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Report

The Snail Repressor Inhibits Release, Not Elongation, of Paused Pol II in the *Drosophila* Embryo

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Summary

The development of the precellular *Drosophila* embryo is characterized by exceptionally rapid transitions in gene activity, with broadly distributed maternal regulatory gradients giving way to precise on/off patterns of gene expression within a one-hour window, between two and three hours after fertilization [1]. Transcriptional repression plays a pivotal role in this process, delineating sharp expression patterns (e.g., pair-rule stripes) within broad domains of gene activation. As many as 20 different sequence-specific repressors have been implicated in this process, yet the mechanisms by which they silence gene expression have remained elusive [2]. Here we report the development of a method for the quantitative visualization of transcriptional repression. We focus on the Snail repressor, which establishes the boundary between the presumptive mesoderm and neurogenic ectoderm [3]. We find that elongating Pol II complexes complete transcription after the onset of Snail repression. As a result, moderately sized genes (e.g., the 22 kb *sog* locus) are fully silenced only after tens of minutes of repression. We propose that this "repression lag" imposes a severe constraint on the regulatory dynamics of embryonic patterning and further suggest that posttranscriptional regulators, like microRNAs, are required to inhibit unwanted transcripts produced during protracted periods of gene silencing.

Results and Discussion

The zinc-finger Snail repressor is one of the most extensively studied repressors in the *Drosophila* embryo. It has been implicated in a variety of developmental and disease processes, including epithelial-mesenchyme transitions and tumorigenesis [3–7]. Snail typically binds to repressor sites located near upstream activation elements within distal enhancers [8, 9]. Repression might result from the passive inhibition of upstream activators, such as the failure of the activators to mediate looping to the core promoter. Alternatively, Snail might alter the chromatin state of the promoter region, resulting in diminished access of the Pol II transcription complex [2, 10]. Such repression mechanisms might cause a lag in gene silencing due to the continued elongation of Pol II complexes that were released from the promoter prior to the onset of repression (Figure 1B). As in the case of the delay in the production of mature mRNAs after initiation (Figure 1A), the lag in repression would be commensurate with the size of the gene, with large genes taking longer to silence than small genes. This can take a significant amount of time due to the surprisingly slow rate of Pol II elongation, only ~1 kb/min [11].

Alternatively, elongating Pol II complexes might be arrested or released from the DNA template due to changes in chromatin structure and/or attenuation of Pol II processivity. Such mechanisms could lead to the immediate silencing of all genes regardless of size (see Figure 1C). Recent studies have documented rapid changes in the chromatin structure across the entire length of genes, exceeding the rate of Pol II processivity [12]. Certain corepressors in the *Drosophila* embryo (e.g., Groucho) are thought to mediate repression by a "spreading" mechanism that modifies chromatin over extensive regions [13]. Indeed, this type of mechanism has been invoked to account for the repression of the pair-rule gene *even-skipped* (*eve*) by the gap repressor Knirps (see below) [14]. The attenuation of Pol II elongation has been implicated in a variety of processes. For example, Pol II attenuation has been documented for the transcriptional repression of MYC [15]. Moreover, the activation of the HIV genome is regulated by Pol II processivity [16]. In an effort to distinguish these potential mechanisms, we visualized the repression dynamics of several Snail target genes, because they are silenced in the presumptive mesoderm of precellular embryos.

short gastrulation (*sog*) encodes an inhibitor of BMP/Dpp signaling that restricts peak Dpp signaling to the dorsal midline of cellularizing embryos [17–19]. The *sog* locus is ~22 kb in length and contains three large introns, including a 5′ intron that is ~10 kb in length and a 3′ intron that is ~5 kb in length (see Figure 2C). The use of separate intronic hybridization probes permits independent detection of 5′ (see Figure 2F) and 3′ (see Figure 2G) sequences within nascent *sog* transcripts (Figure 2). Individual nuclei are then false colored according to the probe combination they contain (see Figure 2H).

sog exhibits synchronous activation at the onset of cell cycle 13 (cc13), ~2 hr after fertilization [20]. There is a lag between the time when nascent transcripts are first detected with the 5′ probe and subsequently cross-hybridize with both the 5′ and 3′ intronic probes (Figures 2A and 2B). This lag is consistent with the established rates of Pol II elongation in flies, ~1.1–1.5 kb/min [11]. cc13 persists for ~20 min [21], and by the completion of this time window, most of the nuclei in ventral and lateral regions exhibit yellow staining, indicating the presence of multiple nascent transcripts containing 5′ and 3′ intronic sequences within each nucleus (Figure 2D). There is little or no repression in ventral regions, presumably due to insufficient levels of the Snail repressor prior to cc14 [8, 22].

As shown previously, nascent transcripts are aborted during mitosis [23, 24]. Consequently, only the 5′ hybridization probe detects nascent *sog* transcripts at the onset of cc14 (Figure 2E). Moreover, a small number of nuclei (at the ventral midline) fail to exhibit nascent transcripts with either the 5′ or 3′ probe, suggesting repression by Snail. This repression becomes progressively more pronounced during cc14 (Figure 3).

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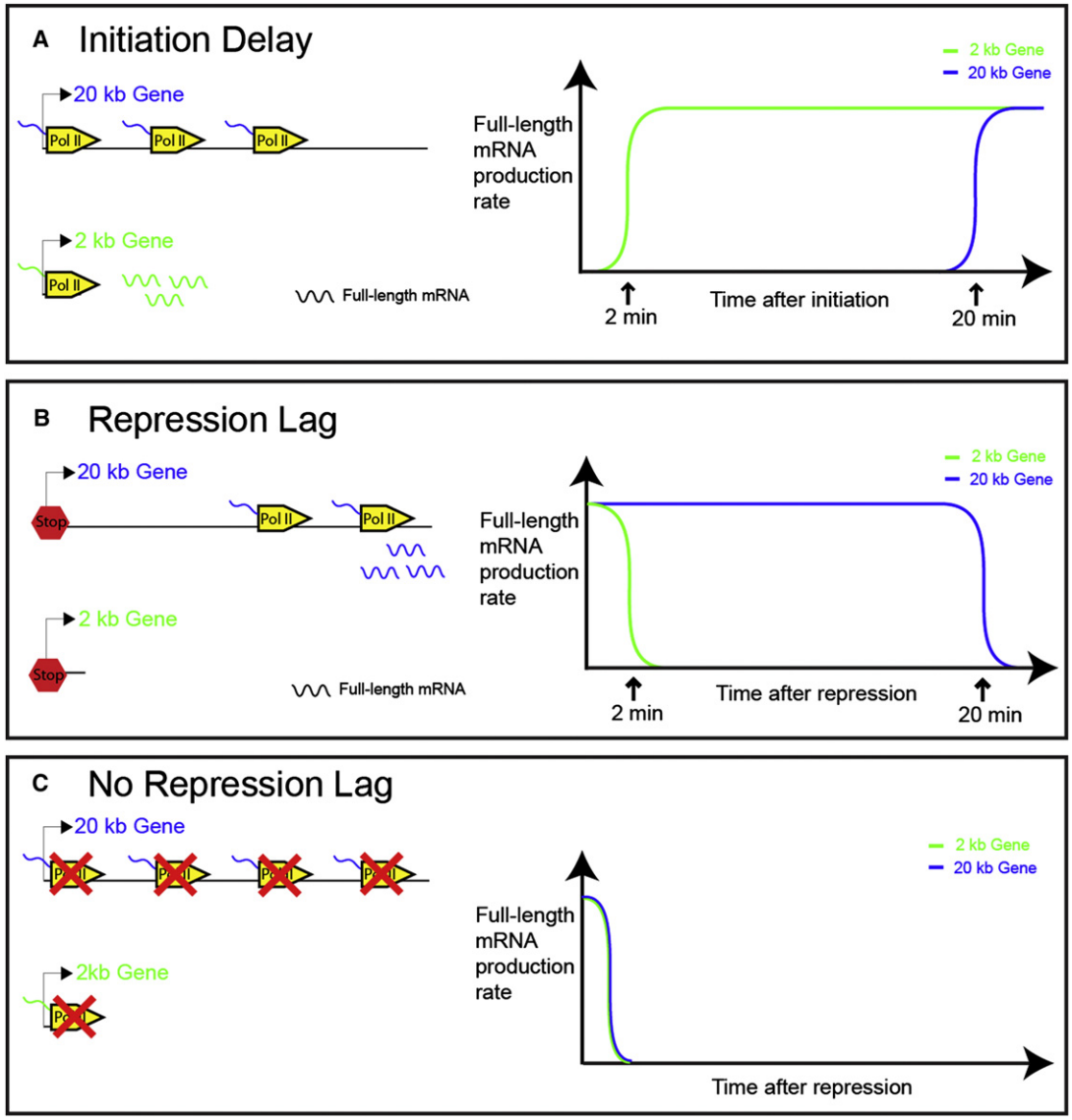

Figure 1. Schematic Showing How the Initiation of Transcription and Different Schemes of Repression Affect the Dynamics of Full-Length mRNA Production

(A) Gene models showing the differences in the distribution of polymerase on a 20 kb and a 2 kb gene and the amount of full-length mRNA produced some time after initiation. Not enough time has elapsed for Pol II complexes to reach the end of the 20 kb gene, but the 2 kb gene is short enough that multiple complexes have already reached it, allowing the production of full-length mRNA. This process is depicted in the graph showing the rate of full-length mRNA production as a function of time after initiation, which shows that there is a significantly longer delay before Pol II complexes can reach the end of the 20 kb gene (20 min) and produce productive transcripts, compared to the 2 kb gene (2 min).

(B) Gene models showing the differences in the distribution of polymerase on the two genes and the amount of full-length mRNA some time after transcriptional repression, assuming that no new Pol II complexes are recruited to the gene after repression but that those on the gene finish elongating. Enough time has elapsed for Pol II complexes to transcribe the length of the 2 kb gene, and hence production of full-length transcripts has ceased. However, not enough time has elapsed for Pol II complexes to reach the end of the 20 kb gene, and so it is still producing full-length mRNA long after the initiation of repression. This process is depicted in the graph showing the rate of full-length mRNA production as a function of time after repression, which shows that there is a significantly longer delay before full-length mRNA production is repressed in the case of the 20 kb gene (20 min), compared to the 2 kb gene (2 min).

(C) As in (B), except that elongating Pol II complexes on the template are arrested or have their processivity attenuated when the genes are repressed. This would result in a rapid cessation of production of full-length mRNA for both the 2 kb and 20 kb genes (assumed elongation speed of Pol II is 1 kb/min throughout).

Within about 10 min of the first detection of nascent *sog* transcripts at the onset of cc14 (Figures 3A and 3G), most of the nuclei exhibiting *sog* expression stain yellow, indicating expression of both 5′ (green) and 3′ (red) intronic sequences (Figures 3B and 3H). During the next several minutes, progressively more nuclei exhibit only 3′ (red) hybridization signals in ventral regions (Figures 3C and 3I). This transition from yellow to red continues and culminates in a "red flash" where the majority of the ventral nuclei that contain nascent transcripts express only the 3′ (red) probe (Figure 3D). As cc14 continues, there is a progressive loss of staining in the presumptive mesoderm (Figure 3E), and eventually, nascent *sog* transcripts are lost entirely in the presumptive mesoderm (Figure 3F).

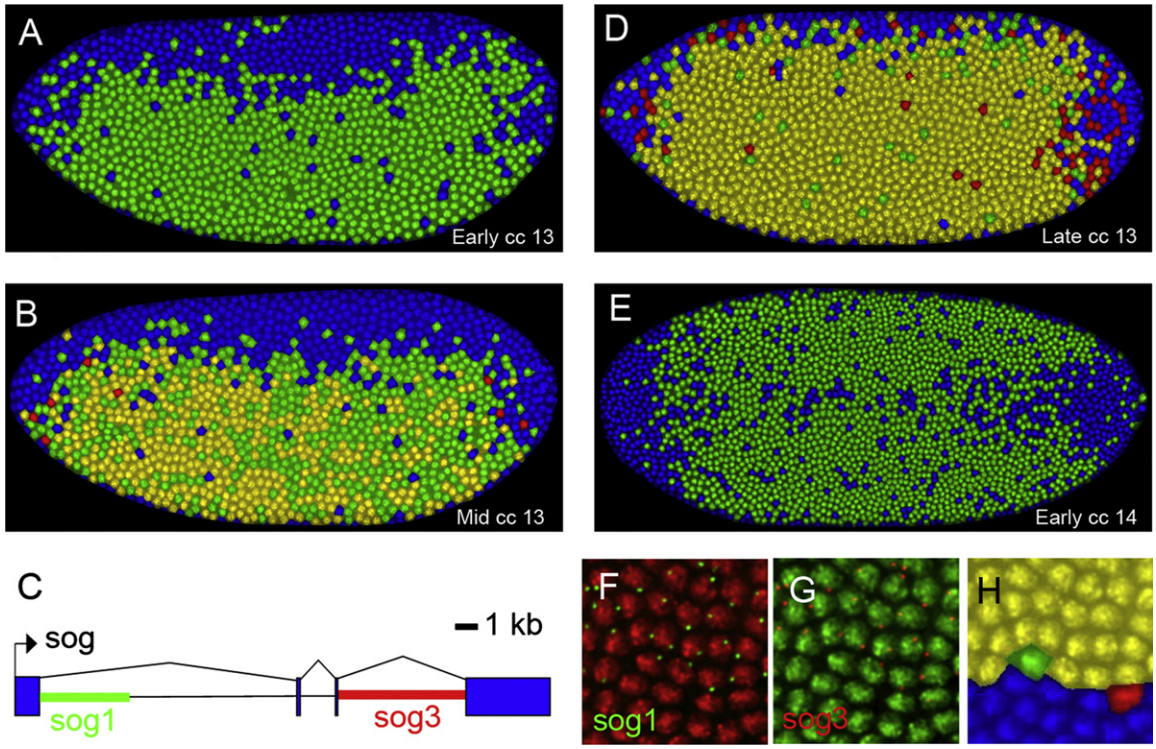

Figure 2. Time Course of *sog* Transcription from Early Cell Cycle 13 to Early Cell Cycle 14

Anterior is to the left. See also Figure S1.

(A) Lateral view of an embryo in the early stages of cc13. Most of the nuclei contain intense dots of in situ hybridization signal that correspond to nascent transcripts. Only the 5′ intronic probe is detected (see C). The nuclei are false colored according to the combination of probes they contain (see F–H).

(B) Lateral view of an embryo midway through cc13. Most of the nuclei show in situ signal for the 5′ probe, and about half of these also show staining for the 3′ probe.

(C) Simplified gene model for the *sog* transcript showing the location of the three biggest introns and the location of the sequences to which the 5′ (green), sog1, and 3′ (red), sog3, intronic in situ probes hybridize.

(D) Lateral view of an embryo in the late stages of cc13. Most of the cells express both the 5′ and 3′ probes.

(E) Ventral view of an embryo in the early stages of cc14. Only isolated 5′ probe is detected.

(F) Zoomed-in section of a cc14 embryo showing the expression of nascent transcript labeled by the 5′ probe in green. The nuclear stain has been false colored red to maximize the contrast.

(G) The same section as in (F), but with the 3′ probe labeled in red and the nuclear stain false colored green.

(H) The same section shown in (F) and (G), but after it has been processed with the segmentation algorithm. Isolated and paired nascent transcripts have been identified and nuclei false colored to reflect which combinations of probes are present in each nucleus. Nuclei that contain only isolated green probes have been false colored green. Nuclei that contain only isolated red probes have been false colored red. Nuclei that contain a coincident red and green dot have been labeled in yellow, and nuclei that contain no detectable probe have been labeled in blue.

These results suggest that after its release from the promoter, Pol II continues to elongate along the length of the *sog* transcription unit, even as Snail actively represses its expression in the mesoderm. The red flash observed during mid-cc14 represents partially processed nascent *sog* transcripts that have lost the 5′ intron (hence no green signals with the 5′ hybridization probe) but retain 3′ sequences (summarized in Figures 3G–3I). Previous studies are consistent with sequential processing of nascent transcripts, beginning with the removal of 5′ intronic sequences and concluding with the removal of 3′ introns [25]. As a control, two separate hybridization probes were used to label opposite ends of *sog* intron 1. As expected, there was no red flash, because both hybridization signals were simultaneously lost when intron 1 was spliced (see Figures S1 and S2 available online).

There is an ~20 min lag between the onset of repression at early cc14 (Figure 3B) and the complete silencing of *sog* expression in the presumptive mesoderm during mid- to late cc14 (Figure 3F). To determine whether this repression lag is a common feature of Snail-mediated gene silencing, we examined additional target genes, including ASPP, *Delta*, *canoe*, and *scabrous* (*sca*). ASPP (Figures 4D and 4E) encodes a putative inhibitor of apoptosis [26], whereas *Delta* (Figures 4A and 4B) encodes the canonical ligand that induces Notch signaling. All four of these genes exhibit repression lag as they are silenced in the presumptive mesoderm of cc14 embryos (Figure 4; Figure S3)

With the notable exception of *Delta*, the genes examined in this study contain promoter-proximal paused Pol II, as do most developmental patterning genes active in the precellular embryo [27]. Moreover, results from whole-genome Pol II binding assays indicate that these genes maintain promoter-proximal paused Pol II in the presumptive mesoderm as they are actively repressed by Snail. These findings are consistent with the observation that the segmentation gene *sloppy paired 1* retains promoter-proximal paused Pol II even after being silenced by the ectopic expression of Runt and Ftz [28]. Thus, the Snail repressor does not appear to affect Pol II

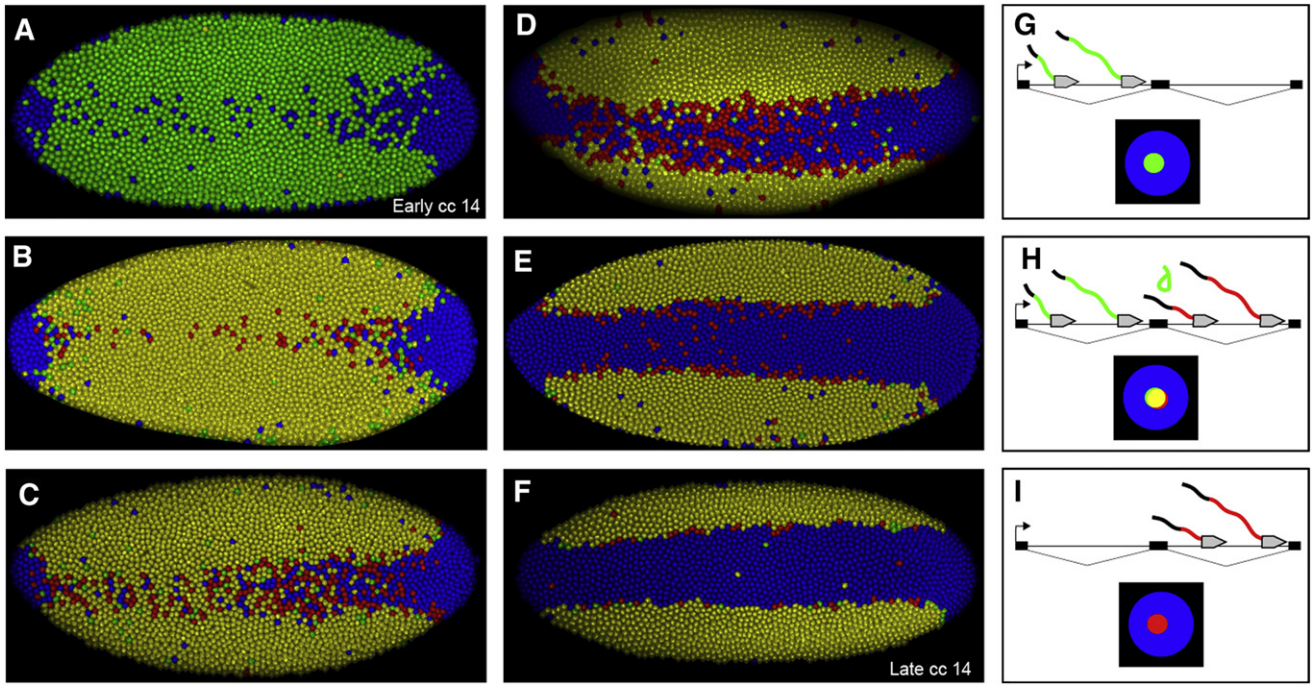

Figure 3. Time Course of *sog* Transcription from Early Cell Cycle 14 to Late Cell Cycle 14 with Schematic Explaining Results

Anterior is to the left, showing a ventral view. See also Figure S2.

(A) Embryo in the early stages of cc14; the embryo is older than the embryo shown in Figure 2E. Most nuclei are only expressing the 5′ (green) probe, but a small number also express 3′ (red) probe.

(B) Within about 10 min of the first detection of nascent *sog* transcripts at the onset of cc14, most of the nuclei exhibiting *sog* expression stain yellow, indicating expression of both 5′ (green) and 3′ (red) intronic sequences.

(C) During the next several minutes, progressively more nuclei exhibit only 3′ (red) hybridization signals in ventral regions.

(D) This transition from yellow to red continues and culminates in a "red flash" where the majority of the ventral nuclei that contain nascent transcripts express only the 3′ (red) probe.

(E) As cc14 continues, there is a progressive loss of staining in the presumptive mesoderm.

(F) Eventually, nascent *sog* transcripts are lost almost entirely in the presumptive mesoderm in late cc14.

(G) Gene model depicting a gene like *sog* with multiple introns, where a 5′ (green) probe recognizes the mRNA coded for by the first intron and a 3′ (red) probe recognizes the mRNA coded for by the second intron. Initially, only the 5′ (green) probe will hybridize to the nascent transcript. This is because not enough time has elapsed to transcribe the mRNA that the 3′ (red) probe hybridizes to. In situ, nuclei where this has occurred will have a green dot at the site of nascent transcription.

(H) After enough time has elapsed for some Pol II complexes to reach the second intron labeled by the 5′ (red) probe, both probes will hybridize and will manifest as a yellow dot in a nucleus. Some of the individual transcripts associated with Pol II complexes that have made it well into the second intron will only hybridize to the 3′ (red) probe because the 5′ (green) probe is cotranscriptionally spliced and degraded.

(I) If repression inhibits new polymerases from initiating transcription but allows elongating polymerases to finish transcription, then after a time, only the 3′ (red) probe will hybridize to nascent transcripts, because all of the intronic sequences containing the 5′ (green) probe will have been spliced out and degraded. In situ, nuclei where this has occurred will have an isolated red dot at the site of nascent transcription.

recruitment but rather inhibits the release of Pol II from the promoter-proximal regions of paused genes. At every round of de novo transcription, each Pol II complex at the pause site must receive an activation signal for its release into the transcription unit. We propose that the Snail repressor interferes with this signal, resulting in the retention of Pol II at the pause site.

It is currently unclear whether repression lag is a general feature of transcriptional silencing. A recent study suggests that the gap repressor Knirps reduces the processivity of Pol II complexes across the *eve* transcription unit [14]. Snail and Knirps might employ distinctive modes of transcriptional repression. Snail recruits the short-range corepressor CtBP [29], whereas Knirps recruits either CtBP or the long-range corepressor Groucho [30]. When bound to certain *cis*-regulatory elements within the *eve* locus, Knirps recruits Groucho, which might propagate a repressive chromatin structure. In contrast, Snail-CtBP might interfere with the release of Pol II from the proximal promoter, as discussed above. There is a considerable difference in the lengths of the genes examined in the two studies. The *eve* transcription unit is only 1.5 kb in length, less than one-tenth the size of *sog*. In fact, many patterning genes active in the early fly embryo contain small transcription units only a few kilobases in length. Small transcription units offer dual advantages in rapid patterning processes: essentially no lag in activation or repression.

All five Snail target genes examined in this study exhibit Pol II elongation after the onset of repression. The number of transcripts produced during repression lag depends on the Pol II density across the transcription unit at the onset of repression. Whole-genome Pol II binding assays suggest that there are at least several Pol II complexes per kilobase [27]. This estimate is based on comparing the total amount of Pol II within these genes to that present at the promoter of the uninduced hsp70 gene, for which there are accurate measurements. As a point of reference, the Pol II density on induced heat-shock

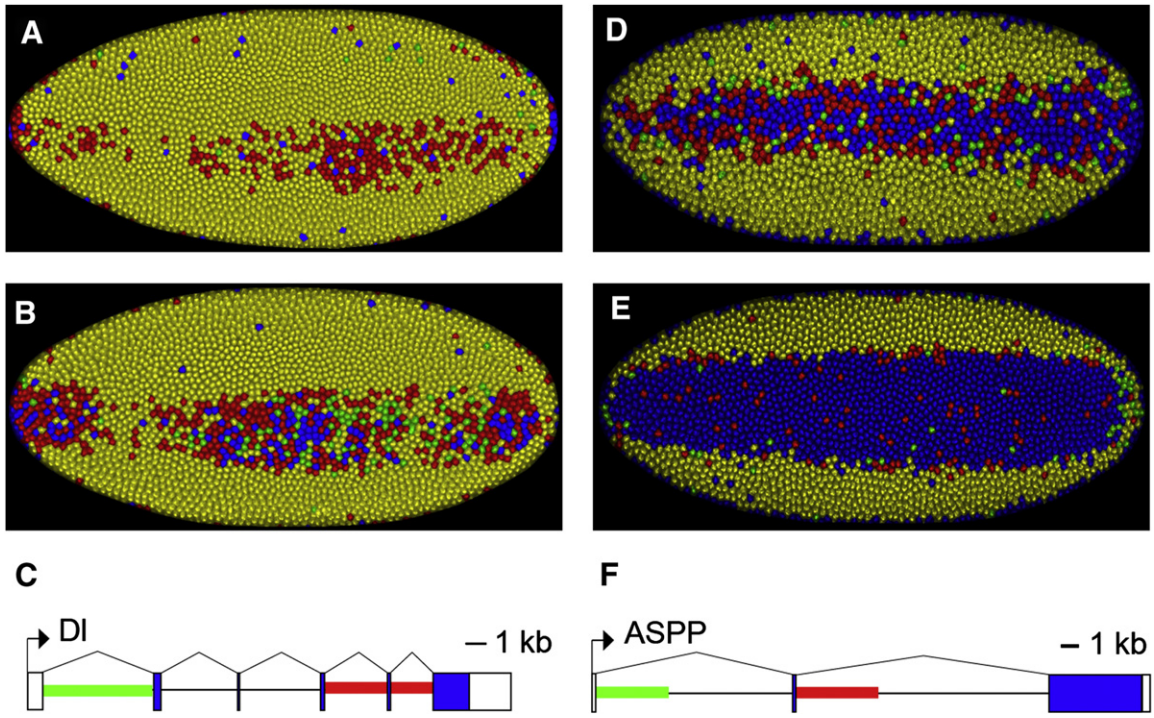

Figure 4. Repression of Delta and ASPP Transcription in the Presumptive Mesoderm

Anterior is to the left, showing a ventral view. See also Figure S3.

(A) cc14 embryo showing staining for Delta. Both the 5′ (green) and 3′ (red) probes (see C) hybridize to the nascent transcripts in most of the nuclei. However, in the ventral regions, a number of nuclei only show the presence of the 3′ (red) probe, consistent with repression. (See Figure 3.)

(B) Older embryo showing more nuclei expressing the 3′ (red) probe, consistent with the continuation of Snail-mediated repression.

(C) Simplified gene model for the *Dl* transcript showing the location of the largest introns and the location of the mRNA sequences to which the 5′ (green) and 3′ (red) intronic in situ probes hybridize.

(D) cc14 embryo showing staining for ASPP. Both the 5′ (green) and 3′ (red) probes (see F) hybridize to the nascent transcripts in most of the nuclei. However, in the ventral regions, there are a large number of nuclei that only show the presence of the 3′ (red) probe, consistent with repression. (See Figure 3.)

(E) Older embryo showing most of the nuclei in the mesoderm without any staining but some isolated nuclei expressing the 3′ (red), consistent with the continuation of Snail-mediated repression.

(F) Simplified gene model for the ASPP transcript showing the location of the largest introns and the location of the mRNA sequences to which the 5′ (green) and 3′ (red) intronic in situ probes hybridize.

genes is one complex per 75–100 bp [31], which is comparable to the footprint size, ~50 bp, of an elongating Pol II complex [32]. Thus, somewhere in the vicinity of ~50 (or more) *sog* transcripts may be produced in a diploid cell after the onset of Snail repression. This represents a significant fraction of the steady-state expression of a typical patterning gene (~200 transcripts per cell [33]).

Repression lag could impinge on a number of patterning processes, such as Notch signaling. The specification of the ventral midline of the central nervous system depends on the activation of Notch signaling in the ventralmost regions of the neurogenic ectoderm [34]. Sca products somehow facilitate the activation of the Notch receptor [35], and repression lag could potentially disrupt this process by producing high steady-state levels of Sca in the mesoderm where Notch is normally inactive. Similar arguments might apply to the unwanted accumulation of Delta products in the mesoderm. Perhaps microRNAs are required to inhibit these transcripts and thereby facilitate localized activation of Notch signaling. Indeed, miR-1 is expressed in the presumptive mesoderm, at the right time and place to regulate Sca and/or Delta [36], and is known to be able to target Delta transcripts [37]. Repression lag is potentially quite severe for Hox genes, particularly *Antp* and *Ubx*, which contain large transcription units (75–100 kb) that could take over an hour to silence after the onset of repression. It is conceivable that miRNAs encoded by the miR-iab4 gene, which are known to target *Antp* and *Ubx* transcripts [38, 39], might inhibit postrepression transcripts.

The precellular *Drosophila* embryo possesses a number of inherently elegant features for the detailed visualization of differential gene activity in development. Indeed, such studies were among the first to highlight the importance of transcriptional repression in the delineation of precise on/off patterns of gene expression. Here we extend this rich tradition of visualization by providing the first dynamic view of gene silencing. The key feature of our method is the use of sequential 5′ and 3′ intronic probes to distinguish nascent transcripts produced by Pol II complexes shortly after their release from the promoter versus mature Pol II elongation complexes that have already transcribed 5′ intronic sequences. We show that elongating Pol II complexes complete transcription after the onset of Snail repression and, as a result, moderately sized genes are fully silenced only after a significant lag. We suggest that this repression lag represents a previously unrecognized constraint on the regulatory dynamics of the precellular embryo.

Experimental Procedures

Fluorescence In Situ Hybridization and Quantitative Imaging Methods

Fluorescence in situ hybridization was performed on *yw* embryos as described in [31], with minor modifications. Embryos were imaged on a Carl Zeiss LSM 700 laser scanning microscope as 20- to 25-section Z stacks through the nuclear layer at 0.5 μm intervals using a Plan Apochromat 20×/0.8 objective lens. Image stacks were maximum-intensity projected and computationally segmented to localize and count nuclei and in situ probes. Nascent transcripts were then assigned to nuclei. In order to visualize the transcriptional state, individual nuclei have been false colored to reflect the transcriptional state as determined by the segmentation of the in situ probes. Extensive control experiments were conducted to show that nascent transcripts could be detected and classified with high accuracy. More details on the image analysis, segmentation, and in situ protocols are included in the Supplemental Experimental Procedures.

Updated versions of the image segmentation routines can be found at https://github.com/JacquesBothma. The source codes used to compute and plot the results from this publication are available at https://github.com/JacquesBothma/Repression_Lag.

Supplemental Information

Supplemental Information includes three figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2011.08.019.

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