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cis-regulatory processing of Notch signaling input to the sea urchin *glial cells missing* gene during mesoderm specification

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Abstract

The *glial cells missing* regulatory gene of *Strongylocentrotus purpuratus* (*spgcm*) was proposed earlier to be the genomic target of Delta/Notch (D/N) signaling required for specification of the mesodermal precursors of pigment cells. Here, we show that microinjection of a *spgcm* antisense morpholino oligonucleotide results in larvae without pigment cells. Microinjection of an mRNA encoding a dominant negative form of Suppressor of Hairless (dn-Su(H)) results in reduced levels of *spgcm* mRNA, disruption of mesodermal founder cell specification and failure to produce pigment cells. These results confirm that this gene is required for pigment cell specification. Three *cis*-regulatory modules of the *spgcm* gene were identified, which when incorporated in a GFP expression construct recapitulate the early expression pattern of this gene. Spatial expression of this GFP expression construct is severely disrupted by co-expression of dn-Su*(H)* mRNA, confirming that *spgcm* is a direct target of canonical N signaling mediated through Su(H) inputs. *cis*-perturbation analysis by mutation of consensus Su(H) sites identified a conserved motif paired-site and a lone site in the middle module that function both to drive expression in SMC precursors which receive the Delta signal and to repress expression in ectopic locations which lack this signal. While these Su(H) target sites provide the *cis*-regulatory architecture with the core of an N signaling transcriptional response switch, both the *on* and *off* outputs from this module require additional inputs.
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Introduction

The endomesodermal gene regulatory network model (EM-GRN) of the sea urchin embryo identifies numerous regulatory nodes central to the mechanism of endomesoderm specification (Davidson et al., 2002a,b; Davidson, 2006). The integrated GRN framework generates testable predictions of regulatory linkages and enables the observations of experimental embryology to be related to the genomic regulatory apparatus (Revilla-i-Domingo et al., 2004; Oliveri and Davidson, 2004; Levine and Davidson, 2005). An essential input in the mesodermal subsegment of the EM-GRN is a signal transduced by the Notch receptor, which is required for induction of the mesodermal founder cells that give rise to the secondary mesenchyme of the embryo (SMCs; e.g., pigment cells and blastocoelar cells; Sherwood and McClay, 1999; Sweet et al., 1999, 2002; McClay et al., 2000; Ruffins and Ettensohn, 1996). The inducing signal is the Delta ligand, expressed by the eight large micromere daughter cells beginning at 7th cleavage. The Delta signal is received directly by the adjacent cells of the macromere lineage. Our objectives in the *cis*-regulatory analysis presented here were to test the prediction in the GRN model that the *spgcm* regulatory gene serves as a primary target for the essential N signaling event; and if so, to demonstrate the *cis*-regulatory logic by which the signal input is transduced into correct spatial *spgcm* expression. The Suppressor of Hairless (Su(H)) transcription factor is the major mediator of N signaling (Wettstein et al., 1997; Morel and Schweisguth, 2000; Furriols and Bray, 2001; Barolo and Posakony, 2002; Barolo et al., 2002; Lai, 2004). Much of what follows concerns the functional roles of the several important Su(H) target sites that these studies revealed in the *spgcm* regulatory apparatus.

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The *spgcm* gene, an orthologue of *Drosophila glial cells missing*, was one of several newly discovered sea urchin regulatory genes identified by Ransick et al. (2002) as participants in the process of endomesoderm specification. Whole mount in situ hybridization (WMISH) revealed that *spgcm* transcripts first appear in 14–16 cells of the Veg2 lineage of 7th cleavage embryos. These constitute the most vegetal tier of macromere-derived cells, and they are in direct contact with the large micromeres, the source of the Delta signal (Sweet et al., 1999, 2002). The *spgcm* mRNA remains detectable in the mesenchyme blastula stage in the non-oral portion of the SMC precursor domain and continues to be expressed by the cohort of cells that during gastrulation ingresses to differentiate into pigment cells. The temporal and spatial expression pattern of *spgcm* is thus consistent with that postulated for a target gene of N signaling. It was furthermore demonstrated that *spgcm* expression is depleted >8-fold after microinjection of mRNA encoding dn-N (Calestani et al., 2003), confirming this gene to be a prime candidate in the EM-GRN as the target of the Delta/N signal required for specification of pigment cells.

Here, we show that expression of the *spgcm* regulatory gene is indeed required for specification of pigment cells, and we describe a *cis*-regulatory analysis of the functional Su(H) target sites of the control modules which govern the expression of this gene. We show that the initial phase of *spgcm* expression in SMC founder cells is a direct consequence of local Delta/N signaling operating through Su(H) inputs into its *cis*-regulatory target sites. These elements function essentially as default-*off*, signaling-*on*, transcriptional toggle switches.

Materials and methods

Morpholino substituted antisense oligonucleotide

To obtain an antisense morpholino substituted oligonucleotide designed to block translation of *spgcm* mRNA, the coding strand sequence around the translation initiation site (from −76 to +30) was provided to GeneTools, LLC. The vendor provided ~300 nmol of a column purified, salt-free, lyophilized 25mer (gcm-mo: 5′-GCTTTGGAGTAACCTTCTGCACCAT-3′), complementary to bases 0 to +25. Gcm-mo and the GeneTools Standard Control Oligo (C-mo: 5′-CCTCTTACCTCACTTACAATTTATA-3′) were resuspended in RNase-free water as concentrated stocks then stored at −20°C as 5 µl aliquots. Stock aliquots were thawed and incubated for 5 min at 37°C just prior to use in preparing injections solutions. Injection solutions containing 200 µM oligo and 125 mM KCl were made fresh for each experiment. Prior to experimental usage, the effectiveness of gcm-mo was confirmed by demonstrating that it blocked translation of a chimeric *spgcm-GFP* [green *f*luorescent *p*rotein] mRNA (Rast et al., 2002).

Dominant negative-Su(H)

The full-length sequence of a sea urchin orthologue of *Su(H)* (from *H. pulcherrimus*, unpublished data) was kindly provided by K. Akasaka (University of Tokyo). This was used to confirm the identity and to characterize an *Strongylocentrotus purpuratus* pSport-cDNA clone 149-E7 (C. Calestani, unpublished data), recovered from an arrayed 20 h embryo cDNA library. Transcripts that would be efficiently translated after microinjection were created by subcloning the ~1.8 kb fragment encoding the entire *Su(H)* open reading frame into the pBluescript-RN3 vector, which adds the 5′ and 3′ globin UTRs onto T3-driven transcripts (Lemaire et al., 1995). An Su(H) construct that produces a dominant negative activity was engineered using the QuikChange Site-Directed Mutagenesis Kit (Stratagene #200519) to replace eight nucleotides in the region encoding the highly conserved DNA binding motif RLRSQTVSTRY, which resulted in four amino acid substitutions that instead encoded *ELE*SQTVST*ES*. Su(H) protein with this modification has been shown to behave as a dominant negative form with respect to blocking transcriptional activation functions, in that DNA binding is blocked while interactions with N-internal fragment are unaffected (Chung et al., 1994; Wettstein et al., 1997). Capped mRNA for microinjection was synthesized in vitro using a T3 Message Machine Kit (Ambion) and was stored at −70°C as a concentrated stock until just prior to use.

BAC clones and Family Relations analysis

Screens of the *S. purpuratus* and *L. variegatus* BAC arrayed libraries (Cameron et al., 2000, 2004) were made with an ~300 b *spgcm* coding region antisense-RNA probe. The positive BAC clones (*Sp*) 33-O18 and (*Lv*) 18-J3 were finally selected for sequencing after mapping had determined the *gcm* coding regions to be centrally located within these BAC clones. To align and search the BAC sequences for conserved elements, the Family Relations software package (Brown et al., 2002) was used in pairwise view, with a 50 bp, 20 bp or 10 bp window size.

Making GFP reporter constructs and microinjections

Regions D (615 bp), E (350 bp) and P (456 bp) were PCR amplified from the *spgcm* BAC 33-O18. The amplicons for D and P are slightly larger than the conserved domains reported in the Results section, due to selection of primer sites slightly outside of the conserved segments. A 1256 bp fragment was PCR amplified from the EpGFPII plasmid (Cameron et al., 2004) containing the basal promoter of *endo16* with the Kozak sequence and the first 14 codons of *cyIIa* fused in frame to the GFP coding sequence and SV40 poly-A sequence of pGreenL3 (Gibco). The 'Spacer' (Sp) region is a ~1 kb segment of genomic DNA amplified from the *spgcm* BAC in a non-conserved region ~10 kb downstream of the 3′ end of exon six and is positioned in all fusion constructs between E and P regions. These amplicons were then merged together by fusion PCR followed by TA cloning into Pgem-T Easy vector (Promega). Several additional constructs, which are discussed in the Results section, were produced from this starting plasmid using a combination of site-directed mutagenesis and fusion PCR. All PCR amplifications used the Expand High Fidelity PCR System (Roche). As an additional precaution, the sequence of each new construct was confirmed (with particular attention to the GFP coding sequence) prior to use in microinjection experiments. In preparation for microinjection, all Pgem-T plasmids were digested with *Eco*RI, cutting in two insert flanking sites to release the intact insert from the plasmid backbone. Reporter construct microinjections were carried according to well-established protocols (McMahon et al., 1985; Arnone et al., 2004). Microinjection solutions were prepared just prior to use and consisted of 30–35 ng *Eco*RI digested GFP construct DNA and 200–225 ng *Hin*dIII digested genomic carrier DNA in 10 µl of 125 mM KCl. Injection solutions of this composition, made with an ~3.7 kb long *D-E-Sp-P-GFP* construct, for example, delivered (per 2 pl injection) 800–950 copies of the construct with a 7–8× molar excess of carrier DNA.

Scoring GFP expression

Embryos microinjected with GFP expression constructs that had successfully progressed through cleavage stages (typically >90%) were collected from injection plates around the time of hatching (15–18 h). Collected embryos were rinsed with fresh, filtered seawater and transferred to relatively deep, U-shaped wells (Falcon #3911). Under these culture conditions, the normally developing cohort of each injected batch (again, typically >90%) would concentrate near the surface of the culture well by virtue of establishing coordinated swimming behavior. For analysis of GFP expression, about 110–130 normally developing embryos (of each injected group) were transferred into an agar tunnel, prepared with 2% Noble Agar in 60 mm petri dishes (Ransick and Davidson, 1995). Once aligned in single file in the tunnels, GFP expression was scored in 21–23 h mesenchyme blastulae by inspecting the rows of slowing, rotating embryos using a Zeiss Axiovert 25 microscope, outfitted with appropriate epifluorescent capabilities and a traversing, cooled stage. Each embryo was scored with respect to the distribution of GFP-positive clones in one or more of five spatial expression domains (see zones 1–5 in Fig. 4A).

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