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Developmental *cis*-regulatory analysis of the cyclin D gene in the sea urchin *Strongylocentrotus purpuratus*

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ABSTRACT

Cyclin D genes regulate the cell cycle, growth and differentiation in response to intercellular signaling. While the promoters of vertebrate cyclin D genes have been analyzed, the *cis*-regulatory sequences across an entire cyclin D locus have not. Doing so would increase understanding of how cyclin D genes respond to the regulatory states established by developmental gene regulatory networks, linking cell cycle and growth control to the ontogenetic program. Therefore, we conducted a *cis*-regulatory analysis on the cyclin D gene, *SpcycD*, of the sea urchin, *Strongylocentrotus purpuratus*, during embryogenesis, identifying upstream and intronic sequences, located within six defined regions bearing one or more *cis*-regulatory modules each.

1. Introduction

Cyclin D family genes link cell cycle control to the genomically encoded program of multicellular development [1–4], by responding to transcription factors activated by intercellular signals [1,2] to promote cell cycle transit from G1 to S phase [3–5], cell growth [6], and differentiation in multiple systems including spermatogonia [7], myeloid cells [8], skeletal muscle [9], and the nervous system [10]. Mis-expression is deleterious: cyclin D genes are overexpressed in many cancers [11–15], and in the sea urchin, ectopic expression during cleavage is lethal [16].

The sea urchin *Strongylocentrotus purpuratus* has one cyclin D gene [17], which is expressed in a dynamic pattern during embryogenesis [16]. At blastula stage, expression is global. During gastrulation, expression restricts to gut, oral ectoderm, and ciliary band [16], a pattern displayed by ~40% of genes expressed in the sea urchin embryo, concordant with continued growth and proliferation [18]. Cyclin D regulates patterning of the embryo, since its knockdown produces malformed larvae [16].

Many developmentally important genes have now been linked into developmental gene regulatory networks (dGRNs) [19–21]. The linkages between genes are mediated by *cis*-regulatory modules (CRMs) that function by binding transcription factors, many of which are activated by intercellular signaling [19,21]. Typically, developmentally regulated genes contain multiple CRMs [21]. Although the promoters of various cyclin D genes have been dissected [2,22], no cyclin D gene has been subjected to a comprehensive *cis*-regulatory analysis to identify CRMs. Such an analysis is a necessary step in elucidating how dGRNs control cell proliferation. We therefore undertook a *cis*-regulatory analysis of *SpcycD* in the sea urchin *S. purpuratus*, which is highly amenable such analyses [23–30]. We used a high throughput method from Nam and colleagues [31] to examine the entire locus.

2. Materials and methods

2.1. Embryo culture

S. purpuratus obtained from the Pt. Loma Marine Invertebrate Lab (Lakeside, CA) were maintained in a seawater aquarium at 12 °C. Gametes were obtained by shaking or injection with 0.55 M KCl [32]. Embryos were cultured at 15 °C.

2.2. Preparing reporter constructs and BAC DNA

Regions to be assayed were amplified from *SpcycD* BAC or genomic DNA template by polymerase chain reaction (PCR) using high fidelity DNA polymerases (from Roche or New England Bioloabs) and the primers in Supplemental Table 1.

'13-tag' reporters were obtained from mini-preps of cultures grown with chloramphenicol (12.5 μg/ml) and Copy Control Induction Solution (Epicentre). These reporters were modified from that

Abbreviations: hpf, hours post-fertilization; CRM, *cis*-regulatory module; GRN, gene regulatory network; 5′ UTR, 5′ untranslated region.

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presented in [31] (J. Nam, personal communication, May 2011) in that the *Sp-gatae* basal promoter was replaced with an *Sp-nodal* basal promoter using primers new_mNBP and end_core-polyA (Supplemental Table 1) in a reaction employing the cycling shown in Supplemental Table 2.

Amplified regions were ligated to EpGFPII [33], after which the resultant constructs were linearized; or linked as directed [31] to the indicated modified 13-tag reporters (Supplemental Table 3). 13-tag-region-linked reporter products visualized using SYBR Safe and a Safe Imager (Invitrogen) rather than UV light were gel purified (Nucleospin Gel and PCR Cleanup, Clontech). Sequencing showed that only 13-tag-linked region 3 showed significant contamination.

BAC DNA was obtained from stab cultures of BAC 4013 F-18 mCherry (from Sp Genome Research Resource, Caltech) grown under chloramphenicol selection and purified as described [34].

2.3. Microinjection

10 μl injection solutions of EpGFPII-linked reporters [33] contained ~10 nmols reporter construct, 165–200 ng HindIII digested genomic DNA, 0.12 M KCl. Injection solutions comprising 13-tag-linked regions were made as described [31], except Master Pool volume in the 10 μl injection mix for all experiments but #8 was increased from 0.5 to 1 μl. In Experiment #8, Master Pool concentration was 5× greater than in other presented experiments. Embryos in seawater containing 1 mM PABA salt were injected with the appropriate reporters using standard methods [35]. About 100–150 embryos were injected with EpGFPII-linked regions, and ⩾200 with 13-tag reporter-linked regions. Hatched embryos were transferred to ~1.5 ml artificial seawater for subsequent culture.

2.4. Preparing RNA, DNA and cDNA

To assay endogenous *SpcycD*, embryos were cultured at ~1200 embryos/4 ml. RNA was obtained (Rneasy Plus mini kit, Qiagen), and DNA was removed as directed. RNA equivalent to 30 ng per 20 μl was converted to cDNA using random hexamers (FirstStrand cDNA Synthesis kit, Invitrogen). RNA and DNA from injected embryos were obtained with a DNA/RNA ALL Prep kit (Qiagen). RNA was treated with DNAse for ⩾30 min as directed. cDNA from injection with EpGFPII-linked regions was made with random hexamers, with 3 μl RNA for each 20 μl reaction. cDNA from injection with 13-tag-linked regions was made from RNA equivalent to 3 μl per 20 μl, and 13-tag vector-specific primer [31] using the FirstStrand Synthesis Kit. The exception was Experiment #5, where random hexamers and the VILO cDNA Synthesis Kit (Invitrogen) were used.

2.5. Real-time PCR

Real-time PCR (relative quantification) was conducted using Perfecta SYBR Green Fast Mix (Quanta BioSciences) and a LightCycler 480 II (Roche). cDNA and DNA equivalent to 1.3 and 1.6 μl, respectively, per 12 μl were used. Unless noted, all reactions were done in duplicate. (Cycling conditions are in Supplemental Table 2.)

Relative levels of *SpcycD* expression with respect to the first time point was determined using the delta-delta Ct method, with ubiquitin as normalization reference [36]. Relative GFP expression from microinjected EpGFPII-linked reporters was calculated by the same method, with further normalization to the amount injected [30]. Activity levels of microinjected BAC 4013 F-18 mCherry were determined the same way, except mCherry sequence was assayed. Expression of each region-linked 13-tag reporter was found using a 13-tag unit-specific primer [31]. Activities were determined as for EpGFPII-linked regions, but were relative to that of empty reporter 1302 for each time point.

2.6. Sequence comparisons

LvcycD sequence was from: a BAC containing 17 kb upstream of exon 1; and *LvcycD* draft sequence from SpBase [37,38]. Comparisons were made using Family Relations II [39,40] to identify sequences of ⩾20 bp in *SpcycD* with ⩾90% similarity to *LvcycD*.

2.7. Fluorescence microscopy

Eggs were arrayed on 50 mm glass bottom dishes (MatTek), fertilized and injected as described above. Injected embryos were visualized with an Axiovert 200 fluorescence microscope (Zeiss).

3. Results and discussion

3.1. Temporal expression of SpcycD

The temporal profile of embryonic *SpcycD* expression was assayed by quantitative RT-PCR. As reported previously by others [16], expression commenced at ~10–12 hpf (early blastula), then increased at least up to pluteus stage (72 hpf) (Supplemental Fig. 1). Interestingly, there was substantial variation between biological replicates.

We also co-assayed the temporal activities of endogenous *SpcycD* and a bacterial artificial chromosome (BAC) bearing *SpcycD* with mCherry knocked into exon 1. This BAC encompassed sequence from ~90 kb upstream of the gene to ~13 kb downstream. Both endogenous *SpcycD* and the injected BAC exhibited similar temporal activities (Fig. 1), suggesting the information needed to regulate embryonic *SpcycD* expression is within this BAC. Our *cis*-regulatory analysis examined from ~13 kb upstream of exon 1 to ~7 kb downstream from the end of exon 5 (Fig. 2A).

3.2. Identification of cis-regulatory regions

We selected 22 regions spanning upstream and intronic sequence of *SpcycD* to assay for regulatory activity (Fig. 2A). The boundaries of most were chosen based on the presence of sequences of ⩾20 bp with ⩾90% similarity to *LvcycD* from *Lytechinus variegatus* (Fig. 2A) [37]. This criterion was based on the fact that sequence comparisons between genes in *S. purpuratus* and *L. variegatus* reliably predict *S. purpuratus* CRMs [40,41]. Our analysis was comprehensive: all non-exonic sequence except 1 bp between the 3′ end of region 10 and the 5′ end of exon 5, and 2 bp between the 3′ end of region 11 and the 5′ end of region 21 was tested.

Candidate *cis*-regulatory regions were assayed for activity using the '13-tag' reporters developed by Nam and colleagues [31]. Representative results are in Fig. 2B and Supplemental Fig. 2. In each experiment, a region was classified as significantly active if activity at one or more time points was ⩾2.5 times that of the mean activity of regions in the middle 40% of the distribution [31].

Several active regions were identified. Region 5, (2.4 kb) in the first half of intron 2 (Fig. 2A) showed the strongest activity, with significant activity at all tested time points from ~10 to 60 hpf. This activity was ~15 times greater than that of empty reporter at its peak, and at least two times higher than those of the next most active regions. The next most active regions were region 2 (~3.6 kb), located ~4.6 kb upstream from the beginning of exon 1; region 6 (2.7 kb), comprising the 3′ half of intron 2; region 19 (4.6 kb), in intron 4; followed by region 4 (2.1 kb), which abuts exon 1; and region 17 (2.1 kb) in intron 1 (Fig. 2A). Regions 2