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How do genomes encode developmental time?

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It is a pleasure to contribute this short perspective to commemorate Terri Grodzicker's remarkable 35 years as Editor of *Genes & Development*, one of the foremost journals at the interface of gene regulation and developmental biology. The Levine lab has published 30 papers in *G&D* during Terri's tenure, and her generous stewardship of these papers attests to her patience, humor, and breadth of scholarship. These studies span gene expression in the early *Drosophila melanogaster* embryo, post-transcriptional processes such as alternative polyadenylation, and the role of gene regulatory networks in the specification of different cell types in the tadpoles of the sea squirt *Ciona intestinalis*. Our heartfelt thanks to Terri for her hard work in improving the quality of our papers over the years.

We can't let Terri skedaddle without bugging her one last time. Most of our earlier papers focused on the spatial control of gene expression in development (e.g., Doyle et al. 1989; Small et al. 1991). In addition to summarizing some of this work, we wish to share a couple of thoughts about an enduring challenge in developmental biology; namely, the temporal control of gene activity. We briefly summarize three potential genome structural mechanisms that modulate the timing of transcription during development: gene length, enhancer proximity, and tethering elements.

Gene length

Since the molecular cloning of key developmental control genes in *Drosophila*, it has been widely noted that large genes take longer to transcribe than short ones (*see panel A in the figure on the following page*). For example, the homeotic genes *Antennapedia* (*Antp*) and *Ultrabithorax* (*Ubx*) contain large introns and transcription units spanning ~100 kb and ~80 kb, respectively (Scott et al. 1983; Hogness et al. 1985). Given the slow rate of eukaryotic RNA polymerase II (Pol II), ~2 kb/min, there is a lag of ~40–50 min between the onset of transcription and the production of nascent RNAs (Jonkers and Lis 2015; Fukaya et al. 2017). This lag has been shown to serve as a filter to exclude *Ubx* RNAs during the extremely rapid cell cycles (~5–10 min) driving the first 2 h of embryogenesis. Only during nuclear cycle 14 (nc14), the final interphase prior to the onset of gastrulation, is there enough time (~1 h) to produce full-length *Ubx* RNAs (Shermoen and O'Farrell 1991). It has been proposed that gene length could also function to control the relative levels of homeotic RNAs and proteins encoded by small and large genes (Gubb 1986). Gene length also influences the differential timing of expression of the paralogous gap genes *knirps* (3 kb) and *knirps-related* (23 kb) (Rothe et al. 1992). Indeed, most of the genes active in precellular *Drosophila* embryos are quite small and often lack introns (De Renzis et al. 2007; Artieri and Fraser 2014), implying that gene length can be a critical structural element under selective pressure in developing embryos with fast cell cycles.

In vertebrate genomes, gene size is generally larger than in *Drosophila*. An extreme example is the human gene *dystrophin*, which is encoded by a 2300-kb transcription unit, requiring an incredible 16 h to complete transcription (Tennyson et al. 1995). A molecular clock mechanism has also been proposed to influence the timing of somitogenesis. In mouse embryos, *Hes7* drives a negative feedback loop that generates oscillating pulses of transcription controlling the formation of somites, thus creating a segmentation clock. Reducing the length of *Hes7* by removing its introns accelerates the production of *Hes7* RNAs and the tempo of the transcriptional oscillations, leading to segmentation defects (Swinburne et al. 2008; Takashima et al. 2011; Harima et al. 2013). The advent of live-imaging methods and CRISPR genome editing should permit a more quantitative assessment of the role of gene length in the temporal control of gene expression during development.

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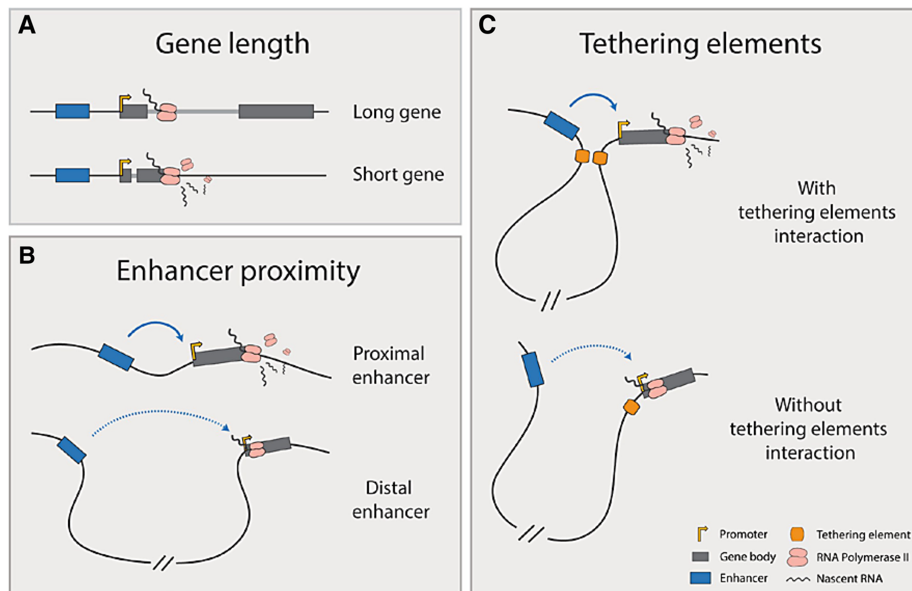

*Summary of three mechanisms that modulate the timing of gene activity. (*A*) Genes with longer transcription units show slower RNA production as compared with shorter genes. (*B*) Genes regulated by proximal enhancers show faster activation dynamics as compared with those regulated by distal enhancers. (*C*) Genes regulated by distal enhancers containing nearby tethering elements show faster activation dynamics as compared with those lacking such elements.*

Enhancer proximity

Developmental enhancers mediate cell-specific patterns of gene activity. Those active during early development are typically larger and more complex than the prototypic enhancers identified in animal viruses such as SV40 (Banerji et al. 1981). The latter are short (~100–200 bp in length) and contain binding sites for pleiotropic activators such as NF-kB. In contrast, developmental enhancers decode complex patterning information such as gradients of transcription factors or cell signaling pathways (e.g., Huang et al. 1997). They are often 300 bp to 1 kb in length and contain binding sites for different classes of transcriptional activators and sequence-specific repressors. For example, the minimal even-skipped (eve) stripe 2 enhancer is 500 bp in length and contains six binding sites for transcriptional activators (e.g., bicoid) and six binding sites for localized "gap" repressors (e.g., giant) (see Small et al. 1991).

Developmental enhancers can map quite far from the genes they regulate. One of the best-characterized examples of long-range regulation is seen for the *Sonic Hedgehog* (*Shh*) gene in vertebrates (Lettice et al. 2003). In tetrapods, *Shh* exhibits localized expression in a specific domain within developing limb buds (the "ZPA"), where it is important for the anterior–posterior patterning of digits. The enhancer responsible for this localized expression, the ZRS, maps 1 Mb away from the *Shh* promoter in the human genome. Despite this large distance, single-nucleotide substitutions in the ZRS cause polydactyly (extra digits) in mice, cats (e.g., "Hemingway's cats"), and humans.

However, developmental enhancers can also map close to their respective promoters. For example, developmental enhancers mediating expression of *rhomboid* in the neurogenic ectoderm of *Drosophila* embryos map within a few kilobases of the transcription start site (Ip et al. 1992). Nonetheless, they exhibit the classic properties of enhancers by working over large distances, in different orientations, and when placed downstream from the transcription unit. Is there a difference in the behaviors of developmental enhancers that map close to or far from their target genes?

There is evidence in the classical literature for "distal delay"; developmental enhancers that map far from their target genes take longer to activate expression than those located in promoter-proximal regions (*see panel B in the figure above*). For example, the *Drosophila* IAB5 enhancer regulating the Hox gene *Abdominal-B* (*Abd-B*) is located ~40 kb downstream from the *AbdB* promoter (Busturia and Bienz 1993). It normally activates gene expression in the primordia of the fifth, sixth, and seventh abdominal segments after the onset of gastrulation. However, when the IAB5 enhancer is placed immediately upstream of a reporter gene, expression is observed at significantly earlier stages, prior to the onset of gastrulation (e.g., Ohtsuki et al. 1998).

These observations raise the possibility of a lag in the activities of distal versus proximal enhancers. The basis for this lag is uncertain, but a reasonable possibility is that it reflects the time it takes for a distal enhancer to search for its target promoter and initiate transcription.

Tethering elements

Most metazoan genomes are organized into a series of sequential topologically associating domains (TADs)

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(Dekker and Heard 2015; Dixon et al. 2016). In *Drosophil*a, a typical TAD is ∼50–100 kb in length and contains several genes and numerous transcriptional enhancers (Rowley and Corces 2018). It is not always obvious how the right enhancer finds the right promoter or how these long-range regulatory connections are established.

Studies on the *Drosophila* Hox gene *Sex combs reduced* (*Scr*) provide evidence for a second class of genome-organizing elements—tethering elements—in addition to TAD boundaries/insulators (Calhoun et al. 2002). The *Scr* gene is contained within a 90-kb TAD that includes an ∼50-kb 5′ regulatory region extending to the neighboring *Antennapedia* locus (Batut et al. 2022). This regulatory region includes several developmental enhancers, including the EE (early embryonic) enhancer that mediates a stripe of expression at the presumptive cephalic furrow during late stages of nuclear cycle 14. The EE enhancer is located ∼35 kb upstream of the *Scr* transcription start site but is separated by an intervening sub-TAD compartment containing the segmentation gene ftz.

Bypass of ftz by the EE enhancer appears to be facilitated by a pair of tethering elements: one located at the *Scr* promoter and another (distal tethering element [DTE]) located ∼5 kb upstream of the EE. High-resolution Micro-C chromosome conformation assays identified a specific association of the two tethering elements. Removal of the DTE via CRISPR–Cas9 editing results in a delay in the onset of *Scr* transcription. Micro-C assays suggest that this delay is due, at least in part, to the loss in directionality of EE–*Scr* interactions (Batut et al. 2022). Tether–tether interactions appear to be important for both the specificity of enhancer–promoter interactions and the timing of expression (*see panel C in the figure on the previous page*). Similar results were observed for other genes active in the early embryo, including *charybde*, which maps nearly 250 kb from a developmental enhancer that mediates expression in the dorsal midline. Deletion of a DTE located near this distal enhancer leads to a significant delay in the onset of *charybde* expression, as well as an overall reduction in expression due to instabilities in long-range enhancer–promoter communication (Levo et al. 2022).

Summary

We have summarized different mechanisms for the temporal control of gene expression based on properties of genome organization. Differences in gene length, enhancer proximity, or tethering element interactions can create temporal differences in gene activity in response to shared activators and regulatory networks. The mechanisms we summarized are probably the tip of the iceberg, and it is easy to anticipate additional features of genome organization that influence the timing of gene activity. This is anticipated by a number of studies suggesting that acute depletion of CTCF leads to a loss of TAD organization without obvious changes in gene expression (e.g., Ghavi-Helm et al. 2019; Hsieh et al. 2022). However, the methods used to measure these changes lack the precision made possible by the use of quantitative live-imaging methods and high-resolution Micro-C assays. It seems likely that TADs and TAD boundaries will prove to be important for the temporal precision of animal development.

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