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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

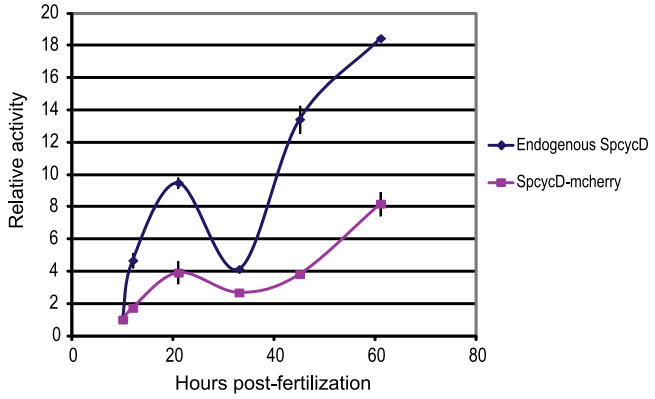

Fig. 1. Expression of endogenous *SpcycD* and microinjected mcherry-linked BAC bearing *SpcycD* plus 90 and 13 kb of up and downstream sequence. Relative levels of SpcycD mRNA were measured at each indicated time point by qRT-PCR as described in the text.

and 6 always showed significant activity for at least one time point when injected without region 5-linked reporter, but not always in its presence (Supplemental Fig. 2).

3.3. Temporal activity profiles of cis-regulatory regions

To gain further insight into the roles of each active region, temporal activity profiles were extracted from experiments in Fig. 2B and Supplemental Fig. 2 (Supplemental Fig. 3). This analysis reveals substantial inter-experimental variation in the temporal activity profiles of each region. An exception concerned region 19, as discussed below. Possible sources of this variation include biological variability, the fact that injection solutions contained different mixtures of 13-tag-linked regions, and the fact that each time point was from a separate injection plate because it was technically possible to inject a maximum of ~200 embryos per plate. To more clearly discern canonical aspects of the temporal activity patterns, the activity values across experiments were averaged (Fig. 3).

From this analysis, we found the following patterns. Region 5's activity was highest at 10–12 hpf, when *SpcycD* is initially activated. As other regions became active, region 5's activity declined somewhat, but remained significant (Fig. 2B). Region 6 likewise showed the strongest activity at ~10 hpf. During the first ~33 h, activities of regions 5 and 6 paralleled each other, then region 6's stabilized, suggesting that region 6 contributes to maintaining *SpcycD* expression after ~33 hpf, corresponding to gastrulation and later stages.

On average, region 2's activity peaked at ~21 hpf (Fig. 3), although peak activity varied from ~12 to 33 hpf (Supplemental Fig. 3). Region 2's activity peak occurred after that of regions 5 and 6. Therefore, region 2's primary role may be to activate transcription during late blastula stage.

Region 4's activity varied considerably (Supplemental Fig. 3), but on average (Fig. 3) increased to low but stable levels by

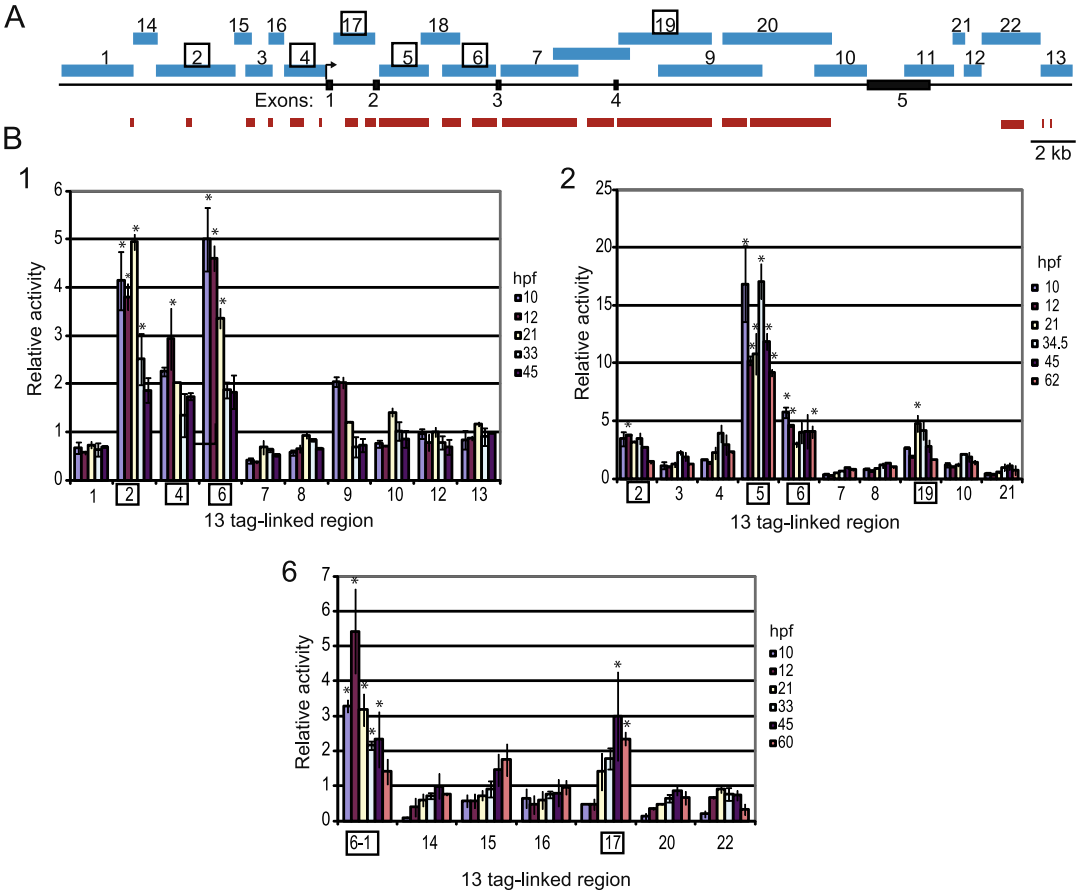

Fig. 2. Identifying *cis*-regulatory sequences. (A) Regions tested for CRM-containing activity. *SpcycD*, plus 13 and 7 kb of upstream and downstream sequence is shown. Exons: black; potential CRM-containing regions: blue; sequences with ⩾90% similarity to *LvcycD*: red; active regions: boxed. (B) Representative activity profiles. Each panel is from the indicated experiment. Asterisks denote significant activity. See Supplemental Fig. 2 for additional activity profiles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

~21–33 hpf. Thus, region 4 may contribute to maintaining *SpcycD* expression.

Region 17's activity slowly increased to stability by ~21–33 hpf (Fig. 3), indicating that this region may contribute to maintenance or lineage-specific activation of *SpcycD* during and after gastrulation.

Region 19's activity peaked at ~21 hpf, the mesenchyme blastula stage (Fig. 3), suggesting that this region may act as a switch that regulates *SpcycD* at the onset of gastrulation. As noted, region 19's activity showed much less variation than those of other active regions (Supplemental Fig. 3; compare Experiments #5, 2 and 3). Therefore, region 19 may be under especially strong control.

As a control, activities of region 2-linked 13-tag vectors at 12 hpf (Supplemental Fig. 4A), and 13-tag vectors linked to unique regions (Supplemental Fig. 4B) were compared. There was significantly less variation between activities of 13-tag reporters linked only to region 2 than between those linked to different regions, indicating that differences in activity among regions could mostly be attributed to region-specific differences rather than 13-tag reporter-specific differences.

3.4. Identification of candidate cis-regulatory modules

Since the sizes of the identified regulatory regions ranged from ~2 to 5 kb (Fig. 2A), additional analysis was needed to identify CRMs, which are generally only up to several hundred bp [21]. By using a combination of computational approaches to analyze each region (Fig. 4; Supplemental Figs. 5 and 6), we identified candidate CRMs within each, the activities of several of which were verified experimentally.

Region 2 contains a 0.5 kb subregion, 2-2, encompassing sequence conserved at ⩾90% with *LvcycD* (Fig. 4A; Supplemental Fig. 5). Experimental analysis using both 13-tag and EpGFPII-linked versions of region 2 and subregion 2-2 showed that subregion 2-2's temporal activity mirrored region 2's (Fig. 4B, panel 1; Supplemental Fig. 7). Further analysis showed that the activities of each were detected at blastula stage by fluorescence microscopy (Fig. 4C, panel 1). Together, these findings indicate that subregion 2-2 contains a CRM.

Region 4 contains two active subregions (4-1 and 4-2; Fig. 4A). Subregion 4-1 overlaps partly with conserved sequence (Fig. 4A; Supplemental Fig. 5), and bears a potential Runx site (Supplemental Fig. 5). Sequence within subregion 4-1 was previously found by chromatin immunoprecipitation to bind the Runx protein SpRunt-1, which was shown to regulate *SpCycD* [42]. Subregion 4-2 contains a 22 bp conserved sequence (Fig. 4A; Supplemental Fig. 5), and a potential Runx site [43] (Supplemental Fig. 5). When tested for activity by fluorescence microscopy, subregions 4-1 and 4-2 were both shown to be active at gastrula stage (Fig. 4C, panel 2), suggesting that both encompass CRMs.

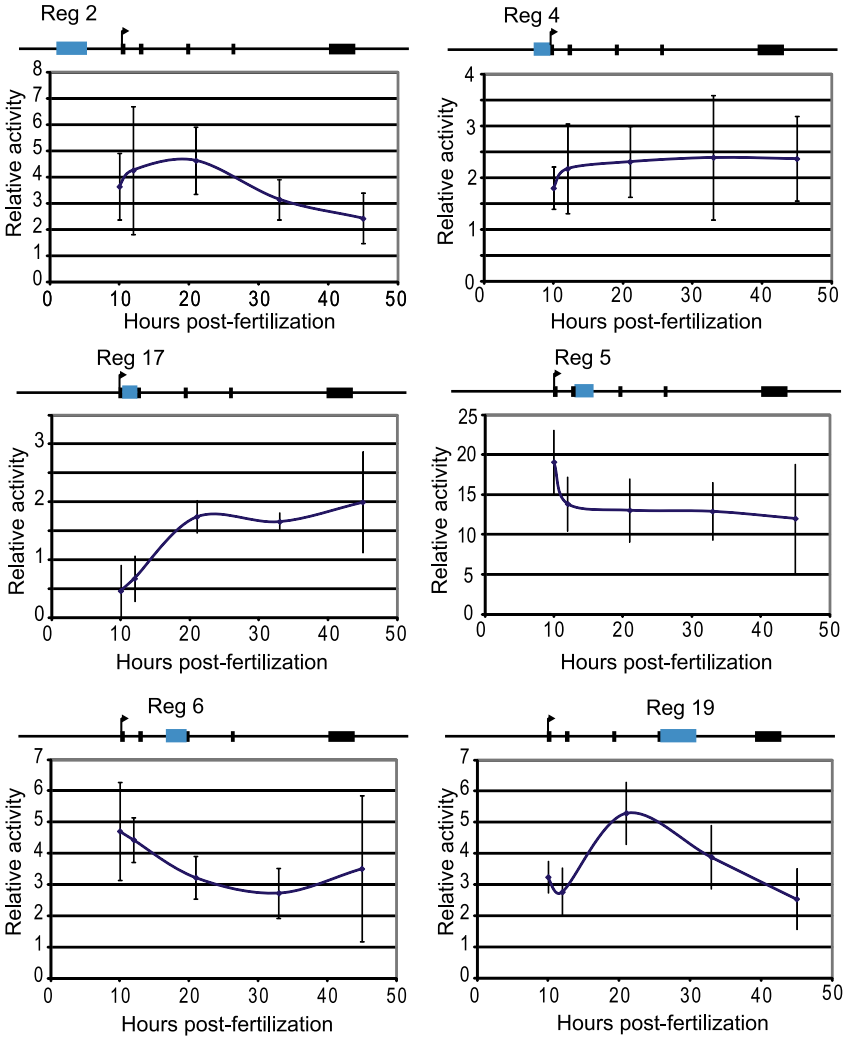

Fig. 3. Averaged temporal activity profiles. Grand means and standard deviations were calculated from the means of all experiments in Supplemental Fig. 3. Small differences between time points in different experiments (for example, 45 and 47 hpf) were ignored.

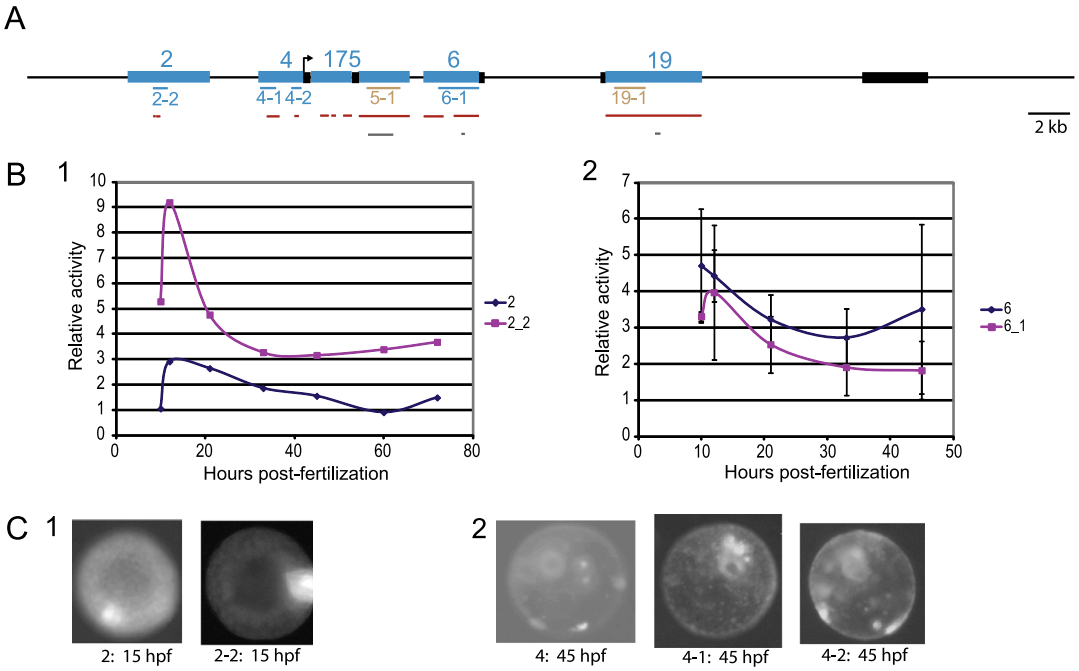

Fig. 4. Identification of *cis*-regulatory modules. (A) *SpcycD* showing active *cis*-regulatory regions. Exons: black rectangles; active regions: blue rectangles; active and inactive subregions: blue and tan lines, respectively; conserved sequences: red; Cluster-Buster-identified sequences: gray. (B) Activities of 13-tag-linked regions 2, 2-2, 6 and 6-1. Panel 1 shows the activities of region 2 and subregion 2-2 in co-injected embryos (one experiment). Panel 2 shows the averaged temporal activities and standard deviations of region 6 and subregion 6-1 from all presented experiments where either region was assayed. (C) Fluorescence micrographs from injection with EpGFPII-linked region 2, 2-2, 4, 4-1 or 4-2. Brightness and contrast were adjusted equally in all images. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Our analysis of the intronic regulatory regions, which contain longer stretches of sequence conservation than the upstream regions (Fig. 4A, red lines), was chiefly computational. In this analysis, a number of sequence elements of interest were identified. Among these, were potential binding sites for TCF and Runx. Wnt-TCF signaling is known to regulate cyclin D expression [44–47]; and, as noted above, the SpRunt-1 protein is known to regulate *SpcycD*. In addition, we searched for sequences with clustered binding sites for transcription factors identified by the program Cluster-Buster, of interest because sequences where transcription factor binding sites cluster are hypothesized to be regulatory [48–50]; and G strings, known to mark regulatory regions in other genes [51–55]. These areas are highlighted on the sequence for each region in Supplemental Fig. 5. Identities of transcription factors identified by Cluster-Buster are in Supplemental Fig. 6.

One candidate CRM in region 5 was subregion 5-1, found 6 bp upstream of a potential transcription factor cluster site to 14 bp downstream from a potential TCF binding site (Fig. 4A, Supplemental Fig. 5). However, subregion 5-1 showed only background activity (Supplemental Fig. 2, Experiments #5 and 9). This was surprising because within its boundaries, which overlapped with conserved sequence, subregion 5-1 contains 6 potential TCF and Runx sites, respectively, most of which overlap with the transcription factor cluster site. Therefore, 5-1 may be necessary but not sufficient for region 5's activity.

Within region 6, we reasoned that the 3′ two-thirds of this region could contain a CRM, as most of the potential regulatory elements of interest were found in that portion (Fig. 4; Supplemental Figs. 5 and 6). This subregion, 6-1, was verified to be active (Fig. 2B, panel 6; Supplemental Fig. 2, Experiments #7, 8 and 9), and its temporal activity closely resembled region 6's (Fig. 4B).

Within region 19, a sequence termed subregion 19-1, which bears few of the potential regulatory elements of interest highlighted in Supplemental Fig. 5, showed only background activity (Supplemental Fig. 2, Experiment #9), indirectly supporting the hypothesis that the highlighted sequence elements shown for this region likely mark one or more CRMs.

4. Conclusions

The entire *SpcycD* locus was analyzed to identify *cis*-regulatory regions and modules (CRMs) that mediate expression. We identified intronic and upstream regions that impart distinct activity patterns, and uncovered likely CRMs in several. A future aim is to determine the specific roles of each regulatory region and candidate CRM by individual deletion of each from a BAC bearing *SpcycD*. Finally, to link *SpcycD* to GRNs that control early embryogenesis, the spatial activity of each CRM should be studied.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.094.

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