

# Promoter strength delimits enhancer threshold in the early *Drosophila* embryo

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**ABSTRACT** The enhancer threshold is defined as the minimum concentration of transcription factors (TFs) required to elicit an enhancer response in a given time and space. Here, evidence is presented that the enhancer threshold is relative to promoter strength in the early *Drosophila* embryo. The apparently inactive *even-skipped* (*eve*) minimal stripe element (MSE), in which a single Hunchback (Hb)-binding site is deleted, is functionally complemented by the *hsp70* promoter in transgenic embryos. Forced pause release of RNA polymerase II (Pol II) and transcription bubble assays show that both *eve* and *heat shock protein 70* (*hsp70*) promoters exhibit paused Pol II. However, bioinformatics analyses and transient transfection assays indicate that the strength of the *hsp70* promoter is much stronger than that of the *eve* promoter. Consistently, inactive MSE function is also restored by promoters stronger than the *eve* promoter. It is conceivable that the functional complementarity between enhancer and promoter strengths defines the enhancer threshold, thus determining whether a genomic locus acts as an enhancer for a particular promoter.

**KEYWORDS:** *drosophila*, embryo, enhancer threshold, enhancer-promoter complementarity

## Introduction

Although there are some quantitative differences in binding strength, chromatin immunoprecipitation (ChIP) followed by DNA microarray or deep sequencing analyses have shown that most genomic regions are occupied by various TFs except for the minority of dense heterochromatic regions (Li *et al.*, 2008; MacArthur *et al.*, 2009; Kaplan *et al.*, 2011). However, not all genomic loci to which TFs bind show *cis*-regulatory activity (Zinzen *et al.*, 2009). Moreover, even among loci containing clusters of different ChIP-binding peaks in close proximity, only a small fraction can function as transcriptional enhancers (Zinzen *et al.*, 2009). The inability to discriminate clusters of functional ChIP-binding peaks from the non-functional is presumably due to our lack of understanding of the minimal requirements between *trans*- and *cis*-acting elements for enhancer activity.

Traditionally, enhancer activity has been explained by the differential affinity hypothesis (Driever *et al.*, 1989). This hypothesis is based on the concept of the enhancer threshold, which is defined as the minimum concentration of TFs required to elicit an enhancer response at a given time and space. According to this hypothesis,

the biochemical affinity between the enhancer and the TFs determines the transcription initiation from its dedicated promoter (Jiang and Levine, 1993). Therefore, any factors that can influence the interaction between an enhancer and TFs, such as a change in the quality of the TF-binding sites within the enhancer or a change in the concentration of TFs, can redefine enhancer activity. In other words, if the concentration of *trans*-acting elements is fixed in a particular time and space, the enhancer threshold will also remain constant unless the nucleotide sequence of the enhancer changes.

**Abbreviations used in this paper:** abd-A, abdominal A; Abd-B, Abdominal B; AED, after egg deposition; AP, anterior-posterior; bcd, bicoid; CDK9, cyclin-dependent kinase 9; ChIP, Chromatin immunoprecipitation; CPE, core promoter elements; EP, enhancer-promoter; *eve*, even-skipped; *ftz*, fushi tarazu; GAL4, GAL4 DNA binding domain; *gt*, giant; *hb*, hunchback; *hsp70*, heat shock protein 70; KMnO<sub>4</sub>, potassium permanganate; *kr*, krüppel; MSE, minimal stripe element; P-TEFb, positive transcription elongation factor b; PCR, polymerase chain reaction; PPM, position probability matrix; *rho*, rhomboid; TF, transcription factor; TSS, transcription start site; UAS, upstream activating sequence.

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We present evidence that promoter strength delimits the enhancer threshold at a given time and space in the early *Drosophila* embryo. Transgenic embryo assays, biochemical and *in silico* analyses show that the defective eve MSE, which has lost its activity, is functionally complemented by a *hsp70* promoter that is stronger than the eve promoter. *eve* is one of the pair-rule genes expressed in seven transverse stripes along the anterior-posterior (AP) axis of the early developing embryo (Harding *et al.*, 1986; Small *et al.*, 1991; Small *et al.*, 1992). The MSE is the minimal *cis*-acting element controlling *eve* expression in the second stripe of the seven (Small *et al.*, 1992). The *eve* gene is activated by maternal TFs, Bicoid (Bcd) and Hb, in almost the anterior half of the embryo along the AP axis (Small *et al.*, 1991), while its expression is restricted within the second strip by transcription repressors, Krüppel (Kr) and Giant (Gt) binding to the MSE (Arnosti *et al.*, 1996; Small *et al.*, 1991). Inactive MSE function is consistently restored by promoters that are stronger than the eve promoter. These results suggest that the functional complementarity between enhancer and promoter strength defines the minimum affinity between enhancers and TFs for productive transcription. Presumably, the enhancer-promoter (EP) complementarity acts through a particular conformation of the massive protein complex formed at the interface between enhancers and promoters, thus determining whether a genomic locus acts as an enhancer for a particular promoter.

## Results and Discussion

### Activity of the defective MSE relative to the *hsp70* promoter

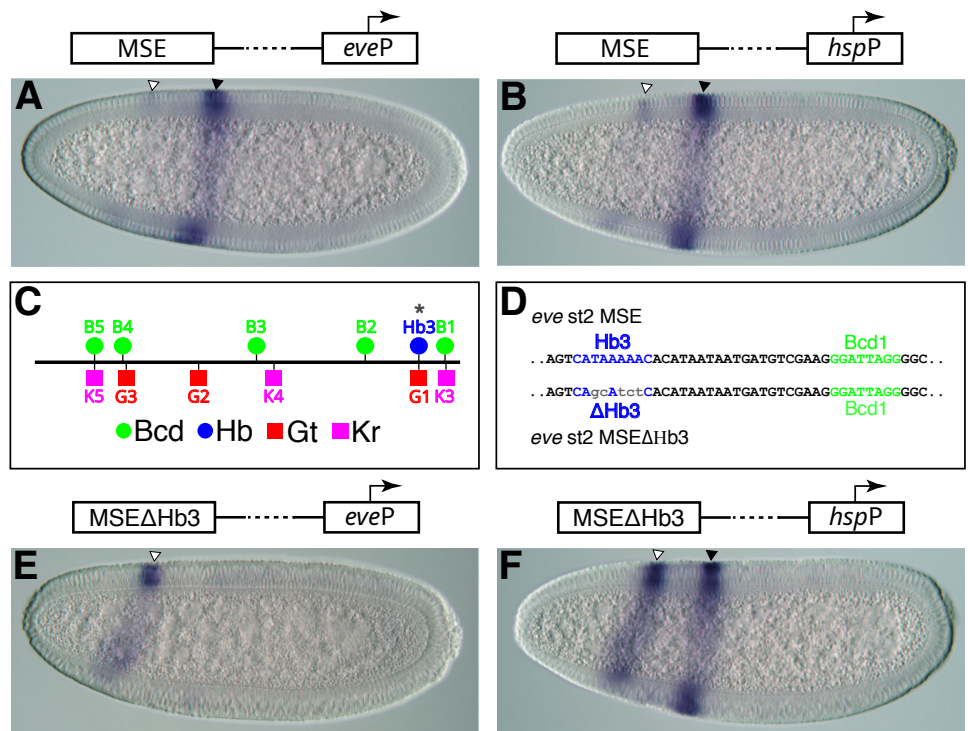
We examined the relative activity of the eve stripe 2 MSE (Small *et al.*, 1992) in transgenic *Drosophila* embryos 2-4 hours after egg deposition (AED). The MSE is a 480 bp region of the eve enhancer that is sufficient to drive the expression of a *lacZ* gene within the

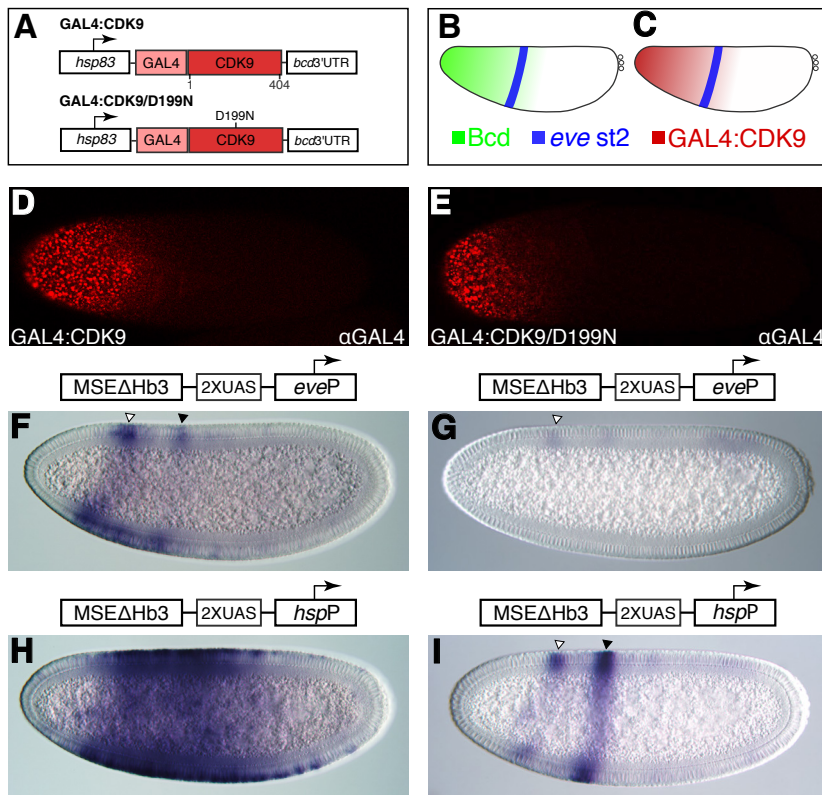
normal limits of the endogenous eve stripe 2. When the MSE was placed upstream of the *eve* (Small *et al.*, 1992) and *hsp70* (Table S1) promoters, it activated the transcription of *lacZ* from both (Fig. 1 A,B). The MSE contains a Hb-binding site (Hb3) (Fig. 1C), which plays a critical role along with a nearby Bcd-binding site (Bcd1) in Bcd-Hb cooperativity (Arnosti *et al.*, 1996). Consistently, mutations introduced into the Hb3 (Fig. 1D) almost completely abolished *lacZ* transcription from the eve promoter (Fig. 1E). The severe reduction in *lacZ* transcription was completely restored by replacing the eve promoter with the *hsp70* promoter (Fig. 1F). These results suggest that the apparent inactivity of the MSE is functionally complemented by the *hsp70* promoter, resulting in productive transcription.

### Pause release from the eve and *hsp70* promoters within the eve stripe 2

What was the difference between the two promoters that produced such disparate outcomes to the defective MSE? The first feasible difference to test was the presence of paused Pol II in its canonical position, because the *hsp70* promoter is a known paused promoter (Kwak *et al.*, 2013), whereas it is unclear whether the eve promoter has a paused Pol II. Previous studies have shown that positive transcription elongation factor b (P-TEFb) is necessary and sufficient for the release of paused Pol II *in vitro* (Bieniasz *et al.*, 1999; Lis *et al.*, 2000), suggesting that the paused Pol II can also be released into productive elongation *in vivo* by cyclin-dependent kinase 9 (CDK9), a component of P-TEFb. P-TEFb is composed of CDK9 and cyclin T (Fujinaga *et al.*, 2023). Since cyclin T is constitutively expressed, ectopic expression of CDK9 is sufficient to generate P-TEFb activity (Lis *et al.*, 2000). Thus, we hypothesized that the pause release by tethering of CDK9 near a promoter in the early embryo could serve as an *in vivo* assessment of a functional paused Pol II.

**Fig. 1. The inactive eve minimal stripe element (MSE) function was completely restored by the *hsp70* promoter.** MSE (A,B) and MSEΔHb3 (E,F) were placed upstream of the eve and *hsp70* promoters in the P-element transformation vector pCaSpeR (Thummel *et al.*, 1988). The *lacZ* fusion genes were expressed in transgenic embryos after P-element-mediated germline transformation (Rubin and Spradling, 1982). 2-4 hour AED transgenic embryos were hybridized with an antisense *lacZ* RNA probe and visualized by immunohistochemical staining. (C) Organization of the enhancer which contains 5 Bcd-binding sites (B1-B5), 1 Hb site (H3), and 3 binding sites for each of the two repressors, Gt (G1-G3) and Kr (K3-K5). The full-length eve stripe 2 enhancer has been reported previously (Small *et al.*, 1991). (D) Several nucleotide substitutions in the single hunchback (Hb) binding site in MSE were generated by site-directed mutagenesis. The enhancer and promoter parts of the transformation vector are depicted at the top of each panel. Solid and open arrowheads indicate *lacZ* expression activated by eve enhancers and unknown sequences of the P-element transformation vector pCaSpeR (Small *et al.*, 1992), respectively. Substituted nucleotides in the Hb3 (ΔHb3) site are shown in gray lowercase.





**Fig. 2. Response of *eve* and *hsp70* promoters to P-TEFb artificially bound in their vicinity.** (A) WT and mutant versions of GAL4:CDK9 coding regions were placed between the *hsp83* promoter and *bcd* 3' UTR. In the mutant GAL4:CDK9 coding region, the 199<sup>th</sup> aspartic acid was replaced by asparagine. The GAL4:CDK9 mRNA gradient (C) distributed along the AP axis recapitulates the endogenous *bcd* mRNA gradient (B) in the early embryo. *In situ* hybridization was performed using antisense RNA probes against GAL4 DNA binding domain (D,E). Male flies carrying a *lacZ* reporter with an upstream activating sequence (UAS) were crossed with females carrying the WT or mutant GAL4:CDK9 fusion (F-I). Two copies of the UAS were inserted between the MSEΔHb3 and the promoter (see the diagram above each panel). The mutant GAL4:CDK9 (GAL4:CDK9/D199N) had no effect on the activity of either promoter (G,I). Solid and open arrowheads are as described in Fig. 1.

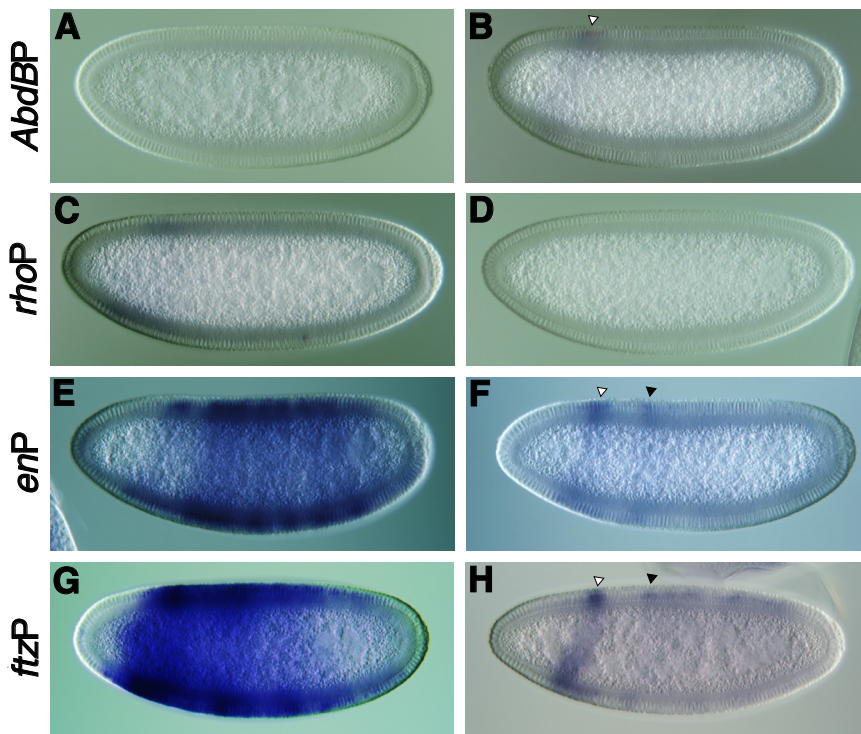
To test if the *eve* promoter has a paused Pol II, an ectopic CDK9 gradient along the AP axis of the early embryo was created. The coding sequence for CDK9 fused with the GAL4 DNA binding domain (GAL4) (Lis *et al.*, 2000) was placed between the *hsp83* promoter and the *bcd* 3' untranslated region (UTR) (Arnosti *et al.*, 1996) (Fig. 2A). This caused the chimeric GAL4:CDK9 mRNA to mimic the *bcd* mRNA gradient (Fig. 2B) and be expressed at a similar concentration to Bcd in the *eve* stripe 2 domain (Fig. 2C). As expected, both wild-type (WT) (Fig. 2D) and mutant (Fig. 2E) versions of GAL4:CDK9 mRNA were most abundant in the head region, progressively decreased along the AP axis of the embryo, and were no longer observed near the middle of the embryo. Male flies from several independent lines carrying *lacZ* fusions with an upstream activating sequence (UAS) were individually crossed with females carrying the WT or mutant versions of GAL4:CDK9 (Fig. 2 F-I). Artificial attachment of GAL4:CDK9 upstream of the *eve* promoter in the presence of MSEΔHb3 resulted in weak *lacZ* transcription in the stripe 2 domain (Fig. 2F). In contrast, GAL4:CDK9 strongly activated *lacZ* transcription from the *hsp70* promoter in almost the anterior three-fifths of the embryo along the AP axis except for some head regions (Fig. 2H). The expression of mutant GAL4:CDK9, in which the 199<sup>th</sup> aspartic acid was changed to asparagine (Lis *et al.*, 2000), did not influence the activity of either promoter (Fig. 2 G,I). These results suggest that CDK9, with the help of a defective enhancer, succeeded in releasing the paused Pol II from both promoters at least within the *eve* stripe 2 domain.

Artificial recruitment of CDK9 is a necessary and sufficient for pause release *in vitro* (Bieniasz *et al.*, 1999; Lis *et al.*, 2000), as mentioned previously. Consistently, tethered GAL4:CDK9 was also functionally necessary and sufficient for the pause release from the *hsp70* promoter in early embryos, even in the presence of

the short-range repressors, Gt and Kr (Fig. 2H). It has been widely accepted that Gt and Kr repress transcription by blocking nearby synergistic cooperativity between activators (Arnosti *et al.*, 1996) and recruiting C-terminal binding protein (CtBP) (Nibu *et al.*, 1998). However, exactly how this short-range repression works is still unknown. This successful efficacy of the tethered CDK9 on pause release completely bypassed Gt and Kr activity. It is conceivable that the short-range repression mediated by Gt and Kr functions chronologically somewhere upstream of the Serine (Ser)-2 phosphorylation within a heptapeptide of Pol II c-terminal domain (CTD). If the short-range repressors function somewhere downstream of Ser-2 phosphorylation, it is evident that the recruitment of CDK9 was not functionally effective in pause release.

As always, one answered question raises another; why did the GAL4:CDK9 fail to bypass Gt and Kr for pause release from the *eve* promoter? Two possibilities should be considered; the presence or absence of paused Pol II in the *eve* promoter. The second possibility should be immediately ruled out because *lacZ* transcription was restored by the tethered GAL4:CDK9 within the *eve* stripe 2, suggesting that the tethered P-TEFb activity caused pause release from the *eve* promoter, at least within this region. The simplest explanation for the first possibility is that there is likely a difference in the response to P-TEFb activity between the paused Pol II in the *hsp70* and *eve* promoters. Pol II pausing can be considered in two broad categories based on its location and distribution: proximal and focused (Prox), and distal and dispersed (Dist) (Kwak *et al.*, 2013). More core promoter elements (CPEs) are concentrated in more precise positions on Prox promoters than on Dist promoters. The mechanistic differences may have regulatory consequences, as the presence and quality of CPE strongly correlate with promoter functionality (Juven-Gershon *et al.*, 2006; Qi





**Fig. 3. Responses of paused promoters to tethered GAL4:CDK9.** The promoter of the pCaSpeR-MSEΔHb3-UAS-eveP construct was replaced with one of the promoters of *Abd-B* (A,B), *rho* (C,D), *en* (E,F) or *ftz* (G,H) (Table S1). All of them, except *Abd-B*, are known as a paused promoters (Zeitlinger *et al.*, 2007; Hendrix *et al.*, 2008). All experiments are identical to Fig. 2 F-I, except for the replacement of the promoter. Consistent with the results in Fig. 2 G,I, the mutant GAL4:CDK9/D199N did not influence the activity of any of the promoters. Solid and open arrowheads indicate *lacZ* expression activated by the MSEΔHb3 enhancer and unknown sequences of the P-element transformation vector (Small *et al.*, 1992), respectively.

*et al.*, 2022). There are four CPEs in the *hsp70* promoter, while the *eve* promoter contains only the TATA box (Fig. 5 and Table S2). More importantly, it is quite different how paused genes respond to genetic perturbation (Boettiger and Levine, 2009). The paused gene, *rhomboid* (*rho*), shows synchronous activation in the neurogenic ectoderm of WT embryos, whereas stochastic induction in Dorsal heterozygous (*dl/+*) embryos. In contrast, a synchronous induction pattern is consistently observed at the locus of another paused gene, *short-gastrulation* (*sog*), in both WT and mutant embryos. Taken together, it is plausible that the failure to bypass Gt and Kr in the *eve* promoter outside of *eve* stripe 2 is at least in part due to the differences in mechanistic features and activation pattern of paused Pol II.

#### Pause release from paused promoters within the *eve* stripe 2

The lack of pause release from the *eve* promoter by GAL4:CDK9 outside the *eve* stripe 2 domain still raises the question of whether the *eve* promoter has paused Pol II or not. To test whether CDK9 recruitment can also facilitate the pause release from other paused promoters, the effect of GAL4:CDK9 expression on known non-paused and paused promoters was examined (Fig. 3). All experiments are identical to Fig. 2 (F-I) except for the replacement of the promoter. As expected, the non-paused *Abdominal B* (*Abd-B*) promoter did not respond to the upstream tethered GAL4:CDK9

(Fig. 3A). The responses of the paused *engrailed* (*en*) and *fushi tarazu* (*ftz*) promoters to GAL4:CDK9 appeared to be similar to that of the *hsp70* promoter (Fig. 3 E,G), except for more posterior expansion of *lacZ* transcription from the *en* promoter (Fig. 3E). The *lacZ* transcription from the *en* and *ftz* promoters was activated in the presence the MSEΔHb3 without the CDK9 activity (Fig. 3 F,H), although the restoration appears to be weaker than that of the *hsp70* promoter (Fig. 1F). However, the *Abd-B* and *rho* promoters did not respond to the MSEΔHb3 in the absence of the CDK9 activity (Fig. 3 B,D). Unexpectedly, the GAL4:CDK9 also failed to release the paused Pol II from the *rho* promoter (Fig. 3C), one of the best known paused promoters (Zeitlinger *et al.*, 2007). These results suggest that tethered CDK9 has the ability to cause paused release from paused promoters *in vivo*, but also that an additional way is needed to verify whether the *eve* promoter has paused Pol II.

In reporter gene assays using transgenic embryos, the *rho* promoter was not responsive to either MSEΔHb3 or tethered CDK9. How can we interpret the insensitivity of the *rho* promoter to any activation stimuli? The three promoters of *hsp70*, *en*, and *ftz* that have responded to both MSEΔHb3 and tethered CDK9 share two common features; first, they each have at least one GAGA site within 50 bp upstream of the TSS (Fig. 5) and second, initiator (Inr) and a downstream promoter element (DPE) or pause button (PB). In contrast, the *rho* promoter shares only Inr with these three promoters (Fig. 5). GAGA-associated factor (GAF) is a pioneer factor that renders a nearby promoter nucleosome-free, making it more accessible to

other *trans*-acting factors (Wilkins and Lis, 1997). Inr and DPE/PB play a critical role in the high attractiveness of transcription factor IID (TFIID) (Fant *et al.*, 2020), which is a hallmark of a strong promoter (Juven-Gershon *et al.*, 2006). It is possible that the open-structured *hsp70*, *en*, and *ftz* promoters strongly attracted the preinitiation complex (PIC) with exposed Inr and DPE/PB, while the relatively closed-structured *rho* promoter was unable to strongly recruit PIC despite having its sufficiently low *E*-value (Table S2). Therefore, this accessibility issue may have made the *rho* promoter more susceptible to subtle fluctuations in activation stimulus. The observation that the genetic perturbation that halves the DI concentration (Boettiger and Levine, 2009) in early fly embryos almost completely abolishes synchronous *rho* expression supports this possibility. Overall, it may be the case that more comprehensive activation mediated by a typical enhancer such as the wild-type MSE is required for productive transcription from the *rho* promoter, since the promoter opening appears to depend on enhancer activity (Beagrie and Pombo, 2016).

#### Transcription bubble assays identified hypersensitive residues in both the *eve* and *hsp70* promoters

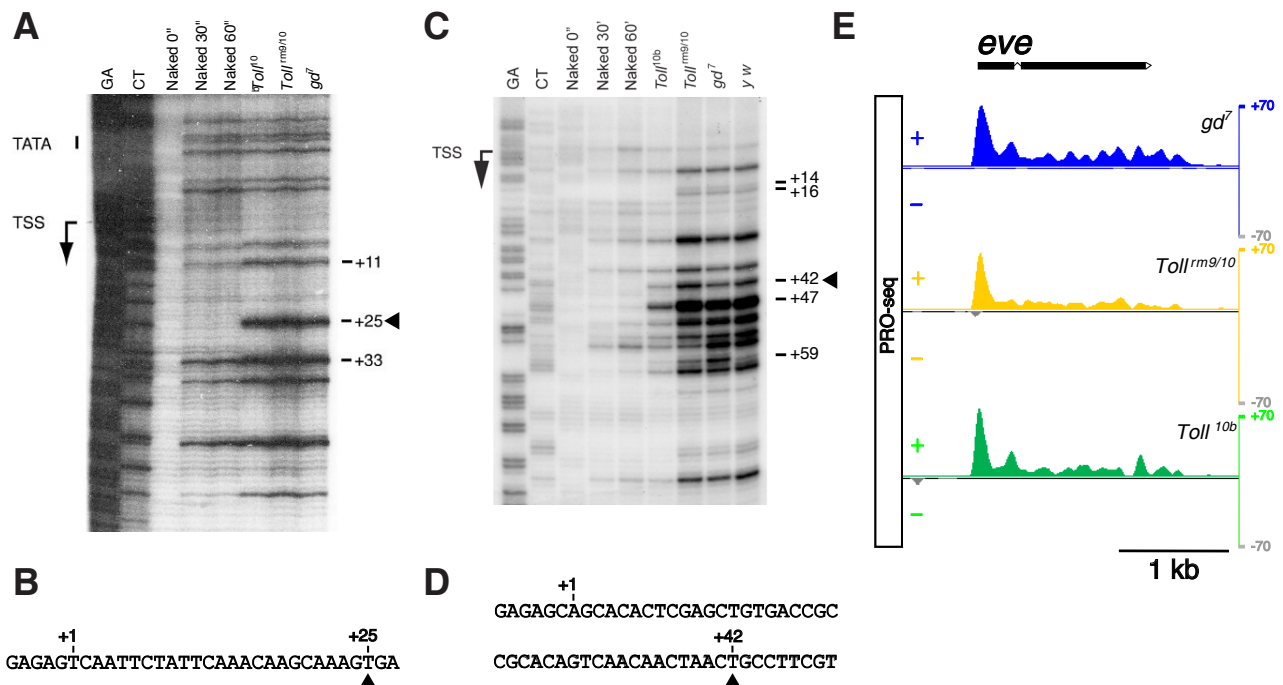
To further test whether the *eve* promoter has a paused Pol II, the potassium permanganate (KMnO<sub>4</sub>) transcription bubble assay (Wang *et al.*, 2005; Wang *et al.*, 2007) was performed with WT

(*yw*<sup>67c23</sup>) and three mutant, *Toll*<sup>10b</sup>, *Toll*<sup>rm9/rm10</sup>, and *gd7* (Hendrix *et al.*, 2008), 2-4 hour AED embryos (Fig. 4, S1 and S2). These three mutant embryos are composed of cells from the mesoderm, neurogenic ectoderm and dorsal ectoderm, respectively. As a result, the patterning genes of the early three germ layers are expressed throughout the embryo, thereby providing a suitable genetic background to demonstrate that the transcription bubble generated by stably paused Pol II is independent of genetic diversity and gene expression status (Fig. S1). For example, *rho* is expressed only in the neurogenic and dorsal ectoderm (Fig. S1C), but its pausing pattern is identical in all mutant embryos regardless of its expression (Fig. S1 A,B). This result suggests that if *eve* were a paused gene, the similar bubble pattern would be also found in WT (*yw*) and the mutant embryos. The KMnO<sub>4</sub> bubble assay, which localizes the transcription bubble, shows that the *eve* promoter, like the *hsp70* promoter (Fig. 4 A,B), has a very distinct bubble band 42 base pairs (bp) downstream of the transcription start site (TSS) (Fig. 4 C,D). Although precision nuclear run-on sequencing (PRO-seq) analysis (Hunt *et al.*, 2024) show that nascent RNAs are detected throughout the *eve* transcription unit in all mutant embryos (Fig. 4E), the higher RNA peak identified at the *eve* 5' end strongly supports the idea that the *eve* promoter has a paused Pol II. In general, the intensity of Pol II near the paused promoter is more than four times greater than the median Pol II occupancy observed throughout the rest of the transcription unit, even when the gene is being expressed (Zeitlinger *et al.*, 2007). The peaks of nascent RNA near the *eve*

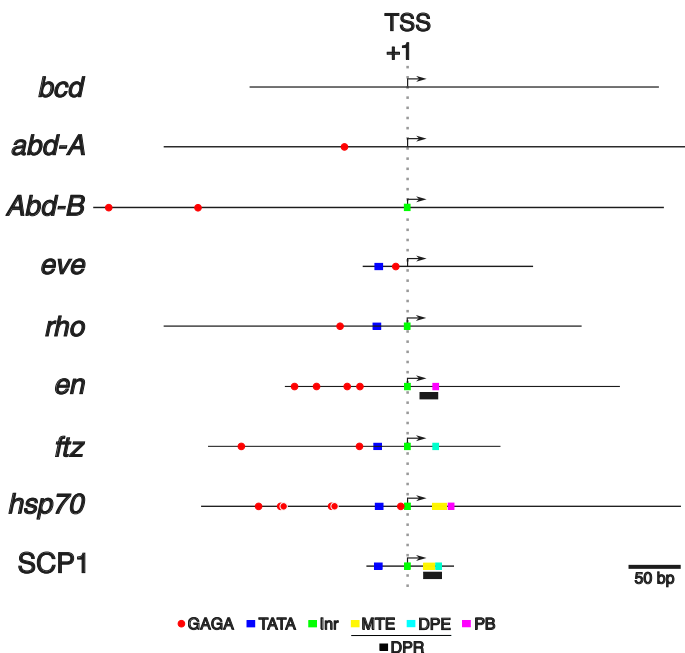
promoter appear to be at least five times higher than the median intensity observed across the *eve* transcription unit regardless of its expression (Fig. 4E). Taken together, since it is reasonable to assume that both the *eve* and *hsp70* promoters have paused Pol II, it is difficult to postulate that the different responses of the two promoters to MSEΔHb3 are solely due to paused Pol II.

#### The *hsp70* promoter is stronger than the *eve* promoter

If not paused Pol II, what else could cause the difference in complementarity of the *eve* and *hsp70* promoters to defective MSEΔHb3? The composition of the CPEs in the *hsp70* promoter is significantly different from that of the *eve* promoter (Fig. 5 and Table S2). The *hsp70* promoter contains the four elements of the TATA box, Inr, motif ten element (MTE), and PB (Kwak *et al.*, 2013), while only the TATA box was identified in the *eve* promoter (Read *et al.*, 1990). In particular, the CPE composition of the *hsp70* promoter is reminiscent of Super Core Promoter 1 (SCP1) (Juven-Gershon *et al.*, 2006), an artificially synthesized promoter containing the TATA box, Inr, MTE and DPE (Fig. 5 and Table S2). The SCP1 is stronger than a cytomegalovirus IE1 core promoter, one of the strongest promoters in mammalian cells (Qin *et al.*, 2010). Furthermore, the *hsp70* promoter contains at least six GAGA sites within a region up to 150 bp upstream of the TSS, but only one GAGA site was identified within a 1 kb region surrounding the *eve* TSS (Fig. 5) (Adato *et al.*, 2024). These findings suggest that the two promoters have significant differences in RNA production and chromosomal



**Fig. 4. A KMnO<sub>4</sub> transcription bubble assay.** Transcription bubble assays were performed for *hsp70A* (A,B) and *eve* (C,D,E) in WT (*yw*<sup>67c23</sup>) and three mutant (*Toll*<sup>10b</sup>, *Toll*<sup>rm9/rm10</sup>, and *gd7*) 2-4 h AED embryos (Fig. S2). The transcription start sites (TSSs) are labeled on the left and the location of prominent bands relative to the TSS (+1) is shown on the right of the autoradiograms. Genomic sequences of G+A and C+T are shown as size markers. As a control, purified genomic DNA from *yw* embryos was either not treated (Naked 0") or treated (Naked 30" and 60") with 20 mM KMnO<sub>4</sub> for 30 and 60 seconds. Solid arrowheads indicate significant pyrimidine residues (T) modified only in chromatin isolated from *yw* and three mutant embryos by KMnO<sub>4</sub> treatment, implying the existence of transcription bubbles *in vivo* (A,C). Promoter sequences downstream of TSS were presented (B,D). Results of precision nuclear run-on sequencing (PRO-seq) analysis (Hunt *et al.*, 2024) on the *eve* locus (E) are shown. + and - represent the mean PRO-seq intensity for sense and antisense strands, respectively, in three mutant embryos. Gene prediction model is displayed above the graphical presentation.



structure, which prompted us to quantify their promoter strengths *in silico* and *in vitro*.

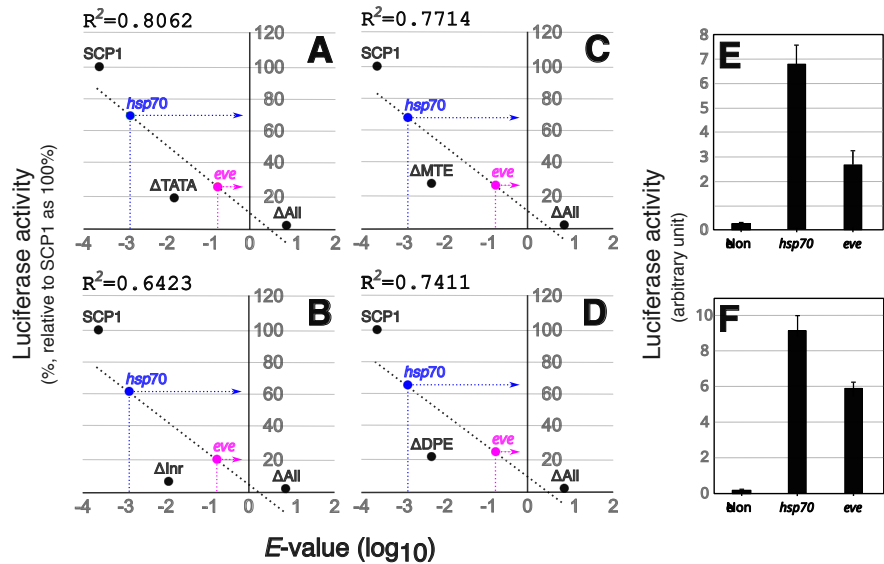
The CPE composition of SCP1 is well documented (Fig. 5 and Table S2) and its promoter strength has been previously quantified *in vitro* (Juven-Gershon *et al.*, 2006). Regression analysis between CPE composition and the rate of mRNA synthesis (Juven-Gershon *et al.*, 2006) of SCP1 revealed a robust correlation (Pearson correlation coefficients of at least 0.8) (Fig. 6 A-D and Table S3). This suggests that the best-fit line (black dotted lines in Fig. 6) generated by the regression analysis can serve as a universal standard for estimating core promoter strength using CPE sequences alone.

**Fig. 5. The composition of the GAGA sites and the core promoter elements (CPEs) in the promoters that were examined in this study.** Various promoters examined in this study (Table S1) were drawn in scale and arranged according to the transcription start site (TSS) (+1). The third nucleotide of Inr and the 5' end of the mRNA of the Flybase (<https://flybase.org>) gene model were designated as TSSs in the presence and absence of Inr, respectively. CPEs (Table S2) were identified by feeding the MEME Suite 5.5.5 (Bailey *et al.*, 2015) with the position probability matrices (PPMs) (Table S4) of CPEs.

The relative strength of the *eve* and *hsp70* promoters was quantified *in silico* by substituting the *E*-values (Table S2) of the CPEs found in the two promoters into the equations (Table S3) of the best-fit lines (Fig. 6 A-D) generated by the regression analyses. The relative strength of the *hsp70* promoter was at least 2.5 times greater than that of the *eve* promoter (Fig. 6 A-D and Table S3). To confirm the *in silico* difference between the two promoters, transient transfection assays were performed in HEK293 cells with constructs containing either the *eve* or *hsp70* promoters (Fig. 6 E,F). The luciferase activity generated by the *hsp70* promoter without enhancer stimulation was almost three times higher than that of the *eve* promoter (Fig. 6E). In the presence of the SV40 enhancer, the *hsp70* promoter produced almost twice as much luciferase mRNA as the *eve* promoter (Fig. 6F). Overall, these results suggest that the disparity in the complementarity of the *eve* and *hsp70* promoters to defective MSEΔHb3 is at least in part due to the divergence in their promoter strengths.

#### Inactive MSEΔHb3 activated transcription from strong promoters

Based on the previous findings, we hypothesized that, for a given concentration of *trans*-acting elements, transcription initiation is determined by the combined strength of an enhancer and a promoter. To test this hypothesis, we investigated whether promoters stronger or weaker than the *eve* promoter are able to carry out productive transcription in conjunction with MSEΔHb3

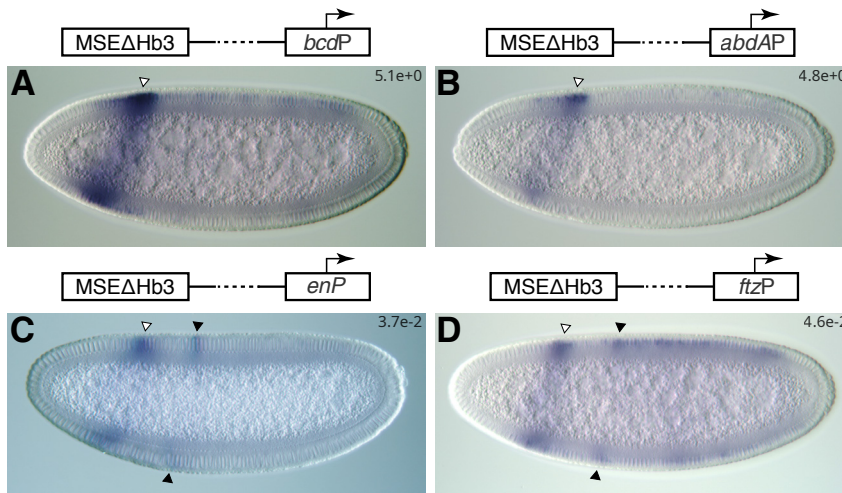


**Fig. 6. The *hsp70* promoter is stronger than the *eve* promoter.**

The best-fit equations (dotted lines) (Table S3) produced by the least squares regression analyses on the SCP1 promoters were used to statistically estimate the strength of the *eve* and *hsp70* promoters. Regression analysis indicates a robust correlation in each of the four combinations of SCP1 promoters [SCP1-SCP1ΔTATA-SCP1ΔAll (A), SCP1-SCP1ΔInr-SCP1ΔAll (B), SCP1-SCP1ΔMTE-SCP1ΔAll (C), and SCP1-SCP1ΔDPE-SCP1ΔAll (D)]. The four response variables (promoter strength) of the *hsp70* promoter are at least 2.5-fold higher than the *eve* promoter. ΔTATA, ΔInr, ΔMTE, ΔDPE, and ΔAll stand for SCP1ΔTATA, SCP1ΔInr, SCP1ΔMTE, SCP1ΔDPE, and SCP1ΔAll, respectively, and refer to mutant versions of the TATA box, Inr, MTE, DPE, or all CPEs (Table S2). *eve* and *hsp70* stand for their core promoters (Fig. 5 and Table S1) used in this study. *R*<sup>2</sup> represents the coefficient of determination, also called R-squared, which is obtained from the least-squares regression analysis of the SCP1 promoters (Table S3). The amount of mRNA synthesized from the WT SCP1 by transient transcription (Juven-Gershon *et al.*, 2006) is considered 100

%. The strength of RNA synthesis of all other SCP1 variants is expressed as a ratio to SCP1. To confirm the results of the regression analyses, transient transfection assays were performed in HEK293 cells with pGL3-basic (E) or pGL3-enhancer (F) constructs containing the *eve* and *hsp70* core promoters (Fig. 5). A β-galactosidase expression plasmid was also cotransfected as a reference. The luciferase activity of each construct was normalized to the corresponding β-galactosidase activity.





**Fig. 7. The relative activity of an enhancer to the strength of its paired promoter.** Based on the functional complementarity between the MSEΔHb3 and the *hsp70* promoter in eve stripe 2 domain, we hypothesized that for a given concentration of *trans*-acting elements, transcription initiation is determined by the combined activities of an enhancer and a promoter. In order to test this hypothesis, it was examined how two promoters weaker (A,B) or stronger (C,D) than the eve promoter respond to the MSEΔHb3. The *E*-values of the two weaker or stronger promoters are higher or lower, respectively, than the *E*-value of the eve promoter (Table S2). The same tests as in Fig. 1 were repeated except that the *hsp70* promoter region was replaced by four promoters (Fig. 5 and Table S1) with different *E*-values. Solid and open arrowheads are as described in Fig. 1. *E*-value for each promoter is shown in the upper right of each panel.

(Fig. 7), in addition to the *rho* and *Abd-B* promoters (Fig. 3 B,D). Identical experiments to those in Figure 1E and F were repeated, except that four promoters (Fig. 5 and Table S1) with different *E*-values (Table S2) were used. The *E*-values of two promoters were higher than (Fig. 7 A,B) that of the eve promoter, while those of the other two were lower than the eve promoter (Fig. 7 C,D). Hereafter, a promoter with an *E*-value higher or lower than that of the eve promoter is referred to as a weak or strong promoter, respectively. As expected, the MSEΔHb3 failed to activate transcription from the weak promoters, *bcd* and *abd-A* (Fig. 7 A,B), which is consistent with *Abd-B* (Fig. 3B). The *Abd-B* promoter contained Inr, which is the only CPE identified among the *bcd*, *abd-A*, and *Abd-B* promoters (Fig. 5 and Table S2), suggesting their weak transcriptional strength. In contrast, MSEΔHb3 distinctly activated *lacZ* transcription from the *en* and *ftz* promoters (Fig. 7 C,D), although the intensities of the transcription were weaker than that of the *hsp70* promoter (compare with Fig. 1F). The *E*-values of both promoters fall between those of the *hsp70* and eve promoters but are much closer to that of the *hsp70* promoter (Table S2). These results suggest that MSEΔHb3-mediated transcription activation is due, at least in part, to a combination with a stronger promoter relative to the eve promoter except for the *rho* promoter.

#### Differential affinity hypothesis versus EP complementarity hypothesis

Over the past four decades of study, it has been widely accepted that neither an enhancer nor a promoter alone is capable of completing productive transcription in metazoan cells (Fig. 8C), although there are very few exceptions (Tippens *et al.*, 2018). In the traditional view, enhancer activity is determined by the concentration of TFs at a given time and space and the quality of the TF binding sites on the enhancer to which they bind (Driever *et al.*, 1989; Jiang and Levine, 1993) (Fig. 8A). In reality, increased binding affinities of the Dorsal and ETS binding sites in the *twist* and *sonic hedgehog* enhancers in fly and mouse embryos, respectively, resulted in the expression of the two genes in otherwise untranscribed regions (Jiang and Levine, 1993; Lim *et al.*, 2024). Here we present evidence that this enhancer threshold response is relative to the strength of the promoter. The lost activity of the defective eve stripe 2 enhancer, in which a single Hb-binding site is disrupted, was fully

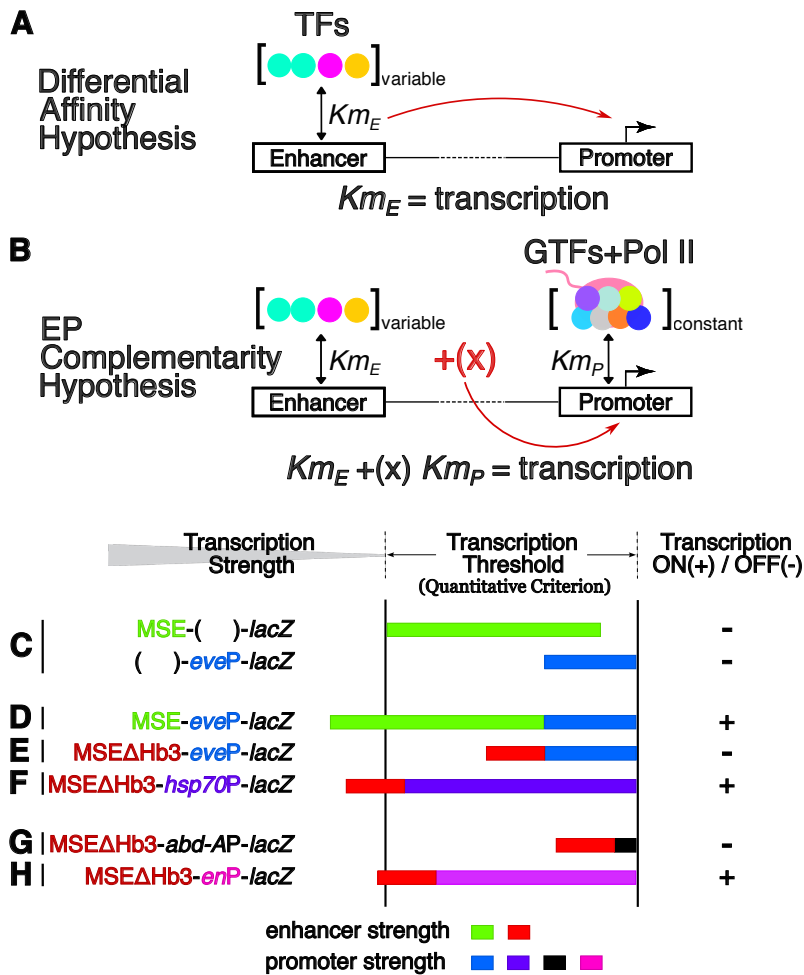
complemented by promoters stronger than the eve promoter. This EP complementarity in function appears to depend more on the strength of the promoter rather than on the presence of paused Pol II. These results suggest that there is a quantitative criterion of the combined activity of enhancers and promoters for transcriptional activation (Fig. 8B). This quantitative criterion reflects a minimum level that the combined strength of the enhancer and promoter must reach or exceed for productive transcription. For example, a weak enhancer may require a strong promoter (Fig. 8 E-H), whereas a strong enhancer may be able to achieve productive transcription even with a weak promoter (Fig. 8D).

If EP complementarity in function can define transcriptional firing, the decision of whether a genomic locus functions as an enhancer for a particular promoter may depend on whether their combined strength meets or exceeds the minimum requirement of the quantitative criterion (compare Fig. 8 E,G with F,H, respectively), given that EP communication is promiscuous (Bergman *et al.*, 2022; Martinez-Ara *et al.*, 2022) and EP contact probability is stochastic (Zuin *et al.*, 2022).

#### Materials and Methods

##### DNA constructs

MSE (Small *et al.*, 1992) and MSEΔHb3 (Arnosti *et al.*, 1996) stand for the wild-type (WT) and the mutant (deletion of the Hb3 site) version of the *even skipped* (*eve*) stripe 2 minimal enhancer, respectively. pCaSpeR-MSE-2xUAS-(-42)eveP-*lacZ* and pCaSpeR-MSEΔHb3-2xUAS-(-42)eveP-*lacZ* constructs (Arnosti *et al.*, 1996) were obtained from Michael Levine laboratory at Princeton University. The MSE-2xUAS-(-42)eveP and MSEΔHb3-2xUAS-(-42)eveP fragments were prepared by digesting pCaSpeR-MSE-2xUAS-(-42)eveP-*lacZ* and pCaSpeR-MSEΔHb3-2xUAS-(-42)eveP-*lacZ* with *Eco*RI and *Bam*HI respectively and cloned into the Agilent™ pBlueScript II vector that had been cut with *Eco*RI and *Bam*HI [pBSKII-MSE-2xUAS-(-42)eveP and pBSKII-MSEΔHb3-2xUAS-(-42)eveP]. All promoter regions except the eve promoter were prepared by polymerase chain reaction (PCR) with fly genomic DNA (Fig. 5 and Table S1). Genomic DNA was isolated from *yw*<sup>67c23</sup> embryos aged 2-4 hours AED, using previously described methods (Hendrix *et al.*, 2008). PCR-amplified promoters were cloned into the Promega™



**Fig. 8. Enhancer-promoter (EP) complementarity hypothesis.** According to the differential affinity hypothesis (Driever *et al.*, 1989), transcription from a promoter is determined by the concentration of transcription factors (TFs) at a given time and space and the quality of the TF binding sites on the enhancer to which these TFs bind (**A**). The current study provides evidence that the enhancer threshold response can be relative to promoter strength (**B**). For example, the inactive *eve* minimal stripe element (MSE), where cooperativity between transcription activators is disrupted, requires a higher level of TFs within *eve* stripe 2 for productive transcription. The defective MSE is completely complemented by the *hsp70* promoter stronger than the *eve* promoter. These results suggest that the combined strength of an enhancer and a promoter determines whether transcription is initiated or not. Since the biochemical affinity ( $Km_E$  and  $Km_P$ ) between *cis*- and *trans*-acting elements highly correlates with their functional strength (Jiang and Levine, 1993; Juven-Gershon *et al.*, 2006),  $Km$ , a measure of biochemical affinity, can represent the strength of the enhancer and promoter once time and space are specified. Consistently, productive transcription does not occur with either an enhancer or a promoter alone (**C**). A strong enhancer may be able to achieve productive transcription even with a weak promoter (**D**), whereas it is possible that a relatively high enhancer threshold (weak enhancer activity) (**E-H**) is functionally complemented by a promoter with relatively high transcriptional strength (**F,H**), resulting in a lower threshold. TF, GTF, Pol II and  $Km$  stand for transcription factor, general transcription factor, RNA polymerase II, and the Michaelis-Menten constant (Michaelis *et al.*, 2011), respectively.  $Km_E$  or  $Km_P$  represent  $Km$  between TFs or GTFs+Pol II and an enhancer or a promoter, respectively.  $+(x)$  represents the sum or product of  $Km_E$  and  $Km_P$ , respectively.

pGEM®-T Easy vector and nucleotide sequences of the cloned fragments were verified by DNA sequencing reaction. Cloned promoters were digested by *Pst*I and *Bam*HI and cloned again into pBSKII-MSE-2xUAS-(-42)eveP and pBSKII-MSEΔHb3-2xUAS-(-42)eveP previously digested with *Pst*I and *Bam*HI. MSE-2xUAS-promoter and MSEΔHb3-2xUAS-promoter fragments were prepared by digesting pBSKII-MSE-2xUAS-promoter and pBSKII-MSEΔHb3-2xUAS-promoter constructs with *Eco*RI and *Bam*HI, and cloned into pCaSpeR-(-42)eveP-lacZ (Small *et al.*, 1992) previously cut with *Eco*RI and *Bam*HI. The GAL4 DNA-binding domain (GAL4):CDK9 and GAL4:CDK9/D199N chimeric coding regions were amplified by PCR (Table S1) with the GAL4:CDK9 and GAL4:CDK9/D199N expression constructs (Lis *et al.*, 2000) as a template, respectively, obtained from John Lis laboratory at Cornell University, and cloned into the pGEM-T Easy vector. A *Not*I fragment containing GAL4:CDK9 or GAL4:CDK9/D199N coding region from pGEM-T Easy-GAL4:CDK9 or pGEM-T Easy-GAL4:CDK9/D199N, respectively, was inserted into a *Not*I site of the *phsp83-bcd*3'UTR transformation vector (Arnold *et al.*, 1996; Huang *et al.*, 1997). Promoter fragments were amplified by PCR (Table S1) and cloned into the pGEM-T easy vector. The cloned promoters were digested with *Not*I, filled with the Klenow fragment and cloned again into pGL3-basic and pGL3-enhancer, which were previously digested with *Sma*I. The orientation of all cloned fragments was confirmed by DNA sequencing reaction.

## Genetic Crosses

To obtain embryos containing the MSEΔHb3 constructs and the maternal expression of GAL4:CDK9 along the AP axis, male flies from several independent lines carrying P-element transposons of the MSEΔHb3-eveP or MSEΔHb3-hsp70P construct were individually crossed with females carrying the WT or mutant versions (D199N) of GAL4:CDK9 fusion (Lis *et al.*, 2000). Three mutant embryos [*Toll*<sup>10b/+</sup>, *Toll*<sup>fm9/Toll</sup><sup>fm10</sup>, *gastrulation defective* (*gd*)<sup>7/gd</sup>] were obtained as previously described (Hendrix *et al.*, 2008). Briefly, *Toll*<sup>10b/+</sup> female flies were generated directly from the balanced stock (*Toll*<sup>10b/+</sup>/TM3 Sb Ser and *Toll*<sup>10b/+</sup>/OR60). *Toll*<sup>fm9/Toll</sup><sup>fm10</sup> female flies were obtained by crossing *Toll*<sup>fm10</sup>/TM3 Sb females with *Toll*<sup>fm9</sup>/TM3 Ser males. *gd*<sup>7</sup>/FM3 male flies were crossed to *gd*<sup>7</sup>/FM3 females and embryos were collected from females homozygous for the *gd*<sup>7</sup> allele (Konrad *et al.*, 1988; Stathopoulos and Levine, 2002).

## KMnO<sub>4</sub> transcription bubble assay

A KMnO<sub>4</sub> transcription bubble assay was performed as previously described (Hendrix *et al.*, 2008) with a slight modification in the preparation of the embryos. Embryos were collected 2-4 hours AED, dechorinated, partially homogenized with a plastic pestle, and treated with 20 mM KMnO<sub>4</sub> for 60 sec on ice. Transcription start site (TSS) of investigated genes were determined by annotated TSSs in Flybase and previous tiling array analysis (Biemar *et al.*,



2006). The linker primers and gene-specific primers were used for ligation-mediated PCR (LM-PCR). All primers used for LM-PCR are listed in Table S1.

### Whole-mount and fluorescent *in situ* hybridization

Whole-mount *in situ* hybridization was performed as previously described (Hong *et al.*, 2013). Briefly, 2-4 hours AED embryos were collected, dechorinated, fixed, and hybridized with a digoxigenin (DIG)-UTP labeled antisense *lacZ* RNA probe. Staining the hybridized antisense *lacZ* RNA probe with nitro-blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) was carried out for exactly 20 min to normalize expression levels. For fluorescent *in situ* hybridization, transgenic embryos containing the GAL4:CDK9 expression construct were hybridized with an antisense RNA probe against GAL4 DNA binding domain labeled with digoxigenin and stained with a secondary antibody conjugated with Alex Fluor 555 dye.

### E-value of the CPE matches in promoter sequences

Position Probability Matrices (PPMs) of GAF-binding motif (GAGA), TATA box (TATA), Inr, Motif Ten Element (MTE), DPE, PB, and Downstream Promoter Region (DPR) were generated by modifying the reported position frequency matrices (PFMs) of GAGA (Adato *et al.*, 2024), TATA (Gershenzon *et al.*, 2006), Inr (Gershenzon *et al.*, 2006), MTE (Sloutskin *et al.*, 2015), DPE (Adato *et al.*, 2024), PB (Hendrix *et al.*, 2008), and DPR (Vo Ngoc *et al.*, 2023) using Kadonaga's *Drosophila* core promoter database (Kutach *et al.*, 2000). The statistical significance of the observed matches found in a promoter DNA sequence to a set of CPE motifs was expressed as the product of the *p*-values of the motif matches, called combined *p*-value (Bailey and Gribskov, 1998). The statistical expression was calculated by MEME Suite 5.5.5 (Bailey *et al.*, 2015) fed with PPMs of CPEs (Table S4) and the DNA sequences of core promoters (Table S1). *E*-value of the sequence match to a group of CPE motifs is defined as the expected number of sequences in a random sequence database of the same size that would have a combined *p*-value equal to or greater than the observed *p*-value by chance (Bailey *et al.*, 2015). It is equal to the product of the combined *p*-value of the sequence and the total number of sequences in the sequence database. Thus, the *E*-value represents the combined statistical significance of multiple matches within the DNA sequence members present in the database.

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### Author contributions

J.W.H. conceived and supervised this study. M.H. performed statistics and bioinformatics analyses. J.W.H. performed all experiments except statistics and bioinformatics analyses. M.H. and J.W.H. contributed to manuscript preparation.

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