtgcctacc | [pEx930[GFP::ceh-6 13kb locus (Δ175-2173 & Δ 3042-7428 + unc-54 3\'UTR; odr-1::rfp] |
|           | U54swapR  |                               |
|           | cagcgaccaatgtaggaacccctcgcaacgagcgagagcgagagcgagagc   |

| pSJ6355  | ceh-6gk769extF | IS2624                        |
|-----------|----------------|-------------------------------|
| (a+i1+i2+i3+i4+i5) | gttggtcgacagcagacgagacaaga   | [pEx924[GFP::ceh-6 13kb locus (Δ-2846 to -215 & Δ175-2173 & Δ2629-2848 & Δ2952-3042 & Δ3178-3223 & Δ3326-3667); odr-1::rfp] |
|           | ceh-6pmidR     |                               |
|           | gcaacagccataatggaacc   |

| pSJ6325  | Ceh-6exon4bf | No lines                       |
|-----------|-------------|-------------------------------|
| (i1+i4)   | gtaactggaaatctctgtcttg   |                               |
|           | Ceh-6exon4br |                               |
|           | ctaactgtctgtctctctctct    |

| pSJ6322  | deletion using two SphI natives sites | Very sick |
|-----------|--------------------------------------|-----------|
| (3\'utr deletion) |                               | No line kept |

**Acknowledgments:** Some strains were provided by the International C. elegans Gene Knockout Consortium and the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440).

**References**

Bürglin TR, Ruvkun G. 2001. Regulation of ectodermal and excretory function by the C. elegans POU homeobox gene ceh-6. Development 128: 779-90. PMID: 11171402.

Fuxman Bass JI, Tamburino AM, Mori A, Beittel N, Weirauch MT, Reece-Hoyes JS, Walhout AJ. 2014. Transcription factor binding to Caenorhabditis elegans first introns reveals lack of redundancy with gene promoters. Nucleic Acids Res 42: 153-62. PMID: 24068555.

Kagias K, Ahier A, Fischer N, Jarriault S. 2012. Members of the NODE (Nanog and Oct4-associated deacetylase) complex and SOX-2 promote the initiation of a natural cellular reprogramming event in vivo. Proc Natl Acad Sci U S A 109: 6596-601. DOI: https://doi.org/10.1073/pnas.1110731109 | PMID: 22493276.

Richard JP, Zuryn S, Fischer N, Pavet V, Vaucamps N, Jarriault S. 2011. Direct in vivo cellular reprogramming involves transition through discrete, non-pluripotent steps. Development 138: 1483-92. PMID: 21389048.

C. elegans Deletion Mutant Consortium.. 2012. large-scale screening for targeted knockouts in the Caenorhabditis elegans genome. G3 (Bethesda) 2: 1415-25. PMID: 23173093.

Tursun B, Cochella L, Carrera I, Hobert O. 2009. A toolkit and robust pipeline for the generation of fosmid-based reporter genes in C. elegans. PLoS One 4: e4625. PMID: 19259264.

Zuryn S, Ahier A, Portoso M, White ER, Morin MC, Margueron R, Jarriault S. 2014. Transdifferentiation. Sequential histone-modifying activities determine the robustness of transdifferentiation. Science 345: 826-9. PMID: 25124442.

**Funding:** This work was funded by an ERC CoG PlastiCell # #648960 to SJ. SJ is a CNRS research director

**Author Contributions:** Arnaud Ahier: Conceptualization, Investigation, Methodology, Validation, Formal analysis, Writing - review and editing. Shashi Kumar Suman: Visualization, Data curation, Formal analysis, Writing - original draft, Writing - review and editing. Sophie Jarriault: Conceptualization, Formal analysis, Supervision, Project administration, Funding acquisition, Writing - review and editing.

**Reviewed By:** Anonymous

**History:** Received October 26, 2020 Revision received December 7, 2020 Accepted December 15, 2020 Published December 21, 2020
Citation: Ahier, A; Suman, SK; Jarriault, S (2020). Gene bashing of ceh-6 locus identifies genomic regions important for ceh-6 rectal cell expression and rescue of its mutant lethality. microPublication Biology. https://doi.org/10.17912/micropub.biology.000339