Complex interactions between cis-regulatory modules in native conformation are critical for *Drosophila snail* expression

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**SUMMARY**

It has been shown in several organisms that multiple cis-regulatory modules (CRMs) of a gene locus can be active concurrently to support similar spatiotemporal expression. To understand the functional importance of such seemingly redundant CRMs, we examined two CRMs from the *Drosophila* snail gene locus, which are both active in the ventral region of pre-gastrulation embryos. By performing a deletion series in a ~25 kb DNA rescue construct using BAC recombineering and site-directed transgenesis, we demonstrate that the two CRMs are not redundant. The distal CRM is absolutely required for viability, whereas the proximal CRM is required only under extreme conditions such as high temperature. Consistent with their distinct requirements, the CRMs support distinct expression patterns: the proximal CRM exhibits an expanded expression domain relative to endogenous *snail*, whereas the distal CRM exhibits almost complete overlap with *snail* except at the anterior-most pole. We further show that the distal CRM normally limits the increased expression domain of the proximal CRM and that the proximal CRM serves as a ‘damper’ for the expression levels driven by the distal CRM. Thus, the two CRMs interact in cis in a non-additive fashion and these interactions may be important for fine-tuning the domains and levels of gene expression.

**KEY WORDS:** Cis-regulatory modules, Gene expression, *Drosophila melanogaster*, snail, Developmental patterning, Repression, Huckebein

**INTRODUCTION**

A number of cis regulatory modules (CRMs) have recently been identified that support concurrent expression of individual genes in similar spatiotemporal profiles in early *Drosophila* embryos, as well as later in development (e.g. Frankel et al., 2010; Hong et al., 2008; Zeitlinger et al., 2007). For the most part, these secondary CRMs were identified as a result of ChIP-chip and ChIP-seq analyses as regions of occupancy located at a distance from genes of interest, up to 10 kb or more (e.g. Li et al., 2008; Ozdemir et al., 2011; Sandmann et al., 2007; Zeitlinger et al., 2007). These newly identified CRMs have been described as being redundant to previously identified promoter-proximal located CRMs and, most recently, it has been proposed that they function to provide robustness to environmental or genetic perturbation (Frankel et al., 2010; Perry et al., 2010). Moreover, in vertebrate genomes it has been shown that many genes have multiple CRMs active concurrently, and that deletion of one cis-regulatory module can have no observable effect on the gene expression pattern (e.g. Ghiasvand et al., 2011; Xiong et al., 2002). Therefore, identifying why multiple CRMs of similar spatiotemporal expression domains are active simultaneously is a problem of general interest.

Here, we focus on analysis of the *snail* (*sna*) locus in *Drosophila*. *sna* encodes a transcription factor containing Zn-finger DNA-binding domains that predominantly functions to repress the expression of a number of genes from ventral regions of the embryo (e.g. Cowden and Levine, 2002; De Renzis et al., 2006; Ip et al., 1992a). As such, Snail is an important patterning molecule that influences the mesoderm-mesectoderm-neurogenic ectoderm boundary (Kosman et al., 1991; Leptin, 1991). Although a CRM supporting expression similar to *sna* was isolated almost 20 years ago by standard *lacZ* reporter gene constructs from a promoter proximal location, even 6.0 kb of upstream sequence failed to completely represent native *sna* expression, which exhibits very sharp anterior-posterior and lateral boundaries (Ip et al., 1992b). Since then, the predominant view in the field has been that synergy between the Dorsal and Twist transcription factors, which is present in ventral gradients within early embryos, functions to specify the sharp *sna* dorsal boundary (Ip et al., 1992b; Zinzen et al., 2006), and that the sharp posterior boundary is defined by the repressor Huckebein (Reuter and Leptin, 1994). Yet the promoter proximal CRM of *sna* does not exhibit either of these sharp borders, despite the fact that it encompasses the region all the way up to the adjacent upstream gene (Ip et al., 1992b).

In general, it is a common assumption in the field that CRMs located in promoter-proximal locations are required to support gene expression. Thus, although it was noticed that the pattern of the promoter-proximal CRM was expanded relative to endogenous *sna*, the existence of another CRM to serve as a vehicle for repressors was not proposed upon the initial characterization of the reporter gene pattern (Ip et al., 1992b). It is a common finding that CRMs do not always support expression in the exact same domain as the genes they regulate, but in the past this was explained away as a flaw inherent to reporter gene assays. For example, the CRM supporting expression within stripes 3/7 of the *even-skipped* (*eve*) gene does not exhibit equivalent effects in *knirps* mutants as does the endogenous *eve* gene: the expression of the reporter gene expands into the midsection, whereas stripes 3/7 associated with the endogenous *eve* gene retain sharp boundaries (Frasch and Levine, 1987; Small et al., 1996).
More recently, however, additional CRMs have been identified sharing similar spatiotemporal profiles to previously characterized CRMs, including one that shares close similarity with the snail expression pattern (Ozdemir et al., 2011; Perry et al., 2010). Another recent study presumably labeled this CRM as a ‘shadow’ enhancer because it is located at a distance from the snail gene, whereas the proximally located CRM was defined as the primary acting enhancer (Perry et al., 2010).

To provide insight into the functions of CRMs associated with the snail locus in the Drosophila early embryo, we undertook a genetic approach towards studying cis-regulatory control using BAC recombineering and site-directed transgenesis to assay the domain and level of expression supported by concurrently functioning CRMs. We focused on the distinction between the proximal and distal snail CRMs, which control early embryonic expression, in particular on the patterns and levels of expression supported by each, as well as their abilities to support Snail function.

**MATERIALS AND METHODS**

**Fly stocks**

Adh
\(^{dr}>\) snai
\(^{en1}\)/CyO, and snai
\(^{18}\)/CyO fly stocks were used (BDSG) after rebalancing with CyO \(\text{fz-lacZ}\) marked balancer. The proximal 2.2 kb and 6 kb lacZ reporter lines and F10 line (hisp83-Toll10B-bcd3) were isolated and electroporated into EPI300 cells (Epicenter) according to the manufacturers instructions. The constructs were inserted as described previously (Lee et al., 2001).

**Cloning and generation of lacZ constructs**

Enhancer sequence for the distal enhancer was amplified from genomic DNA using Sna-Dist 2kb-f (5'-AATTGTACCCACATTA-GCTGCCTTTGGCAG-3') and Sna-Dist 2kb-r (5'-AATTGTGATCCCTGACCGTCAACTGGT-3') and cloned into the pPlm site of the eve promoter-lacZ-attB vector (Liberman and Stathopoulos, 2009). Site-directed transgenesis system was used to create reporter lines (Bischof et al., 2007). The 86Fb fly stock with attP landing site was injected in house with reporter constructs to generate transgenic lines.

**Generation of 25 kb snai rescue constructs**

The 25 kb snai [acman] construct was generated using recombinering mediated gap repair performed using SW105 cells as described previously (Venken et al., 2006). The BAC encompassing the snai gene (BACR23J04) was obtained from the BacPac Resource Center and the attB-[acman]-Ap\(^{R}\) was modified to contain ~600 bp homology arms to the region of interest. Insertion of GFP just before the stop codon of snai was performed using a GFP-frt-kan-frt plasmid and the kan cassette was removed after insertion as described previously (Lee et al., 2001).

Deletion, rearrangement and mutation of the enhancer regions was carried out using the gatk system (Warming et al., 2005). All final constructs were isolated and electroporated into EPI300 cells (Epicenter) and the copy number was induced using Fosmid Autoinduction Solution (Epicentre) according to the manufacturers instructions. The constructs were isolated using Nucleobond EF plasmid midi prep kits (Clontech). P[acman] constructs were injected into line 23648 (BDSC) at a concentration of 0.5-1 \(\mu\)g/\(\mu\)l in water using standard techniques. All primers used for gap repair and recombineering are listed in Table S1 in the supplementary material.

**Rescue experiment**

Lines were created that contained snai
\(^{18}\)/CyO \(\text{fz-lacZ}\) and one of the snai BAC constructs. Males from these lines were crossed to virgin Adh
\(^{dr}>\) snai
\(^{en1}\)/CyO \(\text{fz-lacZ}\). Separate vials were placed at 25°C, 29°C and 18°C. All transgenic flies were counted and the total number of straight wing flies (i.e. snai mutants) was compared with the total number of transgenic flies. The final percentage of straight wing flies for each experiment was then divided by 33%, which would be the expected result were the rescue to be perfect.

We note key distinctions between our construct design and that of another recent study of the snail locus which used a similar approach (Perry et al., 2010); (1) our transgene functions to rescue a snai mutant (i.e. snai
\(^{18}\)/snai
\(^{38}\)) to viability, whereas the other group was limited to assaying early gastrulation defects presumably because a large deficiency background was used; (2) our deletions were guided by our own Twist ChIP-seq data (Ozdemir et al., 2011), effectively guiding definition of the distal CRM as a larger region (~2.0 kb), (3) a spacer sequence (i.e. ampicillin resistance cassette) was not put in place of deletions in our constructs, which allowed us to assay whether native spacing is important; (4) the snai-coding sequence, which may possibly influence cis-regulatory mechanism or stability of transcripts, was left intact within our reporter constructs; and (5) the other group did not assay the gastrulation defects associated with the distal CRM delete large transgene but relied on cDNA rescue data conducted previously (Hemavathy et al., 2004).

**In situ hybridization**

Embryos were fixed and stained following standard protocols. Antisense RNA probes labeled with digoxigenin, biotin or FITC-UTP were used to detect reporter or in vivo gene expression as described previously (Jiang and Levine, 1993; Kosman et al., 2004). Primary antibodies used were: rabbit anti-Eve (provided by M. Frasch, University of Erlangen-Nürnberg, Germany), guinea pig anti-Twist (provided by M. Levine, UC Berkeley, CA, USA), mouse anti-Dorsal (7A4-s from the Hybridoma Bank) and rabbit anti-Histone H3 (Abcam).

**Mean intensity quantification**

Images of three embryos from each construct were taken using identical parameters. From each embryo, a square of 345 \(\mu\)m\(^2\) was extracted and analyzed for mean intensity using the LSM Image Examiner program (Zeiss). This was repeated three times in each embryo within the snail stripe in consistent locations from embryo to embryo. A negative control square of the same size was also analyzed for each embryo. For each measurement within the snail stripe, the negative measurement from that embryo was subtracted and then the measurements were averaged and a standard deviation was determined from the nine measurements.

**RESULTS**

**Multiple CRMs in proximity to the snail gene support expression in overlapping domains**

Previously published Twist-ChIP-seq binding data identified multiple peaks of Twist occupancy to DNA in proximity to the snail gene (Fig. 1A) (Ozdemir et al., 2011). By far, the largest peaks were detected ~7 kb upstream of snai gene within the intron of another gene, Tim17b2. The two proximal Twist occupied regions are covered by the previously studied 2.2 kb and 6 kb enhancer constructs (‘proximal CRM’) (Ip et al., 1992b). A 2.0 kb DNA fragment from the Tim17b2 intronic sequence, containing several closely positioned peaks of Twist occupancy, was also assayed in a reporter context (‘distal CRM’) (Ozdemir et al., 2011).

By analysis of lacZ reporter transgenes, we found that both these CRMs (proximal and distal) supported expression in the ventral region of the early embryo in patterns that are spatiotemporally similar but not identical. In contrast to the broadened expression of the proximal CRM fragment (Fig. 1C,F), the distally located CRM fragment supports high-level expression that is refined, sharp and similar to the endogenous snai expression pattern (Fig. 1D,G, compare with 1B,E). It should be noted that our tested DNA fragment was defined by Twist ChIP-seq analysis and was larger in size than the one recently tested by another group (i.e. 2 kb versus 1.2 kb) (Perry et al., 2010), a study in which no spatial distinctions between the patterns supported by the proximal and distal CRMs was noted.
Cis-regulatory system of the snail gene

**Assay of CRM function using larger reporter transgenes in which native context is retained or modified**

To analyze how expression of the snail gene is controlled in the early embryo, we created a 24.8 kb P[acman] construct encompassing the snail gene, as well as flanking DNA sequences using recombinering methods (Fig. 1H) (Venken et al., 2006). We isolated stable transgenic lines using site-directed methods and determined that this DNA sequence can complement the snail mutant, suggesting that the cis-regulatory information encoded within this ~25 kb DNA segment is sufficient to support the essential aspects of snail expression. To create the reporter construct, we recombinered the gfp cDNA sequence into the snail locus as an in-frame C-terminal fusion to Snail protein (Fig. 1H, ‘sna-gfp’), allowing us to monitor transgenic expression of sna-gfp using a gfp riboprobe (see below).

As our goal was to provide insight into cis-regulatory mechanisms regulating snail expression, we created five deletion constructs within the 25 kb sna-gfp construct using our Twist ChIP-seq data as a guide: (1) a snail promoter proximal deletion of 3.8 kb containing two peaks of Twist binding, including most of the 2.2 kb minimal snail enhancer identified by Ip et al. (Ip et al., 1992b), but leaving the 500 bp promoter proximal region and including more upstream sequence that we found was also bound by Twist in the early embryo (‘Δ Proximal’); (2) a distal deletion of 2.0 kb, which includes three major peaks of Twist binding, located in the intron of the gene upstream of snail, Tim17b2 (‘Δ Distal’); (3) a double-deletion of both the proximal and distal CRMs (‘Δ P and D’); (4) a deletion of the intervening sequence, present between the proximal and distal CRMs (‘squish’); and (5) a construct in which the distal CRM is moved to the proximal position, in a double-delete background (‘D to P’) (Fig. 1H). 500 bp directly upstream of the snail coding sequence was left unmodified in all cases, with the purpose of leaving the promoter intact.

As both the distal and proximal CRMs supported snail expression during early embryogenesis, we investigated whether they function redundantly through analysis of these recombinered reporter transgenes. The proximal CRM deletion (‘Δ Proximal’) supported gfp expression that was comparable with gfp expression from the full sna-gfp rescue construct (Fig. 2B, compare with 2A). Moreover, gfp expression similar to that supported by sna-gfp was detected in the constructs that moved the distal promoter to a proximal location (‘D to P’) and the construct that deleted the intervening sequence (‘squish’) in the early embryo (data not shown). By contrast, deletion of the distal CRM (‘Δ Distal’) supported weaker expression (Fig. 2C), and the construct that deletes both (‘double delete’) lacked early expression altogether (data not shown). Based on pattern alone, the distal CRM appeared more faithful to the snail endogenous expression domain.

**Genetic assay of CRM function by snail mutant rescue**

To determine whether snail expression supported by these transgenes was functionally equivalent, we assayed the ability of these transgenes to rescue a snail mutant. The wild-type reporter and five modified versions, were introduced into a snail mutant background (sna1/sna18) and assayed for their ability to support viability. We found that the native snail gene rescued at 91% (Table...
1) but there was significant, but only partial, rescue with the sna-gfp fusion constructs (76%) (data not shown). For this reason, we assayed the ability of native sna gene constructs, unmodified with gfp, to support rescue.

The 25 kb snail transgene and the delete proximal CRM constructs rescued the sna mutant phenotype; 91% and 82% of expected F1 progeny, respectively, were obtained in rescue crosses (Table 1). By contrast, the distal CRM delete construct completely failed to rescue the sna mutant, as did the double delete ‘ΔP and D’ construct. The ‘squish’ construct, which removes sequence between the proximal and distal CRMs, also failed to complement the mutant. These results support the conclusion that the distal CRM is required to support viability. In turn, the fact that more than 80% of the expected flies emerged from the sna rescue cross with proximal CRM delete transgene suggested that the proximal CRM is not required to support viability.

To further study functional differences between CRMs, we examined the ability of our constructs to support viability at various temperatures: 25°C, 29°C and 18°C. The proximal CRM delete construct showed decreased viability at higher temperature, with 36% viability supported at 29°C when compared with 82% at 25°C; yet at 18°C, we found the rescue was also high at 94% (Table 1). However, we found that the distal CRM delete construct did not rescue at any temperature tested: 0% viability at 18°C, 25°C, and 29°C; further evidence that the distal CRM is the primary CRM responsible for supporting sna expression.

Deletion of the distal CRM, specifically, has consequences on gastrulation

Next, we examined whether these CRMs have similar or different roles during gastrulation. The constructs containing the distal CRM rescued the gastrulation defects of sna mutants [i.e. ‘Δ Proximal’ (Fig. 2E,H) and ‘squish’ and ‘D to P’ (see Fig. S1 in the supplementary material), compare with full length sna-gfp (Fig. 2D,G)]. By contrast, constructs without the distal CRM exhibited gastrulation defects (i.e. ‘Δ Distal’, Fig. 2F,I). In the absence of the distal CRM, not only was single-minded (sim) expression aberrant, with expansion into a broad domain compared with the single line of cells found in wild-type embryos, but invagination was non-uniform and presumably contributed to unequal mesoderm spreading (Fig. 2F,I). As sim is directly repressed by the Snail transcription factor in gastrulating embryos (Kasai et al., 1992), these results indicated that the level of snail expression in the sna mutant background supported by sna-gfp Δ Distal is insufficient to fully support function at this stage of development, resulting in an expansion of the sim domain.

As an assay for possible later phenotypes, we examined expression of even-skipped (eve). eve encodes a homeodomain transcription factor necessary for dorsal mesoderm lineage specification (Frasch et al., 1987), and its lateral expression in 11 clusters of cells on either side of the embryo at stage 11 can be used as an indicator for proper mesoderm spreading. In rescue experiments in which the distal CRM was absent, eve expression was aberrant as gaps in expression were detected in all of the embryos examined (Fig. 2L, arrows). By contrast, constructs that removed the proximal CRM, leaving the distal CRM intact, exhibited normal gastrulation (invagination and sim expression, Fig. 2E,H), as well as normal mesoderm spreading and specification even at later stages of embryogenesis (eve expression; Fig. 2K). Even when the temperature was raised to 29°C, no obvious mesoderm specification defects in the trunk of the embryos were observed in the absence of the proximal CRM (see Fig. S2 in the supplemental material). Our data for rescue of the sna/sna18 background demonstrated that the distal CRM is required to support gastrulation, but that the proximal CRM is not required or supports a minor role (such as supporting expression at the anterior, see below).

The proximal CRM deletion of 3.8 kb removes multiple tissue-specific enhancers, a minimum of three: one module from 1.2 kb to 2 kb supports expression in ventral regions of the early embryo (e.g. Fig. 1C) and two other modules, one from 0.4-0.9 kb and another from 2.2-2.8 kb, support expression in the peripheral nervous system (PNS) and central nervous system (CNS), respectively, at later stages of embryogenesis (Ip et al., 1994; Ip et al., 1992b). We observed changes in the PNS and CNS expression in constructs that delete the proximal CRM, but no effect on expression in these domains was observed in the constructs that delete the distal CRM (see below).

Multiple CRMs support sna expression in germ-band elongated embryos and are organized on the chromosome in a manner that potentially minimizes dominant effects of repressors

In the course of our sna rescue experiments, we found that a construct removing the intervening sequence between distal and proximal CRMs was not able to complement the mutant (Table 1,
The document discusses the role of specific CRM (cis-regulatory modules) in the regulation of the snail gene, which is involved in patterning processes during development. The proximal and distal CRM's were studied for their ability to support expression in different regions of the embryo.

Table 1: The distal enhancer is required for viability at all temperatures, whereas the proximal enhancer is required conditionally at high temperatures

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Percentage rescue</th>
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<tr>
<td>Sna rescue construct</td>
<td>91% (n=170)</td>
</tr>
<tr>
<td>Sna Δ proximal CRM</td>
<td>82% (n=51)</td>
</tr>
<tr>
<td>Sna Δ distal CRM</td>
<td>0% (n=44)</td>
</tr>
<tr>
<td>Sna Δ proximal and distal</td>
<td>0% (n=47)</td>
</tr>
<tr>
<td>Sna squash</td>
<td>0% (n=95)</td>
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Schematics of each of the constructs are shown on the left. Percentage rescue indicates the number of sna/sna18 flies counted out of the total number of flies present, then divided by what would be considered a complete rescue (i.e. 33% of total flies). n is the total number of flies counted. Because the 'Δ proximal and distal' and 'squish' constructs did not rescue at 25°C, they were not further analyzed at the other temperatures.

Repressors predominantly function through the distal CRM to regulate the posterior and dorsal boundaries of the sna expression domain within the early embryo

It has previously been shown that the Huckebein (Hkb) transcription factor, which is expressed at both the anterior and posterior poles, functions as a repressor to define the posterior boundary of sna expression (Goldstein et al., 1999; Reuter and Leptin, 1994). In hkb mutants, posterior sna expression is expanded into the pole and anterior expression is expanded beyond the tip and into the dorsal region of the embryo. Upon examination of the sna-gfp construct in which the proximal CRM was deleted, we found that gfp expression was excluded from the posterior hkb expression domain, similar to endogenous sna expression (Fig. 4B, compare with 4A). This result suggested that Hkb can function to repress the sna posterior boundary, even when the proximal CRM is removed. By contrast, gfp expression was expanded into the posterior end of the embryo upon deletion of the distal CRM (Fig. 4C, compare with 4A).

Sna and hkb expression domains overlap at anterior regions of the embryo. Upon closer analysis of the sna-gfp proximal delete construct, we found that the gfp expression domain recedes relative to sna, such that the boundary of expression was more ventrally located and sharper relative to wild type (Fig. 4E). A similar effect on sna expression has been observed previously in bicoid mutants (Reuter and Leptin, 1994). However, in comparison with the expression domain supported by the sna-gfp distal CRM delete, we found that the sna expression domain was expanded more dorsally at the anterior of the embryo than normal (Fig. 4F), similar to that seen in hkb mutants (Reuter and Leptin, 1994). Collectively, these results suggest the proximal CRM supports Bicoid-mediated activation at the anterior of the embryo and that the distal CRM supports Hkb-mediated repression at both embryonic poles.
The distal CRM supported a refined snail expression boundary not only at the poles where Hkb is functioning but also in lateral regions of the embryo. Because the snail-gfp reporters are also recognized by a riboprobe to snail (such that endogenous and reporter expression cannot be distinguished by in situ hybridization), we chose to assay the lacZ reporter constructs of the two CRMs in comparison with endogenous snail to visualize the patterns at the dorsal boundary of expression. The distal CRM dorsal border was sharp and comparable with the endogenous snail pattern, whereas the proximal CRM supported a patchy expression that was weak and sporadically extended beyond the snail border (Fig. 4H, compare with 4G). Although the dorsal border of the proximal CRM is mostly encompassed by the endogenous snail border, the extension of the proximal CRM even by a few cells beyond the endogenous snail border indicates that this CRM in isolation has no (or reduced) responsiveness to a putative repressor responsible for supporting the sharp boundary (see below).

Previous studies using intersecting dorsoventral patterning cues had provided evidence that one or more repressors might be required for the establishment of the snail lateral border (Huang et al., 1997). In these experiments, an ectopic gradient of nuclear Dorsal (a transcription factor pivotal for dorsoventral patterning) was specified along the anterior-posterior axis by localizing constitutively active Toll receptor to the anterior end of embryos by a transgene, ‘F10’ (Huang et al., 1997). The result was an embryo containing two dorsal-ventral patterning axes that intersect (see Fig. S3A in the supplementary material), with loss of snail expression at the intersection; the existence of a laterally acting repressor was postulated (Huang et al., 1997). Although the identities of such laterally acting repressors (‘repressor X’) have remained uncertain, we nevertheless assayed whether the proximal and/or distal CRMs are required to support the ability to repress snail in this manner.

When the distal CRM lacZ reporter construct was introduced into the F10 background, complete overlap with the endogenous snail expression domain was observed (see Fig. S3B in the supplementary material). By contrast, the expression domain was expanded both laterally and posteriorly when the proximal 2.2 kb lacZ construct was introduced into the F10 background (see Fig. S3C in the supplementary material). These results are consistent with the idea that both repressor X and Hkb function predominantly through the distal CRM to refine snail borders in lateral and posterior regions of the embryo, respectively, although we cannot dismiss a more minor role at the proximal CRM.

Non-additive patterns of expression when proximal and distal CRMs are located in trans suggest cis-interactions are necessary to support patterning

Although both CRMs are present in the wild-type locus, we noticed that the snail gene expression pattern was not simply the summed equivalent of the domains of expression supported by the two
and/or the distal delete CRM reporters, in either cis or trans conformation, and analyzed the gene expression outputs supported by each combination in terms of spatial domain (Fig. 5) and level of expression (Fig. 6).

At two copies, the proximal CRM delete construct supported refined expression (repressed at the posterior and laterally), whereas the distal CRM delete construct supported expanded expression (extending at the poles and laterally) compared with an unmodified reporter construct (Fig. 5A,B, compare with 5D), similar to expression supported by one copy of the transgenes. However, when reporter expression was assayed in an embryo containing one copy of the proximal CRM delete and one copy of the distal CRM delete transgenes, the pattern supported exhibited an expanded expression domain, most apparent at the posterior pole. This result suggested that the expression supported by the proximal CRM is not simply too weak to be observed in the presence of the expression supported by the distal CRM, but that instead repressors associated with the distal CRM normally function to refine expression at the poles and in lateral regions supported by the proximal CRM. Furthermore, these data demonstrate that repressors associated with the distal CRM cannot function in trans, but instead require a cis conformation relative to position of the proximal CRM in order to have an effect. Our results suggest that the normal pattern is a non-additive reflection of the domains of expression supported by each CRM (see Discussion).

Besides differences in domain of expression, we noticed that these constructs supported differences in levels of expression (Fig. 6). When imaged at a power and gain in which all of the constructs examined were not over-exposed, the mean intensity supported by the sna-gfp and sna-gfp Δdistal constructs were comparable, but in comparison the expression levels supported by the sna-gfp Δproximal construct were considerably higher (~3-4 fold). Therefore, in the absence of the proximal CRM, the expression levels increased. At higher gain, however, it was observed that the sna-gfp expression was at least twofold higher than that of the sna-gfp Δdistal (data not shown). Thus, alternately, in the absence of the distal CRM, the expression levels decreased. In addition, the sna-gfp squish construct also supported increased levels of expression relative to the sna-gfp construct (approximately twofold). Collectively, these results suggest that normal levels and patterns of snail gene expression require input from both the proximal and distal CRMs, and that effective regulation of expression levels requires proper organization of these CRMs upon the chromosome.

**DISCUSSION**

In this study, we provide evidence that early snail expression is regulated by two concurrently acting CRMs that support gene expression patterns that are spatially and functionally different. The distally located CRM is necessary to support gastrulation as well as viability of snail mutants, whereas the proximal CRM is dispensable for viability except at high temperature. Furthermore, our data show these CRMs support distinct expression patterns. Although they probably share many transcription factors, the distal CRM alone is responsive to the repressor Huckebein and the unknown laterally acting ‘repressor X’, whereas the proximal CRM alone responds to an anterior activator.

Our data suggest that the proximal CRM functions as a ‘damper’ to reduce the high levels of expression normally supported by the distal CRM. Multiple CRMs associated with a single gene may support spatiotemporally similar expression patterns, but the mean levels of gene expression supported by each can be very different.
In the case of the *snail* locus, our data show that the distal and proximal CRMs drive high or low levels of expression, respectively, within a similar domain in ventral regions of the embryo. Our results support a model in which these two CRMs provide dual-control of expression levels, high versus low, to provide flexibility in terms of levels of *snail* expression (Fig. 6F). The requirement for the proximal CRM at high temperatures could indicate a need to more closely regulate the expression levels of *snail* in stressful environments. Such flexibility is probably advantageous and may explain why two CRMs that support similar expression patterns may be evolutionarily constrained.

Both the proximal and distal CRMs support expression not only during gastrulation in ventral regions of the embryo but in other domains at later stages of development. The distal CRM also supports expression within malphigian tubule precursors (Fig. 3), and, as was previously shown, the proximal CRM supports expression later within neuroblasts (Ip et al., 1992b). Therefore, these elements can be reused during the course of development, and may be evolutionarily retained for reasons beyond a role in canalization.

**CRMs associated with the snail locus function in a non-additive manner to support expression**

Our results show that transcription factors associated with the distal CRM can dominantly affect the other proximally located CRM to support expression of *sna* that is refined and excluded from the posterior pole. Our data support the view that non-autonomous CRM function is responsible for the resulting pattern which is effectively non-additive, i.e. it is not simply the summed equivalent of the domains of expression supported by the two CRMs. Non-autonomous CRM function may be advantageous, providing additional flexibility by allowing individual and combined activities of CRMs based on circumstances, to support canalization. It has been demonstrated that non-additive CRM interactions also play a role defining the expression domain of another *Drosophila* early patterning gene, *sloppy-paired 1* (Prazak et al., 2010). Our data support the view that this is a more common cis-regulatory mechanism than currently appreciated. For example, even in case of the *even-skipped* gene locus that has received considerably focus, questions remain about why particular CRM behaviors are not equivalent to the behaviors of the *eve* gene itself. The expansion of a *eve* stripe 3/7 reporter gene in *knirps* mutants (Small et al., 1996), but not the *eve* gene itself (Frasch and Levine, 1987), suggests that another repressor is required to drive proper *eve* stripe 3/7 expression and that this activity is supported through another DNA fragment. We propose that another CRM associated with the *eve* locus may aid in definition of *eve* stripes 3/7 by serving as a vehicle for additional repressors(s), similar in mechanism to regulation of *snail* gene expression shown here in this study.

**CRMs are organized along the DNA to support effective transcription**

This study also supports the view that CRMs are organized in the context of the gene locus to support proper patterning and to minimize cross-repressive interactions (see also Cai et al., 1996; Small et al., 1993). We believe that the loss of Ect1 expression that we see in the ‘squish’ construct is the result of dominant repression, owing to the fact that the distal enhancer is moved in proximity to the proximal enhancer (see Fig. 3B). This would suggest that the native context of CRMs within a locus can limit interactions between elements, and may go towards explaining why enhancers in diverged species/animals tend to be found in the same general location (Cande et al., 2009; Hare et al., 2008). Similarly, the dampening of all *snail* expression patterns we observe in the ‘D to P’ construct may be due to the repressive activity of the distal CRM being moved near the promoter.

Placing binding sites for repressors near the promoter potentially limits the range of activity of a gene. Many genes involved in early development, such as *snail*, take on different roles later in development and are subject to different molecular inputs during the life of the animal. Like *snail*, the *intermediate neuroblasts defective (ind)* gene also has a distally located enhancer and another that is located in the proximal position. Similar to what we see at the *snail* locus, the distal CRM has documented repression associated with it, whereas the proximally located element functions through positive autoregulatory feedback (Stathopoulos and Levine, 2005; Von Ohlen et al., 2007). We suggest that keeping repressors located at a distance from the promoter supports flexibility in reiterative reactivation of genes throughout the course of development. However, in addition to buffering repressive crosstalk through distance, we propose that linking repression function to the presence of an activator (i.e. between CRMs concurrently active in the same cells) may also serve as an alternate mechanism to moderate non-autonomous CRM interactions; other
studies in the past have suggested that repressors may require activators to bind DNA (i.e. ‘hot chromatin’ model) (see Nibu et al., 2001).

Our data show that expression of the Drosophila snail gene in embryos is established through integrated activity of multiple CRMs that function concurrently and, in part, through non-additive interactions. Non-additive activity of CRMs, through sharing of repressors for example, is likely more commonplace than currently appreciated. It is possible that concurrently acting CRMs function coordinately to regulate spatial domain and levels of expression in general, and may provide one explanation why genes in Drosophila and other animals often have multiple CRMs that support similar spatiotemporal patterns of expression.

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Competing interests statement
The authors declare no competing financial interests.

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