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Mesodermal repression of *single-minded* in *Drosophila* embryo is mediated by a cluster of Snail-binding sites proximal to the early promoter

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***single-minded* (*sim*) is a master regulatory gene that directs differentiation in the central nervous system during *Drosophila* embryogenesis. Recent identification of the mesectoderm enhancer (MSE) of *sim* has led to the hypothesis that two Snail (Sna)-binding sites in the MSE may repress *sim* expression in the presumptive mesoderm. We provide evidence here that three Sna-binding sites proximal to the *sim* promoter, but not those of the MSE, are responsible for the mesodermal repression of *sim in vivo*. Using transgenic embryos injected with *lacZ* transgenes, we showed that *sim* repression in the mesoderm requires the three promoter-proximal Sna-binding sites. These results suggest that Sna represses the mesectodermal expression of *sim* by directly repressing the nearby promoter, and not by quenching adjacent transcriptional activators in the MSE. These data also showed how the MSE, lacking the three proximal Sna-binding sites, reproduced the endogenous pattern of *sim* expression in transgenic embryos. [BMB Reports 2012; 45(10): 577-582]**

INTRODUCTION

The *Drosophila* gene *single-minded* (*sim*) encodes a bHLH-PAS transcriptional activator that functions as a so-called 'master regulatory gene' in differentiation of the ventral midline of the central nervous system (CNS) (1-4). The ventral midline in *Drosophila*, analogous to the floorplate of the vertebrate neural tube, generates a variety of developmental patterning signals (5-7). Sim acts in conjunction with a ubiquitously expressed bHLH-PAS protein, *Tango* (*Tgo*), to regulate target gene expression in the ventral midline (8). The Sim-Tgo heterodimer maintains *sim* expression by directly binding to a cluster of Sim-Tgo binding sites located in a 5′ autoregulatory enhancer element (9).

Sequential expression of patterning genes along the dorsal-ventral (DV) axis of the early embryo is produced by a nuclear gradient of the maternal transcription factor Dorsal (Dl), thereby establishing the presumptive mesoderm, mesectoderm, neurogenic ectoderm and dorsal ectoderm (10, 11). During early embryogenesis, *sim* expression begins before the cellular blastoderm stage, but only in a single row of cells along either side of the embryo (2) (Fig. 1A). This row of cells comprises the boundary between the presumptive mesoderm and the neuroectoderm, and gives rise to the mesectoderm. Although a *snail* (*sna*) repressor is known to repress mesodermal *sim* (3, 12) (Fig. 1B-D), the detailed mechanism for restriction of *sim* expression to the mesectoderm has long been unclear. An enhancer sequence that mediates the mesectodermal *sim* expression was recently identified in genetic and comparative genomic studies (13, 14) (Fig. 1E). This finding suggests that DV patterning determinants Dl, Twist (Twi) and Notch activate the MSE throughout the mesoderm and mesectoderm, while the *sna* repressor specifically inhibits the enhancer activity in the mesoderm by quenching the nearby activities of Dl, Twi and/or Notch (Fig. 1F).

Here, we present evidence that the mesodermal repression of *sim* depends on three Sna-binding sites proximal to the *sim* early promoter, though not on binding sites in the MSE. Transgenic embryos that were injected with *lacZ* transgenes containing wild-type and modified genomic regions showed that removal or mutations of the three promoter-proximal Sna-binding sites led to the derepression of *sim* in the mesoderm. Interestingly, we found that recapitulation of the endogenous pattern of *sim* expression mediated by the 0.6-kb MSE fragment stems from a Sna site, innately distal and non-functional, that was coincidently located proximal to a promoter of the *lacZ* transgene and became transcriptionally functional. We propose that Sna represses mesodermal *sim* expression not by quenching adjacent transcriptional activators in the MSE, but by direct repressive action in the promoter-proximal region.

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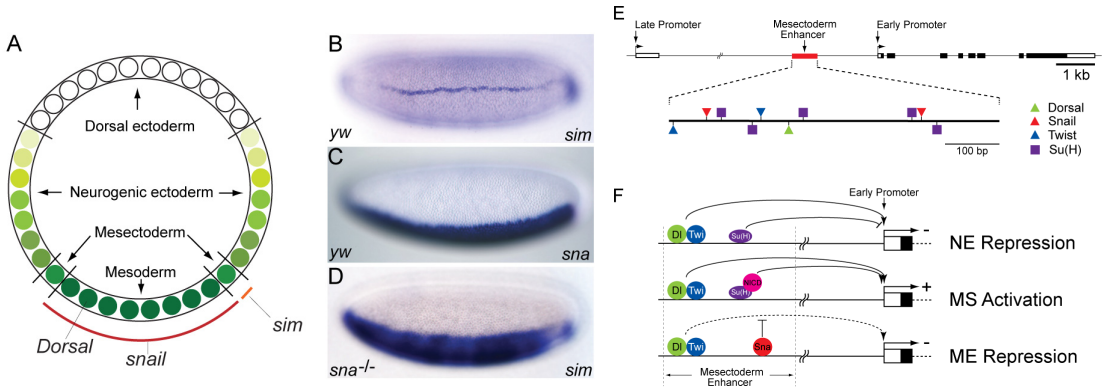

Fig. 1. The zinc finger protein Snail (Sna) is required for mesodermal repression of *sim*. (A) The developmental fate map along the dorsal-ventral (DV) axis and the expression domains of *single-minded* (*sim*) and *sna* are depicted on a diagram representing a cross-section through an early embryo. Filled green circles represent high levels of nuclear *dorsal* (*dl*) protein, and shaded green and yellow circles represent intermediate and low levels, respectively. The differential nuclear gradient of the maternal transcription factor Dl produces sequential patterns of gene expression across the DV axis of early embryos, thereby establishing the presumptive mesoderm, mesectoderm, neurogenic ectoderm, and dorsal ectoderm. The expression of *sna* (red curve) and *sim* (orange curve) are restricted to the prospective mesoderm and mesectoderm, respectively. (B-D) Endogenous *sim* and *sna* expression are visualized by whole-mount *in situ* hybridization with antisense RNA probes. Embryos are oriented with anterior to the left and dorsal up. Only in the mesectoderm, a single row of cells between the mesoderm and neurogenic ectoderm, is *sim* expressed (B); and *sna* expression defines a dorsal borderline of the presumptive mesoderm in wild-type embryos (C). The lack of Sna in the homozygous *sna*-mutant embryo (*sna*$^{-/-}$) (D) permits *sim* expression throughout the presumptive mesoderm and mesectoderm. (E) A *sim* genomic locus is shown schematically. Each box represents an exon and protein-coding regions are shown in black. The ~0.6 kb mesectoderm enhancer is represented by a red box and four transcription factor binding sites mediating mesectodermal *sim* expression are displayed with four different colors: green, Dl; red, Sna; blue, Twist (Twi); and purple, Suppressor of Hairless [Su(H)]. The binding sites identified in sense or nonsense strands relative to the transcription start site are displayed above or below the horizontal line, respectively. The mesectoderm enhancer contains two Sna-binding sites located at ~70 and ~130 bp from either end. (F) A model is proposed for transcriptional regulation of *sim* along the DV axis. Both ends of the mesectoderm enhancer are defined by dotted lines. In the neurogenic ectoderm (NE), Dl and Twi collaborate to activate *sim* transcription. However, Su(H) functions as a repressor to block *sim* transcription because of the absence of Notch intracellular domain (NICD) in the territory (25) (top panel). In the mesectoderm (MS), although the mode of action of the Dl and Twi is *sim*ilar to that in the NE, Su(H) acts together with NICD to activate *sim* transcription (25). Notch signaling is active only in the mesectoderm (middle panel). In the mesoderm (ME), Dl and Twi still activate *sim* transcription but two Sna bound in the mesectoderm enhancer quench a nearby activator of Dl and Twi, resulting in the mesodermal repression of *sim* (bottom panel).

RESULTS AND DISCUSSION

Snail is required for mesodermal repression of sim

It is suggested that *sna* expression in the presumptive mesoderm plays a critical role in mesectoderm-specific *sim* expression (3, 12). Thus, we first tested directly whether sna is required for mesodermal repression of endogenous *sim* in wild-type and homozygous *sna*$^{19/4.26}$ mutant embryos, using whole-mount *in situ* hybridization with antisense RNA probes. Consistent with previous reports, we detected *sim* expression solely in a single row of cells, the mesectoderm, along either side of the early *Drosophila* embryo (Fig. 1A, B). *Sna* expression was restricted to the presumptive mesoderm that lies between the mesectodermal cells of the wild-type embryo (Fig. 1C). The lack of sna protein in the homozygous *sna*-mutant embryo (*sna*$^{-/-}$) allowed *sim* expression to extend throughout the presumptive mesoderm and mesectoderm (Fig. 1D). These findings demonstrated a requirement for *sna* expression in the mesodermal repression of *sim* in precellular embryos.

Snail-binding sites proximal to a sim early promoter mediate mesodermal repression of sim

Sna is a short-range repressor with a zinc-finger motif that can mediate either quenching or direct repression of the transcription complex (15-18). A 2.2-kb genomic region upstream of a *sim* early promoter includes five Sna-binding sites (12) (Fig. 2A). Two of those sites reside in the MSE and the other three are located within a ~230-bp region just upstream of the *sim* early promoter. Recently, an enhancer region (~0.6 kb) directing the mesectodermal expression of *sim* was identified by investigating the clusters of binding sites for Dl, Twi, Suppressor of Hairless [Su(H)] and Sna (13). The ~0.6-kb mesectoderm enhancer (MSE) sequence contains more than one site for Dl, Twi and Su(H) and two for Sna. The two Sna-binding sites are located at ~70 and ~130 bp from opposite ends of the MSE region, flanking sites for the three different activators (Fig. 1E). Distribution of the binding sites across the MSE and the previously observed quenching activity of Sna implied that Dl and Twi activate *sim* transcription, but *sna* proteins bound in the MSE may quench activities of neighboring Dl and Twi, thereby resulting in mesodermal repression of *sim* (Fig 1F, bottom panel).

However, the three other Sna binding sites reside in a ~230-bp region just upstream of the *sim* promoter. Although previous experiments have shown that Sna occupies the three sites (12), the functional activities of the sites are unclear. Proximity to the promoter, actual occupancy of the sites and the direct repressive activity of Sna suggest that Sna may either quench activities of nearby activators in the MSE or directly repress the transcription complex on the *sim* promoter in the presumptive mesoderm. To distinguish these possibilities, germ-line transformations were performed with *lacZ* transgenes containing several *sim* genomic fragments, and the *lacZ* expression was monitored by *in situ* hybridization with an antisense *lacZ* RNA probe. When wild-type (*yw*) embryos were injected with the 2.2 kb-*lacZ* transgene containing five Sna sites (Fig. 2B), *lacZ* expression was detected only in the mesectodermal cells (Fig. 2C). This showed that the *lacZ* transgene recapitulated the endogenous *sim* expression pattern. Deletion of a ~200-bp region containing the three promoter-proximal Sna sites from the 2.2-kb fragment (Fig. 2D) resulted in *lacZ* expression over the presumptive mesoderm and mesectoderm (Fig. 2E). In addition, a mutant 2.2-kb fragment, wherein the DNA consensus sequences of the three Sna-binding sites had been changed by site-directed mutagenesis (Fig. 2F), failed to repress *lacZ* expression in the mesoderm (Fig. 2G). These results demonstrated that the 2.2-kb region contains a complete set of *cis*-acting elements to control *sim* expression in the mesoderm and mesectoderm, and that the three Sna sites in the promoter-proximal ~200-bp sequences mediate *sim* repression in the mesoderm.

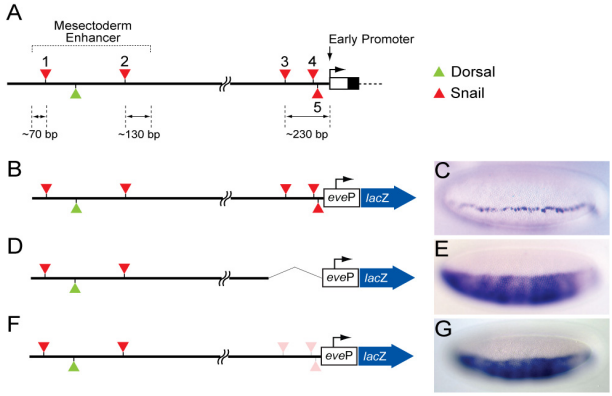

Fig. 2. Mesodermal *sim* repression is mediated by snail-binding sites proximal to the early promoter. (A) Shown is a 2.2-kb genomic region upstream of the early promoter of *sim* that contains five Sna-binding sites. Two of the sites are located in the mesectoderm enhancer and the other three are located in a ~230-bp region upstream of the transcription start site of the early promoter. Green and red triangles represent the binding sites of Dl and Sna, respectively. Four Sna-binding sites (1-4) are identified in the sense strand. The 2.2-kb region containing the mesectoderm enhancer and five Sna-binding sites was fused to an *even-skipped* (*eve*) minimal promoter (eveP) followed by the *lacZ* coding sequences (B). Wild-type (yw) embryos were injected with the 2.2 kb-*lacZ* transgene and tested by hybridization with an antisense RNA *lacZ* probe (C). A 2.0-kb genomic region wherein the ~230-bp region containing three Sna-binding sites had been deleted was directly linked to the eve promoter and the *lacZ* reporter (D). The 2.0-kb *lacZ* transgene was injected into wild-type (yw) embryos and the *lacZ* expression was visualized by *in situ* hybridization with an antisense *lacZ* RNA probe (E). It is worthwhile to note that *lacZ* transcripts were detected throughout the mesoderm and mesectoderm. The consensus sequences of the three Sna-binding sites, 3, 4 and 5, in the 2.2-kb region were changed by site-directed mutagenesis and the *lacZ* transgene containing the mutant 2.2-kb region was injected into wild-type embryos (F). The *lacZ* expression directed by the mutant 2.2-kb region was visualized by *in situ* hybridization with an antisense RNA probe (G).

In an MSE-lacZ transgene, Snail-binding sites located close to the transgene promoter mimic the endogenous promoter-proximal Snail-binding sites

Remarkably, the 2.0- and mutant 2.2-kb genomic regions could not repress *lacZ* expression in the presumptive mesoderm (Fig. 2D-G), although there remained two Sna sites in the MSE. These results implied that the two Sna sites in the MSE are virtually non-functional in repressing mesodermal *sim* expression, and this raised the question of how the previously identified 0.6-kb MSE could restore *sim* expression in the mesectoderm. To address this issue, transcriptional activities of the wild-type and mutant versions of the MSEs were tested by germ-line transformation, followed by *in situ* hybridization.

When the wild-type MSE fragment (Fig. 3A) was fused directly upstream to a *lacZ* transgene (Fig. 3B), *lacZ* expression was found only in the mesectodermal cells (Fig. 3C). Consistent with previous findings (14), this suggested that one or both of the Sna bound in the MSE mediate either quenching or direct repression, so that the *lacZ* expression is repressed in the presumptive mesoderm. To test whether the Sna-binding sites are actually functional, they were mutagenized one at a time and the activities of the mutant MSEs were monitored by *in situ* hybridization. When the DNA sequence of the Sna-binding site 1 in the MSE was changed (Fig. 3D), the *lacZ* expression pattern directed by the mutant enhancer was indistinguishable from the wild-type counterpart (Fig. 3E). However, the MSE containing the mutant version of the Sna-biding site 2 (Fig. 3F) failed to repress *lacZ* expression in the presumptive mesoderm (Fig. 3G). In addition, when the mutant version of the MSE lacking the Sna-binding site 1 (shown in Fig. 3D) was inverted and fused to a *lacZ* transgene (Fig. 3H), *lacZ* expression was also observed in the presumptive mesoderm (Fig. 3I). These findings imply that the MSE-mediated restoration of *sim* expression resulted from direct repression of the transcription complex on the *even-skipped* (*eve*) minimal promoter by the Sna-binding site 2, and not through quenching of activities in nearby activators.

Although Sna can either quench activator activities or directly repress transcription, both of these mechanisms require close linkage (<100 bp) of the repressor with either the nearby activators or the transcription complex. If the Sna-binding sites in the MSE quenched the activities of adjacent activators, the mutant MSE lacking the Sna-binding site 2 (Fig. 3F) and the 5′ *sim* genomic regions lacking the promoter-proximal Sna-binding sites (Fig. 2D, F) could repress the *lacZ* expression in the presumptive

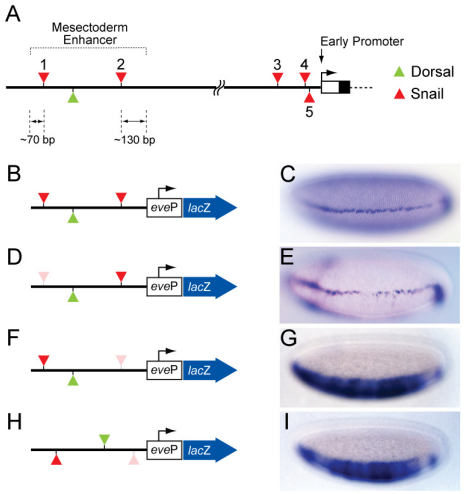

Fig. 3. A Snail-binding site proximal to the *eve* minimal promoter (*eve*P) in the mesectoderm enhancer was sufficient to repress *lacZ* transcription in the mesoderm. (A) The 0.6-kb mesectoderm enhancer is depicted on a *sim* genomic locus. The mesectoderm enhancer contains two Sna-binding sites that are located at ~70 and ~130 bp from either end. (B, D, F, H) The *lacZ* constructs used for P element-mediated germline transformation. Wild-type (B) and mutant (D, F, H) 0.6-kb mesectoderm enhancer sequences were placed upstream of the *eve*P followed by the *lacZ*-coding region. Each of the two Sna-binding sites in the mesectoderm enhancer was mutagenized by site-directed mutagenesis (D, F). The mutant enhancer containing the mutagenized Sna site 1 (D) was inverted and fused to the *eve*P-*lacZ* reporter (H). (C, E, G, I) Embryos injected with the transformation constructs were hybridized with antisense *lacZ* RNA probes to test the pattern of the *lacZ* transgene expression. The absence of a Sna-binding site close to the transcription start site of the *lacZ* transgene led to derepression of *sim* expression in the presumptive mesoderm (F, H).

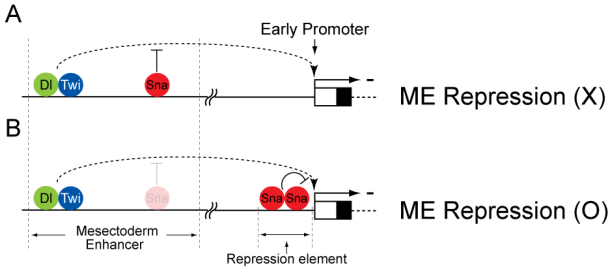

Fig. 4. A new model is proposed for transcriptional repression of *sim* in the presumptive mesoderm. (A) In a current model for mesodermal repression of *sim*, one or both of the Snas bound to sites in the mesectoderm enhancer quenches the activities of Dl and Twi, thereby repressing *sim* transcription in the mesoderm. (B) In the model proposed here, at least two promoter-proximal Sna sites, rather than the two in the mesectoderm enhancer, mediate mesodermal *sim* repression. It is possible that at least one of the Sna bound to the promoter-proximal sites directly represses transcription from the early promoter by interference with activities of the RNA polymerase II initiation complex. Thus, we propose to call the promoter-proximal Sna-binding site a 'repression element' for mesodermal *sim* repression.

mesoderm. The fact that the inverted mutant MSE lacking Sna-binding site 1 (Fig. 3D) lost repressive activity also supports the view that the mesodermal expression of *lacZ* occurs through direct repression mediated by the promoter-proximal Sna-binding site.

A new model for transcriptional repression of *sim* in the presumptive mesoderm

It is postulated that one or both of the Sna bound in the MSE quench transcriptional activities of neighboring activators so as to repress *sim* expression in the presumptive mesoderm (14) (Fig. 4A). The MSE region in the *Drosophila melanogaster* genome was initially identified based on the clustering of Dl-, Twi- and Su(H)-binding sites (13). When the MSE was fused to a *lacZ* reporter, the *lacZ* expression pattern almost completely reproduced the endogenous pattern of *sim* expression. A further analysis of the MSE DNA sequences also identified two Sna-binding sites flanking Dl-, Twi- and Su(H)-binding sites. These observations strongly suggested that the 0.6-kb MSE fragment contains the complete set of elements involved in restricting *sim* expression to the mesectoderm, and that the two Sna-binding sites mediate quenching repression to remove *sim* expression from the presumptive mesoderm. However, our current findings show that the MSE-mediated mesodermal repression of the *lacZ* expression is the result of direct repression by a Sna-binding site that is normally nonfunctional, but coincidentally located close to the promoter of a *lacZ* transgene. In fact, the two Sna-binding sites in the MSE are located ~70 and ~130 bp away from either end (Fig. 3A). Thus, when the MSE fragment is directly fused to a *lacZ* transgene, the transcription complex assembled on a promoter of the transgene is placed under the effective influence of Sna-mediated direct repression so that a single Sna-binding site is sufficient to repress transcription from the *lacZ* promoter.

Based on the results presented here, we propose a new model for the mesodermal repression of *sim* expression (Fig. 4B). In this view, the promoter-proximal three Sna-binding sites, rather than the two Sna-binding sites in the MSE, are instrumental in the mesodermal repression of *sim*. A ~230-bp region located just upstream of the *sim* early promoter includes three Sna-binding sites, and two sites of these sites are located less than 100 bp from the transcription start site of the *sim* promoter (Fig. 2A). Similar to a Sna-binding site in the MSE, the two sites are close enough to directly repress the transcription complex on the *sim* promoter. It is therefore conceivable that at least two promoter-proximal sites directly repress transcription from the *sim* early promoter in the presumptive mesoderm by interfering with activities of the RNA polymerase II initiation complex. Previous studies on direct repression mediated by various repressor proteins strengthen this view.

Direct repression of transcription is demonstrated in both *Drosophila* and mammalian tissue culture assays. When located in promoter-proximal regions, a Sna repressor functions in a dominant fashion and blocks transcription activated by distal en-

hancers (16). Results of transient transfection assays suggest that an *eve* repressor silences transcription, possibly through direct interactions with the TATA-binding protein (TBP) (19, 20). Finally, a glucocorticoid receptor (GR), a member of the nuclear receptor family, can exert repression when bound in promoter-proximal regions (21). In this context, we propose to designate as a 'repression element' for mesodermal *sim* repression, the ~230-bp region that includes the promoter-proximal Sna-binding sites.

MATERIALS AND METHODS

Fly stocks

Drosophila melanogaster strain yw^{67c23} was used for P-element transformation and *in situ* hybridization, as described previously (22). The mutant strain of sna ($b^1sna^{19/4.26}pr^1$ $cn^1wx^{wxt}bw^1/CyO$, $Cy^1dp^{lv1}pr^1cn^2$) was obtained from *Drosophila* Genome Resource Center (stock number 101332) and used for producing the *sna* homozygous mutant embryos.

Plasmid construction, mutagenesis and P-element-mediated germline transformation

Detailed information is described in Supplementary Material.

Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization was performed as previously described (23). Briefly, embryos were collected 2-4 h AED, dechorinated, fixed and hybridized with dioxigenin (DIG) UTP-labeled antisense RNA probes. An antisense *lacZ* RNA probe was used to investigate staining patterns in the transgenic embryos. To examine the pattern of endogenous *sim* expression in wild-type and homozygous *sna*-mutant embryos, an antisense *sim* RNA probe (approximately 1.5 kb) was produced. A 5′-region of the last exon of a *sim* locus (approximately 1.5 kb) was amplified by genomic PCR, introduced into the pGEM®-T Easy vector and used as a template for *in vitro* transcription. Developmental stages during embryogenesis were defined according to Campos-Ortega and Hartenstein (24).

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