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1 **Title:** Constraints and limitations on the transcriptional response downstream of the Bicoid
2 morphogen gradient

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8 **Abstract**

9 The regulation of the *hunchback* promoter expression by the maternal Bicoid gradient has been studied as a model
10 system in development for many years. Yet, at the level of quantitative agreement between data and theoretical
11 models, even the first step of this regulation, transcription, continues to be challenging. This situation is slowly
12 progressing, thanks to quantitative live-imaging techniques coupled to advanced statistical data analysis and
13 modelling. Here we outline the current state of our knowledge of this apparently “simple” step, highlighting the
14 newly appreciated role of bursty transcription dynamics and its regulation.

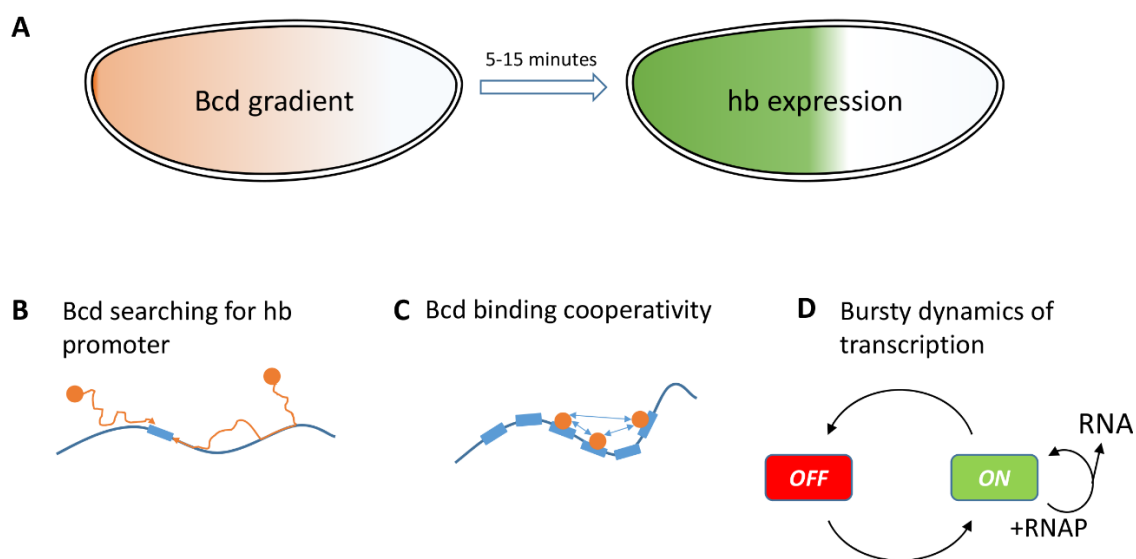
15 **1. Introduction**

16
17 The development of properly proportioned individuals relies on cells precisely reading out positional information
18 and committing to a specific cell fate. In a developing organisms, non-uniformly distributed morphogens
19 gradients provide such positional information to each cell by turning on the expression of specific target genes in
20 a concentration-dependent manner (Karaiskos et al., 2017; X. Liu et al., 2009; Petkova, Bialek, Wieschaus, &
21 Gregor, 2019). Although these gradients are known to be essential since decades in many developmental systems,
22 in most cases we do not know how they are established or how they activate target gene expression in a
23 dose-dependent manner. Here, we discuss recent developments in describing how a noisy transcriptional process
24 can be tuned very rapidly into a reproducible functional developmental pattern by focusing on one well-studied
25 morphogen-target gene pair – the Bicoid (Bcd) morphogen controlling the *hunchback* (*hb*) gap gene in early fly
26 development.

27 The *hb* gap gene is involved in antero-posterior (AP) patterning of young fruit fly embryos. *hb* expression is first
28 detected at the onset of zygotic transcription (nc 8), around one hour after fertilization. No more than 30 min later
29 (nc 11), *hb* transcription occurs in an anterior domain with most nuclei highly expressing the *hb* gene. The border
30 separating this expressing anterior domain from the posterior domain with mostly *hb*-silent nuclei is extremely
31 sharp (Little, Tikhonov, & Gregor, 2013; Porcher et al., 2010) (Figure 1A). This type of expression pattern defines
32 a step-like pattern. Following the *hb* transcription step-like pattern, the Hb protein expression quantified at nc14 is
33 also step-like (Houchmandzadeh, Wieschaus, & Leibler, 2002) and exhibits very low variability in concentration
34 between nuclei at the same position along the AP axis (Gregor, Tank, Wieschaus, & Bialek, 2007). This precise
35 Hb pattern, combined with other gap gene patterns, was proposed to contain enough positional information for
36 nuclei to predict their position in the embryo with ~ 99% accuracy (Dubuis, Tkacik, Wieschaus, Gregor, & Bialek,
37 2013; Petkova et al., 2019).

38 In young embryos, the major regulator of *hb* transcription is the homeodomain-containing Bcd (Driever &
39 Nusslein-Volhard, 1988). Translated from maternal mRNAs anchored at the anterior pole, Bcd proteins form an
40 AP exponential gradient and activate *hb* transcription in the anterior half of the embryo. Increasing or decreasing
41 the amount of Bicoid in the embryo induces a posterior or anterior shift, respectively, of the *hb* step-like pattern,
42 arguing that the expression of *hb* is dependent on Bcd concentration (Driever & Nusslein-Volhard, 1988). The
43 discovery of Bcd’s role in *hb* transcription was exciting as it provided the first example illustrating the elegant idea
44 of the French Flag model for morphogenesis. However, 30 years after its discovery, the mechanisms responsible
45 for the sharpness of the *hb* step-like pattern and its reproducibility are not yet fully understood. The first
46 quantitative studies of the Bcd gradient and Hb protein pattern reproducibility (Gregor, Wieschaus, McGregor,
47 Bialek, & Tank, 2007; Houchmandzadeh et al., 2002) raised doubts about the pattern emerging solely from
48 diffusive biochemical interactions between transcription factors and the gene promoter region. Gregor et al.
49 (2007) used the Berg-Purcell scheme (Berg & Purcell, 1977), in which the concentration of a diffusing ligand is
50 estimated by counting the number of ligand-receptor binding events within a specific time. In this simplest

51 scheme of concentration sensing, the time required for a Bcd binding site of the *hb* promoter to read the Bcd
 52 concentration with 10% accuracy was estimated to be of the order of 2 hours (Gregor, Tank, et al., 2007), much
 53 longer than the interphase duration of nuclear cycles. These early attempts to explain the establishment of the Hb
 54 step-like pattern marked a shift in experimental studies towards more quantitative approaches. The advent of new
 55 measurement methods, based on live imaging especially of transcription dynamics (Bertrand et al., 1998), allowed
 56 for a deeper view of the processes of positional readout in young fruit fly embryos. Specifically it allowed one to
 57 focus on the outcome of the first steps of regulation, transcription at the *hb* locus, to determine how this stage of
 58 early development is controlled. Thanks to advances in imaging discussed below, the pattern of transcription from
 59 the *hb* locus is observed to be established sharply in about 3 min at nc11, despite being generated by noisy gene
 60 expression from a bursty promoter. In this review we discuss recent work and challenges for advancing our
 61 understanding of positional information propagation from the Bcd gradient to the *hb* expression pattern (Figure 1).
 62 We made the choice to only focus on the first step, the transcription process, which in itself contains several steps
 63 (Figure 1B-D), and highlight why this system remains challenging 30 years on. More details about the
 64 establishment of the Bcd gradient can be found in the chapter by Huang and Saunders in the same issue (Huang &
 65 Saunders, 2019).



66
 67 **Figure 1. A)** How is positional information contained in the shallow Bcd concentration gradient transformed into
 68 a robust step-like *hb* expression pattern in the 5 to 15 minutes of the nuclear cycle 11-13? **B-D)** The rate-limiting
 69 steps of *hb* expression in response to the Bcd gradient: **B:** The Bcd searches for the *hb* promoter, which can
 70 involve either 3D diffusion inside the nuclear space or 1D diffusion along the DNA. **C:** Bcd binds cooperatively to
 71 *hb* promoter, separating the embryo into a rich Bcd-bound anterior region and a no Bcd-bound posterior region.
 72 This process can involve exclusively Bcd molecules, other DNA bound transcription factors or factors facilitating
 73 chromatin accessibility. **D:** The production of *hb* RNA was shown to be bursty (Desponds et al., 2016), with
 74 relatively infrequent switching of the *hb* promoter between the ON and OFF expression states.

75

76 2. The Bcd gradient

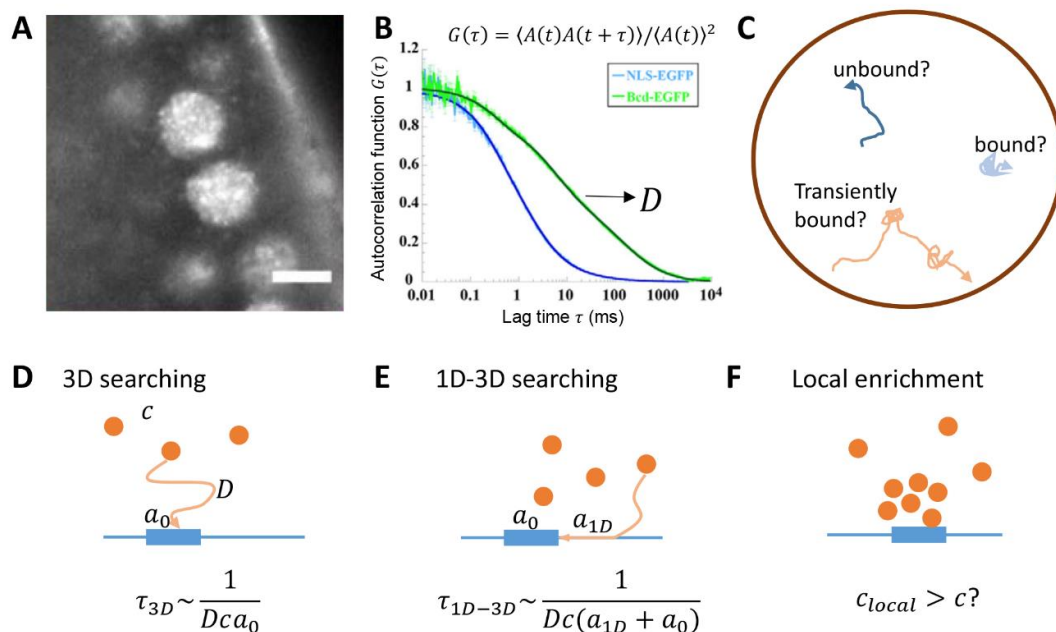
77 2.1. Positional information

78 The Bcd concentration gradient arises from maternal mRNA anchored at the anterior pole of the syncytial embryo
 79 (Grimm, Copepy, & Wieschaus, 2010). Large scale analysis of the Bcd gradient, through immunofluorescent
 80 staining of the endogenous protein or analysis of the fluorescent-fusion Bcd-eGFP protein (Gregor, Wieschaus, et
 81 al., 2007) (Figure 2A), indicated that the Bcd gradient is exponential with a concentration decay length around 80
 82 to 120 μ m (between one-fifth and one-fourth of embryo length) (Abu-Arish, Porcher, Czerwonka, Dostatni, &
 83 Fradin, 2010; Gregor, Bialek, de Ruyter van Steveninck, Tank, & Wieschaus, 2005; Gregor, Wieschaus, et al.,
 84 2007). The concentration of this gradient is a source of positional information for each nuclei along the AP axis.
 85 All the experiments performed to measure the absolute concentration of the Bcd gradient along the AP axis used
 86 the Bcd-eGFP expressed from a transgene in the embryo which rescues the viability of the *bcd^{E1}* null allele. The
 87 total concentration of Bcd at the anterior pole, measured *via* fluorescent Bcd-eGFP ranges from 90 nM, measured

88 by comparing the fluorescence inside nuclei with the fluorescence of an eGFP solution at a given concentration
 89 (Gregor, Garcia, & Little, 2014), to 140 nM (Abu-Arish et al., 2010), equivalent to respectively, 4.4 (estimated to
 90 be ~ 700 Bcd molecules per nuclei at nc14 (Gregor, Wieschaus, et al., 2007)) to 7 molecules/ μm^3 where the *hb*
 91 pattern boundary is established. Even though these measurements are consistent, they were obtained with the
 92 same fluorescent Bcd-eGFP and are likely to be underestimates of the real concentration because a proportion of
 93 the eGFP might not be fluorescent. Further analysis using a Bcd fusion carrying two fluorescent domains (Durrieu
 94 et al., 2018) might help resolve the issue of absolute Bcd concentration measurements in the embryo. Similarly,
 95 taking into account the maturation time of the eGFP points to a slight overestimation of the Bcd gradient decay
 96 length of about 15% (Durrieu et al., 2018; F. Liu, Morrison, & Gregor, 2013). After corrections, the length
 97 constant of the Bcd gradient is estimated as low as one sixth of embryo length (16.5 ± 0.7 % EL).

98 2.2. The motility of Bcd molecules

99 The *hb* locus extracts positional information from the local Bcd concentration via interactions with Bcd
 100 molecules. Given the short time window for positional readout in each interphase, the Bcd search time for the *hb*
 101 promoter τ_{search} is critical in determining the limit of positional readout precision. Therefore, several studies
 102 analyzed Bcd motility, using FRAP (Gregor, Tank, et al., 2007) or FCS (Abu-Arish et al., 2010) on fluorescent
 103 Bcd-eGFP or single-molecule tracking (Figure 2A) (Drocco, Grimm, Tank, & Wieschaus, 2011; Mir, Stadler,
 104 Harrison, Darzacq, & Eisen, 2018)). In the initial FRAP experiments, Bcd motility in the cytoplasm turned out to
 105 be quite slow ($\sim 0.3 \mu\text{m}^2/\text{s}$) (Gregor, Wieschaus, et al., 2007). FCS experiments performed both in the cytoplasm
 106 and the interphase nuclei revealed the existence of Bcd molecules with different motilities: best fitting of the data
 107 to the two-species diffusion model indicated that *i*) in the cytoplasm, 18% of the Bcd molecules are slow-moving
 108 while 82% of the Bcd molecules are fast-moving with an average diffusion coefficient of $\sim 7.4 \mu\text{m}^2/\text{s}$ (Abu-Arish
 109 et al., 2010) and *ii*) in the nucleus, 43% of the Bcd molecules are slow-moving ($\sim 0.22 \mu\text{m}^2/\text{s}$) and 57%
 110 fast-moving ($\sim 7.7 \mu\text{m}^2/\text{s}$) (Porcher et al., 2010). Fast moving Bcd molecules ($\sim 4 \mu\text{m}^2/\text{s}$) were also observed using
 111 a photoactivable Dronpa-Bcd (Drocco et al., 2011) and the existence of at least two populations of Bcd molecules
 112 was further confirmed by high resolution single molecule imaging suggesting that in nuclei, Bcd molecules spend
 113 the same amount of time on nuclear exploration (searching for a binding target) and on binding to chromatin with
 114 surprisingly high unbinding rates, distributed with long tails (Mir et al., 2018).



115

116 **Figure 2. Studying Bcd motility in living fruit fly embryos.** A) Fluorescently-tagged Bcd molecules were shown
 117 to be distributed in foci in the nuclear space of fixed embryos (H. Xu, Sepúlveda, Figard, Sokac, & Golding, 2015)
 118 and hubs of fluorescently-tagged Bcd (Mir et al., 2017, 2018). Figure reused from (Mir et al., 2017). B-C)
 119 Different motilities of Bcd molecules were inferred from Fluorescent Correlation Spectroscopy (FCS) analysis of
 120 the Bcd-eGFP signal in nuclei, with a Bcd population with a high (free) diffusion coefficient (B), Figure reused
 121 from (Porcher et al., 2010) and C, by single-molecule tracking approaches revealing bound, unbound and
 122 transiently bound molecules (Mir et al., 2018). D-F) various scenarios of Bcd searching for its binding sites in the
 123 *hb* promoter. D: Bcd molecules can diffuse in 3D nuclear space to search for Bcd binding sites on the target
 124 promoter. The time, τ_{3D} , for the binding site to be found is inversely proportional to the product of D (coefficient

125 of free diffusion), c (concentration) and a_0 (size of the binding site); **E**: Bcd molecules can “hop” to non-specific
126 locations on the DNA and slide along DNA segments in 1D to search for specific binding sites. The average
127 sliding distance (or footprint) a_{1D} can be much bigger than the size of a Bcd binding site and therefore reduces
128 the search time from the 3D case; **F**: Local enrichment of transcription factor (TF) concentration ($c_{local} > c$) at the
129 promoter region can also reduce the promoter search time.

130

131 2.3. The time it takes for Bcd to find its target sites on the *hb* promoter

132 Transcription factors (TFs) explore, usually by passive diffusion, the nuclear space in “search” of their DNA
133 binding sites on the regulatory sequences of their target genes. In the case of *Drosophila* embryogenesis, the time
134 it takes for the Bcd protein to find its target sites on the *hb* promoter is critical to explain how *hb* expression in
135 response to Bcd can be so fast yet precise despite the limited Bcd concentration in the mid-embryo region.
136 Therefore, many studies have used modeling to estimate the time it takes Bcd to find its target sites and proposed
137 several strategies to shorten this search time (Abu-Arish et al., 2010; Gregor, Tank, et al., 2007; Mir et al., 2017,
138 2018; L. Mirny et al., 2009; Normanno, Dahan, & Darzacq, 2012).

139 In an initial attempt to estimate the Bcd search time for the *hb* promoter, Gregor and colleagues proposed that Bcd
140 molecules can diffuse in 3D inside the nuclear space to come in contact with the specific Bcd binding sites on the
141 *hb* promoter (Figure 2D). They proposed that the lapse time between each contacts was inversely proportional to
142 the diffusion coefficient of Bcd D , the local Bcd concentration c and the size of the Bcd binding site a .

$$\tau_{search} = \tau_{3D} \sim 1/Dca \quad (1)$$

143 The binding site for TFs, including Bcd, are commonly 10bp long which corresponds to ~ 3 nm. Given this value
144 and assuming that the searching Bcd molecules are diffusing freely (i.e. are fast-moving molecules) and that Bcd
145 binding is diffusion limited (each collision between a Bcd molecule and a binding site results in a successful
146 binding event), the binding site search time τ_{3D} is estimated to be on the order of 10 s. However, this is an
147 optimistic estimate given that a is likely 10 fold too large since displacement by a single bp may lead to an entirely
148 different DNA sequence that is not recognizable by the protein (Slutsky & Mirny, 2004). In this later case, a
149 would be ~ 0.3 nm and the search time for Bcd would be 100s. In addition, activation by Bcd requires in general
150 not just the binding of one Bcd molecule to the *hb* promoter but several (Burz, Rivera-Pomar, Jäckle, & Hanes,
151 1998; Driever, Thoma, & Nüsslein-Volhard, 1989; Ronchi, Treisman, Dostatni, Struhl, & Desplan, 1993). Thus,
152 clustering of binding sites in the regulatory sequences of the target genes is another factor that may lengthen the
153 time it takes to bind enough Bcd molecules together to get a transcriptional response.

154 The rapid establishment of the *hb* pattern, despite a long search time in 3D, leads to hypotheses that Bcd molecules
155 may use a combination of 1D and 3D diffusion to search for their target sites (L. Mirny et al., 2009; Slutsky &
156 Mirny, 2004). This mode of searching was first observed in *Escherichia coli* (Elf, Li, & Xie, 2007; Hammar et al.,
157 2012; Riggs, Bourgeois, & Cohn, 1970) for the *lacI* repressor where a combination of a 1D and 3D search was
158 found to reduce the search time by up to 100 fold compared to a pure 3D search. In this scheme, proteins bind
159 non-specifically to DNA and, while doing so, slide along the DNA segment in search for the specific target site
160 (Figure 2E). Therefore, the effective size of the target site is increased by the TF’s sliding footprint along the DNA
161 a_{1D} (up to a hundred bp, compared to the size of a binding site $a = 10$ bp). Another hypothesis is that the target
162 locus could be located in a micro-environments with enhanced TF concentration ($c_{local} > c$) (Figure 2F), thus
163 speeding up the search, as proposed in the case of the Ultrabithorax protein (Tsai et al., 2017). The recent
164 observation of Bcd concentration in dense hubs (Mir et al., 2017, 2018) opens up the possibility that
165 micro-environments that enhance local Bcd concentration could contribute to reduce the Bcd search time for the
166 *hb* promoter. However, one should note that these mechanisms can also introduce non-linearities into the position
167 sensing process: Bcd hubs were found to persist even in the posterior region of low Bcd concentration, leading to
168 a much flatter Bcd concentration profile in hubs than in the cytoplasm. In addition, Hammar *et al.* observed that
169 bound *lacI* molecules may interfere with the 1D-sliding molecules when the distance between the target sites is
170 shorter than the sliding footprint (Hammar et al., 2012). If Bcd employs this mode of searching, the very short
171 distances between Bcd binding sites on the *hb* promoter (as short as 12 bp) may introduce negative feedback to
172 Bcd binding, instead of the positive feedback normally linked to a sharp *hb* pattern (Ma, Yuan, Diepold,
173 Scarborough, & Ma, 1996). It is experimentally challenging to directly observe the scheme of the Bcd target site
174 search. Currently, it is not possible using a confocal microscope to directly identify which one of the Bcd
175 subpopulations with characterized motilities are directly searching for target sites. Observing interactions between
176 Bcd and its target sites is still limited to epigenomics approaches such as ChIP on fixed samples (Bradley et al.,
177 2010; Li et al., 2011) performed on population of nuclei that have very different transcription features. Recent
178 advances in sample preparation (Combs & Eisen, 2013; Karaikos et al., 2017) may allow us to observe how these

179 interactions correlate with the varying degree of inhomogeneity in Bcd motility along the embryo AP axis and
180 help identify the target site search mode.

181 2.4. Activation of transcription by Bcd

182 The Bcd protein is able, on its own, to activate transcription when bound to a promoter containing as few as three
183 of its DNA binding sites (Crauk & Dostatni, 2005; Ronchi et al., 1993). However, how this is achieved remains
184 largely unknown. Structure-function analyses of the Bcd transcription factor indicated that it contains many
185 redundant functional domains (Schaeffer, Janody, Loss, Desplan, & Wimmer, 1999). Besides its homeodomain
186 which allows binding to DNA (Hanes & Brent, 1989; Trelisman, Gönczy, Vashishtha, Harris, & Desplan, 1989),
187 the Bcd protein contains several independent activation domains which can activate transcription on their own
188 when multimerized and fused to a Gal4 DNA binding domain in vitro (Sauer, Hansen, & Tjian, 1995) or in the
189 early embryo (Janody, Sturny, Schaeffer, Azou, & Dostatni, 2001). These include a Glutamine-rich domain, a
190 ST-rich domain and a C-terminal acidic domain. The Bicoid protein also contains independent inhibitory domains
191 which reduce its activation potential (Janody et al., 2001; C. Zhao et al., 2002). Finally, activation by Bcd is
192 enhanced by other TFs binding to the promoter. These include the maternal contribution of the Hunchback protein
193 itself (Porcher et al., 2010; Simpson-Brose, Treisman, & Desplan, 1994) or Zelda (Mir et al., 2017; Z. Xu et al.,
194 2014). Yet, the mechanisms underlying these essential synergistic effects are poorly understood.

195 3. *hb* transcription dynamics

196 3.1. Visualizing *hb* transcription dynamics

197 RNA-FISH on fixed embryos allowed for the monitoring of *hb* nascent transcript accumulation at their site of
198 synthesis inside each nucleus of the embryo, making it an initial marker to study ongoing transcription and
199 promoter dynamics at a given locus. This allowed subsequently for the detection of single mature mRNAs in the
200 cytoplasm and in the nucleus (Little et al., 2013; Perry, Bothma, Luu, & Levine, 2012; Zoller, Little, & Gregor,
201 2018). Distributions of nascent and cytoplasmic *hb* RNA along the AP axis demonstrate sharp step-like patterns at
202 the transcription level, with an expression boundary width varying from 10 % egg length to 8% egg length at nc11
203 and nc13 respectively. The observed data suggested, despite low heterogeneity at the protein level (Gregor, Tank,
204 et al., 2007), a very noisy transcription process occurring with periods of promoter activity and inactivity (H. Xu et
205 al., 2015).

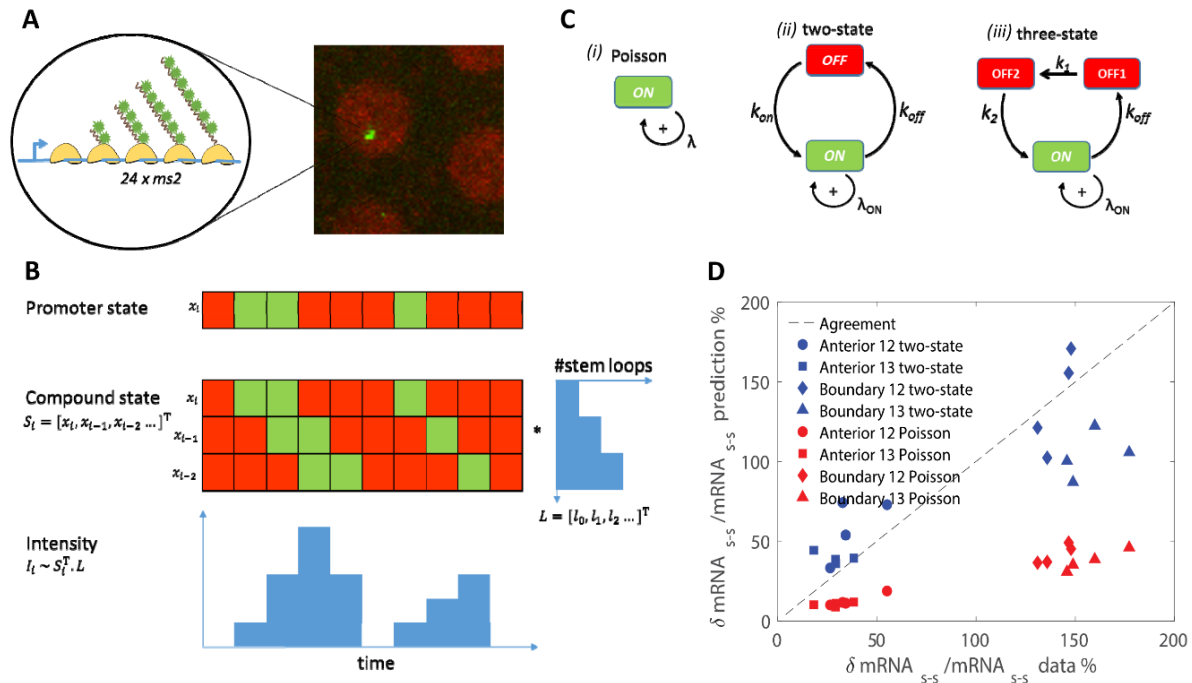
206 RNA FISH requires fixation of the sample and can only provide a snap shot view of the transcription process at a
207 given time (the time of fixation) during nuclear interphase. Following the pioneering work of R. Singer (Bertrand
208 et al., 1998), the MS2 fluorescent RNA-tagging system has been implemented to monitor transcription dynamics
209 in living early *Drosophila* embryo development (Garcia, Tikhonov, Lin, & Gregor, 2013; Lucas et al., 2013). The
210 system takes advantage of strong interactions between the MCP coating proteins and its RNA stem loops from the
211 MS2 bacteriophage. As nascent RNA containing stem loops are being transcribed, they are bound by fusion
212 proteins MCP-GFP, making ongoing transcription at the loci visible as bright fluorescent spots under the confocal
213 microscope (Ferraro, Lucas, et al., 2016).

214 Recently, the MS2 system allowed for the direct visualization in real-time of position-dependent activation of the
215 proximal *hb* P2 promoter (700 bp) (Lucas, Tran et al., 2018): at each nuclear interphase (from nc11 to nc13), *hb*
216 expression (detected via MCP-GFP foci) first occurs in the most anterior then proceeds progressively though very
217 rapidly from the anterior to the boundary region (at ~ 45% of embryo length from the anterior pole). Of note, this
218 progressive appearance of first *hb* foci after mitosis is measured considering as an origin of time the onset of
219 mitosis of each nucleus and is thus not a consequence of the mitotic wave. This dynamics from the anterior pole to
220 the center of the embryo occurs even within the anterior region where Bcd is presumably saturated. These findings
221 suggest that Bcd concentration is not only rate-limiting in the boundary region, as observed from the pattern
222 dynamics at the steady-state, but also within the anterior region. In this region, differences in Bcd concentrations
223 leads to differences in *hb* activation times (up to 3 minutes), which can take significant portion of the early nuclear
224 interphase and consequently affects the total amount of transcripts produced. In addition, analysis of the
225 MCP-GFP time trace of each individual nucleus shows variability in the transcription process even in the anterior
226 region with high Bcd concentration (Desponds et al., 2016), indicating that Bcd is not the sole factor contributing
227 to the noise in *hb* transcription.

228 3.2. Characterizing *hb* transcription dynamics

229 The fluorescent time traces acquired with the MS2 system provide an indirect observation of transcription
230 dynamics. The signal is noisy, convoluting both experimental and intrinsic noise with the properties of the MS2
231 probe. To obtain a specific fluorescent signal sufficiently strong to overcome background fluorescence due to

232 unbound MCP-GFP molecules, a long probe of 24 RNA stem loops was used (Garcia et al., 2013; Lucas et al.,
 233 2013). As the signal is only detected while the probe is being transcribed (Figure 3A and B), this introduces a
 234 significant delay (buffering time) between each instant the promoter is ON and the corresponding fluorescent
 235 detection. This buffering time of ~1 min (Coulon et al., 2014; Fukaya, Lim, & Levine, 2017; Garcia et al., 2013)
 236 and the short length of the traces (5-15 minutes) prevent traditional analysis based on OFF time distributions or
 237 autocorrelation functions to quantify the statistics of the activation and inactivation times.



238
 239 **Figure 3. Inferring promoter dynamics from MS2 loci intensity:** **A**) Visualization of active transcription loci:
 240 as RNAs containing MS2 stem loops are transcribed by RNA Polymerases (RNAP, in yellow), they are bound by
 241 fluorescent MCP-GFP molecules (in green). The succession of several RNAPs transcribing the gene allows for
 242 the accumulation of several fluorescently tagged MS2-containing RNA at the same location which become visible
 243 under the confocal microscope as green bright spots (right panel). **B**) Transformation from the promoter state
 244 dynamics to MS2 spot intensity dynamics: promoter state in discrete time x_i indicates whether the promoter is
 245 ON (green) or OFF (red). This state is encoded in the compound state vector $S_i = [x_i, x_{i-1}, x_{i-2} \dots]^T$, which also
 246 maps the position of RNAP on the MS2 cassette. RNAP arriving at time i will be transcribing a nascent RNA
 247 containing L_j MS2 stem loops at time $i + j$. L depends on the length and on the arrangement of the MS2 stem
 248 loops on the reporter gene. The active loci intensity I_i at time i is given by the product of S_i and L . **C**) Different
 249 models of promoter dynamics: *i*) Poisson model: random RNAP arrival and initiation of transcription; *ii*)
 250 two-state and *iii*) three-state models, where promoters switch successively between ON and OFF states. During
 251 the ON state, RNAPs arrive and initiate transcription in bursts, with maximum rates. **D**) Comparison of readout
 252 noise ($\delta mRNA / mRNA$) at steady state generated from Poisson (red) and bursty two-state models (blue) and data
 253 (dashed). Figure reused from (Desponds et al., 2016).

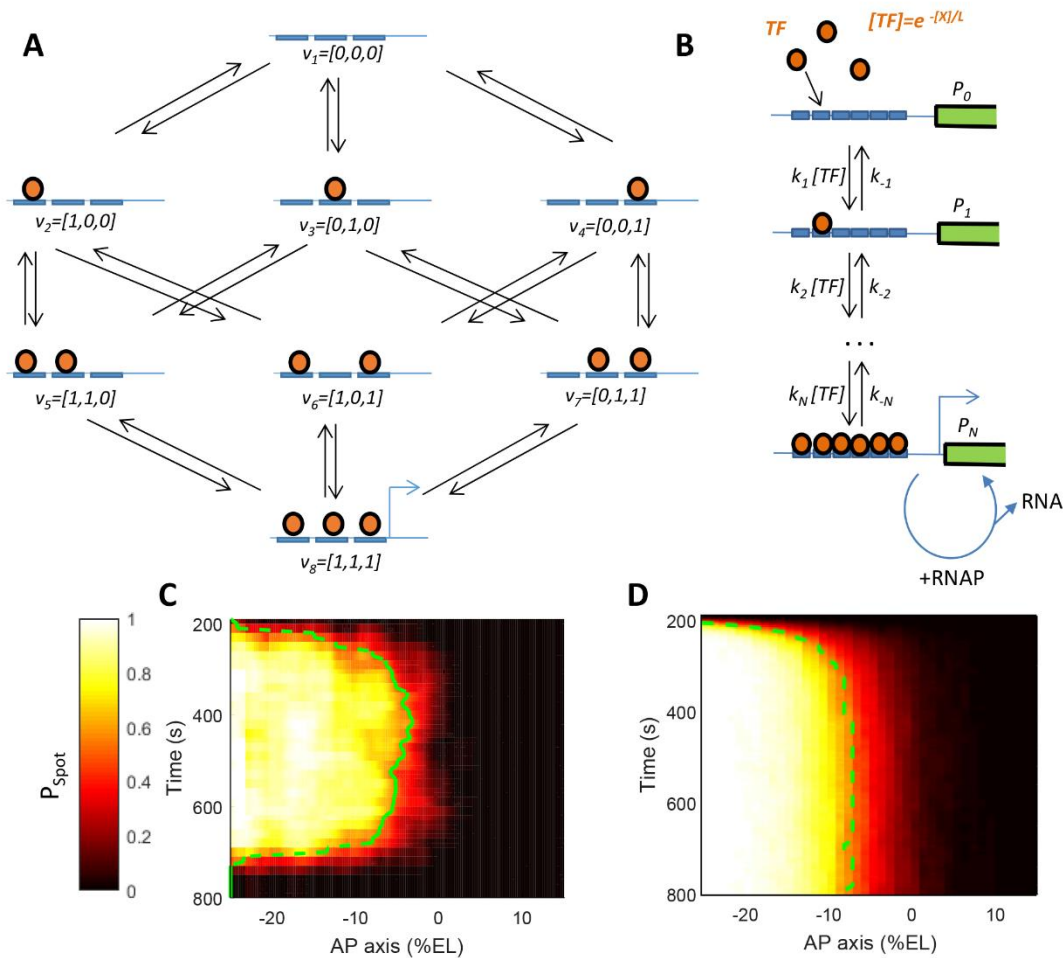
254 Desponds et al. developed a tailored autocorrelation analysis of the fluorescent time traces to overcome these
 255 limitations (Desponds et al., 2016). Combining this analysis with models of transcription initiation (Figure 3C)
 256 and estimates of the precision of the transcriptional readout, provided evidence for bursty transcription initiation
 257 in nuclear cycles 12-13 (Desponds et al., 2016). Namely, they find the dynamics in agreement with a telegraph
 258 model, in which the promoter switches between the ON and OFF states. Only during the ON state can RNA
 259 polymerase arrive and initiate transcription successively (Figure 3C). The switching between the ON and OFF
 260 promoter states can be driven by polymerase pausing which was proposed to prevent new initiation between
 261 transcription bursts (Shao & Zeitlinger, 2017), the binding/unbinding of other TFs required for activation, or DNA
 262 looping for distal enhancer-promoter contacts (Fukaya, Lim, & Levine, 2016). The best-fit switching period (for a
 263 full ON-OFF cycle) is in the order of ~30 s, with the probability to be in the ON state of ~50% at the anterior and
 264 of ~10% at the boundary.

265 It should be noted that the autocorrelation function analysis alone is not able to distinguish reliably between
 266 different models for promoter activation and requires complementary information about the precision of the

267 transcriptional readout to conclude that transcription is most likely bursty (Figure 3D). Recently, an inference
 268 method based on hidden Markov model and maximum likelihood has been developed (Corrigan, Tunnacliffe,
 269 Cannon, & Jonathan, 2016) and tailored for the MS2 system in *Drosophila* (Lammers et al., 2019). This
 270 discrete-time model employs a hidden compound state, which records the previous promoter states during the
 271 elongation time (Figure 3B). This compound state is used to map RNA Polymerase (RNAP)'s position on the
 272 reporter gene segment and calculate the active loci intensity at a given time. The rates of switching between the
 273 promoter states in each time step are fitted based on maximum likelihood. While computationally expensive, the
 274 method allows for direct model selection and shows that transcription bursts are prevalent in stripe gene
 275 expression in later stages of fly development (Berrocal, Lammers, Garcia, & Eisen, 2018; Bothma et al., 2014;
 276 Lammers et al., 2019).

277 3.3. Transcription regulation of *hb* gene by Bcd proteins

278 Data obtained from the MS2 system provided insights not only at the molecular level about the kinetics of the
 279 promoter behavior but also at the cellular level when considering individual nuclei along the AP axis and
 280 individual loci in each of these nuclei. In particular, it was possible to analyze the transcription dynamics of each
 281 *hb*-MS2 locus at the scale of the whole embryo. This analysis indicated that depending on its position along the AP
 282 axis, each locus was able to either turn ON when positioned in the anterior or remain silent when positioned in the
 283 posterior. Surprisingly, the steep border forms in under 3 min at each nuclear interphase 11 to 13 (Lucas, Tran et
 284 al., 2018). This indicates that the system is able to measure extremely rapidly very subtle differences of Bcd
 285 concentration and produce a complete sharp border. This rapid responsiveness is fascinating because it is almost
 286 ten times faster than predicted by previous theoretical models assuming that the Bcd gradient is the only driver for
 287 the *hb* transcription process.



288 **Figure 4: Modeling transcriptional regulation by the Bcd transcription factor through interactions with**
 289 **binding sites on *hb* promoter. A** A general model of transcription factors (TF) (orange) binding/unbinding to N
 290 binding sites of target promoter ($N=3$). Each node v corresponds to a unique ordered TF-bound promoter state.
 291 Figure adapted from (Estrada, Wong, DePace, & Gunawardena, 2016). **B** A simplified promoter binding model
 292 assuming detailed balance to account for energy expenditure in the unbinding process (Estrada et al., 2016),
 293

294 coupled with transcription initiation. When many Bcd binding sites on the *hb* promoter are occupied, RNAP can
295 randomly bind to the promoter and initiate transcription. **C-D**) The probability of an active transcription locus
296 (P_{Spot} , color bar) for the *hb* locus as a function of time in the nuclear cycle and position along the AP axis obtained
297 from the MS2-MCP data (C, nc13) and from the model in B (D, with $N=6$, not accounting for mitosis at the end of
298 interphase). Figure B-D are reused from (Lucas et al., 2018).

299 The steep Bcd-dependent *hb* pattern, given the smooth Bcd gradient, demonstrates a strong nonlinear regulation
300 of the *hb* gene by Bcd. The presence of multiple Bcd binding sites on the *hb* promoter (Driever &
301 Nusslein-Volhard, 1988) suggests that such strong nonlinearities can be achieved by high cooperativity of Bcd
302 binding to the *hb* promoter site. Cooperative binding of Bcd to multimerized binding sites was observed *in vitro*
303 (Burz et al., 1998; Ma et al., 1996) but remains too weak to account for the extremely steep Bcd-dependent *hb*
304 pattern observed *in vivo*. Synthetic reporters with only Bcd binding sites are weakly expressed in very anterior
305 domains which harbor, however, remarkably steep posterior boundaries (Crauk & Dostatni 2005; Ronchi et al.
306 1993). This suggests that Bcd and Bcd binding sites are sufficient to generate a steep posterior border and models
307 of regulation by Bcd binding/unbinding can help understand how this is achieved.

308 Using powerful optogenetics to manipulate Bcd activity in real time, Huang and colleagues demonstrated that the
309 full functional Bcd is critical in the early nuclear cycles (nc11 to 13) for the development of the embryo's
310 mesothorax, where the *hb* boundary is established (Huang, Amourda, Zhang, Tolwinski, & Saunders, 2017).
311 During this time window, light-induced conformational changes of the Bcd molecules, which leads to their
312 functional inactivation, significantly reduces transcription at the *hb* promoter, as shown using the MCP-GFP
313 system. This study links the transcriptional activity of TF bound to their target sites with the temporal dynamics of
314 transcription and provide an unprecedented time resolution of the TF-dependent transcription process. A general
315 model of transcription regulation via binding/unbinding of TF to the binding sites on the target promoter (Figure
316 4A), demonstrates that the pattern's degree of steepness, conventionally characterized by a Hill coefficient H of
317 the fitted Hill function, is limited by the number of TF binding sites N (Estrada et al., 2016). In this model, the
318 promoter states v_i , each associated with a transcription rate, are updated with every TF binding and unbinding
319 event. When the model satisfies detailed balance, the forward and reversed transitions between any two states v_i
320 and v_j are equilibrated:

$$P(v_i) \cdot k_{ij} = P(v_j) \cdot k_{ji}, \quad (2)$$

321 with k_{ij} and k_{ji} the forward and reverse rate constants, $P(v_i)$ and $P(v_j)$ the probability of the promoter states.
322 Thus, the model can be collapsed into the linearized model as in Figure 4B, where only the number of occupied
323 Bcd binding sites and not their identity matters. In this case, H cannot be above N . Experimental H values
324 obtained when observing the protein level (Gregor, Tank, et al., 2007) and several features of the *hb* transcription
325 dynamics (e.g. total amount of RNA produced (Garcia et al., 2013; Lucas, Tran et al., 2018)) range from ~ 5 to ~ 7 ,
326 roughly equal to the number of known Bcd binding sites on *hb* promoter. This leads to assumptions that the 6
327 binding sites, with an unstable first Bcd-bound state (large k_{-1} in Figure 4B) and a stable fully bound state (small
328 k_{-N} in Figure 4B), are sufficient to explain the observed pattern steepness in static measurements.

329 However, Estrada *et al.* did not consider the search time issue (Estrada et al., 2016) and their theory cannot explain
330 how the *hb* pattern can be established in such a short time of 3 minutes following mitosis, as observed in live
331 imaging data (Lucas, Tran et al., 2018) (Figure 4C). Considering a model which accounts for the Bcd search time
332 for the *hb* promoter, Tran *et al.* (Tran et al., 2018) found that, at the mid-boundary position, very high pattern
333 steepness ($H \approx N$) requires a very slow promoter switching time (called τ_{active}) between Bcd-bound states
334 allowing transcription and Bcd-free states prohibiting transcription. Therefore, according to the model, it should
335 take a very long time for the pattern to be established and this is in contradiction with the experimental data from
336 the MS2 system (Lucas, Tran et al., 2018). In addition, slow promoter dynamics results in high nuclei-to-nuclei
337 variability in the amount of total RNA produced in each nuclear cycle. Thus, it would require even more averaging
338 time to achieve the robust protein pattern (10% variability) observed in nuclear cycle 14 (Gregor, Tank, et al.,
339 2007).

340 The failure of the simple model to explain both the observed high pattern steepness and fast establishment time
341 begs for the reconsideration of the model's assumptions. Most obvious candidates are either the underestimation
342 of the number of Bicoid binding sites N or overestimation of the time it takes for Bcd to find the
343 promoter τ_{search} . Estrada *et al.* suggested that energy expenditure, which removes the detailed balance
344 assumption from the binding and unbinding process, can expand the model's limit on pattern steepness H beyond
345 the binding site number N , allowing both high steepness and fast formation time at the same time (Estrada et al.,
346 2016). However, including non-equilibrium binding does not resolve the problem of obtaining a steep yet precise
347 boundary in a short time. Alternatively, Desponds *et al.* (Desponds, Vergassola, & Walczak, 2019) suggested that

348 instead of probing the concentration for a fixed amount of time and then making the decision about the positioning
349 of the nucleus, constantly updating the odds of being in an anterior *vs* posterior position always results in much
350 faster decisions for a fixed accuracy. Assuming a promoter with 6 Bcd binding sites, they showed that the decision
351 time can be reduced by an order of magnitude compared to the classical Berg-Purcell scheme, possibly below the
352 3 minute limit. Unlike the classical time averaging (Berg-Purcell) scheme, which theoretically can reach 100%
353 accuracy given enough sensing time, the target gene “commits” to expression or silence when the odds favoring
354 the anterior or posterior reach an acceptable confidence, even before the end of the exposure window. Possible
355 molecular mechanisms for how this decision scheme could be implemented in fly embryos are still needed.

356 3.4. Dissecting noise in *hb* transcription

357 Noise in transcription dictates the variability of transcript and protein readouts after each interphase and might
358 play a role in determining nuclei identity in downstream processes (Holloway et al., 2011). However, beyond its
359 characterization from observed data, we still lack the mechanistic understanding of processes responsible for this
360 noise.

361 As transcription bursts are prevalent across the embryo in the very short early nuclear cycles, *hb* transcription
362 dynamics is well-fitted by a two-state model, in which the switching rates between the ON and OFF states are
363 modulated by the nuclei’s position or Bcd concentration (Desponds et al., 2016; H. Xu et al., 2015; H. Xu,
364 Skinner, Sokac, & Golding, 2016; Zoller et al., 2018). It should be noted that these ON and OFF states do not
365 correspond to Bcd-free and Bcd-bound states of the promoter as in (Estrada et al., 2016; Tran et al., 2018): in the
366 Bcd-saturating anterior region, the *hb* promoter is constantly active (i.e. bound by Bcd molecules) but
367 transcription still occurs in bursts with the switching time between ON and OFF states $\sim 50s$ (Desponds et al.,
368 2016). This suggests that promoter bursting may be an inherent property of transcription in this phase of
369 development (Bothma et al., 2014; Zoller et al., 2018). The early transcription of *hb* is also regulated by other TFs
370 such as maternal Hb (Lopes, Spirov, & Bisch, 2011; Porcher et al., 2010; Simpson-Brose et al., 1994) or Zelda
371 (Harrison, Li, Kaplan, Botchan, & Eisen, 2011; Lucas et al., 2018; Nien et al., 2011; Z. Xu et al., 2014).
372 Optogenetics was also used to inactivate the transcription factor Zelda in early embryos and reveals that this
373 pioneer factor continuously regulates zygotic gene expression from nc10 to nc14 (McDaniel et al., 2019). Though
374 these factors other than Bcd may not act as a source of positional information, their concentration may be
375 rate-limiting and therefore responsible for bursts.

376 In the *hb* boundary region, where cell fate decision is critical, *hb* transcription readout is more variable than in the
377 anterior region (Desponds et al., 2016; Lucas et al., 2018). This was initially thought to be due to extrinsic noise
378 from Bcd variability (Gregor, Wieschaus, et al., 2007) being amplified in this region. However, given the very
379 high steepness observed from the *hb* pattern (Lucas, Tran et al., 2018; H. Xu et al., 2015), the switching time
380 between Bcd-dependent active and inactive states of the promoter (τ_{active}) is expected to be at least one order of
381 magnitude greater than the Bcd search time τ_{search} (Tran et al., 2018). If the search is done via 3D diffusion
382 ($\tau_{search} \sim 10s$), τ_{active} at *hb* boundary is at least of a similar time scale as the switching time between ON and
383 OFF states at the anterior ($\sim 50s$). In the context of very rapid embryo development (interphase duration of 5 to 15
384 minutes in nc11 to nc13), Bcd-dependent promoter switching becomes a non-negligible source of intrinsic noise
385 that contributes substantially to the higher readout variability observed.

386 4. Perspectives

387 Despite several decades since the identification of the Bcd gradient, we still lack a quantitative description of the
388 process allowing for the transcription of its main target gene *hb* in a step-like pattern. The short timescales of early
389 development and its remarkable precision are questioning how fundamental limits coming from stochastic
390 processes such as diffusion or bursty regulation influence the molecular encoding of regulation. To pursue these
391 issues, recent experimental advances are allowing us to rigorously test theoretical ideas, and call for the creation
392 of new models.

393 This simple example of developmental biology, is turning out to also be an ideal *in vivo* testbed for advances in
394 single molecule techniques to study protein motility (Drocco et al., 2011; Mir et al., 2018) and promises to bring a
395 more definitive view on how TF can find their target promoter and activate transcription. The different motilities
396 of TF (Abu-Arish et al., 2010; Mir et al., 2018) and their inhomogeneous distribution in those so-called hubs (Mir
397 et al., 2017) as seen with the Bcd case support the idea that the search time can be improved by 1D-diffusion along
398 the DNA or via micro-environments of enriched TF concentration. However, with fluorescent-tagged molecules
399 alone, it remains difficult to identify *in vivo* the location of the binding molecule along the chromosome, with
400 enough resolution to detect 1D-diffusion or local enrichment. Conversely, such epigenomics tools as ChIP-seq
401 give information on TF binding along the chromosome, but lack the temporal dynamics of these interactions. With

402 advances in sample preparation and super-resolution microscopy, it might be possible to combine these two types
403 of experiments to understand how TF searches for their specific sites. Another important question is the
404 mechanisms of TF binding “cooperativity” after the search. In the Bcd case, binding cooperativity has been
405 suggested in theoretical work to explain *hb*’s high pattern steepness at steady state (Estrada et al., 2016; Gregor,
406 Tank, et al., 2007; Lopes et al., 2011; Tran et al., 2018). While high steepness is observed experimentally
407 (corresponding to a Hill coefficient ~ 7 to 8) (Lucas et al., 2018), *in vivo* measurements of Bcd binding
408 cooperativity of such high order at the gene loci remains a challenge (Mir et al., 2018; H. Xu et al., 2015). *In vitro*
409 studies showed that Bcd binding cooperativity is of low order (Hill coefficient ~ 2 to 3) and robust to different
410 target sites’ spacing (Ma et al., 1996), suggesting that it may originate from protein-protein interactions rather
411 than from transcription factor-induced binding site conformational changes (Bray & Duke, 2004). More direct
412 experimental evidence is required to fully elucidate high-order cooperativity observed in the expression pattern,
413 possibly coming from different transcription factors.

414 Advances in live imaging of transcription offered a direct view on *hb* transcription pattern establishment in early
415 nuclear cycles: the pattern is established in a considerable portion of the very short interphase and, in contrast to
416 the low variability observed at the protein level, transcription is highly noisy, with prevalent bursty behavior
417 across the embryo. This marks a shift of our approaches towards the understanding of transcription regulation
418 during development from previously static and usually steady-state views to more theoretical and quantitative
419 studies focusing on the out-of-steady-state dynamics of gene expression (Petkova et al., 2019; Tran et al., 2018).
420 The question of TF searching for its targets remains of great interests to explain the rapidity of the *hb* pattern
421 establishment despite a noisy transcription process. Transcription activation following TF binding, even though
422 intensively studied for decades, also remains a mystery. In the context of development, it will be important to
423 understand how the measurement process of positional information manages to combine the rapidity of the
424 response with accuracy (Tran et al., 2018). These observations lead to suggestions that transcription activation
425 may be non-reversible or involve transcriptional memory or mitotic bookmarking (Desponds et al., 2019; Ferraro,
426 Esposito, et al., 2016; R. Zhao, Nakamura, Fu, Lazar, & Spector, 2011). The development of powerful optogenetic
427 tools with high spatial and temporal resolution might shed light on this mechanism, as it is now possible to
428 manipulate TF properties and observe the effects of these manipulations in real time under the microscope (Huang
429 et al., 2017; McDaniel et al., 2019).

430 Finally, *hb* protein expression in *nc14* is still remarkably robust, suggesting that temporal and spatial averaging
431 downstream of noisy transcription is at work. Recent works have shown that positional information can be
432 accurately decoded at the level of the gap genes (Petkova et al., 2019). However, decoding as well as encoding
433 mechanisms in the earlier cell cycles remain unknown and the current experimental and theoretical methods are
434 ready to tackle these questions. At last, it is likely that Bcd may no longer play a major role in maintaining *hb*
435 pattern during the very long nuclear cycle 14 (Durrieu et al., 2018; Perry, Boettiger, & Levine, 2011; Perry et al.,
436 2012). Given this, an intriguing aspect of this system is why it has been selected to provide such fast step-like
437 pattern dynamics in the earlier nuclear cycles.

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