Transcriptional repression via antilooping in the Drosophila embryo

Vivek S. Chopra, Nikki Kong, and Michael Levine

Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720


Transcriptional repressors are thought to inhibit gene expression by interfering with the binding or function of RNA Polymerase II, perhaps by promoting local chromatin condensation. Here, we present evidence for a distinctive mechanism of repression, whereby sequence-specific repressors prevent the looping of distal enhancers to the promoter. Particular efforts focus on the Snail repressor, which plays a conserved role in promoting epithelial-mesenchyme transitions in both invertebrates and vertebrates, including mesoderm invagination in Drosophila, neural crest migration in vertebrates, and tumorigenesis in mammals. Chromosome conformation capture experiments were used to examine enhancer looping at Snail target genes in wild-type and mutant embryos. These studies suggest that the Snail repressor blocks the formation of fruitful enhancer–promoter interactions when bound to a distal enhancer. This higher-order mechanism of transcriptional repression has broad implications for the control of gene activity in metazoan development.

Transcriptional repressors delineate sharp boundaries of gene expression during development (1). Repressor proteins have been proposed to work by a variety of mechanisms, including competition with activator proteins for shared DNA binding sites, inhibition or quenching of adjacent activators bound to a common enhancer, and direct repression of the RNA Polymerase II (Pol II) transcription complex (1–4). A number of sequence-specific repressors have been shown to recruit histone deacetylases, which have been implicated in chromatin condensation (5, 6). Moreover, the recent observation that many developmental control genes in Drosophila contain stalled Pol II raises the possibility that repressors might inhibit Pol II elongation (7–12). To investigate these different mechanisms of transcriptional repression, we used the chromosome conformation capture (3C) technique (13, 14) to examine enhancer-promoter looping at several developmental control genes in the early Drosophila embryo.

Different embryonic patterning mutants were used for this analysis, particularly three maternal mutants that produce abnormal Dorsal gradients controlling dorsal-ventral (DV) patterning. Each mutant transforms all cells into a single embryonic tissue: Toll10b (mesoderm), Toll10a/Toll10d (neuroectoderm), and gd1/gd2 (dorsal ectoderm) (15–17). The short gastrulation ( sog) and brinker ( brk) genes were chosen for 3C assays because they are regulated by well-defined enhancers (18) (Fig. 1 A and B) and are differentially expressed in the three DV mutants. Both sog and brk are activated by low levels of Dorsal in wild-type and Toll10a/Toll10d embryos (16), but are silent in gd1 mutants lacking Dorsal and actively repressed in Toll10b mutants containing high levels of the Snail repressor, as discussed below.

Snail is one of the best-characterized repressors in animal development and disease (4, 19, 20). It is selectively expressed in the presumptive mesoderm of gastrulating Drosophila embryos, where it delineates the boundary between the mesoderm and neurogenic ectoderm by repressing at least 50 target genes that are required for the patterning of the ectoderm (16, 21, 22). Snail is also essential for the formation of the ventral furrow during gastrulation by promoting epithelial-mesenchyme transitions (EMT) through the repression of genes encoding cell-adhesion molecules, such as E-Cad (19). Snail and related repressors (e.g., Slug) have been shown to promote EMT in vertebrate developmental processes, such as the delamination of neural crest (22). Snail repressors have also been implicated in EMT in tumorigenesis; tumors that express Snail repressors display enhanced metastasis (19).

Previous studies have shown that Snail recruits two different corepressor proteins, Ebi and CtBP (5, 23, 24). Snail was classified as a short-range repressor, in that Snail binding sites must map within 100 bp of either upstream activators or the core promoter to mediate effective transcriptional repression (25). Moreover, Snail does not interfere with elongating Pol II complexes released before the onset of repression (26), but blocks the activation or release of Pol II at the promoter. Here, we explore Snail-mediated repression using 3C assays.

Results

Snail Blocks Enhancer–Promoter Interactions. The 3C assays were used to gain additional insights into the mechanisms by which Snail works as a repressor. The 3C technique involves cross-linking embryos with formaldehyde, isolation of cross-linked chromatin, digestion with a restriction enzyme, and detection of hybridization products by PCR (13, 27). Noncross-linked chromatin and nonligated chromatin serve as controls and primer pair efficiency was checked by a genomic DNA control (Fig. S1). PCR amplicons were cloned, sequenced, and mapped to their respective positions in the genome to verify the presence of hybrid DNA products and thereby confirming long-range interactions. In contrast, PCR reactions performed on noncross-linked controls failed to produce amplicons (e.g., Fig. 1C, rows 2, 4, and 6).

These assays suggest looping of both the sog enhancer (primary intronic enhancer) and the 5′ (shadow) enhancer to the sog promoter region (Fig. 1C) in Toll10a/Toll10d embryos, which exhibit constitutive sog expression (Fig. 1C, row 3). Importantly, these loops are lost in DV mutants where sog is inactive (Fig. 1C, rows 1 and 5). The absence of loops in gd1 mutants (Fig. 1C, row 5) presumably results from a failure to recruit appropriate coactivators because these embryos lack nuclear Dorsal. In contrast, the loss of looping in Toll10b mutants (Fig. 1C, row 1) results from active repression by Snail, which is ubiquitously expressed in these mutants. Snail inhibits the expression of neurogenic genes such as sog and brk in the mesoderm (1).

Previous ChIP-chip assays suggest that both Dorsal and Snail co-occupy the sog and brk enhancers in Toll10b embryos (17) (Fig. 1 A and B). Thus, we propose that Snail, along with its corepressors Ebi and CtBP (5, 23, 24), somehow blocks enhancer-promoter looping (summarized in Fig. 4). The extent of looping was investigated in detail for the brk locus (Fig. 2), as discussed below.

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1To whom correspondence should be addressed. mlevine@berkeley.edu.

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brk was selected for in-depth analysis because it is regulated by two well-separated enhancers, located ~10 kb 5′ of the transcription start site (primary enhancer) and ~13 kb downstream of the start site (shadow enhancer) (Fig. 1B). As seen for sog, there is looping of the 5′ primary and 3′ shadow enhancers to the brk promoter region in Toll10b (Fig. 1C, row 1) but not in Toll10b (Fig. 1C, row 3) or gd7 mutants (Fig. 1C, row 5). Systematic 3C assays were performed to visualize the limits of enhancer–promoter looping at the brk locus (Fig. 2). Because the 3C technique depends on the availability of restriction sites, we used two different restriction enzymes (EcoRI and HindIII) to saturate the locus (Fig. 2A). In total, 23 primer pairs were used to examine loop formation across a ~40-kb interval encompassing the brk locus and neighboring genes, including Atg5, which contains the 3′ brk shadow enhancer (18).

The brk promoter region (gray bar) was used as an anchor point for all 3C interactions. As expected, the overall cross-linking frequency decreased with increasing distance from the brk promoter (summarized in Fig. 2B). Nonetheless, augmented looping interactions were observed for both the primary and shadow enhancer regions in wild-type and Toll10b/Tollrm10 mutant embryos where brk is active. In contrast, there was no significant enrichment of looping interactions in either Toll10b or gd7 embryos, which lack brk expression (Fig. 2B). Normalization of the PCR reactions using noncross-link and nonligation controls suggests that the peaks of looping map ~8-kb upstream and ~9-kb downstream of the brk promoter region (Fig. 2B), near the 5′ and 3′ enhancers. However, this is a semiquantitative method that may not accurately identify the exact locations of the accessible regions of the loops (see SI Materials and Methods). To obtain a more accurate mapping of the limits of the loops, we conducted additional assays using more quantitative 3C methods (Fig. 3).

Quantitative Analysis of Enhancer–Promoter Looping. Enhancer–promoter looping interactions were also measured by 3C-
quantitative-PCR (3C-qPCR) assays as described previously (14). These assays were performed with customized Taqman (fluorescent) probes at the anchor points located upstream of the promoter (blue bar, Fig. 3). The fluorophores are released from these probes at the onset of the PCR upon amplification of hybrid DNAs arising from the joining (looping) of distal sequences to the anchor points. Thus, detection of released fluorophores permits quantitative measurements of the amount of hybrid DNAs. As seen with traditional 3C assays (Figs. 1 and 2), enhancer–promoter looping is detected in both Toll10b mutants and wild-type embryos. There is a loss of looping in gd7 mutants, and a significant diminishment in Toll10b mutants, where Snail actively represses brk expression. The residual looping seen for the 3′ shadow enhancer (red line, Fig. 3) is probably a result of transient expression of brk in Toll10b mutants before the accumulation of sufficient levels of the Snail repressor to inhibit expression. The Taqman assays identified the accessible regions of the loops within the limits of the enhancers (yellow bars, Fig. 3).

Discussion

We have presented evidence that Snail represses gene expression by inhibiting the looping of distal enhancers to the promoter regions of target genes (summarized in Fig. 4). Such a mechanism is compatible with the recent demonstration that Snail blocks Pol II initiation, but does not interfere with the elongation of RNA polymerases released before the onset of Snail repression (26). It would appear that Snail blocks enhancer looping, and thereby prevents Pol II release from the promoter region.

Although 3C assays have the potential to produce high background, the use of appropriate controls and the use of different DV mutants provide a critical endogenous control for correlating the loss of specific enhancer–promoter loops and transcriptional repression. There is a loss of looping in two different classes of mutants lacking sog and brk expression. One lacks the Dorsal activator (gd7) and the other exhibits constitutive expression of the Snail repressor (Toll10b).

In summary, the Snail repressor functions via antiloopung, when it is bound to distal enhancers (Fig. 4). We anticipate that
this higher-order mechanism of transcriptional repression will prove to be generally used in a variety of developmental processes. For example, gap repressors controlling segmentation, including Kruppel and Knirps, recruit the same corepressor CtBP that is used by Snail (24). It is possible that CtBP (or Ebi) somehow interferes with the recruitment or function of coactivators [e.g., CBP (28)] or other factors required for the formation of enhancer–promoter loops. Antilooping is a flexible form of repression, in that it need not interfere with the binding or function of Pol II at the core promoter. Such a mechanism might be particularly useful for repressing “poised” genes (containing paused or stalled Pol II) that are rapidly activated during development.

Materials and Methods

Fly Strains. The different alleles of the maternal gene Toll used were Toll10b and Tollrm9/Tollrm10 as described previously (16). The gastrulation defective (gd)7 mutant flies were used as described previously (15, 29). All of the mutant backgrounds were 2- to 4-h staged embryos.

3C Assay. The 3C assay was performed in Drosophila wild-type (yw) and DV mutant embryos, as described previously (30) (see SI Materials and Methods). The 3C-qPCR assays were performed as described in Hagège et al. (14). Briefly, Taqman-MGB probes were designed using Primer Express 3 software and qPCRs performed in ABI Viia7 real-time machine using PerfeCTa FastMix II Low Rox (VWR) master mix. The BAC clone for the brk locus was ordered from http://bacpac.chori.org. The brk BAC DNA was digested with either EcoRI or HindIII restriction enzymes followed by ligation. This BAC template library was used as control quantification template for the standard curves for each primer pair in 3C-qPCR assays. The details of all primers and probes used are illustrated in Tables S1–S4.

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