Tunable Transcriptional Interference at the Endogenous Alcohol Dehydrogenase Gene Locus in Drosophila melanogaster

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ABSTRACT Neighboring sequences of a gene can influence its expression. In the phenomenon known as transcriptional interference, transcription at one region in the genome can repress transcription at a nearby region in cis. Transcriptional interference occurs at a number of eukaryotic loci, including the alcohol dehydrogenase (Adh) gene in Drosophila melanogaster. Adh is regulated by two promoters, which are distinct in their developmental timing of activation. It has been shown using transgene insertion that when the promoter distal from the Adh start codon is deleted, transcription from the proximal promoter becomes deregulated. As a result, the Adh proximal promoter, which is normally active only during the early larval stages, becomes abnormally activated in adults. Whether this type of regulation occurs in the endogenous Adh context, however, remains unclear. Here, we employed the CRISPR/Cas9 system to edit the endogenous Adh locus and found that removal of the distal promoter also resulted in the untimely expression of the proximal promoter-driven mRNA isoform in adults, albeit at lower levels than previously reported. Importantly, transcription from the distal promoter was sufficient to repress proximal transcription in larvae, and the degree of this repression was dependent on the degree of distal promoter activity. Finally, upregulation of the distal Adh transcript led to the enrichment of histone 3 lysine 36 trimethylation over the Adh proximal promoter. We conclude that the endogenous Adh locus is developmentally regulated by transcriptional interference in a tunable manner.

KEYWORDS transcription interference Drosophila Adh CRISPR Cas9 translation chromatin H3K36me3

Transcriptional interference, or cis-mediated downregulation of transcription at a locus as a result of transcription from a nearby location (Shearwin et al. 2005), was initially recognized as a mechanism of gene regulation conferred by retroviral promoters (Cullen et al. 1984).

Since then, transcriptional interference has been observed to endogenously regulate genes in a number of eukaryotic contexts (Martens et al. 2004; Shearwin et al. 2005; Hongay et al. 2006; Bird et al. 2006; Hainer et al. 2011; van Werven et al. 2012; Yu et al. 2016). In particular, transcription of non-coding RNAs is widely associated with interference of promoters or regulatory elements of local coding transcripts (Martens et al. 2004; Hongay et al. 2006; van Werven et al. 2012; Yu et al. 2016; Kaikkonen and Adelman 2018).

In addition to non-coding RNAs, mRNA isoforms have also been linked to transcriptional interference. For genes with more than one promoter, transcription from the distal promoter may not only produce a distinct mRNA isoform, but could also lead to the repression of an mRNA isoform transcribed from the open reading frame (ORF)-proximal gene promoter (Corbin and Maniatis 1989; Moseley et al. 2002; Sehgal et al. 2008; Liu et al. 2015; Chen et al. 2017). In addition, since distinct mRNA isoforms may differ in their
translational efficiency, regulation of promoter choice may impact gene expression at the protein level. In some instances, this difference in translational efficiency is due to the presence of upstream ORFs (uORFs) in the 5' leader of the distal promoter-derived mRNA isoform, which could inhibit translation of the protein-coding ORF (Moseley et al. 2002; Law et al. 2005; Sehgal et al. 2008; Ingolia et al. 2011; Brar et al. 2012; Rojas-Duran and Gilbert 2012; Chew et al. 2016; Chen et al. 2017; Bird and Labbé 2017; Cheng et al. 2018; Zhang et al. 2018). As a result, in these cases, transcription of a distal promoter-derived mRNA isoform causes downregulation of protein expression through the integration of two seemingly disparate mechanisms of transcriptional and translational repression (Chen et al. 2017; Cheng et al. 2018; Van Dalsen et al. 2018; Hollerer et al. 2019).

Transcription can antagonize downstream promoter activity by at least two means: First, the movement of the transcription machinery through the downstream promoter could interfere with transcription factor binding (Shearwin et al. 2005; van Werven et al. 2012; Zafar et al. 2014; Chia et al. 2017). Second, transcription through the downstream promoter could establish a repressive chromatin state (Hainer et al. 2011; van Werven et al. 2012; Woo et al. 2017; Chia et al. 2017). These mechanisms are not mutually exclusive and in fact have been shown to act in concert (van Werven et al. 2012; Chia et al. 2017). In the case of chromatin state changes, co-transcriptional histone modifications such as histone 3 lysine 36 trimethylation (H3K36me3) have been associated with nucleosome stabilization and repression of the downstream promoter (Hampsey and Reinberg 2003; Carrozza et al. 2005; Keogh et al. 2005; Houseley et al. 2008; Govind et al. 2010; van Werven et al. 2012; Ard and Alkshire 2016; Chia et al. 2017). In metazoans, the link between H3K36me3 and transcription-coupled repression has been less clear. In mammalian cells, H3K36me3 has been implicated in Dnmt3b-dependent intragenic DNA methylation and suppression of cryptic transcription (Carvalho et al. 2013; Baubec et al. 2015; Neri et al. 2017). Reduction of H3K36me3 is lethal in Drosophila larvae and leads to elevated levels of histone 4 lysine 16 acetylation, a mark associated with active transcription (Bell et al. 2007; Meers et al. 2017). However, replacement of lysine 36 with a non-modifiable arginine (H3K36R) does not increase cryptic transcription initiation in fruit flies (Meers et al. 2017).

An established example of transcriptional interference in Drosophila is the regulation of the alcohol dehydrogenase (Adh) gene (Corbin and Maniatis 1989). Adh is transcribed from two closely positioned promoters, resulting in the production of at least two distinct mRNA isoforms (Figure 1A). These transcript isoforms are expressed in a developmentally regulated and tissue-specific manner (Ursprung et al. 1970; Benyajati et al. 1983; Savakis et al. 1986; Sofer and Martin 1987; Anderson et al. 1991; Visa et al. 1992). Transcription occurs from the ORF-proximal promoter (hereon referred to as Adh proximal promoter) during the early larval stages and from the ORF-distal promoter (hereon referred to as Adh distal promoter) during late third instar larvae and in adults (Figure 1B, adapted from Corbin and Maniatis, 1989 as well as Sofer and Martin 1987). It has been shown that transcription from the Adh distal promoter is necessary to repress transcription from the Adh proximal promoter (Corbin and Maniatis 1989). However, this previous study employed transgene insertions, and the same allele displayed variable degrees of transcriptional interference, attributed to positional effects (Corbin and Maniatis 1989). Therefore, both the impact and the extent of transcriptional interference at the endogenous Adh locus are currently unknown. It also remains to be tested whether the premature expression of the Adh distal transcript in larvae is sufficient to down-regulate the Adh proximal promoter. Furthermore, whether transcription from the Adh distal promoter is accompanied by downstream changes in H3K36me3 is unknown. Finally, the translational capacity of the two Adh mRNA isoforms has not been investigated. Here, we examined these unexplored aspects of Drosophila Adh regulation. We report that the transcriptional interference at the endogenous Adh locus is tunable and distal promoter activation is associated with H3K36me3 enrichment at the Adh proximal promoter. We further show that the two Adh transcript isoforms are both associated with high polysome fractions, indicating efficient translation.

MATERIALS AND METHODS

Fruit fly stocks, husbandry and larval collection

Fruit flies were raised on standard molasses medium at 25°C. Oregon-R was used as wild type (a generous gift from Don Rio). The tubGAL4 line was obtained from the Bloomington Drosophila Stock Center (ID 5138). All fruit flies in the stock were heterozygous for tubGAL4 and the balancer TM3, Sb' Ser1, as the tubGAL4 chromosome is homozygous-lethal. For experiments requiring adult fruit flies, a mixture of males and females was used. The AdhDIST line was homozygous for the deletion allele. In experiments requiring induction of AdhDIST in larvae, we crossed homozygous AdhAUAS males to virgin female tubGAL4/TM3, Sb' Ser1 or Oregon-R control fruit flies in collection cages with molasses plates spread with live yeast. After 8 hr, plates were removed, and embryos were allowed to age for 72 hr at 25°C. The population consisted of predominantly first and second instar larvae. To collect the samples, larvae were washed off the plates using PBS and then washed three times in PBS. In between washes, larvae were left undisturbed to allow settling by gravity. ~2 mL of larvae were aliquoted, flash-frozen in liquid nitrogen, and stored at -80°C for later processing.

Generation of transgenic fruit flies

We cloned sgRNAs into pCFD4 (Port et al. 2014), which expresses two sgRNAs from U6 snRNA promoters. Two sgRNAs were used to ensure that at least one double-stranded break was formed. The sgRNA plasmid for generating AdhDIST (pUB1041) expressed sgRNAs 5'-AGTGGGCTTGGTCGCTGTTG-3' and 5'-TAATATAGAAAAAGCTTTGC-3'. The sgRNA plasmid for generating AdhAUAS (pUB1038) expressed sgRNAs 5'-CATAAATCGTCCGTGGTAAAT-3' and 5'-ACAATTTGGTAAAAAGCATA-3'. The repair templates were cloned into the pGEX-2TK cloning vector (GE Healthcare). To generate the repair template for the AdhDIST allele (pUB1094), two 1-kb homology arms were amplified from Oregon-R genomic DNA, with the Adh distal promoter region removed. When used as a repair template donor, this results in the removal of the region spanning -387 to -1376 bp upstream of the proximal isoform transcriptional start site. A similar allele was described previously (Corbin and Maniatis 1989). The repair template to generate AdhAUAS (pUB1091) contained two 1-kb homology arms amplified from Oregon-R genomic DNA, flanking a 10xUAS-hsp70 (core promoter) construct amplified from pVALIUM20 (Ni et al. 2011). When used as a repair template donor, this results in the insertion of the 10xUAS-hsp70 (core promoter) construct at the -1 position relative to the distal transcriptional start site.

sgRNA plasmids and their corresponding repair templates were injected into y w M{nos-Cas9}PZH-2A embryos (Bloomington 54591), which express maternal Cas9, by BestGene Inc. (Chino Hills, CA). The resulting mosaic maternal fruit flies were outcrossed to w1118, and the F1 progeny were individually crossed to CyO or CyO, twi > GFP.
profiles for AdhPROX (orange), AdhDIST (blue) and a control αTUB84B transcript (black) in wild-type L1/L2 larvae harvested at 80 hr (E) and wild-type adults (F). RNA was isolated individually from fractions and pooled into four categories: 40S/60S, monosome, low polysome (di- and trisome), high polysome (remaining fractions). Expression levels were obtained using isoform-specific primers and RT-qPCR. Data were first normalized to in vitro transcribed RCC1, which was spiked at equal amounts into each fraction prior to RNA extraction. Normalized data were then plotted relative to the amount present in the monosome fraction for each transcript. Data points represent the mean of 3 independent biological replicates. Error bars represent standard error of the mean (SEM). Two-tailed Student’s t-test was used to calculate the p-values **P < 0.001, n.s. not significant.

RNA isolation, cDNA synthesis and quantitative PCR
Total RNA was isolated using TRIzol (Life Technologies) according to a previously described protocol (Bogart and Andrews 2006). 450 ng of isolated RNA was treated with DNase (TURBO DNA-free kit, Thermo Fisher) and reverse transcribed into cDNA (Superscript III Supermix, Thermo Fisher) according to the manufacturer’s instructions. The RNA levels of specific Adh isoforms were quantified using primers specific to AdhDIST and AdhPROX (Table 1, supplemental file 1). SYBR Green/Rox (Thermo Fisher), and the StepOnePlus Real-time PCR system (Thermo Fisher). AdhDIST and AdhPROX signals were normalized to αTUB84B transcript levels. RT-qPCR for each sample was performed in technical triplicate and the mean Ct value was used for the normalizations. The efficiency value for each oligonucleotide pair was empirically determined and only those pairs that had greater than 90% efficiency were used for the RT-qPCR experiments. The oligonucleotide sequences used for the RT-qPCR experiments are displayed in Table 1, and primer efficiency calculations are shown in supplemental file 1. The raw Ct values and analyses for all the qPCR experiments are shown in supplemental files 2 through 6.

RNaseH digestion of total RNA
To distinguish the size difference between the two Adh isoforms, the total RNA of each sample was treated with RNaseH prior to RNA blot analysis. A total of 15 µg Trizol-extracted RNA was added to 1x RNaseH buffer (New England Biolabs, Ipswich, MA). Next, a site-specific DNA oligo (See Table 1 for sequence) was annealed to RNA by heating to 52° and slowly cooling to 25°. The RNA-DNA hybrid strands were incubated with 1 U RNaseH (New England Biolabs) for 1 hr at 37°. RNA was extracted in phenol: chloroform (1:1) and precipitated in isopropanol with 0.3 M sodium acetate overnight at -20°.

RNA blotting
RNA blot analysis protocol was performed as described previously (Koster et al. 2014) with minor modifications. 15 µg of total RNA was denatured in a glyoxal/DMSO mix (1 M deionized glyoxal, 50% v/v DMSO, 10 mM sodium phosphate (NaPi) buffer pH 6.5–6.8) at 70°
Chromatin immunoprecipitation in larval samples was performed as previously described (Ali et al., 2006) with the following modifications: Chromatin from approximately 2 mL of larval samples was isolated and fixed in 1.0% v/v formaldehyde for 20 min at room temperature and quenched with 100 mM glycine. Crosslinked chromatin was sonicated 12 times with a 30 sec ON/30 sec OFF program using a Bioruptor Pico (Diagenode, Denville, NJ). A fragment size of ~200 bp was obtained. To preclar the lysate, the samples were incubated in pre-RIPA buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.1% SDS) containing cOmplete Protease Inhibitor Cocktail (Roche) and 1 mM PMSF with Protein A Dynabeads (Invitrogen) for 2 hr at 4°C with rotation. After removal of Protein A Dynabeads, pre-cleared lysates were incubated overnight with 4 μg of anti-Histone H3K36me3 (Ab9050, Abcam), anti-Histone H3K4me3 (Ab8580, Abcam), or anti-Histone H3 (Ab791, Abcam). Simultaneously, a new aliquot of Protein A Dynabeads were blocked in pre-RIPA buffer + 1 μg/μL bovine serum albumin overnight at 4°C. The immunoprecipitates were then incubated with the pre-blocked Protein A Dynabeads for 4 hr at 4°C. Reverse crosslinked immunoprecipitated DNA fragments were amplified with Absolute SYBR green (AB4163/A, Thermo Fisher, Waltham, MA) and quantified with a 7500 Fast Real-Time PCR machine (Thermo Fisher). The oligonucleotide sequences of the primers used for ChIP analysis are listed in Table 1. For quantification of enrichment, H3K4me3 and H3K36me3 signal was normalized to H3. Raw data for the qPCR analysis is shown in supplemental file 6.

**Polysome fractionation and RNA extraction**

Whole fruit flies or larvae harvested in 1X PBS were transferred to a microcentrifuge tube on liquid nitrogen. Samples were homogenized on ice in 200 μL cold lysis buffer in the presence of cycloheximide. The lysis buffer for cycloheximide samples is as follows: 500 mM KCl, 15 mM Tris-HCl pH 7.5, 15 mM MgCl2, 0.5 mM Puromycin, 0.02 U SUPERaseIn, 1 cOmplete ULTRA EDTA-free protease inhibitor pill per 50 mL. Samples were centrifuged for 10 min at 15,000 g at 4°C. The aqueous phase was transferred to a new pre-chilled microcentrifuge tube, avoiding the pellet and wax layer. 10% of the aqueous volume was transferred to a new microcentrifuge tube, with 100 μL TRIzol and stored at -80°C for mRNA input sample. A 10% sucrose buffer (500 mM KCl, 15 mM Tris-HCl pH 7.5, 15 mM MgCl2 and 7 μL SUPERaseIn) and 50% sucrose buffer (500 mM KCl, 15 mM Tris-HCl pH 7.5, 15 mM MgCl2 and 7 μL SUPERaseIn) were used to generate a sucrose gradient of 10–40% in a Beckman Coulter 9/16x3.5 PA tube (Cat #331372) SW-41 ultracentrifugation tube. The gradient tube was stopped and the setting “long sur 10–40%” was used to make the gradient. Gradients were centrifuged at 35,000 g using a SW-41 rotor for 3 hr at 4°C and fractionated on a Brandel flow cell (Model #62114007) at 0.75 mL/min with the sensitivity setting at 0.5 Abs. A volume of 750 μL was collected for each fraction. The samples were then pooled as indicated.
in Figure S1. 5 ng roxI(xl)-polyA spike RNA was added to each pooled fraction prior to RNA extraction. RNA was extracted from the fractions using standard acid phenol-chloroform extraction as described in Chan et al. 2018. The RNA pellet was washed with 80% ethanol and then air-dried. After air-drying, the pellet was dissolved in 10 μl of nuclease-free water. The samples were then treated with Turbo DNase prior to cDNA synthesis.

Data availability
All the reagents generated in this study are available upon request. Supplemental material available at figshare: https://doi.org/10.25387/g3.10565984.

RESULTS

The Adh proximal promoter produces a transcript of 1001 nucleotides in length (hereon referred to as AdhPROX), whereas the Adh distal promoter activates a transcription start site (TSS) located 715 base pairs (bp) upstream of the proximal TSS. The resulting transcript from the distal promoter, hereon referred to as AdhDIST, has a unique 5′ leader located in exon 1 (Figure 1A, top). We first measured the relative abundance of the two Adh mRNA isoforms from wild-type embryos, larvae, and adult fruit flies using RNA blot hybridization. Because the two Adh isoforms differ by only 56 nucleotides, we employed an RNaseH digestion strategy to shorten the full-length transcripts so that a clear difference in isoform length could be detected (Figure 1A, yellow line marks the relative location of the oligonucleotide used for RNaseH digestion). Consistent with previous work (Savakis et al. 1986; Corbin and Maniatis 1989; diagrammed in Figure 1B), we observed that both Adh transcripts were undetectable in embryos (Figure 1C). AdhPROX was expressed at high levels in early larval stages, and the AdhDIST transcript was the predominant isoform in adults. To quantify the relative expression levels of each isoform, we used reverse transcription followed by quantitative polymerase chain reaction (RT-qPCR) using isoform-specific primers (supplemental file 1) and normalized AdhDIST and AdhPROX transcript measurements to αTUB84B, a ubiquitously expressed transcript. This analysis revealed that the AdhPROX transcript level was ~20 fold higher in larvae compared to in adults, whereas the AdhDIST transcript had the reciprocal pattern with more than 8000-fold enrichment in adults compared to its expression level in larvae (Figure 1D, supplemental file 2). These data confirm that the Adh locus undergoes developmentally induced transcript isoform toggling, as evidenced by the mutually exclusive expression patterns of the two mRNA isoforms.

To determine the translational status of the two Adh isoforms, we enriched for ribosome-associated transcripts using sucrose gradient fractionation and measured the relative distribution of AdhPROX or AdhDIST across different fractions in larvae and whole adults. AdhPROX was enriched in the high polysome fraction similar to αTUB84B (Figure 1E and Figure S1A, P = 0.2 two-tailed Student’s t-test, supplemental file 3). Interestingly, in the adults, AdhDIST enrichment in the high polysome fraction was more than fourfold higher relative to αTUB84B enrichment (Figure 1F and Figure S1B, P = 0.0006, two-tailed Student’s t-test, supplemental file 3). We conclude that both AdhPROX and AdhDIST are well translated. Furthermore, AdhDIST appears to be noticeably more enriched in the high polysome fractions than αTUB84B, indicating enhanced translational efficiency.

To assess the impact of transcriptional interference on AdhPROX expression at the endogenous locus, we used CRISPR/Cas9-based editing (Jinek et al. 2012, 2013; Cong et al. 2013; Mali et al. 2013) to delete the Adh distal promoter (AdhDISTΔ) (Figure 2A and Figure S2A).

Deletion of the Adh distal promoter resulted in a dramatic reduction of the AdhDIST transcript and led to the expression of AdhPROX in both larvae and adults, albeit at lower levels (Figure 2B and Figure S2B, supplemental file 4). RT-qPCR analysis showed a fivefold increase in AdhPROX abundance in AdhDISTΔ mutants compared to wild-type adults (Figure 2C, supplemental file 4). We conclude that loss of transcription from the Adh distal promoter results in a modest de-repression of AdhPROX, suggesting that, at least in adult fruit flies, transcription from the Adh distal promoter antagonizes the activity of the Adh proximal promoter.

Next, we tested if untimely overexpression of AdhDIST during larval development was sufficient to repress AdhPROX expression. Employing a similar CRISPR/Cas9-based editing strategy, we replaced the endogenous Adh distal promoter with an inducible 10xUAS-hsp70 promoter (AdhUASΔ), transcript produced from this promoter is referred to as AdhDISTΔ) (Figure 3A). The AdhUAS line was crossed to a tub-GAL4 line, which exhibits ubiquitous Gal4 expression driven from the αTUB84B promoter. In the F1 larvae, we observed ~3000-fold increase of the AdhDISTΔ isoform compared to wild type, accompanied by ~10-fold decrease in the AdhPROX isoform (Figure 3B and 3C, supplemental file 5). We noticed that, in F1 larvae from the AdhUAS lines, AdhDISTΔ expression was apparent even without the GAL4 driver, likely due to leaky expression from the hsp70 promoter, located immediately upstream of the AdhDISTΔ TSS (Figure 3C and Figure S3). Comparison of lines with and without GAL4 thus allowed us to achieve a range of AdhDISTΔ expression levels, which provided insight into the dose-dependent relationship between production of AdhDISTΔ and AdhPROX. We found that the degree of AdhDISTΔ overexpression scaled with the degree of AdhPROX repression: the more the distal promoter activity, the less the proximal transcript abundance (Figure 3C, supplemental file 5).
Figure 3 Ectopic expression of AdhDIST is sufficient for downregulation of AdhPROX in larvae. (A) Diagram of GAL4/UAS induction system for Adh. Immediately upstream of the AdhDIST TSS are 10 consecutive Gal4 bind sites (UAS) (shown as yellow bars) followed by the minimal hsp70 promoter (shown in black). Coding (gray) and non-coding (white) exons are shown. Arrows represent TSSs of AdhPROX (orange) and AdhDIST (blue). The yellow line represents the relative location of the oligonucleotide probe used for RNaseH cleavage. Numbers below the Adh locus refer to distance in base pairs (bp) from the AdhPROX TSS. The TSS for the GAL4-induced isoform (referred to as AdhDIST) was determined by 5' RACE (Figure S3). (B) RNA blot analysis confirms that ectopic expression of AdhDIST in larvae is sufficient for AdhPROX downregulation. RNA isoforms were detected using a probe that hybridizes to a common region of all isoforms. Methylene blue staining of rRNA was used as a loading control. (C) Expression levels of AdhDIST and AdhPROX in larvae with varying degrees of AdhDIST induction. Abundances of AdhDIST (left) and AdhPROX (right) in larvae were measured for the following four lines: wild type, heterozygous UAS (UAS/+), homozygous UAS (UAS/UAS), heterozygous GAL4 and heterozygous UAS (UAS+/+, GAL4/+). Expression levels were determined by RT-qPCR using isoform specific primers. All data were first normalized to αTUB84B and then to wild type (1x). The mean of three independent biological replicates is shown. Error bars represent SEM. (D) Induction of distal transcription promotes histone H3 lysine 36 trimethylation (H3K36me3) over the AdhPROX promoter (left panel). Histone H3 lysine 4 trimethylation (H3K4me3) modifications, which are enriched at active promoters, are also shown (right panel). DNA recovered from chromatin IP were quantified using RT-qPCR and 5 primer pairs (A, B, C, D, and E) spanning the region between the Adh promoters as well as 5' end of the gene body. All data were normalized to H3. Data points represent the mean of 3 independent biological replicates. Error bars represent SEM. Two-tailed Student's t-test was used to calculate the p-values ***P < 0.001, **P < 0.01, *P < 0.05, n.s. not significant.

This observation suggests that the antagonistic relationship between the levels of the two transcript isoforms is not binary, but tunable. RNA blotting confirmed that AdhDIST levels were highest in lines carrying the GAL4 driver. AdhDIST was also expressed in AdhUAS homozygous lines without the GAL4 driver (Figure 3B). Even in the AdhUAS heterozygous lines without the GAL4 driver, AdhDIST expression in F1 larvae was still higher than wild-type larvae, consistent with the RT-qPCR data (Figure 3B and 3C). We conclude that AdhDIST transcription is sufficient to downregulate AdhPROX expression in a dose-dependent manner.

To test if transcription from the distal promoter led to changes in chromatin marks at the Adh locus, we performed chromatin immunoprecipitation (ChIP) against H3K36me3 and H3K4me3 in larvae collected from wild type and homozygous AdhUAS lines, where both Adh alleles express AdhDIST. H3K36me3 is a co-transcriptionally established modification that occurs in regions downstream of active promoters (Xiao et al. 2003; Bannister et al. 2005; Mikkelsen et al. 2007) whereas H3K4me3 is highly enriched at active promoters near TSSs (Santos-Rosa et al. 2002). H3K4me3 enrichment was significantly increased near the AdhDIST transcription start site in the homozygous AdhUAS line (Figure 3D, right panel, supplemental file 6), consistent with active transcription. Furthermore, a significant increase in H3K36me3 enrichment occurred over the Adh proximal promoter in these mutants (Figure 3D, left panel, supplemental file 6). We conclude that AdhDIST transcription is accompanied with increased H3K36me3 over the Adh proximal promoter, a chromatin mark that has been previously implicated in co-transcriptional repression in yeast and humans (Carrozza et al. 2005; Keogh et al. 2005; Carvalho et al. 2013).

DISCUSSION

The fruit fly Adh locus, which encodes alcohol dehydrogenase, is a well-established example of transcriptional interference. At the time that it was originally investigated, however, the locus was studied outside of its natural genomic context, using P element transgenes (Corbin and Maniatis 1989). Here, we revisit the regulation of this locus, leveraging CRISPR/Cas9-based editing, reverse transcription coupled with quantitative PCR, and chromatin immunoprecipitation to better define the regulation of this important gene. Although AdhPROX is the predominant transcript isoform encoding the Adh enzyme during normal larval development, we demonstrate that the engineered induction of the AdhDIST transcript is sufficient to repress AdhPROX expression. Importantly, the degree of the distal promoter activity correlates well with the extent of transcriptional interference. Tunable transcriptional interference was first reported in bacteria.
(Bordoy et al. 2016; Hao et al. 2016), more recently in yeast (Chia et al. 2017), and in human cells (Hollerer et al. 2019). All of these studies highlight the notion that gene regulation by transcriptional interference is not binary with an on/off state, but rather can be utilized to tune the expression of regulated mRNAs during developmental gene expression programs.

Even though the untimely expression of AdhDIST in larvae led to a significant decrease in AdhPROX expression, the extent of repression (~10-fold) in the heterozygous GAL4 AdhUAS line appears to be inconsistent with a cis-mediated transcriptional interference mechanism at a first glance. We attribute this unexpectedly high reduction of AdhPROX level in the heterozygous lines to transcription, a common phenomenon in Drosophila in which interallelic promoters are co-regulated due to somatic pairing of homologous chromosomes. It has been shown that the GAL4-UAS system is subject to transcription (Mellert and Truman 2012; Noble et al. 2016). We consider that the transcription auxiliary factor(s) that activate the UAS-hsp70 promoter also activate transcription from the wild-type Adh distal promoter on the homologous chromosome. As a result, the AdhPROX expression can be downregulated by transcriptional interference even at the wild-type Adh locus in these heterozygous lines. Further tests are necessary to determine whether transcription plays a role in this context.

Although deletion of the Adh distal promoter at the endogenous locus de-repressed AdhPROX expression in adult fruit flies, the severity of this phenotype was far less pronounced compared to a previous study (Corbin and Maniatis 1989). A possible explanation for this difference is that position effects arising from differences in P element transgene insertion sites might alter the levels of transcriptional interference that were observed. It is also possible that the transcriptional interference observed in transgene context might be elevated due to the sensitized system. Furthermore, the reduction of the AdhPROX transcript in AdhDIST larvae suggests that the deleted region carries sites for some as yet to be determined positive regulators for AdhPROX expression. Alternatively, the deletion could change the nucleosome positioning in this region, which could impact AdhPROX expression. Regardless of these points, our study demonstrates that at the endogenous Adh locus, distal promoter-driven transcriptional interference is necessary for AdhPROX repression.

Our findings, in conjunction with the data reported in Corbin and Maniatis 1989, are consistent with a transcriptional interference-based mechanism operating at the Adh locus. However, alternative models could also explain why increased transcription from the distal Adh promoter in the UAS lines leads to a reduction in AdhPROX expression. For instance, it is possible that a negative feedback mechanism could exist whereby increasing the expression of the Adh protein indirectly leads to a decrease in expression from the Adh proximal promoter. Overexpression of Adh protein from a transgene could help determine whether such a feedback mechanism indeed exists.

The regulation of the Adh gene described here has some similarities to that found for the NDC80 gene in budding yeast (Chen et al. 2017; Chia et al. 2017). First, both genes have two promoters that are developmentally regulated, with the distal and proximal promoter encoding two distinct mRNA isoforms. Second, transcriptional interference is similar in both cases: transcription from the distal promoter is necessary and sufficient to repress the expression of the proximal promoter-derived isoform. Concomitant with this interference is the enrichment of H3K36me3 marks over the proximal promoter. While the H3K36me3 enrichment is similar between the cases of Drosophila Adh and yeast NDC80, we have been unable to assess causality in the current study. H3K36me3 is deposited by Set2, a highly conserved methyltransferase that physically associates with the elongating RNA polymerase II (Xiao et al. 2003). Set2 is essential for the viability of the fruit fly (Bell et al. 2007). Our attempts to characterize Set2 involvement in Adh regulation using RNA interference were unsuccessful, since these lines did not survive to adulthood. This finding precluded us from determining the impact of H3K36me3 on AdhPROX expression. Furthermore, the observation that replacement of H3 lysine 36 with arginine does not lead to increased cryptic transcription initiation (Meers et al. 2017) suggests that the co-transcriptional repression mechanism in Drosophila is more complex. Therefore, while the enrichment of H3K36me3 over the Adh proximal promoter correlates well with a decrease in AdhPROX levels, this mark does not necessarily need to be involved in co-transcriptional repression in flies.

A key difference between examples of Adh and NDC80 gene regulation is related to the translatability of the distal promoter-derived transcript isoforms. In the case of NDC80, the ORF within the distal promoter-derived mRNA is not translated, due to competing translation of multiple uORFs that are located in the 5′ leader of this transcript. The NDC80 case thus shows an interesting link between transcriptional and translational regulation. In essence, production of the distal promoter-derived transcript results in both transcriptional and translational repression, ultimately resulting in decreased Ndc80 protein production. By contrast, the AdhDIST transcript isoform is well translated, even better than the highly expressed αTUB84B transcript. The lack of translational repression in AdhDIST is consistent with the absence of an AUG start codon within the 5′ leader of this transcript (Figure S3), thus excluding repressive uORF translation. The difference between the apparent regulation in these two cases is important: poor translation in the case of the 5′ extended NDC80UTT isoform and superior translation in the case of AdhDIST. It is interesting to note that an earlier study, which examined the consequences of a natural transposon insertion at the Adh locus in the fruit fly (Laurie and Stam 1988), showed that the insertion of a copia retrotransposon between the Adh adult enhancer and the Adh distal promoter leads to an unusually low level of the Adh protein and enzyme activity. The reduction was found to occur as a result of a decrease in the level of the AdhDIST transcript. Surprisingly though, in this case, the AdhPROX transcript levels were proportionally increased in adults (Laurie and Stam 1988). Given that the levels of the distal and proximal transcripts remain similar between the wild type and the lines carrying transposon insertion, these data suggest that in the adult fruit flies, AdhPROX might not be as efficiently translated as AdhDIST, which is consistent with our polysome analysis. One possibility is that tissue-specific, trans-acting factors could differentially modulate the translation of the two Adh mRNA isoforms. Such spatial effects are likely to be missed by the whole organism polysome fractionation approach that was used in this study.

More broadly, the switch from one mRNA isoform to another may alter not just the translational efficiency of the transcript, but also localization, stability, or alternative splicing as well. In this regard, transcript toggling driven by developmental switches in promoter usage and the subsequent transcriptional interference from distal gene promoters may serve to alter gene expression in respects other than gene silencing. We posit that the Adh example is likely to be one of many cases where developmentally controlled transcriptional interference from ORF-distal promoters can alter genome decoding and cellular function in a manner that has not been anticipated previously.
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